Single cell signalling and immune cell profiling in psoriasis

Anders Krogh Aarebrot

Thesis for the degree of Philosophiae Doctor (PhD)
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Contents

Scientific environment

During the Medical Student Research Programme and throughout this PhD, I have been affiliated with Broegelmann Research Laboratory, Department of Clinical Science, Faculty of Medicine, University of Bergen, Norway, and I have been enrolled at the Bergen Research School in Inflammation. Professor Silke Appel has been my main supervisor, and associate professor Lene Frøyen Sandvik, postdoc Tim Holmes and Professor Roland Jonsson were co-supervisors for my doctoral work.

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Anders Krogh Aarebrot
Bergen, November 2020

List of Publications

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Abstract

Psoriasis is a common, chronic inflammatory skin disease with associated autoantigens, autoantibodies, local and systemic inflammation, and systemic comorbidities, such as cardiovascular disease and metabolic syndrome. Patients with severe disease often need biological treatment targeting the cytokines tumour necrosis factor (TNF), interleukin (IL)-12/23 or IL-17. The lack of precise laboratory analyses to aid selection of drug and monitor treatment effect along with adverse events and loss of treatment efficacy in a significant proportion of patients treated with biological drugs generate a need for robust assays used for monitoring and predicting treatment outcome.

The overall aim of this thesis was to analyse peripheral blood immune cells during active inflammation and treatment with biological drugs and to identify disease-specific immune profiles and biomarkers. In study I and II phospho-specific flow cytometry and mass cytometry were used, respectively, to study phenotypes and function of peripheral blood mononuclear cells (PBMCs) from healthy controls and patients on steady treatment with infliximab (IFX), a TNF inhibitor, and to examine the impact of switching from originator IFX to biosimilar CT-P13. In study III, mass cytometry was exploited to analyse PBMCs from healthy controls and patients starting biological treatment with emphasis on the T cell lineage and its intracellular signalling. In all studies, clinical and standard laboratory parameters were incorporated in the analyses.

In study I and II, increased phosphorylation levels in PBMCs were detected in psoriasis patients compared to healthy controls. The phosphorylation levels decreased during continued treatment, without completely normalising, despite clinical remission. Specifically, in study II, psoriasis patients had higher expression of CD38 in NK and $CD4^+$ T effector memory cells. In neither study I nor II any obvious differences were seen comparing originator IFX and CT-P13, with respect to cell frequencies, phosphorylation levels, clinical or laboratory data.

Study III revealed a shift from helper T (Th)1 to Th2 cells, a transition from naïve/effector to memory predominance, reduction of circulating Th17, Th22, Th9 and $CD8^+$ T cells and enhancement of inhibitory programmed cell death protein 1 (PD-1) expression on T cells after onset of treatment with a TNF-, IL-12/23- or IL-17 inhibitor. Also, the monocyte compartment showed changes favouring reduced cardiovascular risk after treatment onset. Intracellular phosphorylation was higher in psoriasis patients compared to healthy controls and non-responders to treatment compared to responders.

In conclusion, multiple aberrancies were seen in cellular frequencies in patients before treatment with biological drugs. In both untreated patients and patients on originator IFX and biosimilar CT-P13, our findings support a more activated state of PBMCs. These findings provide evidence for an ongoing systemic inflammation both with and without biological treatment, implying an increased risk of comorbidities, such as cardiovascular disease. Further research will help to shed light on the role of PBMCs as potential reporters used for personalised treatment both with regards to the skin disease itself but also the long-term risk of comorbidities.

Abbreviations

ADA	Anti-drug antibodies
APC	Antigen Presenting Cell
BMI	Body Mass Index
CCL20	Chemokine (C-C motif) Ligand 20
CCR4 (6, 10)	C-C Chemokine Receptor type 4 (6, 10)
CD	Cluster of Differentiation
CRP	C-Reactive Protein
CT	Computed Tomography
CTP-13	Infliximab biosimilar
CVD	Cardiovascular Disease
CXCL	Chemokine (C-X-C motif) Ligand
CXCR3	C-X-C Chemokine Receptor type 3
DC	Dendritic Cell
DLQI	Dermatological Life Quality Index
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	exempli gratia
ERK	Extracellular Signal-Regulated Kinases
FC	Fold Change
FlowSOM	Flow Self-Organising Maps
HLA	Human Leukocyte Antigen
i.e.	id est
IFN	Interferon
IFX	Infliximab
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
IL	Interleukin
JAK	Janus Kinase
LL37	Cathelicidin

MAPK	Mitogen-Activated Protein Kinase
MC	Monocyte
mDC	Myeloid Dendritic Cell
MFI	Median Fluorescence Intensity
MSI	Median Signal Intensity
MTX	Methotrexate
NEMO	NF-κB essential modulator
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
PASI	Psoriasis Area and Severity Index
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid Dendritic Cell
PET	Positron Emission Tomography
PMT	Photomultiplier Tube
RNA	Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
Tc	Cytotoxic T cell
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{EMRA}	Terminally differentiated effector memory T cell
Th	Helper T cell
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
Trm	Tissue-resident memory cell
TYK	Tyrosine Kinase
UV	Ultraviolet

1. Introduction

1.1 Background psoriasis

Psoriasis is a common immune-mediated disease, which manifests in the skin, joints, or both, in genetically predisposed individuals.[1]

1.1.1 Epidemiology

The prevalence of psoriasis in adults ranges from 0,5% to 11,4% in different studies, is equally prevalent in men and women and varies between different ethnicities.[1-5] Psoriasis can occur at any age, but there are two incidence peaks for psoriasis debut. Early debut, age 16-22, is called type I psoriasis, and late debut, age 57-66, is called type II psoriasis.[1,6-8] The early form is often more severe than the late form, and a family history with psoriasis in first-degree family members is more common.[6]

1.1.2 Classification

Psoriasis is a heterogenous disease, where several different forms have been described, but the most common subtypes include plaque, guttate, erythrodermic, and pustular psoriasis.[1,9] **Psoriasis vulgaris**, or chronic plaque psoriasis, is the most common subtype. About 90% of psoriasis cases are of the chronic plaque type, which is characterised by sharply demarcated, symmetrically distributed, erythematous lesions with loosely adherent silvery-white scales (**Figure 1A-C**).[1,9] Typical locations include the extensor surfaces on elbows and knees, peri-umbilical, peri-anal, lumbar and retro-auricular regions, and scalp.[1,10-12] It can, however, affect any skin site and the disease can become generalised over time.[1,12] **Inverse psoriasis**, a variant of psoriasis vulgaris, occurs in flexural and intertriginous areas, and lesions are usually deprived of scales due to friction and moisture.[1,10-12] **Guttate psoriasis** is an acute eruption of small papules, typically appearing over a period of one month, persisting for one month and resolving over the third month.[12] It is the most common form of psoriasis in children and young adults, and is often preceded by a streptococcal throat infection.[11,12] One third of children with guttate psoriasis develop plaque psoriasis later in life.[1,11,13] **Pustular psoriasis** is characterised by white coalescing pustules and

can be either localised or generalised.[1] **Erythroderma** is characterised by widespread cutaneous erythema covering > 90% of the body surface.[12] Both erythroderma and generalised pustular psoriasis are life-threatening diseases, as a result of compromised cutaneous functions. Due to loss of fluid, electrolytes, proteins, iron and a reduced temperature control and cutaneous barrier against microorganisms, fever or hypothermia, hypovolemia, electrolyte disturbances, hypoalbuminemia, anaemia, and sepsis can occur.[9] Erythroderma and generalised pustular psoriasis are often preceded by psoriasis vulgaris, but may arise de novo.[1,12] A typical precipitating factor is withdrawal of systemic glucocorticoids.[12]

1.1.3 Histology and inflammatory infiltrate

In psoriatic lesions the epidermis is thickened due to increased keratinocyte proliferation. Epidermal acanthosis, hyperkeratosis and elongation of rete ridges is typical. In the dermis, dilated blood vessels reach the tip of dermal papillae.[1,14,15] A mixed inflammatory infiltrate consisting of epidermal and dermal T cells and an increased number of macrophages, mast cells and neutrophils in the epidermis is often seen. Accumulation of inflammatory cells within the epidermis and formation of micro-abscesses is also typical **(Figure 1 D-G)**.[14,16,17]

A study of human skin grafted on immunodeficient mice revealed that progression towards psoriasiform changes only happened when T cells had entered the epidermis, implying that epidermal T cells are necessary for the development of typical skin pathology.[18]

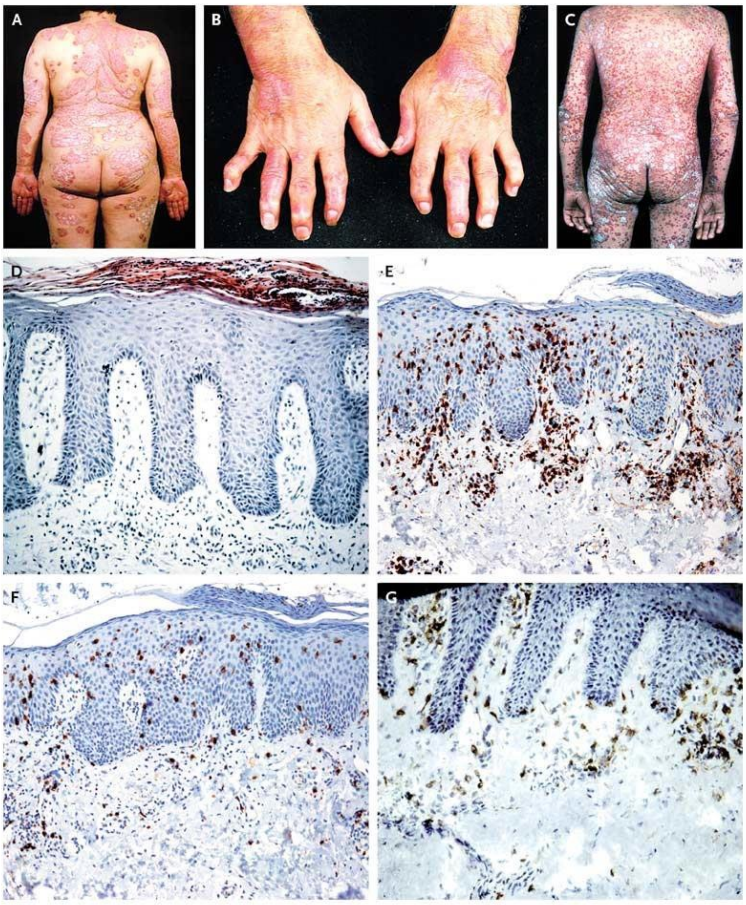

Figure 1. Clinical and Histologic Features of Psoriasis. Erythematous, scaly, sharply demarcated plaques in different sizes and shapes are hallmarks of psoriasis. Although there are predilection sites such as the elbows, knees, and the sacral region, lesions may cover the entirety of the skin (Panels A and C). Concurrent psoriatic arthritis often affects multiple aspects of the interphalangeal joints of the hand (Panel B). The nails are frequently affected, with nail dystrophy and psoriatic lesions of the nail bed. The histopathological picture (Panel D, hematoxylin and eosin) is characterised by thickening of the epidermis, parakeratosis, elongated rete ridges, and a mixed cellular infiltrate. CD3+ T cells (Panel E, 3,3′-diaminobenzidine and hematoxylin) and CD8+ T cells (Panel F, 3,3′-diaminobenzidine and hematoxylin) are detected around capillaries of the dermis and in the epidermis. CD11c+ dendritic cells (Panel G, 3,3′-diaminobenzidine and hematoxylin) are detected mainly within the upper part of the dermis. (Clinical photographs courtesy of St. John's Institute of Dermatology.) Reproduced with permission from Nestle *et al.*, 2009,[15] Copyright Massachusetts Medical Society.

1.1.4 Assessment of disease severity

Psoriasis is classified either as mild, moderate, or severe by evaluating surface area affected and severity of the lesions. Psoriasis area and severity index (PASI) is a

validated tool that combines the area affected and the severity of the lesions into a single score ranging from 0 to 72.[19,20] Scores above 10 are usually considered severe disease.[21] Response to treatment is often presented as a percentage reduction in PASI, *e.g.* PASI75.[22]

Assessment of life quality by Dermatology Life Quality Index (DLQI) is recommended because psoriasis may result in functional, psychological, and social morbidity, even in people with minimal involvement (less than the equivalent of three palm areas).[23] DLQI consists of 10 standardised questions about the patients' impression of how the disease has affected their quality of life over the last week.[24] Moreover, considering prior treatment response, involvement of special areas (face, scalp, palms, soles, and genitals) and presence of comorbidities is also recommended to avoid underestimation of disease severity.[23,25,26] Finally, Physician's global assessment, Body surface area and Nail Psoriasis Severity Index can also be used to assess disease severity.[22]

1.1.5 Genetics

The genetic nature of psoriasis has for long been appreciated, with a concordance rate of approximately 70% and 20% in monozygotic and dizygotic twins, respectively.[14]

The most predominant susceptibility allele for psoriasis, the human leukocyte antigen (HLA) susceptibility locus HLA-C*06:02, accounts for up to 50% of disease heritability and is strongly associated with early onset and more severe disease.[27-29] Moreover, being located in the HLA class I region, which is responsible for antigen presentation to $CD8^+$ T cells, this locus links psoriasis genetics with proposed autoantigens.[14,30]

Two highly penetrant gene mutations have been reported to cause psoriasis, namely *IL36RN* and *CARD14.* Both lead to increased activity of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and excessive production of NF-κB-regulated cytokines.[14,31,32] Otherwise, psoriasis is a complex multifactorial disease with many genes contributing to disease risk, where at least 12 major psoriasis

susceptibility loci and near 40 single nucleotide polymorphisms have been identified,[1,14,33] including genes related to antigen presentation, keratinocyte proliferation and immune regulation.[34]

1.1.6 Triggering factors

In predisposed individuals, several stimuli can trigger psoriasis. Psoriatic lesions can appear in areas of the skin that have recently been injured, for example after mild traumas such as scratching, sunburn, or chemical irritants.[1] Infections can trigger or exacerbate psoriasis. Infection with β-haemolytic streptococci is the prime example of this. It is thought to trigger guttate psoriasis[35] and exacerbations of plaque psoriasis[36] via molecular mimicry where lymphocytes reactive to the M-protein on group A β-haemolytic streptococci recognise human keratin 17, resulting in keratin 17-reactive $CD8^+$ T cells that elicit significant interferon (IFN)-γ responses.[37] Moreover, tonsillectomy in patients with recurring tonsilitis can improve psoriasis.[38] Also, systemic drugs such as β-blockers, lithium, quinolinic anti-malarials and non-steroidal anti-inflammatory drugs can induce or worsen psoriasis,[1,39] and withdrawal of systemic glucocorticoids can induce severe exacerbations, including generalised pustular psoriasis.[39,40] Finally, stress is a known trigger of psoriasis, and tobacco smoking and obesity increase the risk of psoriasis onset.[41,42]

1.2 Immunopathogenesis and inflammation in psoriasis

In genetically susceptible individuals, autoantigens can stimulate the innate and adaptive immune system. In response to the cytokine milieu, mediated by immune cells, keratinocytes proliferate and produce antimicrobial peptides and chemokines, leading to further recruitment of cytokine-producing immune cells which amplifies the pathologic state.

1.2.1 Initiation of psoriatic lesions

In general, naïve T cells require presentation of an antigen by a professional antigen-presenting cell (APC), such as dendritic cells (DC), to become activated.[43] Thus, there must be an upstream initiating event which leads to presentation of autoantigens

to naive T cells. In the epidermis several autoantigens have been identified as objects of T cell-reactivity including cathelicidin (LL37),[44] disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5),[45] keratin 17,[46,47] and phospholipase A2 group IVD (PLA2G4D) **(Figure 2)**.[48] The cationic antimicrobial peptide LL37 is the most studied autoantigen in psoriasis. In response to skin injury or infection, LL37 is produced by several immune cells and keratinocytes.[44,49] Due to its positive charge, LL37 forms complexes with negatively charged DNA and RNA, released from damaged cells, e.g. neutrophil extracellular traps (NET).[16,50,51] LL37 bound to DNA or RNA activates LL37-specific plasmacytoid DCs (pDCs) via toll-like receptor (TLR)9 and TLR7, respectively.[50,52] Activated pDCs produce IFN-α which activates myeloid DCs (mDCs).[52,53] Further, a recent study found that RNA-bound LL37 triggered TLR8-mediated cytokine and NET release by polymorphonuclear neutrophils (PMNs). This uncovers PMNs capability of self-maintaining a vicious cycle through both sensing the inflammatory ligand RNA-LL37 and releasing RNA-LL37 and DNA-LL37 complexes.[51] RNA-bound LL37 also activates mDCs by binding TLR7/8.[52] Hence, mDCs can be activated by both type 1 IFNs and RNA-LL37, driving T cell activation and the production of cytokines found in psoriasis.

1.2.2 Immune cell interplay and cytokines

Psoriasis lesions contain $CD4^+$ T cells that produce IFN-γ, interleukin (IL)-17, and IL-22, labeled as Th1, Th17, and Th22, respectively. There are also $CD8^+$ T cell populations producing the same range of cytokines, termed cytotoxic T cells (Tc)1, Tc17, and Tc22, respectively.[14] Activated DCs facilitate the differentiation of naïve $CD4^+$ T cells into effector cells in the lymph nodes.[54-56] Upon stimulation with IL-12 and IFN-γ, naïve $CD4^+$ T cells will differentiate to Th1 cells.[57] Stimulation with IL-23, IL-1β and IL-6 will generally promote Th17 differentiation,[58] whilst the combination of tumour necrosis factor (TNF) and IL-6 drives the differentiation of Th22 cells.[59] Lastly, Transforming Growth Factor-β (TGF-β), in concert with IL-2, is also involved in the differentiation of regulatory T cells (Tregs), which are important suppressors of the inflammatory response, and thus important for stopping

inflammation.[60] Cytokines produced by Th1, Th17 and Th22 are key factors in the pathogenesis of psoriasis.[15,55,61-63]

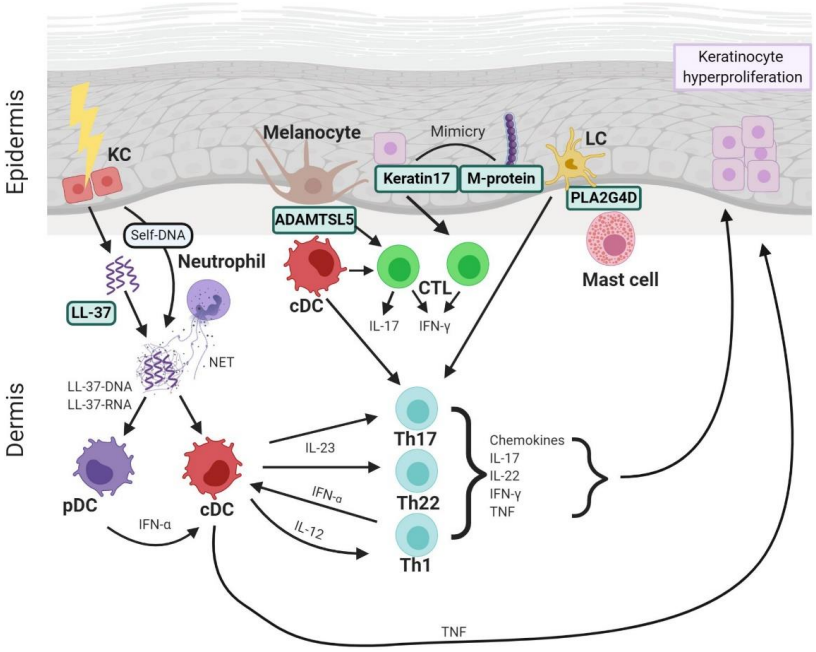

Figure 2. Autoantigens and their potential role in the development of psoriatic skin.
As a result of environmental stimuli (e.g., infection or skin trauma), damaged keratinocytes release LL-37 in genetically susceptible individuals. The antimicrobial peptide LL-37 forms complexes with self-DNA/RNA originating from NETs. LL-37-DNA complexes initiate IFN-α release from pDCs, which in turn activates cDCs. Activation of cDC triggers the expression of TNF, IL-23 and IL-12 inducing Th17, Th22 and Th1 cell subsets, resulting in the production of pro-inflammatory cytokines. Alternatively, ADAMTSL5 in melanocytes results in the activation of intraepidermal $CD8^+$ CTL and increased amounts of IFN-γ and IL-17. Moreover, CTL reactive to the surface M protein from streptococci may recognise keratin 17 via molecular mimicry resulting in IFN-γ production. Finally, psoriatic mast cells are a major source of PLA2G4D that generates neolipid antigens recognized by Langerhans cells, which in response activate lipid-specific T cells. ADAMTSL5 disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5, cDC conventional dendritic cell, CTL cytotoxic T cell, IL interleukin, IFN interferon, KC keratinocyte, LC Langerhans cell, NET neutrophil extracellular trap, pDC plasmacytoid dendritic cell, PLA2G4D phospholipase A2 group IVD, Th T helper lymphocyte, TNF tumour necrosis factor. Figure and text adapted from ten Bergen *et al.*, 2020.[64] Reprinted with permission from John Wiley and Sons.

The local milieu of cytokines, chemokines and antimicrobial peptides favor hyperproliferation,[65] LL-37 production,[66] TLR9 responsiveness[67] and production of

cytokines[68,69] and chemokines[70,71] in keratinocytes, along with attraction of immune cells, including T cells, DCs, neutrophils and monocytes.[15,51,72] The importance of recruitment of immune cells from the circulation is demonstrated by the effect of efalizumab, which blocks the α subunit of CD11a, inhibiting the binding of T lymphocytes to endothelial cells, and their movement from the circulation into dermal and epidermal tissues.[73] The continuous activation of DCs, T cells and keratinocytes, which promotes further recruitment of immune cells, and thereby enhancing the inflammatory response within the psoriatic lesion, results in a self-amplifying feedforward loop.[14,16] Importantly, other cell types than $CD4^+$ and $CD8^+$ T cells can produce cytokines promoting the forementioned local effects, including neutrophils, macrophages, innate lymphoid cells, natural killer (NK) cells and γδ T cells,[74-79] and are, thus, likely to contribute to the chronic inflammation in psoriasis.[80] In addition, Treg function has been reported to be impaired during psoriatic inflammation.[17,81] Usually, Tregs maintain immune tolerance by releasing inhibitory cytokines, inducing apoptosis and by consumption of IL-2.[82,83] However, in psoriasis they are dysfunctional, with reduced suppressive capacity.[14,84,85] Pro-inflammatory cytokines, such as IL-6 and IL-17, which are abundant in psoriatic lesions, have been reported to suppress Treg function, and provide a plausible explanation of the suppressed Treg function and increased proliferation of pathogenic T cells in psoriatic lesions.[17,84,86,87]

Psoriasis lesions often recur in the same locations and regrow to their prior size once therapy is stopped. A possible mechanism is that some T cells progress toward differentiation into tissue-resident memory $CD8^+$ T (Trm) cells after recognition of autoantigens.[88,89] In resolved psoriasis, Trm cells have been detected and are thought to constitute a site-specific disease memory, together with epidermal Tc17 and Th22 cells.[55,62,88,90-93] In addition of innate immune mediators, keratinocytes may act as non-professional APCs and induce immune responses in antigen-experienced Th and Tc memory cells, leading to functional responses like cytokine production or cytotoxic effects.[94,95]

1.2.3 Systemic inflammation

As psoriasis progresses, serum levels of multiple pro-inflammatory cytokines are increased compared to healthy controls, including TNF, IFN-γ, IL-6, IL-8, IL-12, IL-17A and IL-18[96-99] Furthermore, increased frequencies of circulating Th17 cells, and elevated levels of C-reactive protein (CRP) have been reported in psoriasis patients.[100,14] Studies using ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) on patients with moderate-to-severe psoriasis demonstrate subclinical inflammation in the liver, joints and tendons, and significantly increased global arterial and subcutaneous inflammation,[101,102] emphasising a potential benefit of systemic treatment in this patient group.[26] Also, patients with mild psoriasis have been found to have subclinical inflammation in the aorta.[103] After a study of the skin transcriptome and serum protein measurements in 85 patients with moderate-to-severe psoriasis compared with serum proteomics in healthy controls, a model for systemic inflammation in psoriasis was created, which proposed that a subset of inflammatory products is produced at high levels in lesional psoriatic skin and diffuses into the systemic circulation.[14,97] Increased understanding of the roles of these pathogenic molecular pathways has led to an appreciation of the systemic nature of psoriasis and given rise to biological drugs that target cytokines involved in the pathogenesis of the disease.[99]

1.3 Comorbidities in psoriasis

1.3.1 Cardiovascular disease and metabolic syndrome

Psoriasis has for long been thought to be a risk factor of cardiovascular disease (CVD), and many epidemiological studies substantiate this,[104-110] although conflicting findings exist.[111,112] Large meta-analyses and prospective cohort-studies showing increased risk of coronary artery disease, myocardial infarction and stroke in patients with psoriasis, where the risk correlates with disease severity and duration, strengthen the hypothesis of psoriasis being an independent risk factor for CVD.[105,110,113-115] However, the causes of this increased risk are complex and not fully elucidated.[113]

Both psoriasis and metabolic syndrome are associated with CVD. Psoriasis patients are at increased risk of developing metabolic syndrome.[116,117] Furthermore, increasing severity of psoriasis correlates with increasing frequency of dyslipidaemia.[118,119] In addition, smoking is a common risk factor for both CVD and psoriasis.[120] Thus, both smoking and metabolic syndrome are important confounding factors. Patients with severe psoriasis have been estimated to have an additional risk of 6,2% on 10-year incidence of major adverse cardiovascular events, compared with the general population, after adjusting for age, gender, diabetes, hypertension, smoking and hyperlipidaemia.[121] Moreover, studies that have utilised CT and PET/CT have found increased coronary artery and aortic inflammation, respectively, in psoriasis patients compared with healthy controls after controlling for confounding factors.[101,102,122]

A direct link between psoriasis and CVD is increasingly accepted as more knowledge has been gained regarding the immunopathogenesis of the two diseases.[80] Lesional psoriatic skin and atherosclerosis exhibit significant overlap of their transcriptomes, with IFN-γ and TNF being inflammatory mediators shared between the two disease processes. Moreover, IFN-γ and TNF, which are elevated in sera of psoriasis patients, synergistically increase the inflammatory responses in endothelial cells and atherosclerotic tissue, and may therefore provide a link between the two diseases.[123] Because of the link between IL-17A and neutrophil infiltration in atherosclerotic plaques and its importance in the pathogenesis of psoriasis the IL-17A/neutrophil axis has been suggested to take part in the atherogenesis associated with psoriasis.[124,125] Studies of IL-17A in atherosclerosis are contradictory,[126] and although it seems to be mostly pro-atherogenic it might exert both anti- and pro-atherogenic effects, depending on the inflammatory context.[127] Neutrophils are important for atherosclerosis through their interactions with damaged endothelium, recruitment of other leukocytes and development of foam cells.[113,128,129]

The concept of the “psoriatic march” suggests a pathogenetic link between psoriasis and cardiometabolic comorbidities **(Figure 3)**. According to this hypothesis, the systemic inflammation in psoriasis induces insulin resistance. Insulin resistance triggers widespread endothelial cell dysfunction, which leads to atherosclerosis and

myocardial infarction or stroke.[130] The hypothesis of inflammation spreading from psoriatic plaques through the vessels is supported by the parallel alteration of cytokine patterns in the skin and blood compartment. Mediators originating from the skin may include cytokines from infiltrating immune cells and molecules secreted from resident cells in response to the cytokine milieu, such as abnormal amounts of pro-inflammatory adipokines (adiponectin, leptin, and resistin)[131] produced by altered dermal fat underlying psoriatic lesions,[132] and adipocytes. These adipokines may thus synergise with psoriatic cytokines and drive a local inflammatory condition, which over time leads to a widespread vascular and systemic inflammation **(Figure 3)**. However, neither the precise role of local adipose tissue in psoriatic pathogenesis nor the quantitative impact of fat depots on the inflammatory burden has been determined.[133]

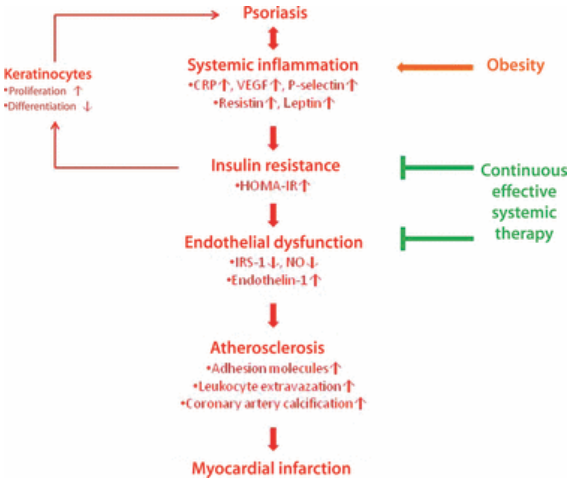

Figure 3. The concept of the 'psoriatic march'. It suggests a causal link between psoriasis as a systemic inflammatory condition and cardiovascular comorbidity, as systemic inflammation may cause insulin resistance, which in turn triggers endothelial cell dysfunction, subsequently leading to atherosclerosis and finally myocardial infarction or stroke (red, bold). This 'backbone' may be developed further by adding additional 'modules', such as a possible feedback of insulin resistance to epidermal homeostasis (red, fine). Obesity is a known risk factor for psoriasis and may induce the phenotype through systemic inflammation (orange, bold). Continuous effective systemic therapy may stop the 'psoriatic march' through interference with insulin resistance and endothelial dysfunction (green). Figure and text adapted from Boehncke *et al.*, 2011.[130] Reprinted with permission from John Wiley and Sons.

Besides potential systemic effects, the impact of the local inflammatory milieu on the vessel walls in the immediate vicinity is of importance. It is well known that inflammatory mediators induce and modulate the expression of adhesion molecules and influence local recruitment of circulating inflammatory cells to the respective sites.[134-136] At this level, the development of a psoriatic plaque and an atherosclerotic plaque show many similarities.[137] Of note, pro-inflammatory cytokines with known relevance in psoriasis are capable of inducing insulin resistance in endothelial cells,[130,138,139] referring to the observation that a number of pro-atherogenic effects are still being triggered within these cells, while anti-atherogenic effects are blocked. Dermal endothelial cells are particularly sensitive to this phenomenon.[139] The anatomical proximity between cytokine-producing psoriatic plaques and dermal blood vessels, exposes these cells to risk of turning insulin resistant, resulting in a state of dysfunction and stiffness. This results in an augmented peripheral resistance in the circulation, increased heart work, arterial hypertension, and finally macroscopic vascular disease in the form of atherosclerosis. Thus, high systemic levels of inflammatory mediators produced in psoriatic skin are not required for a plausible explanation of how psoriasis may drive cardiovascular disease.[80,140]

1.3.2 Other comorbidities

Psoriasis patients can have a severely reduced quality of life,[141-144] and the risk of depression is increased, with possible biochemical links.[145-147] Although many factors can contribute to depression in psoriasis patients, a growing body of evidence indicates that inflammation plays a role in pathophysiology of mental illnesses, including major depression.[148] Numerous studies demonstrate elevated levels of CRP and pro-inflammatory cytokines in patients with depression, including TNF, IL-1β, and IL-6.[146,148,149] These cytokines, which are involved in the psoriatic pathogenesis, cross the blood brain barrier and interact with pathophysiological spheres involved in depression.[148] Psoriasis is an independent risk factor of non-alcoholic fatty liver disease (NAFLD) in patients who are 55 years or older.[150] NAFLD is also associated with a chronic inflammatory state with increased levels of CRP, IL-6 and TNF, although a causal relationship has not been proven.[150] Psoriasis is also associated with

Crohn´s disease with probable shared features with respect to cytokines, T cell involvement and amelioration of symptoms by neutralising TNF and IL-12/23.[151-156]

1.4 Treatment

1.4.1 Non-biological treatment

Most people with psoriasis have localised disease where topical therapy forms the cornerstone of treatment. Glucocorticoids, vitamin D3 analogues, and keratolytic agents such as salicylic acid and urea, are used for local treatment. Several combinations and formulations (creams, ointments, solutions) are available.[157,158] The topical retinoid tazaroten is also an effective treatment option for psoriasis patients.[158] However, in patients with extensive disease (>10% of body surface area affected) or affection of areas where topical treatment is ineffective, such as nail disease, a second line treatment is recommended, either phototherapy or systemic non-biological therapy.[23]

Ultraviolet (UV) light is a commonly used treatment modality. 311 nm narrowband UVB light is particularly effective, with 70% of patients reaching PASI75.[159-162] By using a combination of topical or systemic psoralen and UVA (PUVA), up to 90% achieve PASI75 response.[163] Further, a combination of coal tar and UVB light, named The Goeckerman Regimen, is an effective and safe, although time-consuming option.[164]

Methotrexate (MTX), a folic acid antagonist which inhibits DNA synthesis and cell replication but also has specific T cell-suppressive effects, is a widely used systemic drug for psoriasis treatment.[158] Up to 50-60% of patients achieve PASI75, but severe side effects can limit the use. These include teratogenicity, bone marrow suppression, liver fibrosis and cirrhosis and rarely pulmonary fibrosis.[1,158]

1.4.2 Biological treatment

Biological drugs are protein-based drugs derived from living cells cultured in a laboratory,[165] including fusion proteins, recombinant proteins, and monoclonal

antibodies. Biological drugs have revolutionised the treatment of patients with moderate-to-severe psoriasis and are given to those who do not respond to other treatments. Biologics are given by subcutaneous injections or intravenous infusions to patients with moderate-to-severe psoriasis that have not responded due to earlier treatment failure, intolerance or contraindication to conventional therapies or phototherapy. Biologics are more expensive than conventional systemic drugs, but the clinical efficacy is higher and risk of adverse events lower.[166] In psoriasis, the biologicals used target the TNF/IL-23/IL-17 axis **(Figure 4)**.

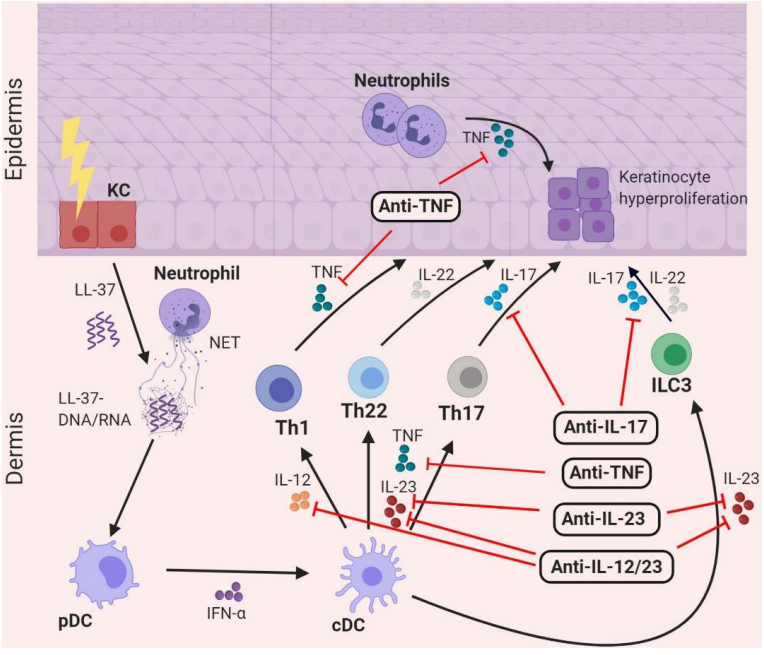

Figure 4. Biologic treatments targeting the TNF/IL-23/IL-17 axis in psoriatic skin. An initial trigger (e.g. biochemical stimuli, infections) induces cell damage, NETs formation and increased production of antimicrobial peptides (e.g. LL-37). Self-nucleic acids and LL-37 complexes induce IFN type I production of activated pDCs stimulating maturation of cDCs followed by production of IL-12, IL-23, and TNF. The pro-inflammatory cytokine IL-23 drives T cell differentiation and stimulates production of Th22/ILC3 (IL-22) and Th17/ILC3 (IL-17) cytokines, while IL-12 initiate Th1 differentiation and subsequent TNF secretion. The released cytokines stimulate the proliferation of keratinocytes with neutrophilic inflammatory infiltrate. The schematic depicts biologics targeting TNF, IL-12/23, IL-23, and IL-17 cytokines highlighting the central role of this signalling pathway in psoriasis. cDC conventional dendritic cell, IFN interferon, IL interleukin, ILC innate lymphoid cell, LL37 cathelicidin, NETs neutrophil extracellular traps, pDC plasmacytoid dendritic cells, Th T helper lymphocyte, TNF tumour necrosis factor. Figure and text adapted from ten Bergen *et al.*, 2020.[72] Reprinted with permission from John Wiley and Sons.

In the early 2000s the pathogenesis of psoriasis was proposed to rely on Th1 responses, due to the increased lesional expression of Th1 cytokines, such as IFN-γ, TNF and IL-12.[61] Moreover, the astonishing therapeutical efficacy observed when blocking TNF[167,168] and the p40 subunit of IL-12 strengthened this hypothesis.[61,169] However, concomitant to the development of a monoclonal antibody targeting p40, IL-23 was found to contain the identical p40 subunit.[170] Later, IL-23 was established to induce IL-17-producing $CD4^+$ T cells, later named Th17 cells.[171,172] The enhanced efficacy of novel biologics targeting IL-17 and the specific p19 subunit of IL-23 along with the effects of IL-17 in murine models of psoriasiform inflammation, stresses the importance of IL-17 in psoriasis.[61] Moreover, the primary mechanism of action by TNF inhibitors in psoriasis is thought to be an indirect inhibition IL-17 signalling via the regulation of IL-23 production from DCs.[173] Altogether, these findings indicate that psoriasis is a disease driven by IL-17 rather than Th1 cytokines, and that successful biological treatment mainly depends on its ability to block IL-17 signalling.[61,173]

TNF inhibitors

The first generation of anti-psoriatic biologics targets the cytokine TNF. By inhibiting TNF they break the self-sustaining cycle of DC- and T cell-activation, and cytokine, growth factor and chemokine production by multiple cell types including lymphocytes, neutrophils, DCs and keratinocytes.[174] Neutralisation of TNF with etanercept leads to a reduced cytokine production by inflammatory DCs and Th17, indicating that TNF directly regulates DC and Th17 cell function.[14,175,176] TNF inhibitors are also effective as treatment of psoriatic arthritis.[177] Currently, four TNF-inhibitors are approved for the treatment of moderate-to-severe psoriasis, namely etanercept, infliximab (IFX), adalimumab and certolizumab pegol. Etanercept is a recombinant human TNF-receptor and Fc-fragment fusion protein, that neutralises the effects of endogenous TNF by competitively inhibiting its interaction with cell-surface receptors.[178] It is self-administered subcutaneously and about 50% of patients achieve PASI75.[178-182] IFX is a chimeric monoclonal antibody, administered as intravenous infusion, which binds and neutralises TNF. It is the most effective TNF

inhibitor[183] with PASI75 ranging from 75% to 88% in different randomised controlled trials.[184-186] Adalimumab is a fully human monoclonal antibody, which is self-administered subcutaneously. 54-80% of patients reach PASI75.[187,188] Certolizumab consist of Fab fragments from humanised monoclonal antibodies conjugated to polyethylene glycol. As it does not have an Fc fragment, it does not cross the placenta.[189] Patients using IFX have an increased risk of serious infections.[190-193] All patients evaluated for therapy with a TNF inhibitor needs to be tested for latent tuberculosis, as there is an increased risk of re-activation.[194] Another side effect observed in 2–5% of patients treated with a TNF inhibitor is "paradoxical psoriasis",[195] with inflammatory skin lesions caused by overactive innate inflammatory response, driven by pDC-derived type I IFNs that does not lead to T-cell autoimmunity which is common in plaque psoriasis.[196] Due to the involvement of TNF in body weight homeostasis, weight gain is frequently seen in IFX-treated patients.[197]

IL-12/23 inhibitor

Ustekinumab is a human monoclonal antibody which binds to the p40 subunit on IL-12 and IL-23. About 70% of patients reach PASI75. When comparing ustekinumab to IFX, PASI75 does not differ significantly, but in contrast to IFX, ustekinumab does not induce weight gain.[198] Drug survival of ustekinumab is better than that of TNF inhibitors both for biologic-naive and biologic-experienced patients with psoriasis.[199]

IL-23-p19 inhibitors

The humanised monoclonal antibodies tildrakizumab and risankizumab and the fully human monoclonal antibody guselkumab bind to the p19 subunit of IL-23. In head-to-head trials the IL-23-p19 inhibitors are superior to ustekinumab with regards to clinical efficacy,[200,201] suggesting that neutralisation of p19 more potently inhibits IL-23,[202] and indicating that IL-12 has a less important role in maintaining the psoriatic plaque. During clinical trials, no significant safety issues were observed. In guselkumab-treated patients, the most common adverse events were non-serious upper respiratory tract infections, nasopharyngitis, headache and arthralgias.[203] There are no reports of increased rates of malignancy, serious and opportunistic infections

or major cardiovascular events.[204] However, as these agents are novel there is a lack of long-term follow-up studies and experience regarding their use.

IL-17 inhibitors

Three monoclonal antibodies against IL-17 have been approved for the treatment of psoriasis, namely secukinumab, ixekizumab and brodalumab. Ixekizumab and secukinumab target IL-17A directly. Brodalumab blocks the receptor subunit IL-17RA. Secukinumab,[205-207] brodalumab[208] and ixekizumab[209] have all demonstrated superiority to ustekinumab for achieving PASI75 and PASI90. Thus, neutralising IL-17 is more effective than neutralising IL-12/IL-23. Moreover, beneficial efficacy of both secukinumab[210] and ixekizumab[211] compared to etanercept, has been observed.

In brodalumab-treated patients with underlying psychiatric disorders there have been a few cases of suicides, raising concerns regarding its safety, although causality never has been established.[212] Inhibition of IL-17 slightly increases the risk of opportunistic infections, especially mucocutaneous candida and slightly increases the risk of triggering or worsening inflammatory bowel disease.[213] Loss of IL-17 may facilitate inflammation in the gastrointestinal tract by favoring Th1 pathways or by changing the human mycobiome balance in the gut.[214] A likely cause is that IL-17 has an important function in gut homeostasis and resistance to extracellular microorganisms.[215-218]

Biosimilars

Biosimilars are biotechnologically manufactured products entering the market when the original biological loses its patent. Biosimilars have the same amino acid sequence as the originator drug but are not exact copies of the originator product. Due to the complex structure and manufacturing process, the biosimilars are prone to variation, and regulatory rules are more extensive than those of generic versions of chemically synthesised drugs.[219,220] However, no long-term efficacy and safety studies, which are required for originator biologicals, are performed for biosimilars prior to their approval, raising concerns regarding their efficacy and safety.[221]

1.4.3 Personalised treatment

For every single patient that undergoes a consultation with a physician, resulting in some sort of treatment, the received treatment is personalised. However, in the context of biological treatment, a lack of robust methods to guide choice of treatment is evident, and the clinical treatment strategies are largely empiric.

Some patients are primary non-responders to biologics. A significant proportion of psoriasis patients experience loss of effect of biological treatment, more frequently if concomitant MTX has not been used.[222-224] The efficacy of biologics can be limited by immune reactions in patients. This can result in anti-drug antibodies (ADA) that neutralise the drug and reduce its efficacy, by altering pharmacodynamics and pharmacokinetics, with subsequent loss of response and increased risk of allergic or infusion reactions, serum sickness, bronchospasm, and vasculitis, some with fatal outcome. ADAs and a decline in serum trough levels are sometimes detected.[225-227] The different biological drugs have various risk of inducing neutralising ADAs. Neutralising ADAs have been identified in patients treated with IFX, adalimumab and ustekinumab.[228] Etanercept, however, is not associated with neutralising ADAs. Anti-etanercept antibodies have been identified, but they have all been non-neutralising.[224] Neutralising ADAs to secukinumab have not been identified either, though caution, due to shorter experience with secukinumab, must be taken.[229] Structural components of biological drugs have been proposed as causes of immunogenicity. The murine components of IFX at the drug target's binding sites, likely induces an antigenic reaction specific to them.[230] Contrarily, the binding sites of etanercept are naturally occurring type II (p75) receptors. This suggests immunogenicity against other portions that do not compromise the therapeutic activity of etanercept.[230] In vitro, neutralising anti-adalimumab antibodies specifically confined to the TNF-binding region of adalimumab has been identified.[231] Further, it has been speculated that ADAs can form immune-complexes with adalimumab, leading to accelerating adalimumab clearance, due to shorter half-lives of immune complexes compared to free-standing antibodies.[224,232] Although the mechanisms behind ADAs are not fully understood, their effects are clinically significant.[224]

Measurements of trough level and ADAs have been outlined for clinical use **(Figure 5)**.[227] However, they leave room for misinterpretation due to different and non-standardised methodologies and recommendations, which can be confusing. Some prediction of treatment responses within an ongoing treatment course is possible by utilising drug trough level and ADA measurements,[227,233] but they cannot be used as predictive tools before treatment with a biological drug.

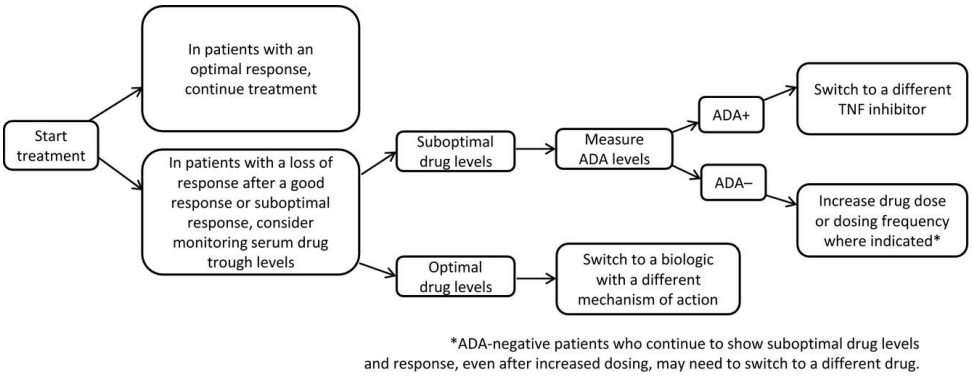

Figure 5. Proposed treatment decision tree when monitoring drug levels in patients on biologics starting with TNF inhibitors. Figure and text adapted from Carrascosa *et al.*, 2014.[227] Reprinted with permission from John Wiley and Sons.

Still, when a patient experiences worsening of the disease, it is difficult to know if the problem is a flare of the disease, loss of efficacy or poor treatment adherence. Thus, there is a clinical need for better tailored therapy, based on predictive assays for monitoring and understanding disease and drug reactions in patients.

A method used to measure levels of functionally available drug, reporter-gene assay, utilises a genetically TNF-sensitive reporter gene cell line to quantify residual drug activity and levels of neutralising ADAs in sera from anti-TNF-treated patients.[234] This leads to the question if a patient's own cells can be used as reporters of TNF activity, and moreover, reporters of IL-12, IL-23 and IL-17 activity. Can the activity of a psoriasis patient's circulating cells serve as a measurement of disease activity and treatment efficacy?

1.5 Signalling pathways

When thriving to gain knowledge about the immunopathogenesis of a disease, knowing which types of cells that are present and if any of these cells are abundant or deficient is of course important, but rarely sufficient. Often it is important to find out what the cells do. Cytokine levels supply information about the signalling environment between cells in the tissue. However, to find out which state each individual cell or cell population is in, measurement of molecules expressed by the cells only when in a certain state is necessary. The following paragraphs are limited to the molecules associated with immune cell activation that were used in experiments included in this thesis.

1.5.1 Intracellular signalling pathways

In response to a stimulus on the cell surface, the cell utilises many mechanisms to respond appropriately. To allow changes to occur in the timeframe necessary for cellular responses, the intracellular signalling systems use many enzymes as catalysts, including protein kinases that catalyse phosphorylation and adenylyl cyclase which catalyses formation of cyclic adenosine monophosphate. With respect to the cytokines TNF, IL-12, IL-23, and IL-17 which are targets for biological treatment of psoriasis, several signalling cascades are relevant. TNF signals via TNF-receptor 1 and 2, which are expressed on all nucleated cells,[235] and induces phosphorylation of NF-κB, a transcription factor, and p38 mitogen-activated protein kinase (MAPK). TNF signalling can result in phosphorylation of signal transducer and activator of transcription 3 (STAT3), another transcription factor, and extracellular signal-regulated kinases (ERK).[236-239] IL-17 signals via somewhat similar pathways, with engagement of the IL-17 receptor leading to activation of TNF receptor-associated factor 6 (TRAF6), and subsequent phosphorylation of NF-κB as well as p38 and ERK.[240] Binding of IL-12 and IL-23 induces receptor dimerisation of IL-12Rβ1/IL-12Rβ2 and IL-12Rβ1/IL-23R, respectively, leading to activation of the kinases Janus kinase (JAK)2 and tyrosine kinase (TYK)2. Downstream, IL-12 leads to phosphorylation of STAT4 and IL-23 leads to phosphorylation of STAT3 and NF-κB (**Figure 6**).[241-243]

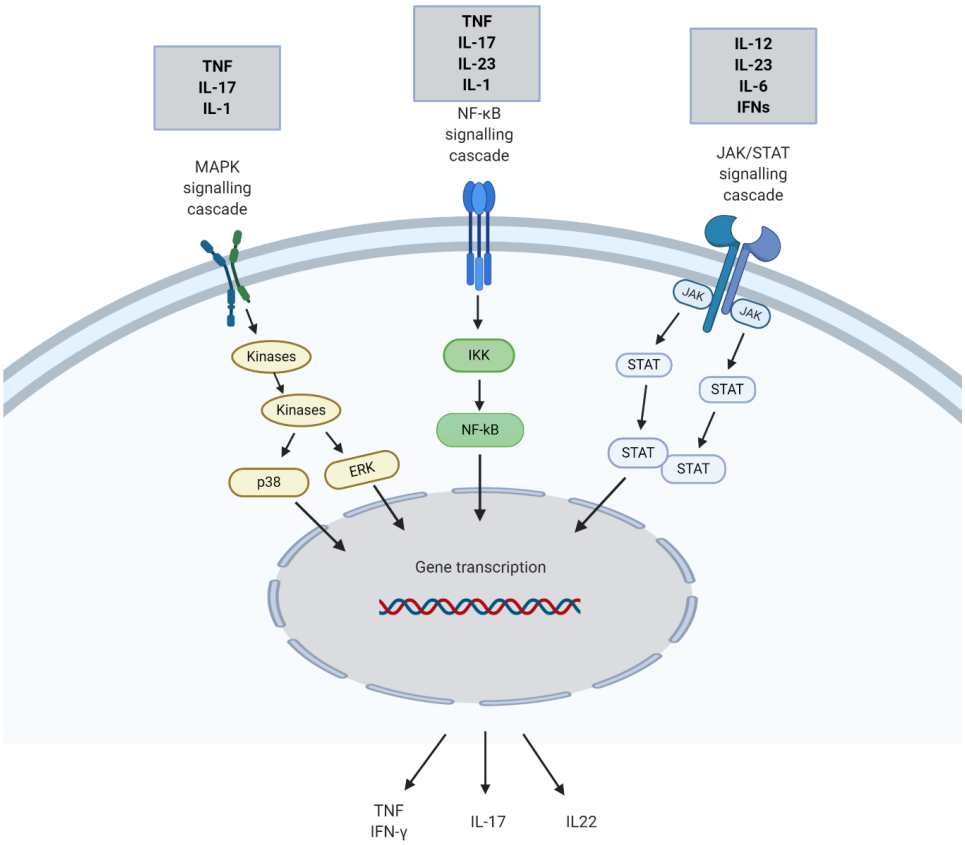

Figure 6: The intracellular signalling pathways involved in the pathogenesis of psoriasis. Cytokines involved in the pathogenesis of psoriasis induce different intracellular signalling transduction pathways, leading to gene transcription and subsequent production of cytokines, such as TNF, IFN-γ, IL-17 and IL-22. The MAPK signalling cascade can be induced by TNF, IL-17 and IL-1, which results in activation of kinases and downstream activation of the MAPKs p38 and ERK that translocate to the nucleus. The NF-κB signalling cascade can be activated by TNF, IL-17, IL-23 and IL1, and leads to nuclear translocation of NF-κB. IL-12, IL-23, IL-6 and IFNs all stimulate the JAK/STAT signalling cascade, leading to activation of JAKs, which in turn activates STATs that translocate to the nucleus. ERK; extracellular signal-regulated kinase, IFN; interferon, IKK; inhibitor of κB kinase IL; interleukin, JAK; Janus kinase, MAPK; mitogen-activated protein kinase, NF-κB; nuclear factor kappa-light-chain-enhancer of activated B cells, STAT; signal transducer and activator of transcription, TNF; tumour necrosis factor. **Created with BioRender.com**

JAK/STAT

The JAK/STAT signalling pathway regulates the cellular responses many cytokines and growth factors. By utilising intracellular tyrosine kinases called JAKs and

transcription factors called STATs, the pathway transduces a signal from the extracellular receptor to the nucleus. Binding of a ligand to the extracellular domain of a JAK-associated membrane-bound receptor results in JAK phosphorylation, which subsequently leads to phosphorylation, dissociation, dimerisation, nuclear translocation and DNA-binding STATs.[244] The 4 different JAKs and 7 different STATs are functionally heterogenous and are activated by different ligands. Furthermore, serine kinases can phosphorylate the serine residue of STAT1 and -3, independently of tyrosine phosphorylation mediated by JAKs. Altogether, the JAK/STAT pathway, depending on extracellular ligands, results in differing signalling specificity.

Cytokines elevated in psoriatic patients are prototypical stimuli of several STATs. For example, IL-12 and IFN-γ are strong activators of STAT4 and STAT1, respectively, but both can also activate STAT3.[245-247] IL-6 and IL-2 are strong activators of STAT3 and STAT5, respectively.[248,249] However, multiple cytokines transduce signals through the different STATs and the downstream cellular effects differ depending on the ligand. For example, both IL6 and IL10 signals through STAT3 but elicits distinct cellular effects, evidenced by their respective pro- and anti-inflammatory activities in myeloid cells.[247,250] Some mechanisms by which the JAK-STAT pathway can be modulated are through the production of suppressor of cytokine signalling (SOCS) proteins, microRNAs, internalisation of the cell-surface IFN receptors and ubiquitin carboxy-terminal hydrolase 18.[251]

In psoriasis, STAT3 has emerged as a key player in the development and pathogenesis, due to hyperactivation in lesional skin and its prominent role in Th17 cell differentiation.[252-254] Following IL-6-stimulation of naïve $CD4^+$ T cells, STAT3 upregulates IL-23R which is essential for IL-23 responsiveness and full effector functions and maintenance of Th17 cells.[255-257] Moreover, phosphorylation of STAT3 is thought to mediate the impaired function of Tregs seen in psoriasis patients.[81] With regards to IL-17A production, STAT3 and -5 are functionally competitive. Both bind to the *IL17a* promoter. However, while STAT3 induces *IL17a* transcription, STAT5 is a negative regulator.[258,259]

Signalling through STAT1 is generally pro-inflammatory, anti-proliferative and pro-apoptotic.[260] STAT1 has also been postulated as a suppressive factor for *IL17a* expression and Th17 cell development, [259,261,262] supported by impaired IL-17-mediated immunity and STAT1 gain-of-function mutation in patients with chronic mucocutaneous candidiasis.[263] Moreover, STAT1 activation has been reported to repress IL-22 gene expression and psoriasis pathogenesis in a imiquimod-induced murine psoriasis model.[264] Also, in lesional psoriatic skin, increased level of phosphorylated STAT1, both serine and tyrosine phosphorylation, has been detected.[265] STAT1 may therefore play a part in regulating the psoriatic inflammation through suppressing IL-17. However, as STAT1 is a key factor in the signalling of IFN-γ, which is abundantly expressed in psoriatic lesions, the role of STAT1 in psoriatic inflammation needs to be investigated further.

Both STAT4 and STAT5 augment survival, proliferation and differentiation.[260] Signalling through STAT4 is activated by a variety of cytokines, including IL-12, IL-23 and type I IFNs. In response to IL-12, STAT4 promotes IFN-γ production and Th1 differentiation.[266-268] In psoriatic skin, T cells have been described to have higher levels of phosphorylated STAT4 than those of non-psoriatic skin, increasing their response to IFN-α and subsequent upregulated IFN-γ production.[266,269] STAT5 signalling plays an essential role in the differentiation of Tregs by inducing the expression of the transcription factor, FoxP3,[259,270,271] and is, thus, a potential negative regulator of psoriatic inflammation.

In psoriasis patients treated with the IL-12/23 inhibitor ustekinumab, the transcriptional activity of *STAT1, -3,* and *-4* was silenced in peripheral blood.[272] Thus, the JAK/STAT pathway plays an important role in the psoriasis pathogenesis, and further knowledge is needed to fully elucidate its role in the delicate interplay.

MAPK

Being among the most prevalent cascades in eukaryotes, the MAPK cascade regulates several fundamental cellular processes including proliferation, differentiation, and

apoptosis.[273-275] Members of the MAPK family are typically activated by serial phosphorylation and activation of upstream kinases. MAPKKK phosphorylates MAPKK which in turn phosphorylates MAPK.[274,275] MAPK, the effector of the pathway, acts on hundreds of substrates including transcription factors and -suppressors, and chromatin remodelling proteins. MAPKs comprise four subfamilies, namely ERKs, JUN N-terminal kinases (JNK), p38 and ERK5.[274,276]

The ERK1/2 pathway can enhance the production of pro-inflammatory cytokines, such as TNF,[277] IL-6[278] and anti-inflammatory cytokines including IL-10.[279] Moreover, inhibition of ERK phosphorylation by chloroquine reduces TNF gene expression in several cell types.[277] ERK signalling is typically triggered by growth factors, such as epidermal growth factor. Interestingly, the epidermal growth factor receptor is up-regulated in active psoriatic lesions, and inhibition of this receptor in cancer patients with psoriasis has improved their psoriatic lesions.[280]

Activation of p38 is associated with production of cytokines including IL-6, IL-1β and TNF[281]. Phosphorylated p38 has been detected in lesional psoriatic epidermis.[282] Activation of p38 is mainly induced by environmental stress and inflammatory stimuli and cytokines, such as IL-1 and TNF.[274] Fumaric acid esters, used in the treatment of psoriasis, effectively inhibit the activity of p38 MAPK, decreasing pro-inflammatory cytokine production.[283]

NF-κB

The NF-κB protein complex regulates genes that control transcription of cytokines important in psoriasis pathogenesis and genes regulating cellular differentiation, survival, and proliferation.[284-286]

The NF-κB network consists of five family member protein monomers, p65 (RelA), RelB, cRel, p50 and p52. These monomers form homodimers or heterodimers that bind DNA differentially[287-289] and are regulated by two pathways – the canonical (classical) or non-canonical (alternative) pathway.[290]

In the canonical, NF-κB essential modulator (NEMO)-dependent pathway, receptor activation leads to activation of kinase complexes consisting of the adaptor protein NEMO and two inhibitors of κB (IκB) kinases (IKKα and IKKβ). This IKK-complex, once activated, phosphorylates IκB, leading to ubiquitination and proteasomal degradation. Following degradation of IκB, associated NF-κB dimers, predominantly RelA/p50, are released and translocated to the nucleus. After IκB release, the NF-κB subunits are subjects to a variety of posttranslational modifications, e.g. phosphorylation[286,291], that fine-tune gene expression control.[290,292] The canonical pathway responds to diverse stimuli, including ligands of various cytokine receptors, TNF receptor (TNFR), TLR and B and T cell receptors. In contrast, the non-canonical, NEMO-independent pathway, responds to a more specific group of stimuli, which includes ligands of a subset of the TNFR superfamily. It relies on the activation of IKKα which phosphorylates p100, resulting in its ubiquitination and proteasomal processing to p52. This process results in the nuclear translocation of RelB/p52 dimer and the following upregulated gene transcription.[293] Functionally, the canonical pathway is involved in almost all aspects of the immune responses, whilst the non-canonical pathway regulates specific processes, such as immune cell maturation and differentiation and lymphoid organogenesis. However, the evident crosstalk between these two pathways[290] has led to the suggestion of viewing them as a single NF-κB signalling system.[294-296]

NF-κB is one of the most important regulators of pro-inflammatory gene expression. Synthesis of cytokines important in psoriasis, such as TNF[297,298], IL1β[299], IL6[300] and IL8[301], is mediated by NF-κB.[285] Suppression of apoptosis has been proposed as a mechanism responsible for epidermal thickness in psoriasis. Altered NF-κB signalling changes the balance of signals from B-cell lymphoma (Bcl)-proteins and cyclins. Survivin, an anti-apoptotic factor, increased in psoriasis also relies on NF-κB.[284,302-306] Furthermore, psoriatic skin displays increased level of phosphorylated NF-κB/RelA[307] and positive epidermal staining of NF-κB.[285] Also, NF-κB-induced microRNA-31 promotes epidermal hyperplasia.[308] Altogether, the literature implies that NF-κB is a key player in the pathogenesis of psoriasis.

1.6 Literature search

The literature search was completed 29th of November 2020.

2. Aims

In this thesis we wanted to combine immunological assay development as well as clinical and biological investigations with the overall aim of gaining insights to psoriasis treatment and deepening understanding of disease pathophysiology.

The specific aims were to:

- Develop robust functional cell-based assays to monitor drug-targeted intracellular signalling pathways in psoriatic peripheral blood immune cells.
- Investigate frequencies of peripheral blood cell subpopulations and their respective activity of drug-targeted intracellular signalling pathways in psoriasis patients with active or biopharmaceutical-treated disease, and healthy controls.
- Compare clinical parameters, and intracellular signalling activity in peripheral blood cells, in psoriasis patients treated with originator infliximab and the biosimilar CT-P13.
- Search for psoriasis-specific peripheral blood immune signatures and biomarkers of treatment response.

3. Materials and methods

3.1 Materials

3.1.1 Study population

The biobank used for these studies was started and organised by Dr Silje M. Solberg. 101 patients diagnosed with psoriasis and found eligible for biological treatment at the Department of Dermatology, Haukeland University Hospital, Bergen, Norway, were included from April 2015 to September 2018. To be included in the study, patients needed to be at least 18 years old, have moderate-severe psoriasis and being prescribed biological drugs. About half the cohort were included prior to initiation of biological treatment. The rest were already treated with a biological drug at inclusion. Blood samples from healthy controls were obtained, spread throughout the year, from the blood bank at Haukeland University Hospital, Bergen, Norway. All healthy controls were non-psoriatic. Further, they were age-, sex- and BMI-matched. The studies presented here, and the biobank were approved by the regional ethics committee (2014/1489 and 2014/1373). All patients and controls signed a written informed consent. External funding sources did not influence planned methods, data analyses of presentation of results.

3.1.2 Sample collection, handling and storage

Blood was collected at the laboratory of the Department of Dermatology, Haukeland University Hospital, to be further processed and stored at Broegelmann Research Laboratory. Blood samples from each patient were collected in lithium-heparin tubes at inclusion, and after approximately 3 and 12 months. Peripheral blood mononuclear cells (PBMCs) were isolated by immediate density gradient centrifugation with Lymphoprep (Axis-Shield Ltd, Dundee, Scotland). The cells were added to a mixture containing 42,5% freezing medium (Profreeze^{TM} CDM), 50% serum-free media (X-VIVO-20^{TM}, Lonza, Basel, Switzerland), and 7,5% dimethyl sulfoxide (DMSO). At a concentration of $5x10^6$/ml, the PBMCs were stored in aliquots of 1 ml in a freezing

chamber (CoolCell®) at -70°C allowing reproducible freezing rate of 1°C/min. The following day the PBMCs were moved for long-time storage in liquid nitrogen.

3.1.3 Collection and storage of patient data

Patients filled out DLQI themselves, whilst the clinical evaluation, including PASI, was performed by a medical doctor. Patients were, most often, evaluated by the same clinician at every visit, to avoid inter-individual variation. Gender, age, and BMI were registered as well as clinical and biochemical data. Collected data was stored, unidentifiable, on the research server from Helse Vest with the opportunity of patient identification by a digital key, as required by the regional ethics committee.

3.2 Methods

3.2.1 Phospho-specific flow cytometry

Flow cytometry is a laser-based technology, which allows rapid, high-content multiparameter analysis of single cells. Cells in fluid suspension are hydrodynamically focused to separate from each other.[309] The fluid stream passes through one or more lasers, with the resulting fluorescent and scattered light detected by a photomultiplier tube (PMT), where it is amplified, and converted to a voltage pulse which is converted to a digital value.[310] Each measurement is performed on a single particle, allowing identification, and grouping of individual particles, for example as cellular subpopulations. Fluorescent agents, most often fluorochrome-conjugated monoclonal antibodies, are used to further identify the characteristics of individual particles. The fluorochrome is excited by a specific wavelength of light provided by a laser and emits light with a lower specific wavelength. The emitted light of specific wavelength is discriminated by use of optical filters before detection of the emitted light, which greatly increases the number of parameters which can be detected. Flow cytometry is well suited for measuring frequencies of cellular subtypes in peripheral blood **(Figure 7)**.[311]

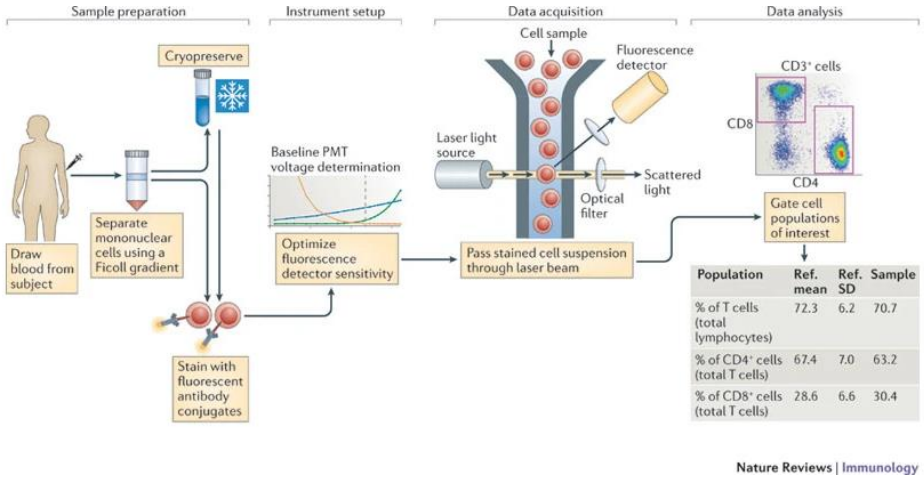

Figure 7: A typical flow cytometry experiment. Sample preparation from blood often involves Ficoll gradient separation of mononuclear cells, and sometimes cryopreservation, before staining with fluorescent antibody conjugates. Each of these steps can introduce variability in the assay results. Instrument setup involves setting voltage gains for the photomultiplier tubes (PMTs) to achieve optimal sensitivity. To the extent that this is not standardised, it becomes a source of variability as well. Data acquisition involves passing the stained cells through a laser beam and recording the fluorescence emission from all the bound antibody conjugates. Here, the main variable is the type of instrument, including the lasers and optical filters used. This is followed by data analysis. Figure and text adapted from Maecker *et al.*, 2012.[310] Reprinted with permission from Springer Nature.

By use of phospho-specific flow cytometry, it is possible to measure the phosphorylation status of proteins critical to intracellular signalling cascades at the single-cell level **(Figure 8)**. It combines identification of cellular subtypes through CD-markers, with the analysis of intracellular signal transduction pathways, thereby identifying the activation of different cell functions, such as transcription of pro-inflammatory cytokines. [312,313]

By phospho-specific flow cytometry we measured frequency and intracellular phosphorylation of PBMC subpopulations from psoriasis patients and healthy controls. Cryopreserved PBMCs from patients and healthy controls were thawed, and incubated for 2 h at 37 °C, 5% CO2. Cells were divided and either stimulated for 15 min with TNF or left unstimulated. Following fixation and permeabilisation with paraformaldehyde and methanol, the cells were stained according to a 4 x 2 barcoding grid using 4 and 2 different concentrations of the succinimidyl ester dyes pacific blue

and pacific orange, respectively. 8 barcoded samples were combined into one tube, allowing simultaneous antibody staining and data acquisition, with the different samples being distinguishable during software analysis based on their fluorescence intensities of the barcoding dyes. After staining cells with antibodies reactive to cell surface- and intracellular antigens, acquisition on a LSRI Fortessa flow cytometer with BDFACSDiVa™ Software (both BD Biosciences) was performed. An internal control, which was a buffy coat from one healthy donor, was used for every experiment.

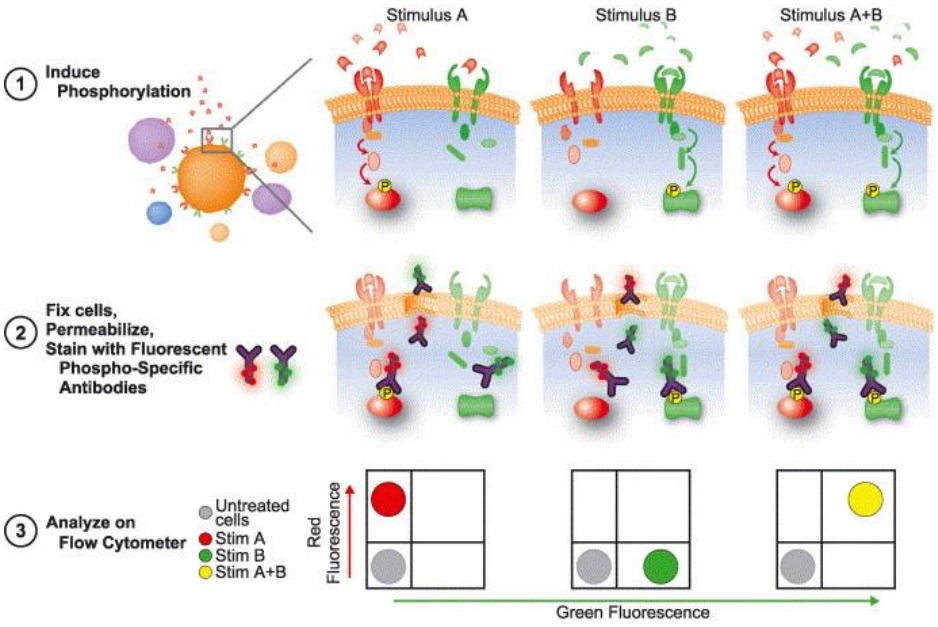

Figure 8. Basic steps in phospho-specific flow cytometry. 1: A heterogeneous sample of cells is treated with different stimuli, to induce distinct signalling cascades and phosphorylation of target proteins. 2: The cells are then fixed, permeabilised, and stained with fluorophore-conjugated phospho-specific antibodies and surface markers to identify cell types. 3: The cells are then analysed on a flow cytometer. Figure and text adapted from Krutzik *et al.*, 2004.[312] Reprinted with permission from Elsevier.

3.2.2 Mass cytometry

Mass cytometry couples flow cytometry with mass spectrometry[314,315] and has expanded the number of detectable targets to > 40 on a single cell level **(Figure 9)**.[316,317]

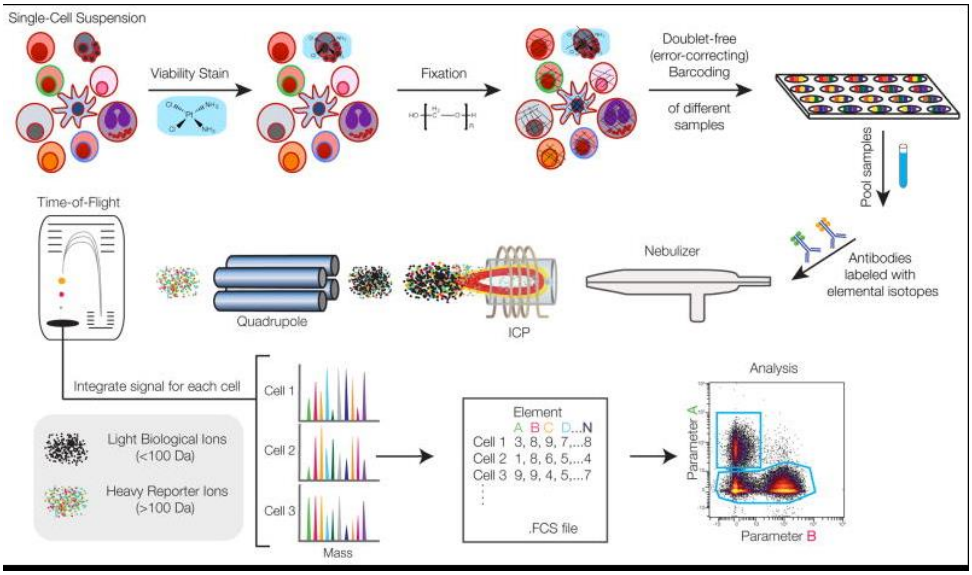

Figure 9. Workflow of a Typical Mass Cytometry Experiment.
Single cells are acquired, and a viability stain is applied to mark dead cells for exclusion from analyses. Fixation can optionally be applied at this point to preserve the cell state. Multiple samples can be barcoded with unique combinations of heavy metal tags, enabling them to be pooled together prior to staining to minimise technical variability at this step. After pooling samples into one tube, cells are then incubated with antibodies targeted against proteins of interest. Cell permeabilisation can be performed if intracellular targets are to be measured. Cells are nebulised into droplets as they are introduced into the mass cytometer. They then travel into an inductively coupled argon plasma (ICP), in which covalent bonds are broken and ions are liberated. The ion cloud is filtered by a quadrupole to remove common biological elements and enrich the heavy metal reporter ions to be quantified by time-of-flight mass spectrometry. Ion signals are integrated on a per-cell basis, resulting in single-cell measurements for downstream analysis. Data are compiled in an FCS file that can then be parsed and plotted in a variety of ways. Figure and text adapted from Spitzer *et al.*, 2016.[317] Reprinted with permission from Elsevier

Mass cytometry uses heavy metal isotopes not commonly found in nature, instead of fluorophores, for labelling probes, thereby typically avoiding overlap between detection channels.[317] Cells in suspension are nebulised, and travel through inductively coupled argon plasma, where covalent bonds are broken, resulting in free

ions.[318,319] The ion cloud is filtered by a quadrupole for removal of common biological elements, which enriches the quantification of heavy metal reporter ions by time-of flight mass spectrometry. The velocity of lighter ions is higher and reach the detector before the heavier ions which have lower velocity.[314,318] The ion counts are converted into electrical signals and into a data matrix where every column represents a distinct isotope and each row represents a single mass scan of the detector. Isotopes with different atomic masses can be identified with minimal signal spillover between channels.[320]

The actual work in the lab is very similar for mass cytometry as for phospho-specific flow cytometry. However, it differs slightly. Firstly, cisplatin was used as an identifier of dead cells, [321] and was added before cells were stimulated and fixed. Secondly, staining of surface antigens, was done before permeabilising the cells, and in study III the chemokine receptors were stained before fixation. Thus, 2-3 steps of staining were needed. Thirdly, iridium, which intercalate DNA, was used as a cell identifier.

3.2.3 Data processing and statistical analysis

Initial visualisation and processing of data was done in FlowJo version 10.2. For flow cytometry experiments compensation was done in FlowJo, by use of compensation beads. Deconvolution of barcoded samples was done by gating on relevant combination of barcoding dyes. Gating on monocytes (MC) and lymphocytes and removal of doublets was based on light scatter properties. Further identification of PBMC subpopulations was based on relative expression of light from fluorochrome-conjugated antibodies bound to CD-molecules. Further analysis of flow cytometry data was performed in cytobank by analysing median fluorescence intensities (MFI).

Mass cytometry data was normalised to beads. Deconvolution of barcodes was done using Fluidigm software and the debarcoding module in the Astrolabe Cytometry Platform[322] in study III. In study III, removal of beads, doublets, dead cells, and debris was done in FlowJo by manual gating, before uploading the clean fcs-files to Cytobank Cellmass v7 for further data analysis.

Historically, single-cell data is examined in two dimensions at a time in scatter plots, but with increasing number of parameters the number of scatter plots becomes overwhelming, and the risk of overseeing multivariate relationships increases. Due to the complexity of mass cytometry data, computational tools for dimensionality reductions and cell clustering have been developed.[324] To project high-dimensional data to lower dimensionality, which is more easily interpreted, dimensionality reduction algorithms are needed. The t-distributed stochastic neighbour embedding (t-SNE) algorithm measures pairwise distances between all cells in the high-dimensional space to determine each cell's location on a two-dimensional plot. The result is a mapping of high-dimensional data onto two dimensions yet conserving the dimensional structure of the data.[325,326] Clustering algorithms segregate similar cells into groups that can be evaluated as one entity. FlowSOM uses self-organising maps (SOM), which is an unsupervised technique for clustering and dimensionality reduction, and can reveal how all the markers behave on the individual cells and identify subsets easily missed by manual gating.[327] In FlowSOM, all cells from all samples are brought together in a matrix. The matrix is used to train a SOM, which results in a grid of nodes corresponding to clusters of cells. Approximate nodes represent more similar populations than distant nodes. The clustering is visualised by a minimum spanning tree. The assigning of a metacluster to each cluster, effectively groups them into populations.[327] In study III, FlowSOM was used to identify the PBMC subpopulations **(Table 1)**.

For study II, single-cell data was clustered using the *FlowSOM* R package[327] and labeled using the Ek'Balam algorithm.[322] Cell subset definitions followed Maecker et al.,[310] and Finak et al. **(Table 2)**,[328] with the addition of mDCs. Cluster labeling, method implementation, and visualisation were done through the Astrolabe Cytometry Platform (Astrolabe Diagnostics, Inc.). Differential abundance analysis was done using the *edgeR* R package[329,330] following the method outlined by Lun et al..[331] Differential expression analysis was done using the *limma* R package[332] following the method outlined by Weber et al..[333] Inverse hyperbolic sine (asinh) calculations all used a cofactor of 5.

Data from study 1 and 3 were transferred to the statistical package for social science (SPSS) Statistics 24 for statistical analysis. Since flow and mass cytometry generally is not normally distributed, non-parametric tests were used for comparison; Mann-Whitney U test for independent, unpaired data, and Wilcoxon signed-rank test for paired data (between inclusion and follow-up). For correlation analyses in study 3, the strength of correlations revealed by Spearman's rank order test were interpreted according to the recommendation from British Medical Journal (https://www.bmj.com/about-bmj/resources-readers/publications/statistics-square-one/11-correlation-and-regression), with rho 0.00-0.19 regarded as very weak, 0.20-0.39 as weak, 0.40-0.59 as moderate, 0.60-0.79 as strong and 0.80-1.00 as very strong correlation.

Fold changes (FC) of MFI and median signal intensities (MSI) for samples relative to corresponding internal control was used in study I and III, to overcome inter-assay variation. FC of follow-up samples relative to corresponding inclusion value (Timepoint 2/Timepoint 1 or Timepoint 3/Timepoint 1) were also used in study I and III, to analyse individual variation over time.

Figures in study I and III were made in GraphPad Prism v8.0 and Cytobank Cellmass v7. Figures in study II were generated in Astrolabe Cytometry Platform (Astrolabe Diagnostics, Inc.).

Table 1. Immune cell subset definitions in study III.

Population	Defining markers
T cells	CD3+CD19-
T helper cells	CD3+CD19-CD4+CD8-
CD8 positive cells	CD3+CD19-CD4-CD8+
B cells	CD19+CD3-
Monocytes -Classical -Non-classical -Intermediate	CD3-CD19-CD56- CD14++CD16- CD14+CD16++ CD14++CD16+
NK	CD3-CD19-CD14-CD16+CD56+CD161+
NKT-like	CD3+CD56+
Tfh	CD3+CD19-CD4+CD8-CXCR3-CXCR5+ PD-1+/-ICOS+/-
Treg	CD3+CD19-CD4+CD8-CD25+CD127-
Th1	CD3+CD19-CD4+CD8-CXCR3+CCR4- CCR6-
Th2	CD3+CD19-CD4+CD8-CXCR3- CCR4+CCR6-
Th9	CD3+CD19-CD4+CD8-CCR4-CCR6+
Th22	CD3+CD19-CD4+CD8- CCR4+CCR6+CCR10+CXCR3+/-
Th17	CD3+CD19-CD4+CD8-CXCR3+/-CCR4+/- CCR6+CCR10-CD161+

Table 2. Immune cell subset definitions in Study II:

Population name	CD molecules
B cells	CD3-CD14- CD19+CD56-
B naive	CD27-
B memory	CD20+CD27+
Plasmablast	CD20-CD27+CD38+
T cells	CD3+CD14-CD19-CD56-
Double negative	CD4-CD8-
CD4+	CD4+CD8a-
Naive	CD27+CD45RA+
Central memory (CM)	CD27+CD45RA-
Effector memory (EM)	CD27-CD45RA-
Terminally differentiated (EMRA)	CD27-CD45RA+
CD8+	CD4-CD8a+
Naive	CD27+CD45RA+
Central memory (CM)	CD27+CD45RA-
Effector memory (EM)	CD27-CD45RA-
Terminally differentiated (EMRA)	CD27-CD45RA+
NK cells	CD3-CD14-CD19-CD56+
Natural killer (CD56+CD16+) (NK)	CD16+
Natural killer (CD56+CD16-) (NK)	CD16-CD56++
NKT-like cells	CD3+CD14-CD19-CD56+
Dendritic cells	CD3-CD14-CD19-CD56-
Conventional dendritic cells (cDC)	CD11c+CD16-CD123- HLA-DR+
Plasmacytoid dendritic cells (pDC)	CD11c-CD123+HLA-DR+
Monocytes	CD3-CD19-
Classical monocytes	CD14+CD16-
Intermediate monocytes	CD14+CD16+
Non-classical monocytes	CD14-CD16+CD56-CD123-

4. Results

4.1 Study I

Phosphorylation of intracellular signalling molecules in peripheral blood cells from patients with psoriasis on originator or biosimilar infliximab

In this study, we included 19 healthy controls and 25 patients with psoriasis vulgaris who had all been diagnosed with severe psoriasis at an earlier timepoint but were now in or close to remission (PASI 0–4) with frequent infusions of originator IFX. In total, 22 patients were randomised, either to continue originator IFX or to switch to the biosimilar CT-P13 as part of another study,[154] and therefore these samples were analysed double blinded. The final three patients continued treatment with originator IFX. PBMCs from healthy controls and patients at three timepoints (0, 3 and 12 months), were analysed by use of phospho-specific flow cytometry. The MFI of fluorochromes conjugated to antibodies against phosphorylated NF-κB, ERK1/2, p38 and STAT3 was used as a measurement of phosphorylation level and signalling activity.

The basal phosphorylation of NF-κB, ERK1/2, p38 and STAT3 was significantly higher in patients at inclusion compared to healthy controls in almost all cell populations analysed, despite having achieved clinical remission by treatment with IFX. At 12 months, patients still displayed significantly higher basal phosphorylation levels than healthy controls, but the number of epitopes with significant differences was decreased.

The FC (TNF-stimulated MFI divided by corresponding unstimulated cells) which is the phosphorylation level after stimulation with TNF relative to the basal phosphorylation level, was significantly lower for pSTAT3 in MCs, T-, B- and NK cells, pERK in T cells and pNF-κB in NK cells at inclusion compared to healthy controls. After 12 months, only FC of pNF-κB in MCs and NK cells in addition to pSTAT3 in MCs were decreased in patients compared to healthy controls.

No differences in basal phosphorylation of NF-κB, ERK1/2, p38 or STAT3 were detected between patients treated with originator IFX and the biosimilar CT-P13. FC of pSTAT3 in B cells was increased in patients on CT-P13 compared to patients on IFX after 3 months, but this difference was not present after 12 months.

4.2 Study II

Mass cytometric analyses of peripheral blood mononuclear cells from psoriasis patients on anti-TNF treatment

PBMCs from 10 patients and 6 controls, included in Study I, were used for mass cytometric analyses of cell frequencies and differential expression of molecules associated with cell activation. In the patient cohort, 5 of the patients continued originator IFX, the other 5 switched to CT-P13 after the first blood sample was obtained.

No obvious differences in cell frequencies were observed between patients and controls, nor between the two treatment cohorts.

Differential expression analyses of asinh-transformed MSI displayed a significantly higher expression of CD38 on NK cells from patients compared to healthy controls. This was seen in both $CD16^+$ and $CD16^-$ NK cells, but the difference between patients and healthy controls was statistically significant only in the parent population. A trend with higher expression of CD38 on T cells from patients was also observed, particularly on $CD4^+$ effector memory T (T_{EM}) cells , but also on $CD4^+$ central memory T (T_{CM}) and terminally differentiated effector memory T (T_{EMRA}) cells this was seen. A higher expression of CD38 on mDCs was also seen in patients compared to healthy controls. The expression HLA-DR was slightly increased in $CD16^+$ NK cells and decreased in mDCs and pDCs from patients compared to healthy controls.

Patients at inclusion displayed a higher basal level of pSTAT3 in $CD16^-$ NK cells, $CD4^+$ T_{EM} and $CD4^+$ T_{EMRA} than healthy controls. Basal phosphorylation level of NF-kB was higher in MCs and DCs from patients than those from healthy controls, particularly in $CD14^-CD16^+$ MCs and mDCs. In patients, higher levels of pERK in NK cells, both $CD16^-$ and $CD16^+$, than in healthy controls, were observed. $CD4^+$ T_{EM}, $CD14^-CD16^+$ MCs and pDCs from patients had higher basal levels of pp38 than those from healthy controls. However, none of these results were statistically significant after adjusting for multiple comparisons.

No obvious differences were observed between the patients continuing IFX compared those switching to CT-P13 with regards to basal levels of CD38, HLA-DR, pERK, pNF-κB, pp38 and pSTAT3. However, the CT-P13-treated patients had a decreasing trend of most activation markers, which was not obvious in the IFX-treated patients.

Upon stimulation with TNF patients displayed lower FC (TNF-stimulated asinh-transformed MSI value minus basal asinh-transformed MSI value) of pNF-κB and pp38. The most obvious differences were for pNF-κB in $CD16^+$ NK cells, $CD8^+$ T_{EMRA} cells and $CD14^+CD16^-$ MCs, and pp38 in $CD8^+$ T_{EMRA} and $CD16^+$ NK cells.

4.3 Study III

Mass cytometry analysis of blood immune cells from psoriasis patients on biological therapy

PBMCs from 32 patients with severe psoriasis were analysed using mass cytometry before and 4 and 12 months after initiation of biological therapy using IFX, etanercept, ustekinumab or secukinumab.

After receiving biological therapy, a shift in the Th1/Th2 balance was detected in psoriasis patients. At inclusion patients had a higher number of Th1 cells and lower number of Th2 cells compared to healthy controls and follow-up. Patients had higher fractions of Th22 and Th9 cells at inclusion compared to 1-year follow-up. Patients

tended to have reduced frequency of Tregs at inclusion and 4 months, but after 1 year the level had normalised. A shift from naïve/effector ($CD45RA^+$) to memory ($CD45RO^+$) predominance in the $CD4^+$ population, including Tregs, was seen during biological treatment. Further, the frequency of and $CD8^+$ memory T cells ($CCR4^+$) was higher in patients at inclusion compared to healthy controls.

Programmed cell death protein 1 (PD-1) expression was higher on Th2 cells and lower on $CD8^+$ T cells from patients with active disease than those from healthy controls. After 1 year, the expression of PD-1 on $CD4^+$ T cells, including Tregs, from patients had an increased.

At inclusion, patients displayed increased levels of phosphorylated p38 in Th2 cells and Tregs, ERK in $CD4^+$ T cells and STAT1 in classical MC compared to healthy controls. Increased phosphorylation was still evident at both follow-up time points in Th2 cells and after 1 year and for ERK in $CD4^+$ T cells. The level of phosphorylated STAT1 and p38 decreased in classical MC during the first 4 months of treatment and the level of pSTAT1 in Th17 cells was reduced after 1 year.

Responders had a negative correlation between PASI improvement and FC of Th17 cell frequency after 4 months (Timepoint 2/Timepoint 1), and FC of $CD8^+$ T cells after 1 year (Timepoint 3/Timepoint 1). Non-responders had a decrease of NK and NKT-like cells after 4 months relative to inclusion. Moreover, CD45RA/RO ratio in Tregs at inclusion was lower in responders compared to non-responders. Responders had higher FC of PD-1 on $CD4^+$ T cells after 4 months and on NK cells after 1 year. The expression of the epithelial-homing receptor CCR10 on B cells was higher in patients than healthy controls at inclusion. Responders had higher FC of epithelial-homing CCR10 on Th cells, $CD8^+$ T cells and B cells after 4 months and on NK and Th17 cells at 1 year follow-up compared to non-responders. Non-responders had higher pSTAT1 in Th17 cells, pp38 in classical MC, and pNF-κB and pp38 in intermediate MC, at inclusion, than responders. Responders had higher FC of pp38, pSTAT1 and pNF-κB in non-classical MC and lower FC of pSTAT3 in intermediate MC compared to non-responders after 1 year.

5. Discussion

5.1 Methodological considerations

5.1.1 Common considerations for single cell analyses

Patients and controls: Study I included 25 patients and 19 healthy controls, study II included 10 patients and 6 healthy controls and study III included 32 patients and 10 healthy controls. In all three studies, cryopreserved PBMCs from a single donor buffy coat were included in each experimental run as an internal control to check and adjust for inter-assay variation. In all three studies, a large number of variables from relatively small patient cohorts were analysed with the risk of false positive results due to multiple comparisons.[334,335] no corrections for multiple comparisons were made in study I and III. No power calculations were done for any of the three studies. Nevertheless, these studies aimed to assess biologically relevant differences in PBMCs and should therefore be considered despite low power. PBMC isolation: To minimise basal phosphorylation, a colleague previously tested how different anticoagulants in test tubes affected basal phosphorylation. Lithium-heparin tubes followed by density gradient separation of PBMCs showed significantly lower basal phosphorylation of ERK, p38, STAT1, STAT3 and STAT5 in multiple cell types than those isolated through CPT sodium citrate and sodium heparin tubes.[336]

The effect of freezing and thawing cells: To enable simultaneous analysis of many samples, PBMCs were cryopreserved. To optimise viability and recovery of cryopreserved cells, serum support media, such as foetal bovine serum (FBS), are commonly used as freezing media. To avoid unspecific stimulation caused by FBS, our freezing mixture contained a non-animal origin chemically defined freezing medium, ProFreeze^{TM}-CDM, a serum-free media, X-VIVO-20^{TM} and cryoprotectant DMSO. To ensure a constant freezing rate of 1°C/minute, CoolCell® freezing chambers were used for freezing of samples.[337] The combination of cooling cells at a controlled rate and the use of DMSO minimises damage to the cells by increasing permeability of the plasma membrane and preventing the formation of ice crystals.[337,338] To avoid ice re-crystallisation and osmotic stress and to maximise cell

recovery and viability, the cell were thawed rapidly at 37°C.[337] After thawing, a resting period of 2 hours was included, to allow cells to recover.[336,339,340]

Antibody selection and titration: When designing the panel, the surface antigens were selected based their ability to differentiate PBMC subpopulations. The choice of phospho-epitopes were in study I and II based on relevant signalling pathways through TNF-stimulation. In study III, more phospho-epitopes were included to account for T cell differentiation and the general cytokine milieu. All antibodies were titrated for their specific experimental conditions to find their optimal staining concentrations. For all surface molecules, the lowest concentrations that resulted in clear separations between negative and positive populations were chosen. For phospho-specific antibodies the optimal dilutions were defined as the lowest concentrations resulting in clear separations between unstimulated and stimulated populations. Correct titrations of antibodies and suitable choices of metal isotopes or fluorochromes for antibody conjugations are crucial for acquiring precise single cell data by flow and mass cytometry, particularly when measuring changes in intracellular signalling transduction. Unwanted interactions may occur, despite extensive efforts to reduce experimental artefacts. In addition, purity of isotopes/fluorochromes, density of the measured molecule, and the sensitivity of different channels can influence the signal detection. A general rule is, therefore, to assign low-frequency molecules to sensitive channels and vice versa, and to avoid spillover from abundant molecules or bright channels to neighbouring channels.[341]

Stimulation: The stimulation of cells is also a crucial step. Both concentration and timing need to be perfected. The cells were rested for 120 minutes at 37°C, 5% CO_2, to reduce the basal signalling levels following the process of thawing. TNF was diluted in X-VIVO-20™. The cells were stimulated for 15 minutes with TNF at a concentration of 50 ng/ml. Cells that were not stimulated were added the same volume of X-VIVO-20™ to ensure equal cell concentration but also to expose them to the same drop in temperature as the stimulated cells, by leaving the incubator during the stimulation step.

Fixation and permeabilisation: The recognition of signalling activation is through detection of phosphorylation of proteins. Detection of phosphorylated proteins brings challenges different to detection of surface proteins alone. These proteins are localised intracellularly and are therefore inaccessible to the fluorochrome- and metal-conjugated antibodies. Moreover, after stimulation, phosphorylation is a transitional event, i.e. the phosphorylated protein is not stable.[312] To overcome these problems, the cells are fixed and permeabilised to stabilise the phosphorylated proteins for detection and to allow entry of phospho-specific antibodies.[312] Choosing fixation and permeabilisation agents are based on optimising the detection of phosphorylation while preserving the stability of molecules. Consequently, all antibodies used for antigen detection were validated on cells following fixation and permeabilisation.

Barcoding: Barcodes label individual samples with a unique signature of dyes/metals and enables multiplexing of the samples prior to staining and acquisition on a flow or mass cytometer. Fluorescent cell barcoding was used in study I and mass-tag cell barcoding was used in study II and III. Following barcoding, the samples can be pooled together into one tube, allowing simultaneous staining and data acquisition.[342,343] This reduces antibody consumption, increases throughput, shortens acquisition time and reduces staining variability. For fluorescent cell barcoding, used in flow cytometry, pacific orange and pacific blue were utilised. Pacific orange and pacific blue are fluorescent dyes that react to amine groups on protein lysine residues and at the N-terminus.[342] Barcodes used for mass cytometry were palladium (Pd)-based. Using different combinations of the six isotopes of Pd, were each sample is either positive or negative for each of the six epitopes, twenty different combinations are possible. Thus, a unique combination is generated for each sample which is used to identify the sample when deconvoluting the barcodes **(Figure 10)**.[343]

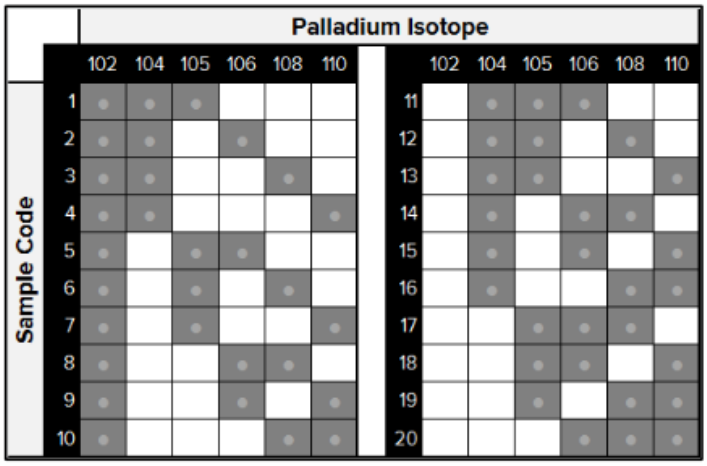

Figure 10. Palladium (Pd) barcoding. Each kit has 20 unique barcodes generated from 6 Pd isotopes, where combinations of 3 isotopes are used to identify each sample. This is called a "6-choose-3" strategy. Figure downloaded from https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/cell-id-20-plex-pd-barcoding-kit-ug-prd023/cell-id-20-plex-pd-barcoding-kit-ug-prd023/fluidigm%3Afile.

5.1.2 Flow versus mass cytometry

Developments in the field of conventional flow cytometry are constantly increasing the dimensionality of acquired data, both through novel hardware and novel probes with reduced promiscuity.[344] However, conventional flow cytometry panels exceeding 18 channels are reserved for the few, due to spectral overlap.[345] Further, a flow cytometry panel with many channels will need compensation to deal with the spectral overlap which can interfere with the accuracy of the results. With mass cytometry, this is barely an issue, and panels exceeding 40 markers is possible.[317,320] However, with mass cytometry many of the events will be lost before it reaches the detector, making it a less sensitive method than flow cytometry. Moreover, as the biological material is lost during a mass cytometric analysis, sorting of cells after analysis is not possible. Also, mass cytometry is more expensive. Another difference between flow and mass cytometry is the range of signal intensities, where the metal isotopes used in mass cytometry all have similar signal intensities, and the fluorophores used in flow cytometry have a larger range of signal intensities. A large range of signal intensities can complicate panel design but can also be an advantage if

detection of a lowly expressed molecule, is needed, e.g. a phospho-epitope. Altogether, mass cytometry is a better tool for explorative studies, whilst flow cytometry should perhaps be the preferred method if you know what you are looking for. Finally, flow cytometric assays are already established in clinical practice. Thus, flow cytometric research results are yet more easily translated to clinical practice.

5.1.3 Phospho-specific flow cytometry: Special considerations

The laborious process of designing a panel for phospho-specific flow cytometry had already been accomplished by a colleague in our group to study PBMCs from patients with Sjögren's syndrome.[336] With small modifications, his method was implemented to our patient samples. Precautions had to be taken regarding signal spillover when designing the antibody panel. The choice of fluorophores was therefore a compromise between the wavelengths of the available lasers, the filters on the flow cytometer, availability of the chosen fluorochrome-conjugated antibody. The phospho-epitopes where expected to display very subtle differences, making it very vulnerable to compensation. Therefore, minimising spillover into important and sensitive channels, such as the phospho-antigens, was key to reduce compensation. However, due to availability some concession had to be made. For example, the anti-STAT3 (Y705) was conjugated to PerCP-Cy5.5, which is a relatively dim fluorophore, and thus, not best for use on a sparsely expressed molecule.

Single fluorescent-stained compensation beads were used for the measurement and compensation of fluorescent spillover. Further, as fluorescent cell barcoding included two very bright fluorophores (pacific blue and pacific orange), a residual vial holding barcoded cells, without fluorescent antibodies, was analysed to control fluorescent spillover from the barcoding. Moreover, BD cytometer setup and tracking beads were used for determining minimum baseline PMT voltages and monitoring cytometer setup and performance (laser alignment, laser time delay, and sensitivity).[346]

5.1.4 Mass cytometry: Special considerations

Panel design: The MaxPar panel designer and panel wheel were used to check for signal spillover and tolerance for each channel. Although no major spillover was

detected, spillover from channel +/-1 and +16 which corresponds to oxidated metals should be considered. The purity of isotopes, to which degree a molecule is present and different sensitivity of channels can influence signal detection.[341] Barium contamination has for long been an issue, as it may interfere with analytical readout. Barium occurs naturally as a mix of several stable isotopes, though mainly 138 Ba and 137 Ba.[347] Many sources of barium contamination have been identified, including purified bovine serum albumin (BSA), commercial salts used to prepare phosphate-buffered saline (PBS) and dish soaps used to clean buffer containers. Moreover, other researchers using the Core Facility for Flow Cytometry have seen that latex powder free gloves also are a significant source of barium. To overcome this issue great precautions were taken on choice of material and washing procedures prior to sample acquisition.

To identify dead cells when analysing the data, cisplatin had to be added prior to fixation and permeabilisation. Thus, in study II, cisplatin was added to the cells before stimulation with TNF. This could affect basal phosphorylation levels and how the cells responded to TNF-stimulation. This was, however, a necessary compromise.

In study II and III, slightly different definitions for non-classical MCs were applied. In study II non-classical MCs were defined as $CD14^-$, whilst in study III they were defined as $CD14^{low}$. However, they stand for the same population, despite differing terminology.

In study III, the antibodies for CXCR3, CCR4, CCR6 and CD127 were added to live cells before fixation, barcoding and permeabilisation, because they were sensitive to fixation.[348] Moreover, for study III some markers in the panel were prone to some signal overlap. A lot of effort was put in to optimise this panel, but due to availability of antibodies, some compromises had to be made. CCR4 (175Lu) and pSTAT3 (158Gd) had medium tolerance, were in the medium sensitivity area of the machine and had medium overlap. CD45RA (143Nd), CCR10 (164Dy), PD-1 (155Gd), pERK (167Er) and pNF-κB (166Er) all have low tolerance for spillover. However, except

for CD45RA, these markers were assigned to relatively sensitive channel. Further, we did not experience any obvious problems with CD45RA.

As mass cytometry overcomes the limitations of spectral overlap associated with flow cytometry, compensation controls are not required. Polystyrene bead standards, called EQ™ four element calibration beads (containing 140/142Ce, 151/153Eu, 165Ho and 175/176Lu) were used to monitor changes in instrument performance and signal fluctuations during acquisition of data and across assays, to minimise inter- and intra-sample variations.

5.1.5 Data analysis

Multivariate data can be difficult to visualise and interpret, merely due to the number of data points and dimensions. With an increasing number of variables, it is increasingly clear that you are overpowered by the sheer load of information. In the flow cytometric analysis, the number of variables were limited, and the PBMCs were therefore, by manual gating, divided into lymphocytes and MCs based on their size and granularity in forward vs. sideway scatter plots, before being divided into B cells, T cells, NK cells and NKT-like cells based on their expression of CD19, CD3, CD56 and CD56/CD3, respectively. For the mass cytometry data in study II and III, however, manual gating on biaxial plots was not feasible and would involve high risk of error. Analysis of high-dimensional cytometric data bring computational challenges in terms of data pre-processing, normalisation, dimensionality reduction and clustering.[349] Many of the algorithms used for high-dimensional data circumvent the traditional approaches for flow cytometric data, fundamentally altering the processing and interpretation. The large number of available algorithms, such as viSNE, SPADE, X-shift, PhenoGraph, FlowSOM and Citrus, and the missing consensus on best practices for data pre-processing and analysis are issues that need to be addressed.[350,351] Pipelines using different tools are frequently developed, making it increasingly challenging to comprehend the considerations for each approach and understand what kind of biological insight you can obtain from it. In study III FlowSOM was the chosen algorithm for defining the PBMC subpopulations used for downstream functional analysis, largely due to the availability in cytobank and the

ability to combine dimensionality reduction and clustering in an unsupervised manner, but still being able to decide the number of clusters. In study II, a cloud-based data analysis pipeline[322] was used, which both clustered and defined the PBMC subpopulations but also directly did the group comparisons we sought.

5.2 Biological and clinical implications of the results

There is convincing support in the literature for systemic inflammation and increased risk of comorbidities like CVD in psoriasis patients.[113,352] Biological therapy has been proven as very effective treatment of immune-mediated diseases like psoriasis, and the clinical efficacy of the four biological drugs used in our study, as measured by PASI and DLQI, was excellent. The following section will address biological interpretations of the single cell analyses and clinical implications of the findings.

5.2.1 Abberant frequencies of immune cell subsets in psoriasis patients improves with biological treatment

In study III, initiation of biological therapy led to a decreased fraction of Th1 cells, along with a significant increase in the Th2 fraction, i.e. a shift in the Th1-Th2 cell balance, which is in concordance with the findings of others.[353] Increased fractions of Th1 cells has previously been detected in the blood of psoriasis patients with active disease[354] and are thought to be recruited to the skin.[124] Moreover, in psoriasis patients $CD4^+$ T cells producing IFN-γ, the key cytokine of Th1 cells, have been shown to be increased.[355] Contrarily, others have found circulating Th1 cells to be reduced and negatively correlated with PASI in psoriasis patients.[356,357] Indeed, the hypothesised recruitment of Th1 cells from the circulation to the psoriatic lesion,[124] could result in a time-dependently reduced fraction of Th1 cells in the circulation, undetectable in our cohort. Also, in these studies, slightly different cell definitions were applied, and the cohorts differed both in size and disease activity.

Th9 and Th22 displayed higher frequencies at inclusion compared to 1-year follow-up, but not compared to healthy controls. Both Th22 and Th9 have been associated with active psoriasis, and are thought to actively participate in cutaneous inflammation.[62] Aberrant frequencies of circulating Th22 cells but not Th9 cells have

previously been described in psoriasis patients.[355] Moreover, Th22 cells has, along with Tc17 cells, been proposed as part of the site-specific disease memory in recurrent psoriasis,[88,91] and could therefore be an interesting target to prevent chronic disease.

Surprisingly, no changes were seen in the Th17 cell frequency, which previously has been described to be increased in patients compared to healthy controls.[100] This study, however, defined Th17 cells based on the expression of IL-17A, while we defined the Th17 cells based on the expression of the chemokine receptor CCR6. Moreover, only patients with milder disease, based on PASI, not treated systemically were included. In our cohort, all patients had severe disease and 12 out of 34 patients were treated with MTX. Altogether, this can very well explain differing results.

Patients with active psoriasis had reduced frequencies of Tregs. This was, however, normalised with biological treatment. This pattern has also been detected by others.[353] Tregs are important suppressors of inflammation.[358,359] In psoriatic skin, Tregs are outnumbered by effector T cells and display a deficient suppressor activity. This combination leads to insufficient peripheral tolerance and hyperproliferation of autoreactive T cells.[84] Interestingly, Tregs have been described as dysfunctional and low-frequent in peripheral blood from patients with acute coronary syndrome.[360]

The elevated levels of skin-homing B cells in untreated patients compared to healthy controls is highly relevant in light of the recent discovery of antibodies against the autoantigen LL37.[44,361] These cells could, possibly, be involved in an autoimmune response against skin autoantigens, and thus, represent a new treatment target.

Although others have found $CD8^+$ T cells in abundance in psoriasis patients compared to healthy controls, our comparison of psoriasis patients and healthy controls did not reach statistical significance.[362,363] However, we did find that reduction of circulating $CD8^+$ T cells correlated with PASI-reduction. Of note, the Tc17 phenotype ($CD8^+CCR6^+CD161^+$) is thought to be involved in the autoantigen detection and IL-17 production in the early stage of the disease, and thus, is forwarded as a potential treatment target.[364-366] In our study, a circulating Tc17

population was detected, but no statistically significant differences were observed between groups. $CD8^+CCR4^+$ memory T cells were significantly increased in untreated patients compared to healthy controls. Others have found that $CD8^+$ T_{EM} predominantly express CCR4.[362] Interestingly, it has been postulated that a fraction of resident $CD8^+$ memory cells is released from chronically inflamed tissue to the circulation, contributing to the systemic inflammation associated with psoriasis.[367]

After treatment initiation, $CD4^+$ T cells including Tregs displayed an expression of CD45RO and CD45RA which increased and decreased, respectively. This demonstrates a shift towards an increased fraction of memory cells compared to naïve/effector cells, and is also in accordance with the findings of others.[356]

5.2.2 Originator infliximab versus biosimilar CT-P13

Clinically, switching from originator IFX to CT-P13 has been reported as non-inferior to continued treatment with originator IFX for treating inflammatory bowel disease, spondylarthritis, rheumatoid arthritis, psoriatic arthritis and chronic plaque psoriasis. However, the study was not powered to show non-inferiority in the individual diseases.[154] Comparing the two patient groups receiving originator IFX or CT-P13 by flow and mass cytometry, there were no significant differences in basal phosphorylation after 3 and 12 months. In study I, FC of pSTAT3 (Y705) in B cells was significantly increased after 3 months in patients who switched to CT-P13 compared to those continuing treatment with originator IFX. As basal phosphorylation was similar between the two groups, the difference is most likely due to an increased activation upon TNF stimulation. However, this difference was not evident after 12 months. Of note, patients in the CT-P13 group had a tendency of a reduction in the activation molecules when comparing the 12 months sample to inclusion, which was not present in the originator IFX group to the same degree. This might reflect differences in structure and efficacy of the two drugs. This was not a phenomenon exclusively present in NK cells, which could have been predicted due to difference in afucosylated glycans and affinity for FcgRIIIa and FcgRIIIb. Altogether, our results strengthen the hypothesis of CT-P13 being a non-inferior treatment alternative to originator IFX.

5.2.3 Increased expression of molecules associated with cell activation in immune cells from psoriasis patients

T cell activation requires interactions between the T cell receptor on the T cell and HLA, which have strong genetic association to psoriasis, on the APC.[14] Simultaneously, co-stimulatory signals are required for T cell activation. Conversely, co-inhibitory signals can stop the activation. PD-1 is one of the molecules which silences the power of this mechanism. Furthermore, inhibition of PD-1 or its ligand, PD-L1, releases the "break" on the immune system, and leads to a stronger immune response. This principle is exploited in cancer immune therapy.[368] In our study, patients with active psoriasis displayed a decreased expression of PD-1 on $CD8^+$ T cells and an upregulation of PD-1 on $CD4^+$ T cells including Tregs during the follow-up period after treatment initiation. Moreover, responders had a significantly higher FC of PD-1 in $CD4^+$ T cells and NK cells than non-responders, which means they had a higher increase of PD-1 in the follow-up period after treatment initiation. These findings implicate an impaired interaction between PD-1 on T cells and its ligand on the APC, which in turn leads to an up-regulated immune response, a pattern previously described in psoriasis patients.[369] Blockade of PD-1 has been demonstrated to augment Th1 and Th17 responses and suppress the Th2 response.[370] To find out whether this pattern of differing PD-1 expression is a specific disease mechanism or a mere consequence of chronic inflammation, more research is needed.

Patients with severe psoriasis had elevated levels of intracellular phosphorylation in Th2 cells and Tregs, in addition to classical MCs. Increased phosphorylation in $CD4^+$ T cells from psoriasis patients has also been found by others,[362] and earlier studies of inflammatory disorders have found that activation levels of PBMCs decrease with successful treatment.[353,371]

In study I and II, our findings of elevation of phosphorylation levels in distinct immune cell subsets in IFX-treated patients support that there is an ongoing systemic inflammation which persists after resolution of skin manifestations, without correlation to trough level or length of IFX treatment prior to inclusion. Possibly, ceasing systemic inflammation is a slower process than ceasing skin inflammation.

Another possibility is that signalling of the measured pathways is maintained by other mediators than TNF,[371,372] as neither of the pathways are TNF-specific. Also, mutations in genes encoding NF-κB and MAPK or inhibitors of NF-κB could explain increased activity of the signalling pathway, despite years of treatment.[34] Hence, therapy targeting intracellular signalling cascades could be of interest. However, these signalling cascades are involved in general cell processes, and serious side effects of such therapies are, thus, likely.

The reduction, but not complete normalisation of the intracellular signalling cascades underlines the chronicity of the disease. An important question is therefore if long-term systemic treatment is advisable, not only to treat the skin lesions, but also to reduce the risk of comorbidities.

In study II, higher expression of CD38 and HLA-DR in PBMC subsets from patients than healthy controls, were found. In general, CD38 and HLA-DR are more highly expressed on activated immune cells. HLA-DR is a major histocompatibility complex, constitutively expressed on DCs, MCs, macrophages and B cells, which typically appears at the late stages of NK and T cell activation[373-375]. Moreover, the less mature $CD56^{bright}$ population of NK cells with high functional activity also co-expresses HLA-DR.[376] CD38 is a multifunctional ecto-enzyme, predominantly expressed on immune cells. It metabolises nicotinamide nucleotides to adenosine diphosphate ribose (ADPR) and cyclic ADPR (cADPR), which are potent intracellular Ca^{2+}-mobilising compounds.[377] Indeed, CD38 is present on activated immune cells. This is possibly due to its function of promoting metabolic collapse in pathogens by degrading nicotinamimide adenine dinucleotide (NAD^+) and its precursors.[378] Further, CD38 binds to CD31, and thus, confers lymphocytes with the ability to adhere to endothelial cells, a necessary step in extravasation. The increased expression of CD38 particularly on NK and $CD4^+$ T_{EM} cells, most likely represents an activated state of these cells, despite being in clinical remission.

5.2.3 Stratification of responders and non-responders

The findings in study I and II implicate an ongoing systemic inflammation despite amelioration of skin lesions. Due to low PASI, absence of ADAs, normal drug trough level, and normal blood values, a correlation between functional markers by flow and mass cytometry and routine clinical and biochemical markers was not possible to detect. In study III, some predictive potential for disease severity and treatment effect was detected.

Levels of circulating NK and NKT cells have been reported to be lower in psoriasis patients than in healthy controls,[379-381] although contradictory findings exist.[382,383] During follow-up of the patients in study III, responders had a higher increase in the frequency of NK and NKT-like cells. The increase in NKT-like cells during follow-up also correlated with PASI improvement. Interestingly, responders had a significantly higher fraction of memory Tregs than non-responders during active disease. Thus, the fraction of memory Tregs serves as a possible biomarker for prospective stratification of treatment response.

At inclusion, non-responders had higher pSTAT1 in Th17 cells, pp38 in classical MC, and pp38 and pNF-κB in intermediate MC than responders. This indicates that early stratification based on cell-based immune profiling may be used to predict treatment response, and perhaps also to steer the choice of biological agent. However, our data cannot be used to identify cut-off values of different markers, which is necessary for translation to clinical practice. For this, larger studies are needed.

Surprisingly, responders had a higher expression of the epithelial-homing marker CCR10 on circulating $CD4^+$ T cells including Th2 and Th17 cells and on $CD8^+$ T cells, B cells and NK cells, compared to non-responders. This could, however, represent sustained production of skin-homing PBMCs despite reduced expression of ligands in the skin, or it could represent efflux from healed skin.[356,367]

5.2.4 Relation to comorbidities

Being a systemic immune-mediated disease with associated comorbidities, there has been an increasing interest regarding the risk-lowering impact of biological treatment on comorbidities in psoriasis patients.[130,367]

Inflammation is important in CVD, highlighted by the protective effects of aspirin, colchicine and canakinumab in CVD patients.[113,384-386] Large observational studies have reported reduced incidences of cardiovascular diseases in patients treated with MTX[387] and TNF inhibitors.[388,389] Although others have failed to document such protective effects, the control groups in these studies have either received phototherapy[390] or other systemic agents.[391] Further, in a study comparing the rate of cardiovascular events in patients treated with TNF inhibitors (etanercept, IFX or adalimumab) and MTX, only 1,5% of patients in the TNF inhibitor cohort experienced a cardiovascular event within 12 months compared to 4,1% in the MTX group,[392] suggesting that the risk of cardiovascular events reduces over time with continued TNF neutralisation.

Complementary to these results, several small, controlled trials have evaluated changes in level of biomarkers for CVD during systemic psoriasis treatment. Indeed, several groups reported amelioration of such markers, including cytokines, adipokines and endothelial dysfunction.[113,393-396] Moreover, studies using FDG PET and FDG PET/CT have found decreased inflammation in the ascending aorta and carotid arteries in psoriasis patients treated with biologicals,[397] but also here contradicting reports exist, and the only randomised, double-blinded study found no difference between patients treated with a TNF inhibitor and placebo.[398,399] Also, carotid intima/media thickness has been reported to decline with systemic treatment of moderate-to-severe psoriasis.[400]

Although the literature is still heterogenous, in part due to methodological differences, treatment with TNF inhibitors most likely exert some CV-protective effect.

In study III, patients with active disease had elevated frequencies of intermediate MCs compared to healthy controls, which decreased after treatment onset. Moreover, non-responders had higher levels of pNF-κB and pp38 in intermediate MCs than responders. An elevated level of intermediate MCs is associated with CVD,[401,402] and could, thus, provide a immunological mechanism of CVD-risk in psoriasis patients. Also, the increased activation level of signal transduction pathways in non-responders compared to responders may also indicate a larger risk of CVD for non-responders.[403] The frequency of classical MCs, which also are associated with atherosclerosis, was lower in patients with active disease at inclusion than in healthy controls, but normalised after treatment onset. A possible explanation is that the MCs are recruited to the sites of inflammation, including the skin and the vessel wall, in patients with active psoriasis.[404] Contrarily, non-classical MCs, which had higher basal phosphorylation levels in responders than non-responders after 1 year of treatment, are thought to have athero-protective effects.[404] Altogether, the distribution of MC subpopulations indicates that biological treatment protects psoriasis patients against CVD.

In study I and II, no differences in cell frequencies were seen between patients and healthy controls, which was expected as they were clinically in remission. However, increased levels of molecules associated with cell activation were observed, including CD38, HLA-DR and pNF-κB, pSTAT3, pERK and pp38. This indicates an ongoing systemic inflammation. Indeed, evidence points towards a decreased risk of CVD in biologically treated patients with severe psoriasis, as compared to patients receiving topical treatment only. However, the literature does not supply evidence showing that biological treatment of severe psoriasis reduces CVD-risk to that of the general healthy population. Indeed, our findings of ongoing systemic inflammation despite normalisation of skin lesions, together with contradictory reports regarding vessel inflammation,[397-399] may represent that patients treated with biologics still have an increased risk of comorbidities, including CVD, compared to healthy controls, although reduced compared to active disease.

CD38 has been proposed as a possible aggravator of atherosclerosis, by mediating production of pro-inflammatory cytokines and inflammatory cell infiltration throughout the development of CVD, though further research is needed to confirm this hypothesis.[405] NF-κB can also play a role in CVD. Its function in myeloid cells of promoting pro-inflammatory gene expression and conversion of macrophages into foam cells supports that it does. Moreover, in low-density lipoprotein (LDL) receptor-deficient mice, transgenic expression of a nondegradable IκBα in macrophages reduces lipid loading and foam cell formation, whereas myeloid cell-specific IκBα deletion sensitises atherosclerosis development.[294] Thus, the activity in the NF-κB pathway in myeloid cells can be an important factor for determining CVD risk. Activation of p38 is also known to play a crucial role in cardiovascular disease, and occurs in response to stress mediators, such as hypertension, oxidised LDL cholesterol, ischemia and vascular injury. Moreover, inhibition of p38 has been considered due to beneficial effects in cell culture and animal models in a number of CVDs.[406,407] Increased levels of phosphorylated STAT1 can reflect an increased level of IFN-γ. Importantly, both phosphorylated p38 and NF-κB are downstream events of TNF binding. As previously mentioned, IFN-γ and TNF are thought to contribute to inflammatory atherogenesis in a synergistic manner.[123] Thus, level of phosphorylated STAT1, p38 and NF-κB can possibly serve as biomarkers of cardiovascular risk in psoriasis patients.

5.2.5 NK cells: A forgotten cell population in psoriasis?

The role of NK cells in psoriasis is not fully elucidated and has been little in focus so far. NK cells are capable of producing cytokines central in the psoriatic pathogenesis.[408] This combined with NK cells expression of two receptors that bind HLA class I (killer-cell immunoglobulin-like receptors and the C-type lectin-like molecule CD94/NKG2),[409] which is closely associated with psoriasis susceptibility,[8,27] highlights the likelihood of NK cell-involvement in the pathogenesis of psoriasis.[382] Adoptive transfer of NK cells from patients with psoriasis into beige-SCID mice with transplanted autologous skin grafts has been reported to cause classic psoriasis histology,[410] and activated NK cells in psoriatic

lesions have been reported to contribute to disease progression.[411] Altogether, these findings suggest that NK cells may have been underappreciated contributors to the pathogenesis of psoriasis.

As mentioned previously, a significant reduction in NK and NKT cell numbers in peripheral blood of psoriasis patients with a negative correlation of NK cell number and disease severity has been reported in previous studies.[380] However, incongruous findings have been published.[382,383] In our studies, NK cell frequencies were similar between patients and controls. However, most patients received systemic treatment, either MTX, a biologic, or both, which can affect the cell frequencies.[412-414]

NK cells in peripheral blood has been demonstrated to have an increased expression of Fas and downregulated NKG2A in patients with new-onset psoriasis. This was speculated to be early markers NK activation and represent a pro-inflammatory phenotype of NK cells in psoriasis patients.[382] In our studies, we found increased expression of pERK, pp38, which can mirror an increased cytotoxic activity.[415,416] Interestingly in this regard, a recent study reported an increased frequency of granulysin-expressing NK cells in peripheral blood of patients with severe psoriasis, which strengthens this hypothesis.[417] Increased expression of pSTAT3 in $CD16^-$ NK cells can be a sign of an increased transcription of mediators important for inflammation, such as TNF and IFN-γ.[418] $CD56^{bright}CD16^-$ NK cells have previously been found to be actively recruited to psoriatic lesions and release high amounts of IFN-γ, contributing to disease progression.[411]

In general, CD38 and HLA-DR are more highly expressed on activated immune cells. In study II, NK cells displayed increased levels of these markers, where CD38 was significantly increased in patients compared to healthy controls. In cytokine-activated NK cells CD38 expression is associated with an increased cytotoxic activity, most likely through mediating calcium-signalling which causes degranulation and release of granzymes and perforin.[419,420] Further, increased expression of perforin in psoriatic epidermis[421,422] and blood has been demonstrated.[383] Also, a recent study demonstrated that $CD38^+$ NK cells reduced the proportion of Tregs in patients with

rheumatoid arthritis.[423] Thus, increased CD38 expression on NK cells could be an alternative explanation of the previously described reduced proportion of Tregs in psoriatic patients.[424] CD38 expression has also been shown to be increased in NK cells during pregnancy, possibly explaining their enhanced response to influenza.[419] Importantly, psoriasis patients have a higher risk of serious infections and the risk increases with disease severity, either due to biologics or the disease itself, and are often recommended vaccination against influenza and S. Pneumoniae.[425]

5.3 Limitations of the study

In all three studies, due to expensive and work-laborious methods, sample size, i.e. number of patients and controls, was a compromise between chance of statistically significant results and expediency. Nevertheless, all studies, especially study II, would benefit from including additional patients and controls. Study II was the only study where results were adjusted for multiple comparisons, which is needed when analysing high-dimensional datasets. After these corrections, few significant differences were found, even though differences were seen with unadjusted p-values below 0,05. With such small sample size, it is probable that these corrections, although statistically correct, negatively affects the p-value, and thus, conceal biologically relevant results.

Cryopreservation of blood samples can possibly affect cell recovery and intracellular signalling. However, it was required to combine the collection of blood samples during routine consultations with simultaneous analysis. Moreover, all samples from patients and controls underwent the same procedure, and the inter-individual variety of storage duration, did not reveal obvious effects on phosphorylation levels. Any error arising due to this would be a systematic one.

As mentioned previously, for flow cytometry experiments it is advantageous to use dim fluorochrome-conjugated antibodies against abundant antigens and bright fluorochromes conjugated to antibodies against rare antigens. Therefore, the use of

anti-STAT3 (pY705) conjugated to PerCp-Cy5.5 was not optimal, but unfortunately a necessary compromise due to availability.

When discussing the frequency of the cell subsets, it is important to be aware of that the measurement is merely the fraction of a larger parent population. Therefore, an increased frequency of a certain cell subset is not necessarily due to an increased number of the respective cell subset. It may as well be due to a decreased number of another cell subset. Thus, caution must be taken when interpreting frequencies of cell subsets, rather than true count comparisons. The importance of true or absolute counts is exemplified by the counting of $CD4^+$ T cells in patients with HIV to stratify their risk of opportunistic infections.[426,427]

As mentioned, in study III, antibodies for CXCR3, CCR4, CCR6 and CD127 were added before barcoding. This introduces the risk of inter-sample variation of staining. Moreover, in study III, Barcode Perm Buffer was likely to interfere with the detection of CCR10 and CD161, as their expressions were increased compared to test runs without barcoding.

In study III, we defined Tregs as $CD3^+CD4^+CD25^+CD127^-$. To identify Tregs with confidence, FoxP3 should be included. Unfortunately, this was not possible due to its need for a specialised lab-protocol incongruous with the detection of phospho-epitopes.

In study I we did not include a live-dead marker. Pacific orange was of little value as the cells were permeabilised. A live-dead marker compatible with permeabilisation was tested, but due to fluorescent spillover it was excluded from the experiments. Our approach was therefore to gate very strictly on the lymphocyte and MC population. In a parallel assay we determined the viability in these gates by staining with 7-aminoactinomycin D. The viability of the cells was above 98% in the lymphocyte gate and 95% in the MC gate. The addition of cisplatin in study II and III enabled exclusion of dead cells from the analysis. In all studies we can assume sufficient removal of dead cells. However, as these methods of dead cell exclusion do not differentiate between the different cell subsets, there is a risk of higher levels of

apoptosis in some subpopulations, particularly the rarer ones. Therefore, alterations of signalling caused by apoptotic cells cannot be ruled out, especially in the rare populations.

A high inter-individual variation was observed for all studies with regards to phosphorylation levels and the differences observed between groups were subtle. Thus, caution must be taken when interpreting these results. More studies with increased number of patients and controls are needed to confirm our results.

6. Conclusions

The three studies included in this thesis promote single-cell analyses as useful methods to approach an understanding of the complex immunological network in psoriasis. Also, these studies imply that patients with severe psoriasis have systemic inflammation, also when treated with biological drugs, with potentially increased risk of comorbidities.

Study I:

Psoriasis patients treated with IFX have higher phosphorylation levels in PBMCs than healthy controls, which does not completely normalise with ongoing treatment. This may reflect systemic inflammation despite treatment with a TNF inhibitor and amelioration of skin lesions. Switching to biosimilar CT-P13 did not affect clinical parameters or phosphorylation levels, supporting the hypothesis of CT-P13 being a non-inferior treatment alternative.

Study II:

Based on the expression of CD38 and HLA-DR, and the levels of phosphorylated NF-κB, p38, ERK and STAT3, NK cells, $CD4^+$ T_{EM} and T_{CM}, mDCs, pDCs and non-classical MCs from psoriasis patients are in a more active state than those from healthy controls, despite treatment with IFX or biosimilar CT-P13, with no obvious differences detected between the two treatment cohorts. This suggests an ongoing systemic inflammation, with sustained risk of comorbidities, such as CVD.

Study III:

Biological therapy facilitates a shift in the fraction of T cell subsets, an increased expression of PD-1 on T cells, and a less atherogenic MC-distribution. Intracellular phosphorylation level of PBMCs is higher in psoriasis patients than healthy controls and in non-responders than responders. Altogether these changes indicate reduction of systemic inflammation in response to treatment, and that broad immune profiling may enable stratification of patients with respect to ongoing and future treatment response.

7. Future perspectives

Our findings of increased CD38, particularly in NK cells, but also in T_{EM}, warrant further investigations, both due to a low number of patients and weak staining of CD56. Therefore, we are planning a flow cytometry assay looking at different surface activation markers for NK cells and T cells in a larger group of patients and controls. Importantly, as CD38 expression is easily quantified by flow cytometry, it could serve as a potential biomarker in the future.

Further comparisons of responders and non-responders are needed. With basis in study III, a functional analysis of Tregs, comparing responders and non-responders would be interesting. Also, as the fraction of Tregs was higher in responders, than non-responders, further analyses of Treg fraction which includes analysis of FoxP3 would be beneficial. Moreover, analysing absolute counts of Tregs could also generate important results. An absolute count is more likely to be robust in clinical practice because it is less prone to interference by the quantites of other cell subsets. Also, identifying patients who are non-responders to several biologics, with subsequent functional analysis of immune cells in peripheral blood and skin are in the planning.

The aberrant frequencies in the MC compartment in untreated patients should be analysed further. Analyses of absolute counts would be preferred to analyses of cell fractions. Also, as NF-κB signalling in myeloid cells could be an important mediator of CVD risk,[294] a focused assay looking at the NF-κB pathway in MCs and DCs could provide useful information regarding risk stratification.

With regards to the signalling assays, we only measured a small fraction of each signalling cascade, where none of the phospho-epitopes are specific for an upstream event. In the future it would be advantageous to analyse larger fractions of the different cascades. When measuring pNF-κB, only the RelA-phosphorylation, the prototypical marker of the canonical NF-κB cascade, is analysed. However, by adding IκB and perhaps NEMO to the analysis, the activation level of these proteins these proteins would more certainly mirror the activity in the canonical NF-κB

signalling pathway. Moreover, when analysing the NF-κB signalling activity in dendritic cells, phosphorylated RelB should be included in the analysis. Although canonical NF-κB signalling often is thought to be synonymous with RelA activation, inflammatory dendritic cells also render RelB as a key transcriptional effector of the canonical pathway.[290]

Peripheral blood cells and cytokines are promising biomarkers for estimating systemic inflammation in psoriasis and treatment response.[371,428] In rheumatoid arthritis, a whole blood flow cytometric assay measuring baseline JAK phosphorylation has been speculated to enable prediction of treatment response of synthetic disease-modifying anti-rheumatic drugs.[429] Moreover, a flow cytometric sputum assay measuring STAT1-phosphorylation has been reported to enable assessment of sensitivity to and efficacy of JAK-inhibition in chronic obstructive pulmonary disease.[430] In this regard, testing a phospho-specific flow cytometry assay, measuring STAT phosphorylation in psoriasis patients before and during treatment with a JAK inhibitor could provide useful information.

Finally, all our studies were based on peripheral blood, mainly due to the availability and the obvious advantages regarding biomarker discovery. Peripheral blood immune cells may be used as reporters of drug responses give insight to the pathophysiology of psoriasis. However, the primary site of inflammation is in the skin. Therefore, we plan to collect biopsies from psoriasis patients, for analyses by imaging mass cytometry. Imaging mass cytometry enables visualisation of many markers simultaneously, while preserving the tissue architecture.[431] Thus, it can reveal the interplay of the many cell subsets involved in psoriatic plaques.

8. References

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Phosphorylation of intracellular signalling molecules in peripheral blood cells from patients with psoriasis on originator or biosimilar infliximab*

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Summary

Background Psoriasis vulgaris is a chronic, inflammatory skin disease characterized by a dysregulated immune response and it is associated with substantial systemic comorbidities. Biological drugs such as tumour necrosis factor (TNF)-α inhibitors can ameliorate the disease but are expensive. Biosimilar drugs have the same amino-acid sequence as the originator, but differences in manufacturing can affect biological activity, efficacy and tolerability.

Objectives To explore potential differences in intracellular phosphorylation of signalling molecules in peripheral blood cells from patients with psoriasis treated with the TNF-α inhibitor infliximab compared with healthy controls, and to investigate if the phosphorylation pattern was influenced by switching from the originator infliximab to the biosimilar CT-P13.

Methods By flow cytometry, we measured phosphorylation of nuclear factor kappa B, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase and signal transducer and activator of transcription 3, before and after TNF-α stimulation in monocytes and T, B, natural killer and $CD3^+$ $CD56^+$ cells from 25 patients with psoriasis treated with infliximab and 19 healthy controls.

Results At inclusion, phosphorylation levels of peripheral blood mononuclear cells (PBMCs) were increased in patients with psoriasis compared with healthy controls, even though clinical remission had already been achieved. Phosphorylation levels declined in patients on both originator infliximab and biosimilar during continued treatment. No significant differences were detected between the two medications after 12 months.

Conclusions Patients with psoriasis on infliximab have higher activation levels of PBMCs than do healthy controls, possibly reflecting systemic inflammation. Switching from the originator infliximab to biosimilar CT-P13 did not affect phosphorylation levels or clinical parameters, suggesting that CT-P13 is a non-inferior treatment alternative to the originator infliximab.

What's already known about this topic?

- The pathogenesis of psoriasis encompasses interactions between dendritic cells, T cells, keratinocytes and neutrophils. Certain cytokines, including tumour necrosis factor (TNF)-α, from these cells activate intracellular signalling cascades, which can be measured using phospho flow cytometry.
- Infliximab and the biosimilar CT-P13, both effective in psoriasis, act by binding TNF-α.

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Conflicts of interest
None to declare.

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What does this study add?

- Peripheral blood mononuclear cells (PBMCs) from patients with psoriasis are more activated with higher intracellular signalling activity than PBMCs from healthy controls.
- This elevated activation level declines during infliximab treatment with no significant differences between originator and biosimilar infliximab.

What is the translational message?

- Higher activation levels of PBMCs implies an ongoing systemic inflammation, possibly related to cardiovascular disease and obesity associated with psoriasis.
- Long-term infliximab treatment may be beneficial in preventing such comorbidities.
- Switching from originator to biosimilar infliximab does not seem to influence intracellular signalling activity of PBMCs.

The pathogenesis of psoriasis encompasses interactions between dendritic cells, T cells, keratinocytes and neutrophils.[1–3] Cytokines released from these cells initiate and perpetuate the inflammation that is characteristic of psoriasis.[4] Patients have increased risk of cardiovascular disease and diabetes,[5–7] indicating systemic inflammation. This is supported by higher numbers of circulating lymphocytes[8,9] and increased gene expression of transcription factors and cytokines involved in differentiation of T helper (Th)1, Th17 and Th22 cells.[10,11] Furthermore, patients with psoriasis display elevated levels of inflammatory cytokines in blood.[12,13]

Among the cytokines involved in psoriasis pathogenesis, tumour necrosis factor (TNF)-α is regarded as one of the most predominant.[4] Levels of TNF-α are elevated in lesional psoriatic skin and plasma, and peripheral blood mononuclear cells (PBMCs) express high levels of *TNF*-α mRNA. Moreover, patients with psoriatic arthritis have increased synovial TNF-α.[14] TNF-α signals via TNF-receptor 1 and 2, which are expressed on all nucleated cells,[14] and induces phosphorylation of nuclear factor kappa B (NF-κB), a transcription factor, and p38 mitogen-activated protein kinase (MAPK). TNF-α signalling can result in phosphorylation of signal transducer and activator of transcription 3 (STAT3), another transcription factor, and extracellular signal-regulated kinases (ERKs).[15,16] NF-κB regulates genes involved in inflammation, cell survival and proliferation. It promotes expression of cytokines involved in the pathogenesis of psoriasis, and *NFKB1* and *NFKBIL1* gene variants are associated with severe psoriasis.[17,18]

The targets of STAT3 are mainly genes that are anti-apoptotic or pro-proliferative, or which regulate angiogenesis and cytokine production.[19] TNF-α induces activation of STAT3 via phosphorylation of two of its residues, tyrosine (Y705) and serine (S727).[15,20] STAT3 is required for the development of Th17 cells.[21] Phospho flow cytometry has been used to show that phosphorylation of STAT3 (at Y705) is increased in regulatory T cells of patients with psoriasis, resulting in impaired suppressive function.[22] The Janus kinase–STAT pathway is considered a promising drug target in psoriasis.[23,24] Phosphorylated p38 MAPK can lead to activation of promoters of genes involved in inflammation and production of inflammatory cytokines such as interleukin (IL)-6, IL-8 and IL-12.[25]

Fumaric acid esters, used in the treatment of psoriasis, effectively inhibit the activity of p38 MAPK, decreasing pro-inflammatory cytokine production.[26] ERKs are MAPKs with substrates that include transcription factors and immediate early gene products involved in gene expression and cell function. Hyperactivity of this pathway is associated with unregulated cell proliferation.[27] Phosphorylation of the abovementioned intracellular epitopes can be quantified by phospho flow cytometry, potentially serving as a measure of cytokine stimulation.[28] Hence, this method can also gauge the cytokine-blocking effect of biological drugs.

Biological drugs aimed at the cytokines TNF-α, IL-12/23 and IL-17A have revolutionized the treatment of psoriasis.[29,30] Infliximab is a monoclonal antibody against TNF-α and CT-P13 is a more recently licensed, cheaper biosimilar assumed to have comparable efficacy and safety.[31–33] However, despite the same amino-acid sequence of the originator infliximab and its biosimilar, differences in levels of afucosylated glycans and binding affinity for FcγRIIIa and FcγRIIIb have been detected, implying a potential difference in natural killer (NK) cell activation.[34] This might influence biological activity, tolerability and efficacy.[35,36]

The aim of the present study was to compare phosphorylation levels of intracellular epitopes in PBMCs from patients with psoriasis treated with infliximab and healthy controls using phospho flow cytometry, and to investigate if switching from originator infliximab to biosimilar CT-P13 affected clinical parameters and intracellular phosphorylation patterns.

Materials and methods

Patient characteristics

In this observational study, at the Department of Dermatology, Haukeland University Hospital, we included 25 patients with psoriasis vulgaris who had all been diagnosed with severe psoriasis [Psoriasis Area and Severity Index (PASI) > 10] at an earlier time point but were now in or close to remission (PASI 0–4) with frequent infusions of originator infliximab. In total, 22 patients were randomized either to continue infliximab or to switch to the biosimilar CT-P13 as part of another study[31] and therefore these samples were analysed double blinded. The final three patients all continued on infliximab. Patients gave written informed consent at the Department of Dermatology, Helse Bergen (regional ethics committee approvals 2014/1373 and 2014/1489). There were 19 healthy controls (age-, sex- and body mass index-matched, Table 1) included, and samples were collected from the blood bank at the Haukeland University Hospital.

Blood sampling

Blood was collected at inclusion and after approximately 3 and 12 months, just before patients were to receive the next infusion. The samples were collected between April 2015 and September 2016 in lithium-heparin tubes (BD 367526, Becton Dickinson Ltd., Reading, U.K.). PBMCs were isolated by density gradient centrifugation with Lymphoprep (Axis-Shield Ltd, Dundee, Scotland) and cryopreserved in liquid nitrogen until use, as described previously.[37] Trough level and antidrug antibodies were measured with immunofluorometric assay as a routine analysis.[37] The therapeutic range of the trough level is above 2–3 mg L^{-1}.

Cell culture and stimulation

PBMC samples were thawed and rested in serum-free media (X-vivo-20™, Lonza, Basel, Switzerland) for 2 h at 37 °C, 5% CO_2. Cells were divided and either stimulated for 15 min with TNF-α (50 ng mL^{-1}; Immunotools, Friesoythe, Germany) or left unstimulated. Next, samples were fixed with 1·5% paraformaldehyde (37 °C) incubated for 10 min at room temperature and permeabilized with ice cold 100% methanol for 30 min on ice, as described previously.[36,38] The cells were washed with phosphate-buffered saline (PBS), then stained according to a 4 × 2 fluorescence cell barcoding (FCB) grid (three time points and one internal control with two stimulation conditions) with different concentrations of Pacific Blue (100, 25, 6·3 and 0 ng mL^{-1}) and Pacific Orange (70 and 0 ng mL^{-1}; both Life Technologies, Grand Island, NY, U.S.A.),[40] then incubated in the dark at 4 °C. Further, cells were washed and resuspended in fluorescence activated cell sorter (FACS) buffer (PBS with 1% bovine serum albumin) before combining the eight FCB combinations. FcR blocking reagent (1 : 21) (Miltenyi Biotec, Bergisch Gladbach, Germany) was added, cells were divided into two panels followed by staining with titrated amounts of fluorochrome-conjugated antibodies for 30 min in the dark at room temperature. Cells were then washed with FACS buffer and resuspended in 200 μL FACS buffer + 2 mmol L^{-1} ethylenediamimetetraacetic acid.

Antibodies used for flow cytometry

The following monoclonal antibodies were used: PE conjugated anti-CD56 (clone N901, Beckmann Coulter, CA, U.S.A.), BV786 conjugated anti-CD3 (clone SK7), Alexa Fluor 488 conjugated anti-CD20 [clone H1(FB1)], PE-Cy7 conjugated anti-NF-κB p65 (pS529, clone K10-895·12·50) and

Table 1 Characteristics of patients and healthy controls at inclusion

	Originator infliximab	CT-P13	Healthy controls
Sex, *n*			
Women	2	3	3
Men	10	10	16
Age, years			
Mean ± SD	50·83 ± 11·14	51·53 ± 15·98	47·32 ± 14·64
Range	28–65	29–79	24–70
BMI kg m^{-2}			
Mean ± SD	27·67 ± 4·62	28·15 ± 4·41	26·27 ± 3·91
Range	21–38	23–40	20–38
Duration of psoriasis, years			
Mean ± SD	27·91 ± 10·55	25·61 ± 10·15	NA
Range	11–49	8–38	NA
Psoriatic arthritis, *n*	5	3	NA
Duration of originator infliximab treatment before inclusion, months			
Mean ± SD	86·83 ± 39·35	68·92 ± 45·75	NA
Range	40–177	18–175	NA

NA, not applicable.

Table 2 Clinical parameters, treatment doses and intervals of the patients included in the study

	Originator infliximab			CT-P13		
	Inclusion	3 months	12 months	Inclusion	3 months	12 months
Psoriasis Area and Severity Index						
Mean ± SD	1·36 ± 0·69	1·02 ± 0·88	1·20 ± 0·99	2·28 ± 1·11	1·68 ± 1·22	1·38 ± 0·83
Range	0·00–2·20	0·00–2·40	0·00–3·00	0·80–4·20	0·00–3·60	0·00–2·90
Dermatology Life Quality Index						
Mean ± SD	1·08 ± 1·73	1·25 ± 1·71	0·6 ± 0·89	1·12 ± 2·04	1·92 ± 2·78	1·17 ± 2·59
Range	0–5	0–6	0–3	0–6	0–10	0–9
Infliximab dose, mg						
Mean ± SD	590 ± 104	590 ± 104	590 ± 104	638 ± 296	638 ± 296	638 ± 296
Range	400–700	–	–	300–1500	–	–
Interval, weeks						
Mean ± SD	7·82 ± 1·08	–	–	6·54 ± 1·13	–	–
Range	6–10	–	–	5–9	–	–
Methotrexate mg						
Mean ± SD	10·00 ± 5·11	10·00 ± 5·11	10·00 ± 5·11	14·38 ± 5·44	14·38 ± 6·23	14·38 ± 6·23
Range	0·00–20·00	0·00–20·00	0·00–20·00	7·50–25	7·50–25	7·50–25
Antidrug antibodies	1	1	1	–	–	–
Prior use of other biological, n						
Adalimumab and etanercept	1	–	–	0	–	–
Adalimumab	1	–	–	1	–	–
Etanercept	2	–	–	7	–	–
Efalizumab	0	–	–	1	–	–

anti-p38 (pT180/pY182, clone 36/p38), Alexa Fluor 647 conjugated anti-ERK1/2 [(pT202/pY204),20A,RUO – 612593] and anti-STAT3 (S727; clone 49/p-STAT3) and Per-CP-Cy5.5 conjugated anti-STAT3 (Y705; clone 4/p-STAT3) (all from BD Biosciences, San Jose, CA, U.S.A.).

Data acquisition

All samples from the same patient were stimulated, stained and analysed under the same conditions on the same day. An internal control, buffy coat from one healthy donor, was used for every experiment, to account for interassay variation. All the experiments were performed by one person within 2 weeks in July 2016 to minimize inter- and intra-assay variation in the laboratory and on the flow cytometer. Samples were acquired on a LSRI Fortessa flow cytometer with BDFACSDiVa™ Software (both BD Biosciences). The flow cytometer was equipped with 407, 488, 561 and 635 nm lasers. Further specifications are given in Table S1 (see Supporting Information). A minimum of 200 000 events was acquired in the intact cell gate. A representative gating strategy is shown in Figure S1 (see Supporting Information).

Phosphorylation of NF-κB, ERK, p38, STAT3 (S727) and STAT3 (Y705) were quantified in immune cell subsets. Beads were used for compensation, which was performed in FlowJo version 10.2. Cells within each subtype were analysed in Cytobank[40] in each stimulation condition. Basal phosphorylation was defined as raw median fluorescence intensity (MFI) of the inspected phosphoprotein in unstimulated cells, divided by raw MFI of the corresponding phosphoprotein in unstimulated internal control cells. The fold change was defined as raw MFI of the inspected phosphoprotein in TNF-α-stimulated cells, divided by raw MFI of the corresponding phosphoprotein in unstimulated cells. The viability of the cells was above 98% in the lymphocyte gate and 95% in the monocyte gate as determined by 7-aminoactinomycin D staining (data not shown).

Statistical analysis was carried out using SPSS Statistics 23/24 (IBM, Armonk, NY, U.S.A.) with the Mann–Whitney U-test for independent, unpaired data and the Wilcoxon signed-rank test for paired data.

Results

In total, 12 of the 25 patients continued on the originator infliximab and 13 switched to the biosimilar CT-P13. Duration of psoriasis, incidence of psoriatic arthritis, dose of infliximab and concomitant methotrexate were comparable between the two treatment groups (Table 1). There were no significant differences between the groups regarding sex, age and body mass index, PASI, Dermatology Life Quality Index (DLQI; Table 2) or routine laboratory analysis at inclusion, after 3 and 12 months (Table S2; see Supporting Information). One patient had antidrug antibodies at inclusion (originator infliximab group) and no patients developed antidrug antibodies during the study.

Increased basal phosphorylation in peripheral blood mononuclear cells from patients with psoriasis treated with infliximab

The basal phosphorylation of NF-κB (S529), ERK1/2 (T202/Y204), p38 (T180/Y182) and STAT3 (S727 and Y705) was significantly higher in patients at inclusion than in the healthy

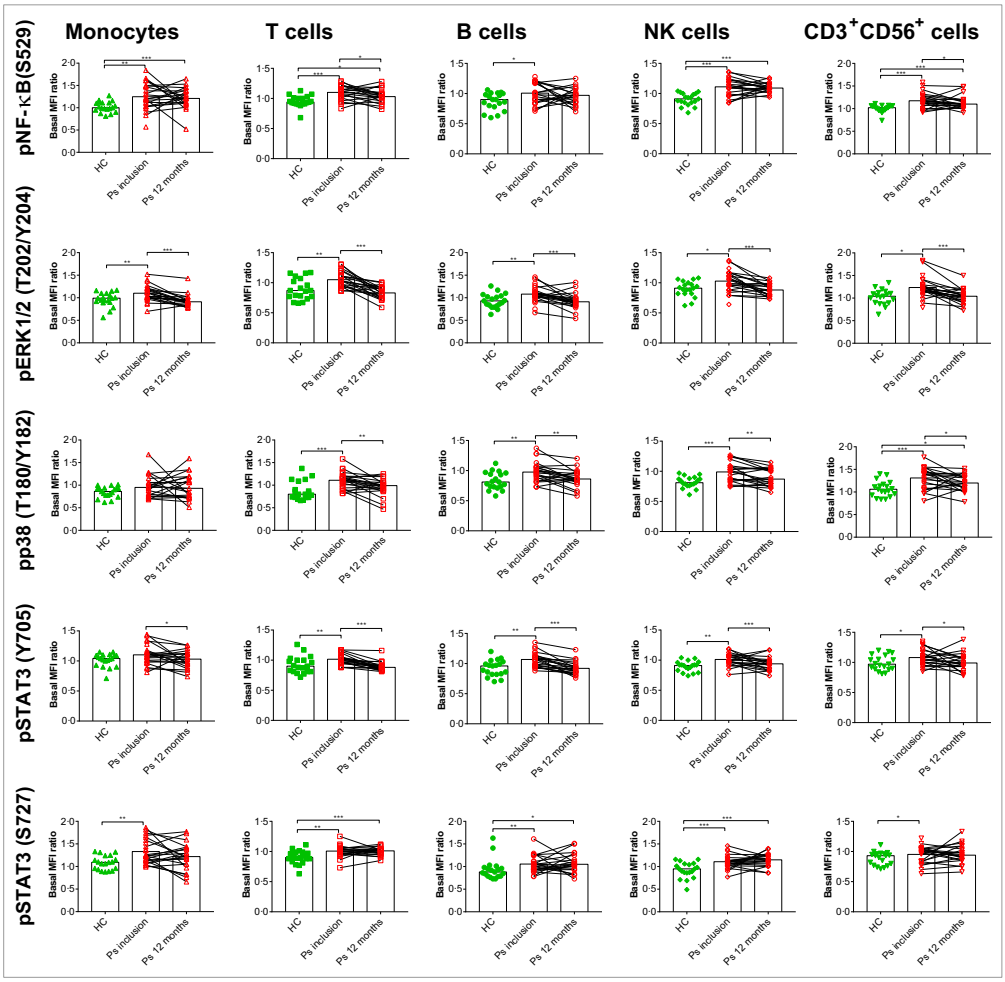

Fig 1. Phosphorylation of intracellular epitopes in unstimulated peripheral blood mononuclear cells from the psoriasis and healthy control groups at inclusion and after 12 months. Basal median fluorescence intensity (MFI) is shown as a ratio of raw MFI of unstimulated cells relative to raw MFI of unstimulated internal control for phosphorylated nuclear factor kappa B (pNF-κB), phosphorylated extracellular signal-regulated kinase (pERK), phosphorylated p38 (pp38), phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (Y705) and pSTAT3 (S727). HC, healthy control, green; Ps, patient with psoriasis, red. Each symbol represents one individual, the bar shows the median. NK, natural killer. *P ≤ 0·05; **P ≤ 0·01; ***P ≤ 0·001

controls in almost all cell populations analysed. Twelve months after inclusion, patients still displayed significantly higher basal phosphorylation levels than healthy controls, but the number of epitopes with significant differences was decreased (Fig. 1).

Further, we analysed phosphorylation patterns upon TNF-α stimulation, relative to a corresponding unstimulated sample; the fold change. As anticipated, because of the higher basal phosphorylation levels at inclusion, the fold change of pSTAT3 (Y705) upon stimulation was significantly lower in monocytes and T, B and NK cells from patients at inclusion compared with healthy controls. Fold changes of pERK in T cells and pNF-κB in NK cells were also significantly lower for patients than healthy controls. After 12 months, there were fewer significant differences in fold change between patients and healthy controls compared with at inclusion (Fig. S2; see Supporting Information). Only fold changes of pNF-κB in monocytes and NK cells in addition to pSTAT3 in monocytes were decreased in patients compared with healthy controls.

Patients treated with CT-P13 displayed similar basal phosphorylation and fold change to those treated with the originator infliximab after 3 and 12 months

Next, we compared phosphorylation levels in patients continuing on the originator infliximab to those who switched to CT-P13. The basal phosphorylation of NF-κB, ERK, p38 and STAT3 (S727 and Y705) showed no significant differences at inclusion, after 3 or 12 months. The only exception was lower levels at inclusion of pSTAT3 (Y705) in NK cells (Fig. S3a; see Supporting Information) and pSTAT3 (S727) in $CD3^+$ $CD56^+$ cells (data not shown) from patients who continued treatment with the originator infliximab compared with those who switched. Although no significant difference was detected in basal phosphorylation, fold change of pSTAT3 (Y705) in B cells was significantly increased in patients on CT-P13 relative to the originator infliximab 3 months after switch (Fig. 2a, b). After 12 months, this difference was no longer significant (Fig. 2c).

The phosphorylation of peripheral blood mononuclear cells from patients with psoriasis on infliximab and CT-P13 decreased during the study period

All patients were in clinical remission and treated with the originator infliximab when included in the study. Nevertheless, when investigating the change of phosphorylation over time separately for the originator infliximab and the biosimilar CT-P13, there were significantly lower basal pSTAT3 (Y705) levels in T, B and NK cells (Fig. 3a, Fig. S3a; see Supporting Information) and pERK in all cell types 1 year after inclusion in both treatment groups (Fig. 3b, Fig. S3b–f).

As the phosphorylation levels decreased over time for all epitopes except STAT3 (S727) (Figs 1 and 3), we questioned if infliximab treatment length prior to inclusion had an effect. We therefore analysed basal and stimulated pSTAT3 (Y705) levels in T cells in relation to duration of infliximab treatment before inclusion for individual patients in the two treatment groups. No obvious correlation between the length of infliximab treatment prior to inclusion and basal phosphorylation levels was detected (Figs S4a, b, d and e; see Supporting Information). In order to exclude *ex vivo* effects of the medication, drug trough levels were compared with basal and TNF-α stimulated levels of pSTAT3 (Y705) in T cells in both patient groups at three time points. All patients had trough levels within the recommended range, and trough levels did not correlate with phosphorylation levels (Figs S4c and f, Table S2).

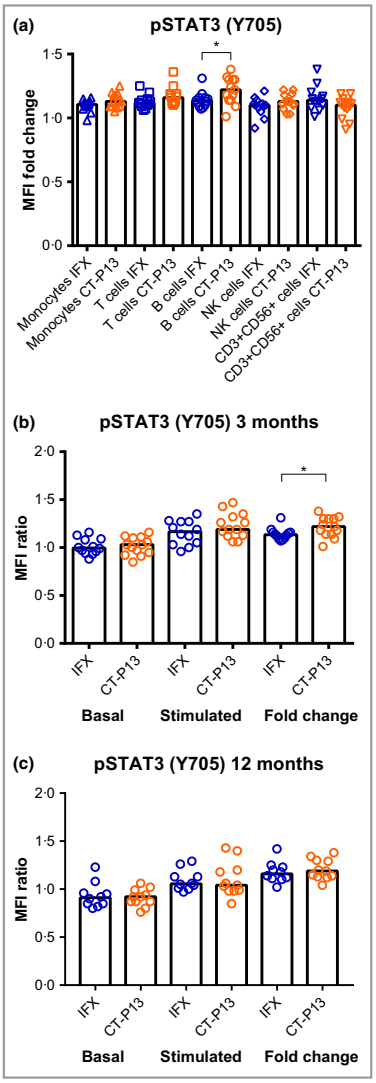

Fig 2. Significant difference in phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Y705) in B cells between patients on infliximab and CT-P13 3 months after switch. (a) Fold change of pSTAT3 (Y705) in immune cell subsets 3 months after inclusion. (b) Basal median fluorescence intensity (MFI) ratio, TNF-α-stimulated MFI ratio and fold change 3 months after inclusion. (c) Basal MFI ratio, TNF-α-stimulated MFI ratio and fold change 12 months after inclusion. Basal MFI ratio: unstimulated sample/unstimulated internal control. Stimulated-MFI ratio: TNF-α-stimulated sample/unstimulated internal control. MFI fold change: TNF-α-stimulated cells/corresponding unstimulated cells. IFX, originator infliximab, blue; CT-P13, orange; IC, internal control. Each symbol represents one individual, the bar shows the median *$P \leq 0{\cdot}05$

Discussion

To the best of our knowledge, phospho flow cytometry has not been used previously on a broad panel of intracellular epitopes in PBMCs comparing patients with psoriasis and healthy controls. Neither has it been used to monitor patients on biological drugs over time, including switching from an

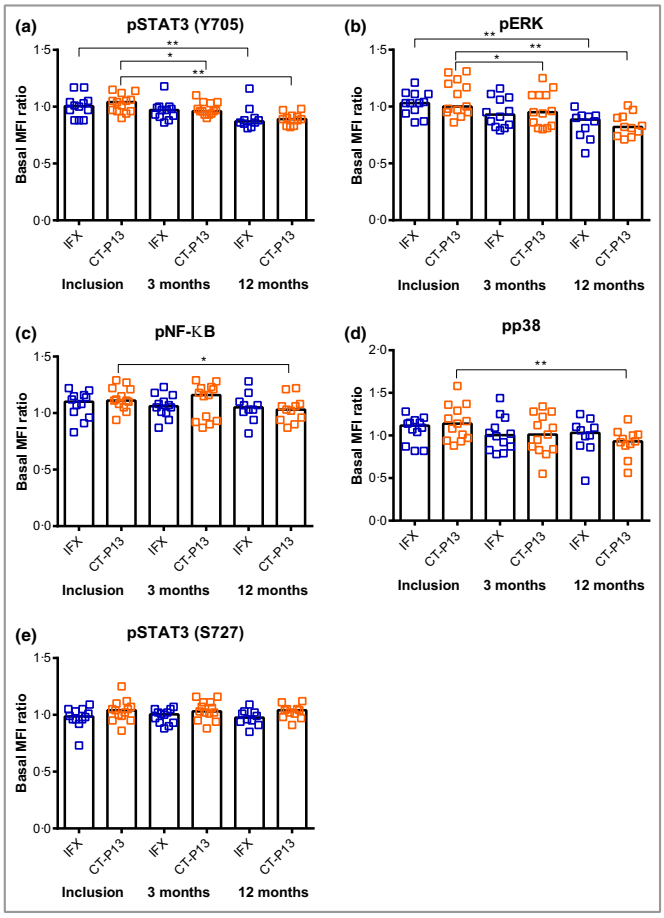

Fig 3. Basal phosphorylation over time for both patient groups, exemplified with T cells. (a) Phosphorylated signal transducer and activator of transcription 3 (pSTAT3, Y705); (b) phosphorylated extracellular signal-regulated kinase (pERK); (c) phosphorylated nuclear factor kappa B (pNF-κB); (d) phosphorylated p38 (pp38); (e) pSTAT3 (S727). The basal median fluorescence intensity (MFI) ratio is defined as raw MFI of measured phosphoprotein in unstimulated sample cells relative to unstimulated internal control cells. IFX, originator infliximab, blue; CT-P13, orange; IC, internal control. Each symbol represents one individual, the bar shows the median. *P ≤ 0·05, **P ≤ 0·01.

originator to a biosimilar. Whereas there were significant differences in intracellular phosphorylation of PBMCs between patients with psoriasis and healthy controls, switching of patients from the originator infliximab to the biosimilar CT-P13 did not cause any major differences.

The basal phosphorylation levels in PBMCs from the psoriasis group were significantly increased in almost all analysed phospho-epitopes and immune cell subsets at inclusion compared with healthy controls, even though patients had been treated with the originator infliximab for a minimum of 18 months and had no or low-grade skin inflammation. This may indicate that systemic inflammation takes a longer time to cease than skin lesions or that signalling through the investigated pathways is maintained, at least in blood, by cytokines besides TNF-α. Basal levels of pSTAT3 (Y705) in patients with psoriasis have been reported to be similar to those observed in healthy volunteers.[42] The discrepancy between these findings and ours may be explained by different methodological approaches (enzyme-linked immunosorbent assay was used in the study by Punwani *et al.*[42]) and differences in patient characteristics.

Our study would have benefitted from evaluation of phosphorylation levels and corresponding clinical parameters before the patients started treatment. As the clinical effect was sustained throughout the study period, PASI and DLQI were of little value when interpreting how the phosphorylation levels of PBMCs related to clinical parameters. Moreover, it would have been advantageous to have follow-up samples of

healthy controls to evaluate normal variation over time. Most of the patient samples at inclusion were collected almost a year before the healthy controls samples, thereby increasing duration of cryopreservation. At the same time, there was broad interindividual variation for each time point regarding duration of storage in liquid nitrogen without having an obvious effect on phosphorylation levels. Our findings of gradual decreases in PBMC phosphorylation during treatment merit further longitudinal studies including pretreatment samples.

In vitro inhibition of NF-κB or STAT3 (with parthenolide or Stattic) blocks cytokine production by both Th1 and Th17 cells from patients with psoriasis. The same study also found that infliximab reduced the number of Th1 and Th17 cells *in vivo*.[13] The psoriasis group in our study had increased pNF-κB and pSTAT3 levels in addition to p38 and pERK in T cells compared with healthy controls even though they were treated with infliximab at inclusion. However, patients had a reduction of activated epitopes during the follow-up period. The phosphorylation level of STAT3 (Y705) in T cells did not seem to be influenced by drug trough level, most probably because all of our patients had trough levels in or above the recommended range. As infliximab treatment reduced levels of pNF-κB and pSTAT3 in T cells over time, a gradual reduction in Th1- and Th17-associated cytokines is plausible.

Our findings of elevated phosphorylation levels in distinct immune cells support that systemic inflammation is increased in psoriasis and may persist upon treatment even after resolution of skin manifestation. Systemic inflammation promotes cardiovascular disease. The extent to which systemic treatment reduces this risk is currently debated. Investigation of inflammation in aortic and carotid arteries with positron emission tomography revealed no differences after 16 weeks between patients with psoriasis treated with TNF-α inhibitor adalimumab or placebo, and a small increase of inflammation in the carotids after 1 year.[43] Contrarily, another recent study found decreased aortic inflammation in patients with severe psoriasis who had at least 75% improvement of skin lesions 1 year after commencing biological drugs.[44] In these studies patients were treated differently, making it difficult to reach conclusions. Our study supports the notion that use of systemic rather than local treatment in patients with psoriasis at risk of cardiovascular disease might be beneficial.

Comparing the two patient groups receiving the originator infliximab or the biosimilar CT-P13, there were no significant differences in basal phosphorylation after 3 and 12 months. Surprisingly, fold change of pSTAT3 (Y705) in B cells was modestly increased after 3 months in patients who switched to CT-P13 compared with those continuing on the originator infliximab. However, no difference was detected after 12 months.

There was a notable tendency that basal- and TNF-α-stimulated phosphorylation were decreased in more immune cell subsets and epitopes in the CT-P13 group compared with the originator infliximab group 3 and 12 months after inclusion. This might reflect differences in the structure and effect of the two drugs. Notably, this was not exclusively observed in NK cells, which could have been predicted because of differences in afucosylated glycans and affinity for FcγRIIIa and FcγRIIIb. Studies with greater numbers of patients are needed to ascertain potential differences in intracellular phosphorylation of PBMCs between the two drugs.

Evaluation of immune-mediated diseases such as psoriasis should ideally aim at the molecular aberrancy in each patient for personalized treatment.[45–49] To this end, assays capable of predicting which treatments are most likely to be beneficial for each individual are required, before commencing therapy.[50] Phospho flow cytometry may be a promising tool for estimating systemic disease activity and treatment response of people with psoriasis in the future[51,52] and might be helpful in the quest for new potential drug targets.[53]

In conclusion, relative to healthy controls, patients with psoriasis displayed higher activation levels of PBMCs, and this systemic inflammation decreased gradually with time on infliximab treatment. Switching from infliximab to CT-P13 did not worsen clinical parameters or increase intracellular phosphorylation of NF-κB, ERK, p38 or STAT3. Our data indicate that phospho flow cytometry might represent a promising tool for monitoring disease activity and treatment efficacy.

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We thank all patients and blood donors who participated in the study, the laboratory at the Department of Dermatology, Haukeland University Hospital, for taking blood and routine laboratory samples, Marianne Eidsheim and Kjerstin Jakobsen at Broegelmann Research Laboratory for expert technical assistance. The flow cytometry analysis was performed at the Flow Cytometry Core Facility (http://www.uib.no/en/clin2/flow) Department of Clinical Science, University of Bergen, Norway.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. Representative gating strategy used for the flow cytometry data.

Fig S2. Fold change of intracellular epitopes in peripheral blood mononuclear cells from the healthy control and psoriasis group at inclusion and after 12 months.

Fig S3. Significant changes in basal phosphorylation of intracellular epitopes with time in both treatment groups.

Fig S4. Basal median fluorescence intensity ratio and fold change for signal transducer and activator of transcription 3 (STAT3, Y705) in T cells from the originator infliximab and CT-P13 groups compared with duration of treatment before inclusion and trough level.

Table S1 Specifications of the LSRI Fortessa flow cytometer.

Table S2 Laboratory analysis: laboratory values from patients at inclusion, 3 and 12 months.

Powerpoint S1 Journal Club Slide Set.

Video S1 Author video.

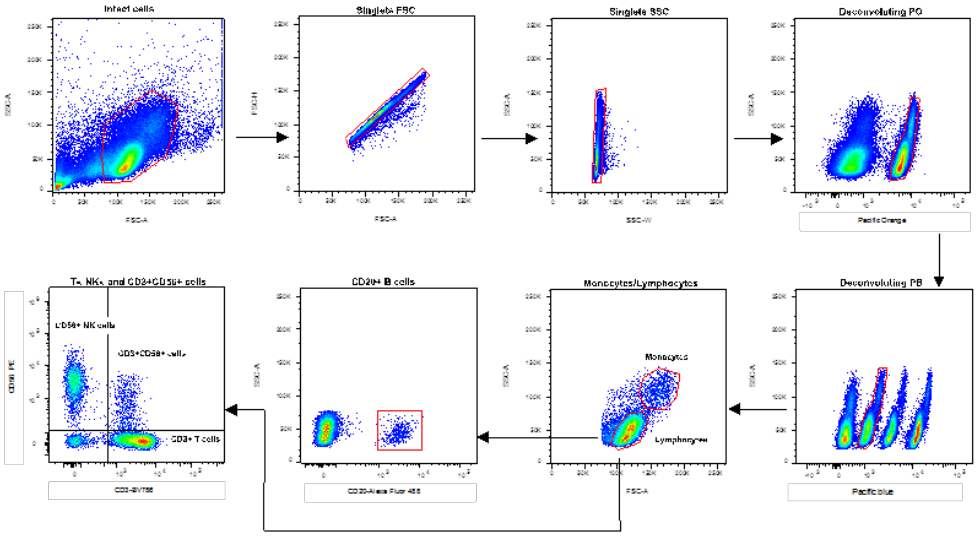

Figure S1: Representative gating strategy used for the flow cytometry data. Intact cells were gated based on forward scatter area (FSC-A) and side scatter area (SSC-A) followed by single cell gating. The different samples were then identified through the intensities of their pacific blue (PB) and pacific orange (PO) stains. Cell subtypes were identified based on their FSC-A and SSC-A scatter properties as either monocytes or lymphocytes. Lymphocytes were then subtyped as B cells ($CD20^+$), T cells ($CD3^+/CD56^-$), NK cells ($CD3^-/CD56^+$) or $CD3^+/CD56^+$ cells based on surface antigen expression.

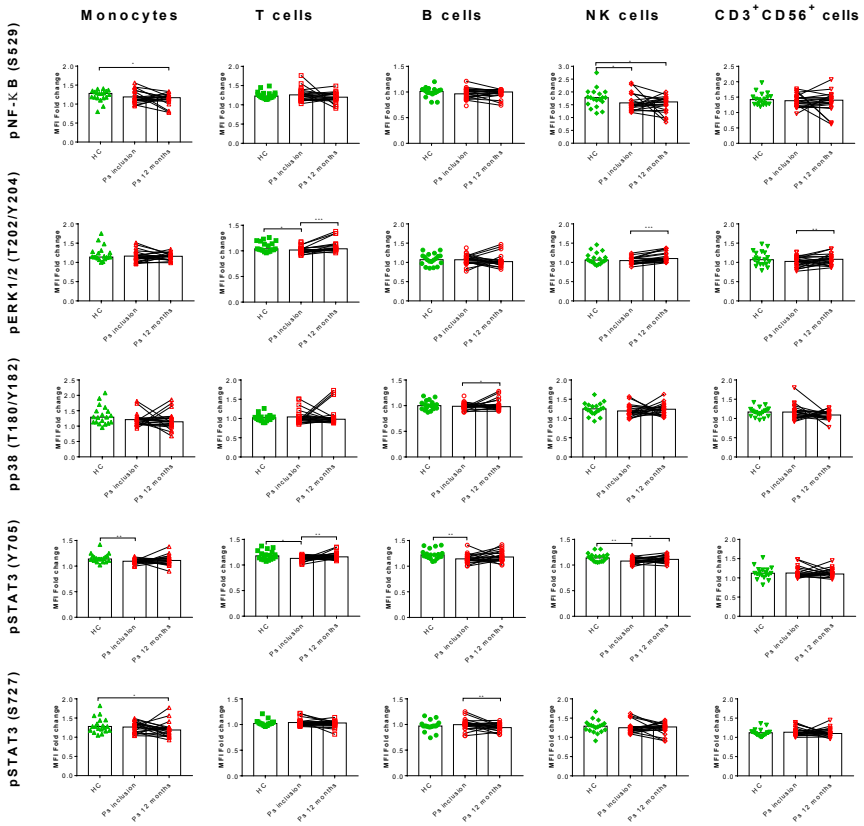

Figure S2: Fold change of intracellular epitopes in PBMC from healthy controls and psoriasis patients at inclusion and after 12 months. Fold change was defined as raw MFI of measured phosphoprotein in TNF stimulated cell subset relative to raw MFI of corresponding phosphoprotein in unstimulated cell subset. Immune cell subsets with corresponding pNF-κB, pERK, pp38, pSTAT3 (Y705) and pSTAT3 (S727) are shown. HC = healthy controls, green. Ps = psoriasis patients, red. Each symbol represents one individual, the bar shows the median. * = $P \leq 0.05$, **=$P \leq 0.01$

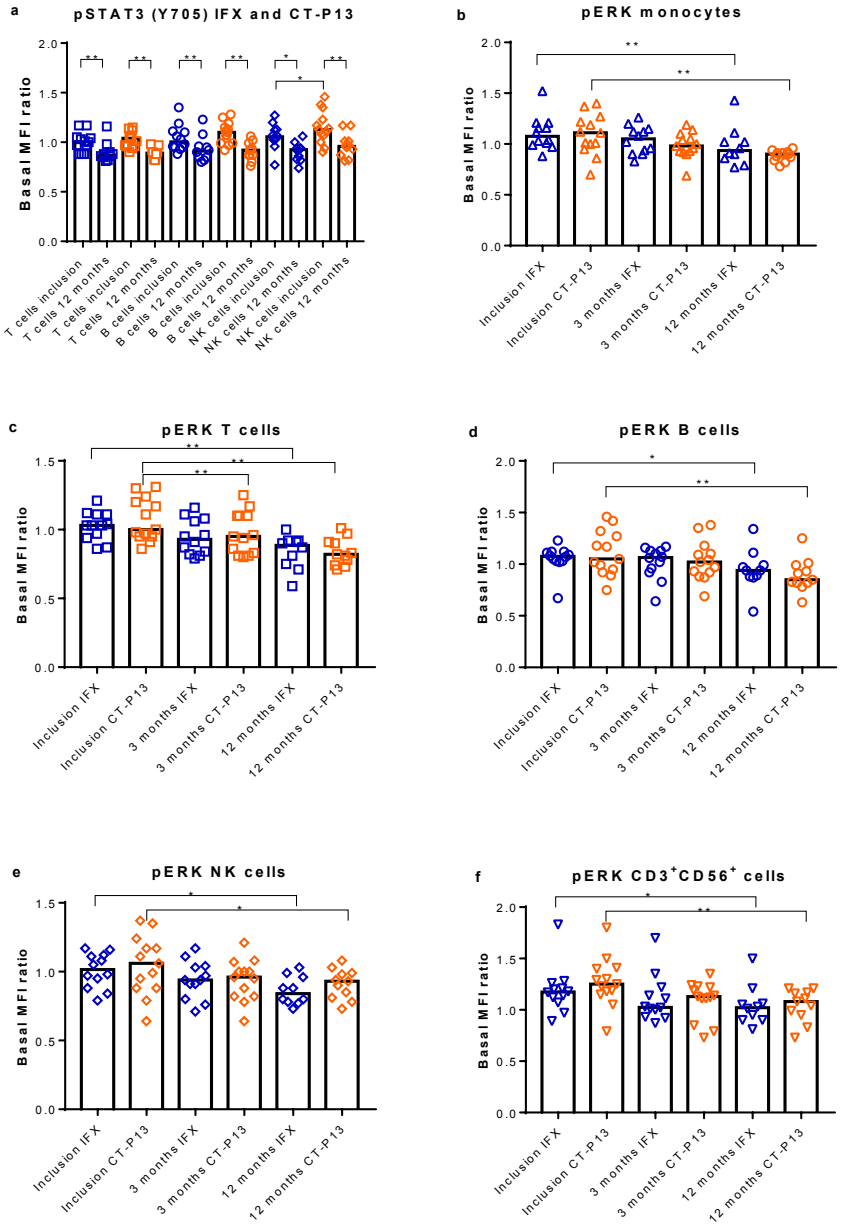

Figure S3: Significant changes in basal phosphorylation of intracellular epitopes with time in both treatment groups. a: pSTAT3 (Y705) in T, B and NK cells at inclusion and after 12 months. b-f: pERK in immune cell subsets at inclusion, after 3 and 12 months follow-up. b: monocytes, c: T cells, d: B cells, e: NK cells, f: $CD3^{+}CD56^{+}$ cells. IFX= originator infliximab, blue. CT-P13 = orange. * = $P \leq 0.05$, ** = $P \leq 0.01$

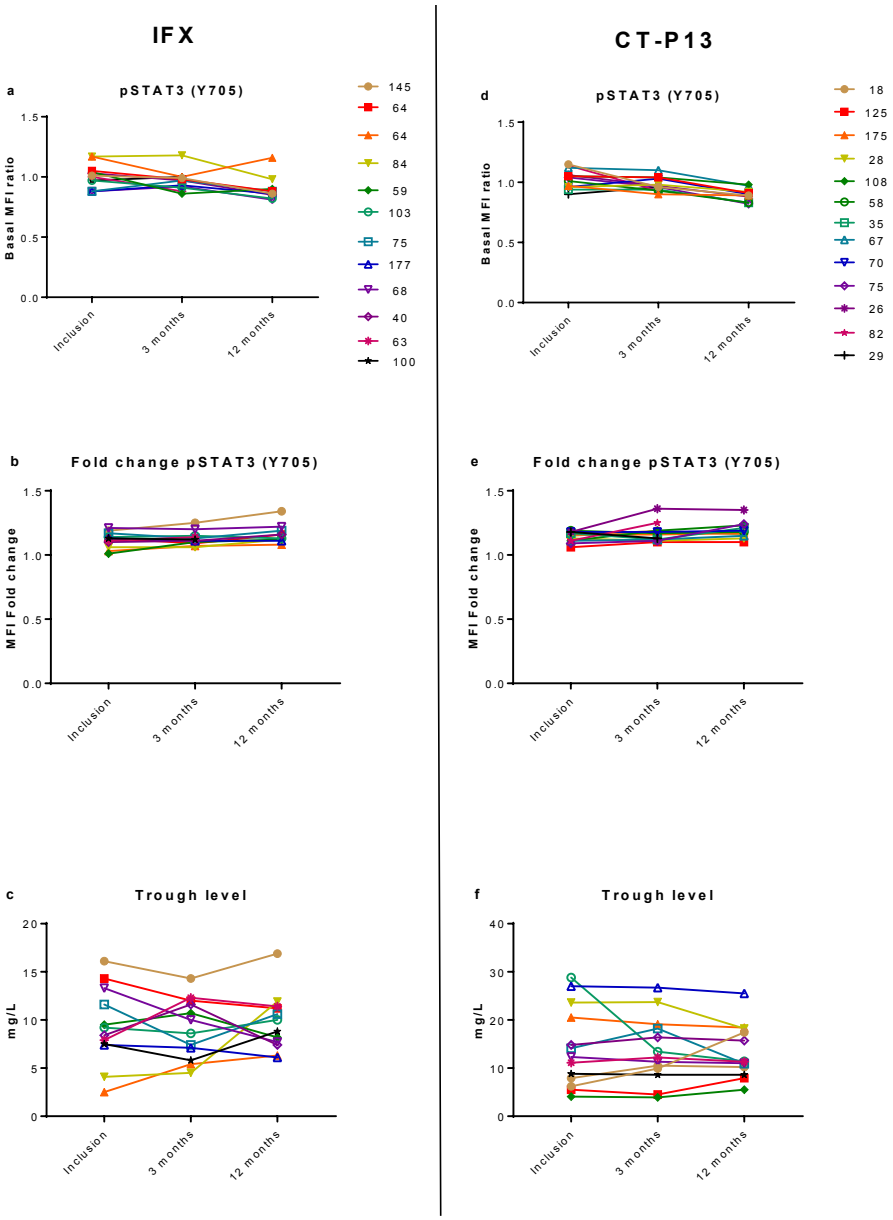

Figure S4: Basal MFI ratio and fold change for STAT3 (Y705) in T cells from originator infliximab (IFX) and CT-P13 patients compared to duration of treatment before inclusion and trough level. a and d: Basal MFI and duration of IFX treatment before inclusion (months; shown at the right side of graph); a: IFX and d: CT-P13. b and e: MFI fold change; b: IFX and e: CT-P13. c and f: Trough levels; c: IFX and f: CT-P13. Each patient represented by a single color and symbol.

Table S1: Specifications of the LSRI Fortessa flow cytometer

Fluorochrome	Emission filters
PerCP-Cy5.5	LP: 685, BP: 695/40
Alexa fluor-488	LP: 505, BP: 530/30
PE-Cy7	LP: 750, BP: 780/60
PE	LP: –, BP: 582/15
APC	LP: –, BP: 670/14
Pacific blue	LP: –, BP:450/50
Pacific orange	LP: 570, BP: 585/42
BV 786	LP: 750, BP: 780/60

Table S2: Laboratory analysis: Laboratory values from patients at inclusion, 3 and 12 months.

	Originator infliximab			**CT-P13**		
	Inclusion	3 months	12 months	Inclusion	3 months	12 months
CRP [a]	1-25 3,83±6,79	1-5 1,91±1,37	1-27 3,83±7,42	1-67 6,61±18,17	1-7 1,92±1,66	1-4 1,53±0,88
Sedimentation Rate [b]	2-110 22,80±31,70	3-64 17,83±18,21	3-60 18,33±17,01	2-67 20,00±21,08	2-33 10,45±10,13	2-36 10,75±10,20
Leukocyte Count [c]	4,00-13,60 6,81±2,59	3,70-8,20 6,28±1,32	0,80-8,60 5,45±1,96	3,60-10,80 7,40±2,00	3,70-9,10 6,96±1,71	3,00-10,00 6,81±1,69
Lymphocytes[c]	1,40-3,30 2,15±0,49	1,60-3,20 2,20±0,55	1,5-3,4 2,27±0,56	0,90-3,90 2,16±0,78	0,90-3,60 2,06±0,66	0,80-3,60 2,13±0,76
Monocytes[c]	0,32-1,09 0,58±0,21	0,16-0,80 0,52±0,20	0,33-0,95 0,62±0,18	0,32-1,16 0,66±0,26	0,29-1,02 0,59±0,20	0,37-0,86 0,58±0,18
Eosinophiles[c]	0,10-0,50 0,20±0,13	0,00-0,30 0,16±0,09	0,00-0,60 0,20 ±0,18	0,10-0,40 0,16±0,10	0,10-0,30 0,15±0,07	0,00-0,30 0,16±0,09
Basophiles[c]	0,00-0,10 0,04±0,05	0,00-0,10 0,05±0,05	0,00-0,10 0,05±0,05	0,00-0,10 0,04±0,05	0,00-0,20 0,07±0,05	0,00-0,20 0,06±0,05
Trough level[a]	2,50-16,10 9,31±3,99	4,50-14,30 9,14±3,13	6,10-16,90 9,70±3,00	4,10-28,80 14,20±8,31	3,90-26,70 13,72±6,86	5,50-25,50 13,23±5,49

Values are listed as range and mean ± SD. [a]: mg/L, [b]: mm/h, [c]: 10^9/L

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Mass cytometry analysis of blood immune cells from psoriasis patients on biological therapy

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Abstract

Psoriasis is a chronic immune-mediated skin disease accompanied by systemic inflammation and comorbidities. We analyzed peripheral blood mononuclear cells (PBMCs) in the search for immune signatures and biomarkers related to psoriasis severity and treatment effect. 32 patients with psoriasis and 10 matched healthy controls were included. PBMCs were collected before and after initiation of anti-TNF, anti-IL-17 or anti-IL-12/23 treatment and analyzed utilizing 26-parameter mass cytometry. The number of circulating Th17, Th22, Th9 and cytotoxic T cells were increased in severe psoriasis. Intracellular pp38 and pERK in T helper cells were associated with disease severity. Differences between responders and non-responders regarding cell composition and intracellular signaling were identifiable already at inclusion. Biological treatment induced memory cells, restored inhibitory PD-1 function of T cells and reduced a potential pro-atherogenic profile in monocytes. In conclusion, these results indicate amelioration of systemic inflammation in psoriasis after biological treatment. Such broad immune profiling may enable prospective stratification of patients regarding future treatment response. Successful early intervention may lead to a healthier trajectory with favorable implications on later comorbidities.

Key words

Psoriasis; mass cytometry; systemic inflammation; biological therapy; cardiovascular risk; PD-1

Abbreviations

BC	Barcode
BMI	Body Mass Index
CAS	Cell Acquisition Solution
CCL20	Chemokine (C-C motif) ligand 20
CCR4 (6, 10)	C-C chemokine receptor type 4 (6, 10)
CD	Cluster of Differentiation
CD8+	CD8 positive T cells
CSB	Cell Staining Buffer
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
CXCR3	Chemokine receptor type 3
DC	Dendritic cell
DLQI	Dermatological Life Quality Index
ERK	Extracellular signal–Regulated Kinases
FC	Fold Change
FlowSOM	Flow Self-Organizing Maps
HC	Healthy Controls

IC	Internal Control
IFN	Interferon
IL	Interleukin
LL37	Cathelicidin
MC	Monocyte
MSI	Median Signal Intensity
MST	Minimum Spanning Tree
MTX	Methotrexate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cell
NKT	Natural Killer T cell
PASI	Psoriasis Area and Severity Index
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Death-1 receptor
pSTAT	Phosphorylated Signal Transducer and Activator of Transcription
ROS	Reactive oxygen species
RT	Room temperature

SPSS	Statistical Package for the Social Sciences
STAT	Signal Transducer and Activator of Transcription
Tbet	T-box expressed in T cell
Tc	Cytotoxic T cell
Th	Helper T cell
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
T1, T2, T3	Timepoint 1, 2, 3

Introduction

Immunopathogenesis of psoriasis reaches beyond skin lesions [1-5]. Crosstalk between innate and adaptive immune system leads to self-sustaining inflammatory loops with keratinocytes, dendritic cells, T cells and the TNF/IL-23/IL-17 axis playing central roles [6-9]. Autoreactive cytotoxic T (Tc) cells and autoantibodies against LL37 are involved in initiation of plaques [7, 10, 11]. Th1 cells are recruited in initial phases, while Th17 and Th22 cells predominate in chronic plaques [6]. Epithelial Th22 and Tc17 cells constitute disease memory and the Th9 subset exert a role in induction and maintenance of inflammation [8, 12, 13]. Comorbidities including cardiovascular disease (CVD) share susceptibility genes and mechanistic links with psoriasis [3, 4, 14-16].

Flow cytometry is traditionally used for phenotyping of peripheral blood mononuclear cells (PBMCs) but is limited by overlapping fluorescence spectra [17-19]. Increasing number of molecules are currently applied to define cell subsets. Mass cytometry has expanded detectable targets, facilitated broader characterization of immune cells, and given new insights to immunopathology of inflammatory diseases [20-25]. Extra- and intracellular molecules are labeled with heavy metal-tagged antibodies before single cells travel through argon plasma, evaporating biological material, leaving only ions to be identified depending on time of flight as a function of atomic mass [20]. Since mass cytometry can be applied on blood and tissue, it is particularly advantageous in dermatological research [26].

Immunoprofiling enables individualized therapeutic strategies [27, 28]. Targeting systemic inflammation might alter the course of psoriasis with positive implications on comorbidities [14, 29].The purpose of this study was to characterize PBMC subsets by use of mass cytometry, in the search for immune signatures and systemic biomarkers related to psoriasis severity and treatment effect.

Results

Psoriasis and life quality improve with biological treatment

At inclusion patients had severe psoriasis with reduced quality of life. Psoriasis Area and Severity Index (PASI) and Dermatological Life Quality Index (DLQI) were significantly improved at follow-up (Table S1). 15 of 32 patients reached PASI75 (responders), while the 17 non-responders improved moderately (< PASI75) (Table S2).

Abundance of pathogenic PBMC subsets normalizes with biological treatment

The frequency of Th2 cells was significantly lower at inclusion than at follow-up, and Th1 cells tended to decline to level of HC during treatment (Figure 1). The fraction of Th9 and Th22 cells was higher in patients at inclusion compared to 1-year follow-up (Figure 2). However, patients tended to have reduced levels of Tregs at inclusion and 4 months, but after 1 year the level had normalized.

At inclusion, the number of Th cells expressing CD45RA was significantly higher and Th cell expressing CD45RO lower compared to follow-up time points (Figure S1). Further, patients at inclusion had a higher frequency of memory (CCR4+) CD8 positive cells (CD8+) than HC. Also, CD45RA+ $CCR4^{low}$ Tregs were more and CD45RO+ $CCR4^{high}$ Tregs less frequent at inclusion than at follow-up**.** The frequencies of classical and intermediate monocytes (MC) decreased during the first 4 months of treatment but reached level of HC after 1 year (Figure S1). In order to exclude a possible influence of the BMI we investigated the different cell subset frequencies in the patients with the highest and the ones with lowest BMI at initiation. No typical patterns were seen. In addition, the BMI of the patients changed very little during the study in most of the patients (30/32). The remaining 2 had an increase of 7-10 units in BMI, and both showed an increase in Treg frequencies.

Expression of inhibitory PD-1 on T cells is influenced by biological treatment

At inclusion, PD-1 expression on Th2 cells was higher and on CD8 cells lower in patients compared to HC (Figure S2). After 1 year, the expression of PD-1 on Th cells and Tregs in patients had increased.

Intracellular phosphorylation of PBMCs normalizes during biological treatment

At inclusion patients had higher intracellular phosphorylation of p38 in Th2 cells and Tregs, ERK in CD4 cells and STAT1 in classical MC than HC (Figure S3). The phosphorylation was still higher at both follow-up time points for Th2 cells and after 1 year for ERK in CD4 cells, implying improvement, but not complete normalization during biological treatment. Increased intracellular phosphorylation is exemplified with one patient at inclusion compared to one HC in Figure S4.

Basal intracellular phosphorylation of STAT1 and p38 decreased in classical MC from patients during the first 4 months of treatment and the level of pSTAT1 in Th17 cells was reduced after 1 year (Figure S5).

Stratification of responders and non-responders

Responders had a negative correlation between PASI improvement and FC of Th17 cell frequency after 4 months (T2/T1) ($p \leq 0.04$, rho -0.535), and FC of CD8+ cells after 1 year (T3/T1) ($p \leq 0.04$, rho -0.545) (data not shown).

Non-responders had a decrease of NK and NKT-like cells after 4 months relative to inclusion (Figure S6). Further, CD45RA/RO ratio in Tregs at inclusion was lower in responders compared to

non-responders. Responders had higher FC of PD-1 on CD4 cells after 4 months and on NK cells after 1 year.

The expression of the epithelial-homing marker CCR10 on B cells was higher in patients than HC at inclusion (Figure S6). Interestingly, responders had higher FC of epithelial-homing CCR10 on Th, CD8+ and B cells after 4 months and on Th17 and NK cells after 1 year of treatment compared to non-responders (Figure 3).

At inclusion non-responders had higher pSTAT1 in Th17 cells, pp38 in classical MC, and pp38 and pNF-κB in intermediate MC than responders (Figure 4). Responders had higher FC of pNF-κB, pSTAT1 and pp38 in non-classical MC (increased activation) and lower FC of pSTAT3 in intermediate MC (reduced activation) compared to non-responders after 1 year (Figure S7).

Discussion

Exploiting mass cytometry, this study gives a novel, in-depth characterization of multiple blood immune cell subsets in psoriasis. The detected shift in Th1-Th2 balance and naïve/effector-memory predominance during treatment is in agreement with earlier reports [4, 7, 30-34]. The elevated levels of circulating Th9 and Th22 associated with active disease further supplements the current view on psoriasis pathogenesis. Increased level of CD8+ cells in active psoriasis, including memory CD8 (CCR4+) cells, is also confirmed by this study [24, 35-37]. Even though the Tc17 phenotype (CD8+CCR6+CD161+) is of pathogenic interest in psoriasis [7, 38-40], we did not detect significant differences between groups for this subset. However, reduction in CD8+ and Th17 subsets correlated with improvement of PASI. In light of the recently discovered autoantibodies against LL37, the higher expression of epithelial-homing CCR10 on B cells in psoriasis is especially interesting [10, 41]. The results also indicate that after response to biological treatment, there is a decline in recruitment of inflammatory cells to the skin.

In psoriasis and coronary artery disease, Tregs are reduced in number with deficient suppressor activity. In psoriasis, this leads to insufficient peripheral tolerance against autoreactive T cells [15, 30, 42]. In addition, differentiation into IL-17 producing Tregs contribute to chronic inflammation [42-44]. At inclusion, our patients had a reduced fraction of Tregs, however, after 1 year with biological treatment, the level was normalized. Interestingly, responders had relatively more memory than naive/effector Tregs initially than non-responders, implying that prospective stratification of treatment response might be possible.

PD-1, a co-inhibitory molecule important for immune response silencing, is downregulated in psoriasis, resulting in immune overstimulation [45]. Blockade of PD-1 augments Th1 and Th17, but suppresses Th2 responses [46]. Our patients had reduced expression of PD-1 on CD8+ cells at inclusion, however, the opposite was detected for Th2 cells. After 1 year on biological therapy, the expression of PD-1 on Th cells and Tregs was increased. Further, responders had significantly larger increase of PD-1 on Th and NK cells at follow-up than non-responders. PD-1 constitutes a potential treatment target in psoriasis.

Upon cell activation, intracellular molecules are phosphorylated to propagate signals [6, 47]. Patients with severe psoriasis had elevated levels of intracellular phosphorylation in Th2 cells and Tregs, in addition to classical monocytes. Further, intracellular phosphorylation was higher in CD4+ and Th2 cells after months on biological treatment, but with a decreasing trend. This reduction, but not complete normalization, might be related to genetic predisposition, underlining the chronicity of psoriasis [6, 12, 35, 48, 49]. Studies of inflammatory diseases have shown that phosphorylation in PBMCs decreases with treatment [24, 30, 50]. Interestingly, a recent study showed increased ROS production in activated memory CD4+ T cells [51]. Our patients had a significant reduction of phosphorylation in Th17 cells after 1 year. Further, we detected higher intracellular phosphorylation in Th17 cells, classical and intermediate monocytes from non-responders than responders at

inclusion, indicating that stratification based on immune-profile may predict future treatment response.

Cardiovascular risk is linked to psoriasis by mechanistic pathways [15, 29, 52]. It is hypothesized that recirculating T cells lead to amplification of inflammation and contribute to comorbidities [13, 53]. Th1 and Th17 cells produce inflammatory mediators attracting monocytes, neutrophils and Tc cells [15]. Monocytes can be polarized towards pro-atherogenic phenotype by skin-inflammation, and recruitment to vessel walls is an early event in atherosclerosis [54, 55]. Elevated levels of classical and intermediate monocytes have been shown in CVD [56, 57]. Our psoriasis patients had a tendency of lower level of classical monocytes with higher intracellular phosphorylation than HC during the first 4 months of follow-up, possibly representing recruitment to vessel walls [58]. After 1 year, the frequency of classical monocytes was normalized. In addition, patients at inclusion had elevated levels of intermediate monocytes, which decreased during the first 4 months of treatment.

Non-classical MC, however, have been postulated to exert an atheroprotective effect [58]. Responders had higher intracellular phosphorylation in non-classical MC after 1 year than non-responders. The distribution of subtypes in the monocyte compartment indicates that systemic treatment of psoriasis may lower CVD risk [59].

This study is unique in exploring many PBMC subsets simultaneously in psoriasis, however, more markers could have been included. Antibodies sensitive to fixation were added before barcoding, increasing staining variability [60]. Live barcoding might interfere less with sensitive epitopes [61, 62]. Patients not naïve to biological treatment underwent recommended wash-out period before inclusion, however, some used MTX. Despite being the largest mass cytometry study on psoriasis, sample size was relatively limited. Because of the small groups of comorbidities and

different treatments given to individual patients, it was not possible to evaluate the effect of comorbidities or its treatment on the cell composition.

Conclusions

Mass cytometry enables a comprehensive description of systemic inflammatory cells and can be used for biomarker discovery linked to disease severity and treatment effect. This study simultaneously detects shifts in the balance and activation of PBMC subsets important for the pathogenesis of psoriasis and cardiovascular disease. Immune-profiling is a promising avenue for patient stratification and personalized treatment that may alter the chronic course of psoriasis with positive implications on comorbidities.

Methods

Characteristics of patients and healthy controls

32 patients with severe psoriasis vulgaris were included at the Department of Dermatology, Haukeland University Hospital, Norway. They were prescribed infliximab (anti-TNF-α antibody), ustekinumab (anti-IL12/23 antibody), secukinumab (anti-IL17A antibody) or etanercept (TNF-receptor blocker), eight in each group. Blood samples and clinical data were collected before initiation of biological therapy, after 4 and 12 months.

10 age, sex and body mass index (BMI) matched healthy controls (HC) without psoriasis were included from the Blood bank at Haukeland University Hospital. The study was approved by the regional ethics committee (approvals 2014/1373 and 2014/1489). Written informed consent was obtained from all participants. Demographic characteristics are summarized in Table S3.

Blood sampling

Blood was collected in Lithium-heparin tubes (Becton Dickinson Ltd., UK) and PBMCs were cryopreserved in liquid nitrogen following density gradient centrifugation as described previously [63]. The study encompassed 120 samples (96 patient samples, 18 HC samples and 6 internal control samples (IC)), divided on six 20-plex barcodes (BC). The ICs were PBMCs from the same donor and included in every BC. A schematic of the workflow is presented in Figure 5.

Sample preparation and viability staining

Cells were thawed and washed in serum-free medium X-VIVO-20™ (Lonza, Switzerland) containing endonuclease (Pierce™ Universal Nuclease for Cell Lysis; Thermo Fisher Scientific, MA, USA), followed by resuspension in X-VIVO-20™ at room temperature (RT) before 120 min incubation (37°C, 5% CO_2). Equal number of cells from each sample was transferred to a MegaBlock® 96 deep well plate (Sarstedt, Germany). After centrifugation, cells were resuspended in RPMI-1640 (Lonza, Switzerland) before 5 min incubation, 37°C, with Cell-ID™ Cisplatin (Fluidigm, California, USA) at a final concentration of 2.5 μM.

Antibody staining and barcoding

Epitopes sensitive to fixation (CXCR3, CCR4, CCR6 and CD127) were stained on live cells. After wash with X-VIVO-20™, cells were resuspended in Maxpar Cell Staining Buffer (CSB) containing FcR Blocking Reagent (Miltenyi Biotec, Germany) (2 μl per 1 million cells) and incubated 30 min on ice with Antibody Mix 1 (Table S4). Cells were washed twice with CSB followed by fixation with 1 ml Fix I Buffer from Fluidigm (10 min, RT). Barcoding was conducted in accordance with protocol for Cell-ID

20-Plex Pd Barcoding kit. The 20 samples were combined in a 5 ml Polystyrene Round-bottom Tube (BD Biosciences, MA, USA), and cells counted.

For additional surface staining, 9 million barcoded cells were washed and resuspended in CSB followed by incubation with Antibody Mix 2 (Table S4) for 30 min (RT). After two washes and 10 min rest on ice, permeabilization with ice-cold Methanol (Merck, Germany) was performed for 15 min on ice. For intracellular staining, cells were washed twice resuspended in CSB, followed by incubation with Antibody Mix 3 (Table S4) for 30 min on ice. Between additional washes, the samples were fixed in 1.6% Formaldehyde solution (Methanol-free, Pierce™, diluted in PBS, RT, 10 min). Samples were stored over night at 4°C in Cell-ID™ Intercalator–Ir diluted in Maxpar Fix and Perm buffer (final concentration 66.7 nM).

The following day cells were centrifuged and washed prior to resuspension in Maxpar Cell Acquisition Solution (CAS), before final wash. Cells were left pelleted until acquisition. Unless otherwise stated, all products were from Fluidigm (California, USA) and washes done in CSB for 5 min (453g/RT before, 800g/4°C after fixation).

Mass cytometry acquisition, normalization and debarcoding

Prior to acquisition, cells were suspended in 0.5 ml CAS solution with 1:10 concentration of EQ™ Four Element Calibration Beads. Samples were passed through a 35 µm cell-strainer (Falcon®, New York, USA) and analyzed on a Helios™ Mass Cytometer with WB injector (Fluidigm Corporation, California, USA). The resulting FCS files were normalized to beads (140Ce, 151Eu, 153Eu, 165Ho, 175Lu), concatenated per barcode and debarcoded (Fluidigm software) (Figure S8).

Gating, clustering and dimensionality reduction

Clean-up gates for live single cells and elimination of beads were drawn in FlowJo 10.2 (Figure S9). Defining markers for identification of PBMC subpopulations [17-19, 22] are listed in Table 1. Manual gates were tailored per file and population according to gating strategy shown in Figure S10. In addition, FlowSOM analysis including 20.000 events (randomly subsampled) from each sample, was conducted [64]. Adjusted meta-clusters were tailored from FlowSOM clusters by use of Star plots and Minimum Spanning Tree (MST) and visualized after dimensionality reduction in Figure 6.

Statistical analyses

Wilcoxon and Mann-Whitney *U* test were conducted in SPSS v24. PASI75 was set as limit for responders (≥) and non-responders (<). Fold changes (FC) of Median Signal Intensity (MSI) at inclusion relative to corresponding IC was used to compare samples from different barcodes to overcome inter-assay differences. To compare individual variation over time, FC of follow-up samples relative to corresponding inclusion values were used. The relationship between PASI improvement and FC of marker expression was investigated with Spearman's correlation coefficient. A p-value less than 0.05 was considered statistically significant. GraphPad Prism v8.0 and Cytobank Cellmass v7.0 were used for figures.

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Conflict of Interest statement

The authors state no financial or commercial conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Table 1. Defining markers for identification of PBMC subpopulations.

Population	Defining markers
T cells	CD3+CD19-
T helper cells	CD3+CD19-CD4+CD8-
CD8 positive cells	CD3+CD19-CD4-CD8+
B cells	CD19+CD3-
Monocytes	CD3-CD19-CD56-
-Classical	CD14++CD16-
-Non-classical	CD14+CD16++
-Intermediate	CD14++CD16+
NK	CD3-CD19-CD14-CD16+CD56+CD161+
NKT-like	CD3+CD56+
Tfh	CD3+CD19-CD4+CD8-CXCR3-CXCR5+ PD-1+/-ICOS+/-
Treg	CD3+CD19-CD4+CD8-CD25+CD127-
Th1	CD3+CD19-CD4+CD8-CXCR3+CCR4-CCR6-
Th2	CD3+CD19-CD4+CD8-CXCR3-CCR4+CCR6-

Th9	CD3+CD19-CD4+CD8-CCR4-CCR6+
Th22	CD3+CD19-CD4+CD8- CCR4+CCR6+CCR10+CXCR3+/-
Th17	CD3+CD19-CD4+CD8-CXCR3+/-CCR4+/- CCR6+CCR10-CD161+

Figure legends

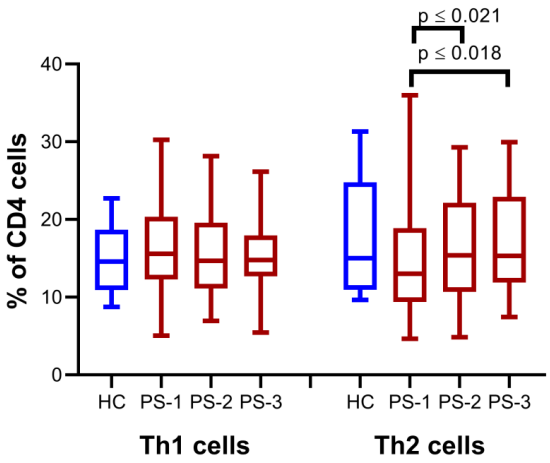


Figure 1. Frequencies of Th1 and Th2 subsets in healthy controls (n=10) and psoriasis patients (n=32) at inclusion and follow-up during biological treatment. Percentage out of CD4 population is displayed**.** Box and whisker plots show quartiles with median, ends of whiskers represent min-max.

HC: healthy controls. PS-1: inclusion. PS-2: after 4 months and PS-3: after 1 year on biological therapy. Wilcoxon test was used to compare abundance of subsets of PBMCs at different time points for patients. Significant differences were defined as $p \leq 0.05$.

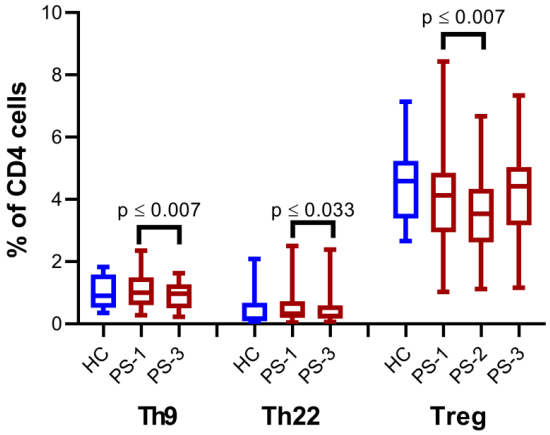


Figure 2. Frequencies of Th9, Th22 and Treg subsets in healthy controls (n=10) and psoriasis patients (n=32) at inclusion and follow-up during biological treatment. Percentage out of CD4 population is displayed. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. HC: healthy controls. PS-1: inclusion. PS-2: after 4 months and PS-3: after 1 year on biological therapy: PS-2 and after 1 year: PS-3. Wilcoxon test was used to compare abundance of subsets of PBMCs at different time points for patients. Significant differences were defined as $p \leq 0.05$.

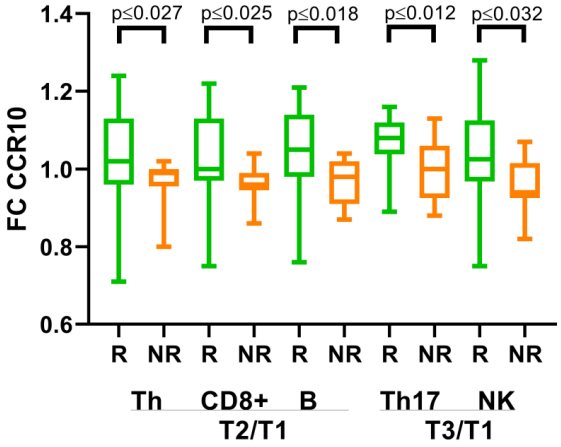


Figure 3. Change of CCR10 expression on PBMCs during biological treatment is compared for responders (R, n=15) and non-responders (NR, n=17). Box and whisker plots show quartiles with median, ends of whiskers represent min-max. Th: T helper cells. CD8+: CD8 positive cells. B: B cells. NK: Natural killer cells. T1: inclusion. T2: after 4 months and T3: after 1 year on biological therapy. Mann-Whitney test was used to compare expression on PBMCs for responders and non-responders. Significant differences were defined as $p \leq 0.05$.

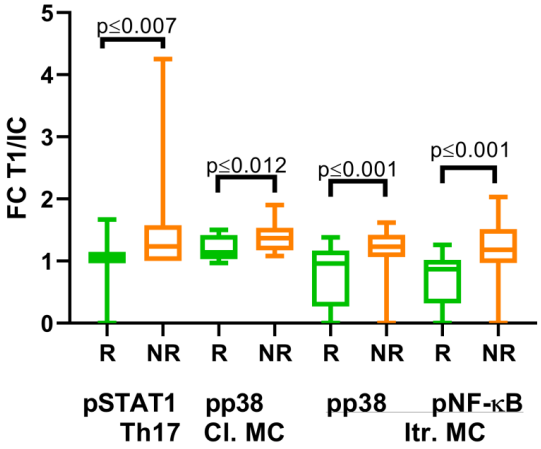


Figure 4. Intracellular phosphorylation at inclusion in Th17 cells, classical and intermediate monocytes is compared for responders (R, n=15) and non-responders (NR, n=17) to biological treatment. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. FC: Fold change. T1: inclusion. IC: internal control. Cl. MC: Classical monocytes. Itr. MC: Intermediate monocytes. Mann-Whitney test was used to compare expression on PBMCs for responders and non-responders. Significant differences were defined as $p \leq 0.05$.

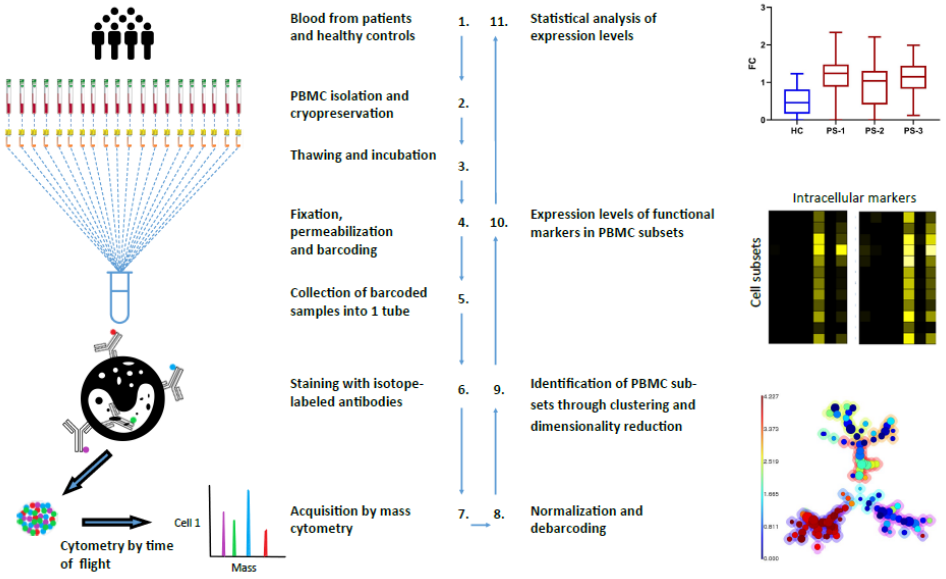


Figure 5. Schematic representation of the workflow. PBMCs from 10 healthy controls and 32 psoriasis patients before and after onset of biological therapy (4 and 12 months) were isolated and cryopreserved. Due to practical reasons, 20 samples were thawed together in six consequtive experiments, each including 19 PBMC samples (mix of patients and healthy controls) and one internal control. For each experiment, samples went through thawing, fixation, permeabilization, barcoding and staining with isotope-labeled antibodies. Acquisition was performed on a Helios mass cytometer, followed by normalization and debarcoding before data from all six experiments (120 samples) were pooled. Identification of PBMC subsets from all 120 samples was done using the FlowSOM and viSNE algorithms. Finally, the median signalling intensities of extracellular and intracellular markers were analysed, comparing patients and healthy controls as well as the different treatments and timepoints.

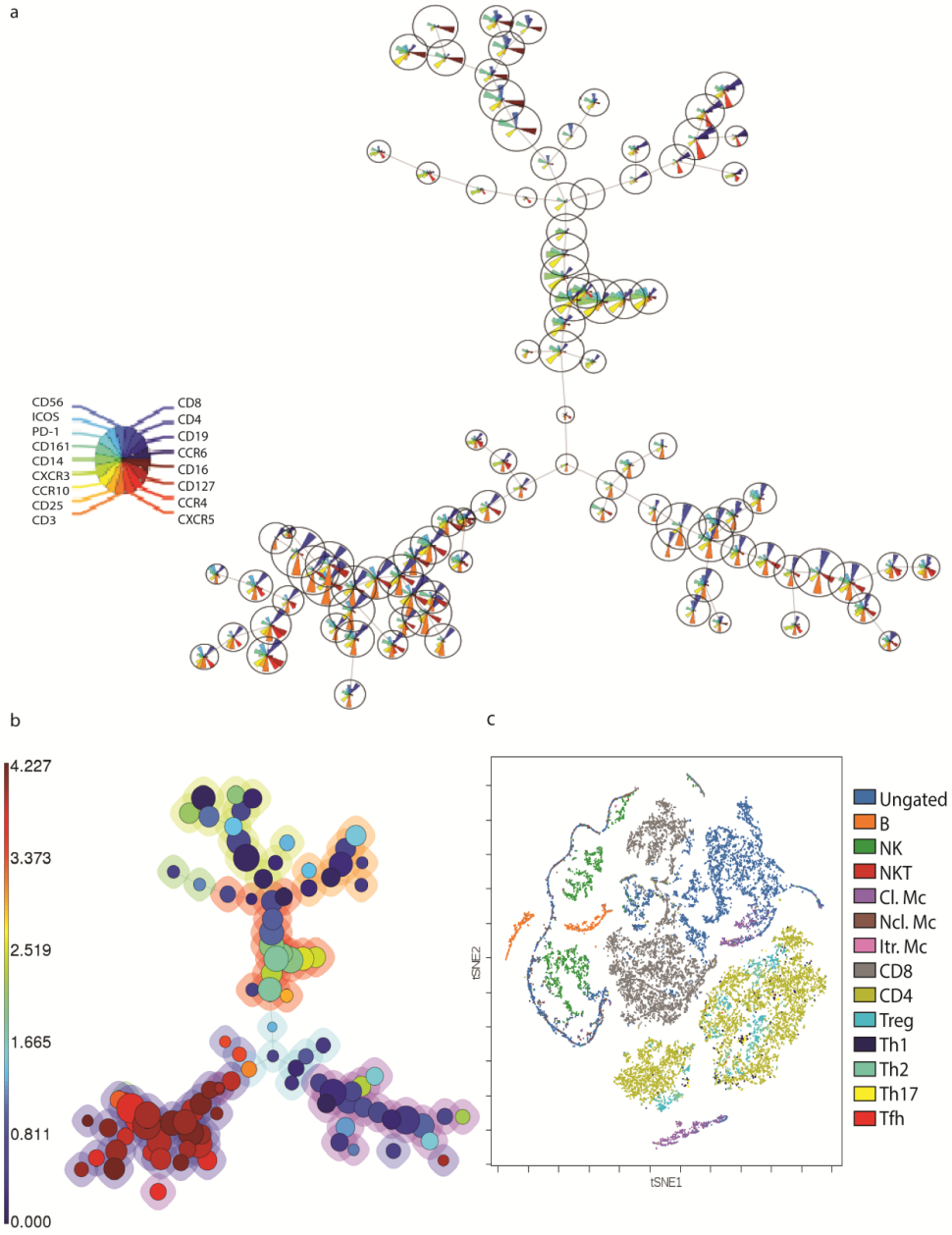
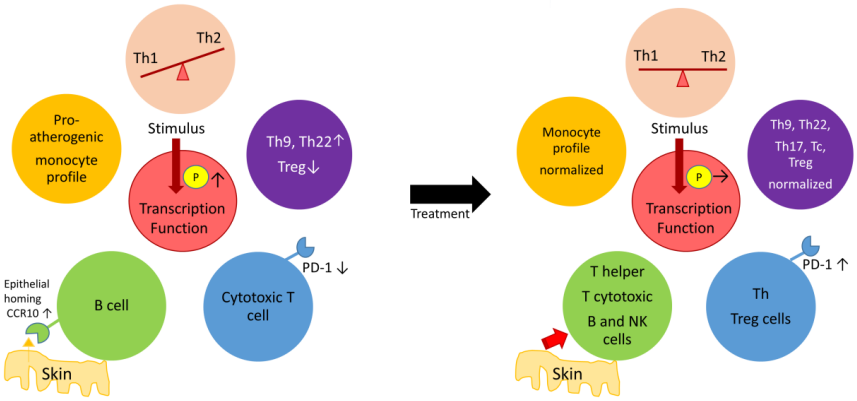


Figure 6. Clustering and dimensionality reduction methods used in analysis. a: Star plot generated from FlowSOM. Mean signal intensity of all clustering markers for cells in each cluster is shown. Height of tile corresponds to intensity. Clusters have relative size. **b:** Channel colored Minimum Spanning Tree displaying clusters from FlowSOM analysis, exemplified with CD4 expression on cells; red indicates high expression, and color specter down to blue indicates progressively lower expression. **c:** Colored cluster overlay showing the 13 tailored meta-clusters in FlowSOM based on clinically relevant PBMC subsets.

Graphical abstract



Patients with severe psoriasis have alterations in peripheral immune cells that are restored upon biological treatment, indicating amelioration of systemic inflammation after biological treatment.

Online Repository Material

Table S1. Clinical data for patients (n=32): PASI and DLQI at inclusion, 4 and 12 months after starting biological treatment, mean ± S.E.

	PASI incl.		PASI 4M		PASI 12M		DLQI incl.		DLQI 4M		DLQI 12M	
Infliximab	11.7	±1.1	2.2	±0.6	1.4	±0.5	15.3	±2.3	1.5	±0.5	1.9	±0.5
Ustekinumab	11.1	±3.4	4.3	±1.8	2.9	±1.1	10.0	±2.2	3.1	±0.8	2.5	±0.8
Secukinumab	8.0	±1.8	1.9	±0.5	1.9	±0.4	13.3	±3.1	4.6	±1.8	4.1	±2.0
Etanercept	9.5	±1.5	3.5	±0.8	2.9	±0.7	16.0	±1.6	4.9	±1.3	3.8	±1.3
All patients	10.1	2.0	3.0*	0.9	2.3*	0.7	13.7	2.3	3.5*	1.1	3.1*	1.2

*= $p \leq 0.001$

4M= 4 months, 12M= 12 months

Table S2. Improvement of PASI for non-responders (NR, n=17) and responders (R, n=15) after 4 and 12 months on biological therapy. Mean and min-max are listed.

		% PASI improvement	Min-max
4 months	NR	52	43-62
	R	90	84-95
12 months	NR	57	46-67
	R	92	89-96

Table S3. Characteristics of patients and controls: sex, age, weight, body mass index (BMI), psoriatic arthritis (PsA) and methotrexate (MTX) use; values are listed as min-max and mean.

	Patients (n=32)	Controls (n=10)
Sex	24 ♂ 8 ♀	7 ♂ 3 ♀
Age, years	18-74 (43)	29-58 (41)
Weight, kg	61-133 (96)	65-122 (94)
BMI	21-42 (30)	23-38 (30)
PsA	13	0
MTX (dose)	12/32 (12 mg/week)	0/10

Table S4. Antibody panel for mass cytometry with target, antibody clone and conjugated metal listed.

	Target	Clone	Metal tag
Antibody Mix 1	CCR4	L291H4	175Lu
	CXCR3	G025H7	163Dy
	CCR6	G034E3	141Pr
	CD127	A019D5	176Yb
Antibody Mix 2	CD8a	RPA-T8	146Nd
	CD19	HIB19	142Nd
	CD45RA	HI100	143Nd
	CD45RO	UCHL1	165Ho
	CD3	UCHT1	170Er
	CD4	RPA-T4	145Nd
	PD-1	EH12 2 H7	155Gd
	CD14	M5E2	160Gd
	CXCR5	RF8B2	171Yb
	CD25	2A3	169Tm
	ICOS	C398.4A	151Eu
	CCR10	314305	164Dy
	CD161	HP-3G10	159Tb
	CD16	3G8	209Bi
	CD56	NCAM16.2	149Sm
Antibody Mix 3	pNFkB	K10x	166Er
	pp38	D3F9	156Gd
	pERK	D1314.4E	167Er
	pSTAT1(Y701)	58DG	153Eu
	pSTAT3(Y705)	4/PStat3	158Gd
	pSTAT4 (Y693)	38/p-Stat4	174Yb
	pSTAT5 (Y694)	47	147Sm

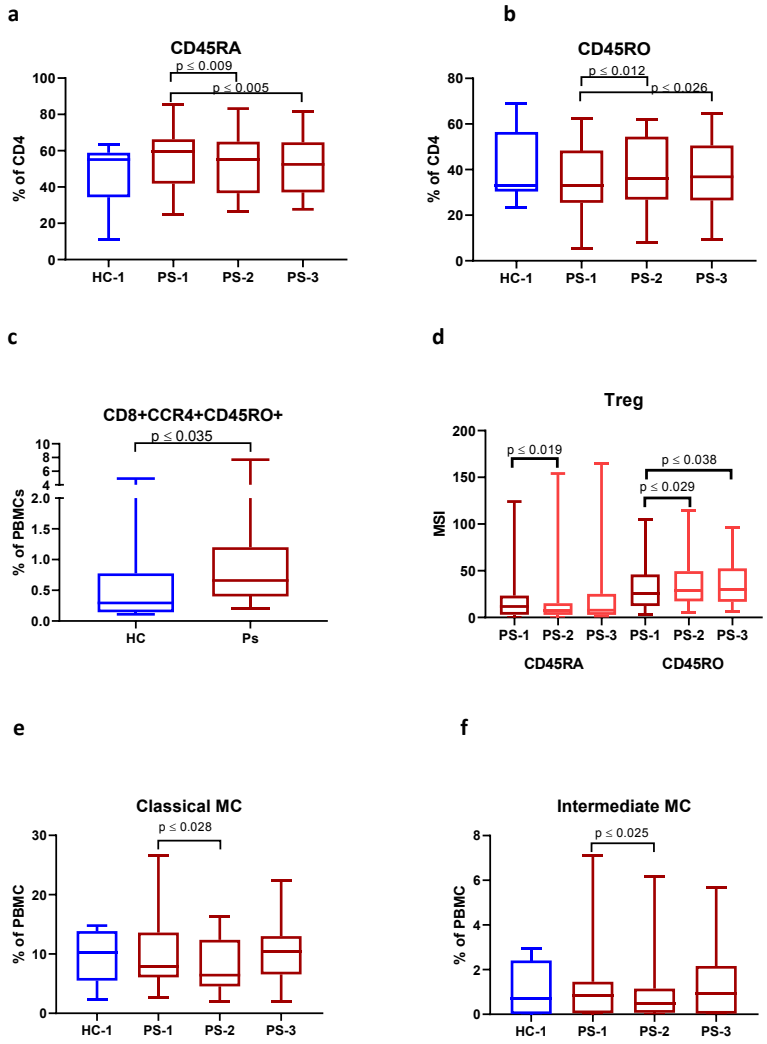


Figure S1. Frequencies of immune cell subsets in healthy controls (n=10) and psoriasis patients (n=32) at inclusion and during biological treatment. a and **b**: Percentage of CD4 cells expressing CD45RA (**a**) and CD45RO (**b**). **c:** Frequency of memory CD8 (CCR4+) cells as % of PBMCs in healthy controls and psoriasis patients at inclusion. **d:** Treg expression of CD45RA and CD45RO in patients at inclusion and follow-up. **e** and **f**: Frequency of classical (**e**) and intermediate (f) monocytes (MC) in total PBMCs. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. HC: healthy controls. Psoriasis patients at inclusion: PS-1, after 4 months on biological therapy: PS-2 and after 1 year: PS-3.

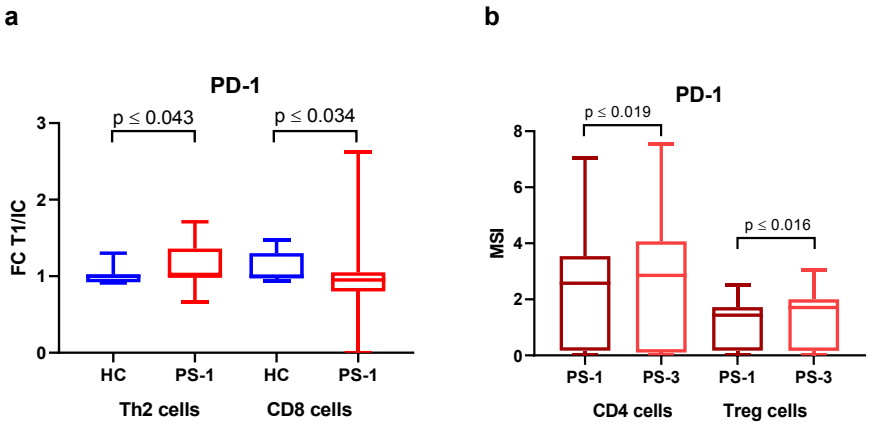


Figure S2. PD-1 expression on T cells. a: Differences between healthy controls (n=10) and psoriasis patients (n=32) regarding PD-1 expression on Th2 and CD8 cells at inclusion. **b:** Change in PD-1 expression on CD4 cells and Tregs from patients at inclusion and after 1 year of biological treatment. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. HC: healthy controls. PS-1: psoriasis patients at inclusion. PS-3: psoriasis patients at 12 months follow-up. FC T1/IC: fold change timepoint 1/internal control. MSI: median signal intensity.

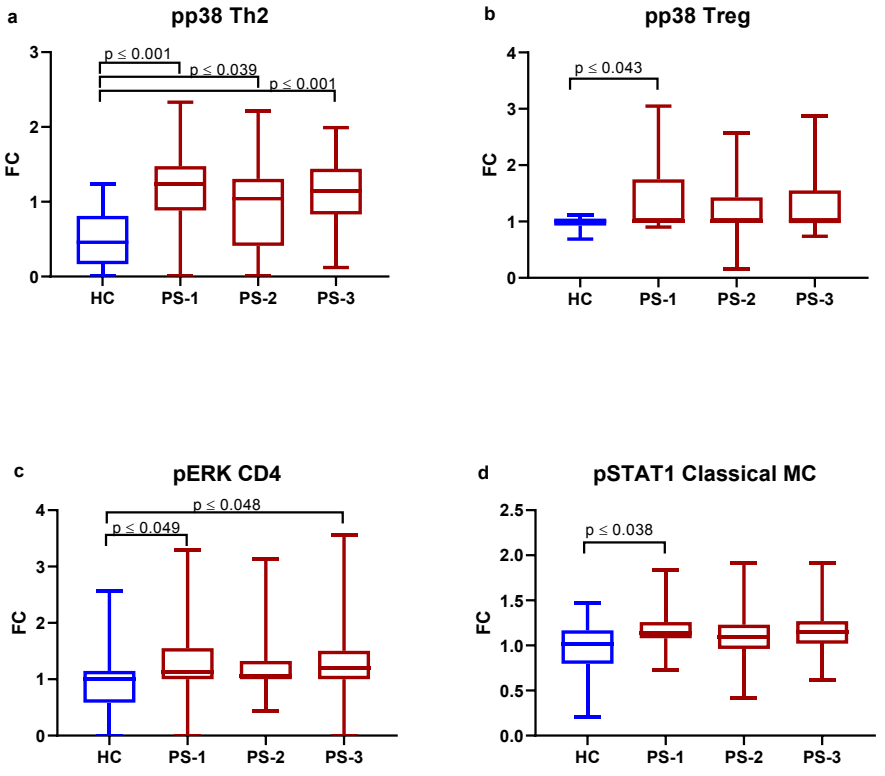


Figure S3. Comparison of intracellular phosphorylation in PBMCs from healthy controls (HC, n=10) and psoriasis patients (PS, n=32). Values are displayed as fold change (FC) of median signal intensity (MSI) from HC or PS normalized against MSI from corresponding internal control. **a:** pp38 in Th2 cells; **b:** pp38 in Treg cells; **c:** pERK in CD4 cells; **d:** pSTAT1 in classical monocytes. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. Psoriasis patients at inclusion: PS-1, after 4 months on biological therapy: PS-2 and after 1 year: PS-3.

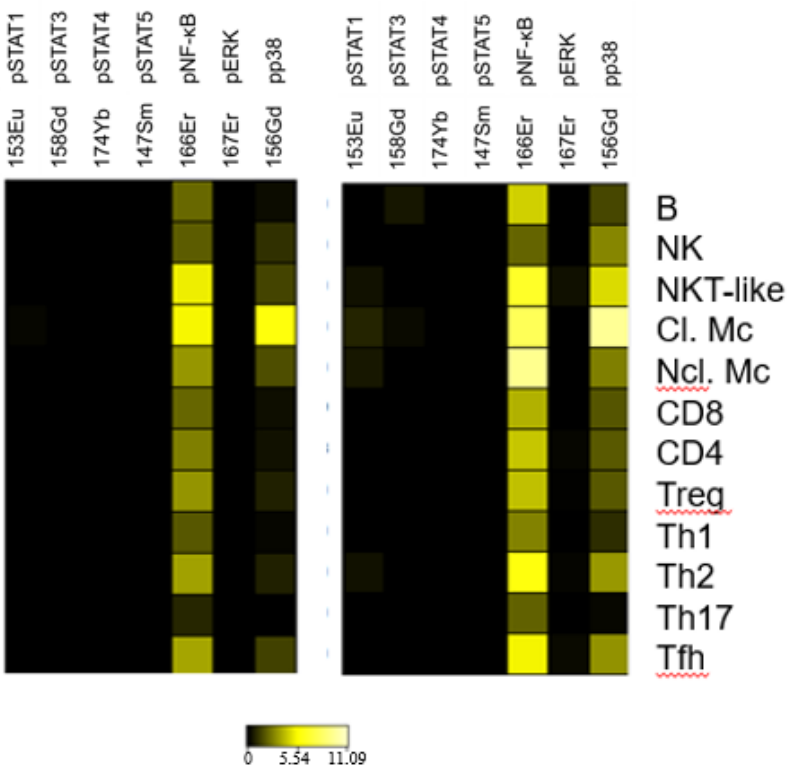


Figure S4. Heatmap of basal level of intracellular phosphomarkers, exemplified with one healthy control (left) and one patient (right). Cl. Mc: classical monocytes. Ncl. Mc: non-classical monocytes. Tfh: T follicular helper cells.

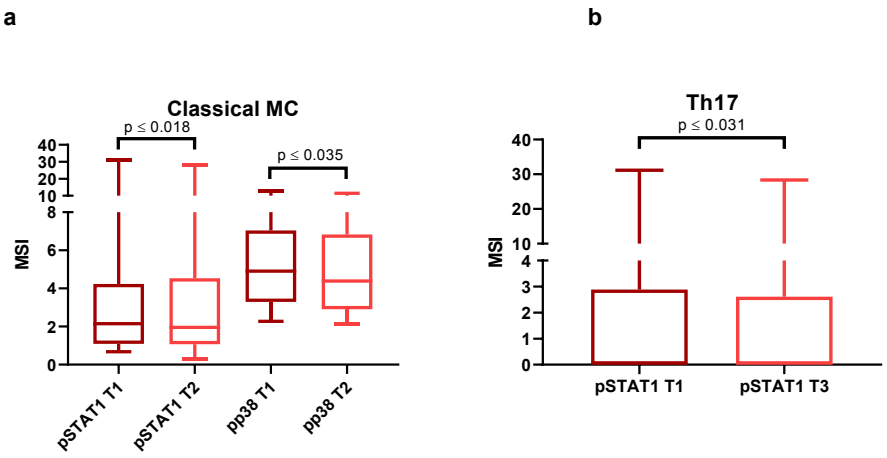


Figure S5. Intracellular phosphorylation in PBMCs from psoriasis patients (n=32) during biological treatment. a: Phosphorylation of STAT1 and p38 in classical monocytes. **b:** Phosphorylation of STAT1 in Th17 cells. T1: inclusion. T2: after 4 months, and T3: after 1 year of biological therapy.

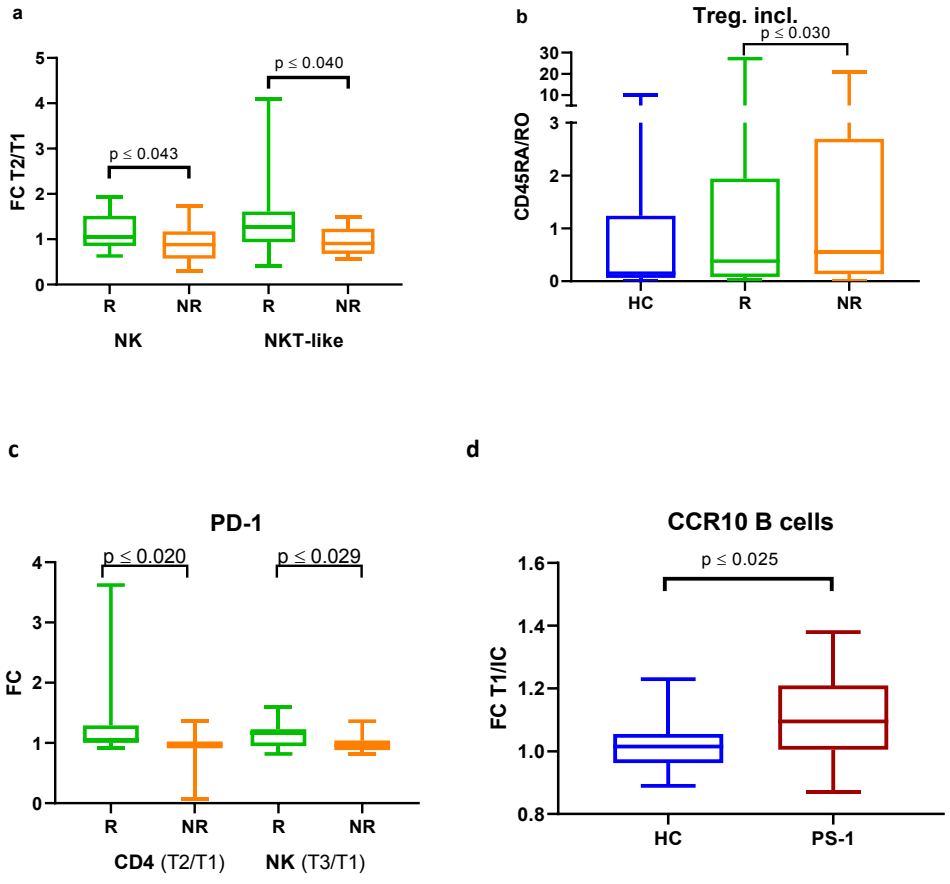


Figure S6. Frequencies of immune cell subsets in responders (n=15) and non-responders (n=17) to biological therapy and of epithelial-homing B cells between healthy controls (n=10) and psoriasis patients (n=32). a: Fold change (FC) after 4 months of NK and NKT-like cell frequencies after 4 months in responders (R) and non-responders (NR). **b**: Ratio of CD45RA/RO expression in Tregs at inclusion for healthy controls (HC), R and NR. **c**: Change in PD-1 expression for R and NR displayed as FC for CD4 cells after 4 months and for NK cells after 1 year. **d:** Expression of CCR10 on B cells at inclusion for HC and patients. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. T1: inclusion. T2: 4 months follow-up. T3: 1 year follow-up. IC: internal control.

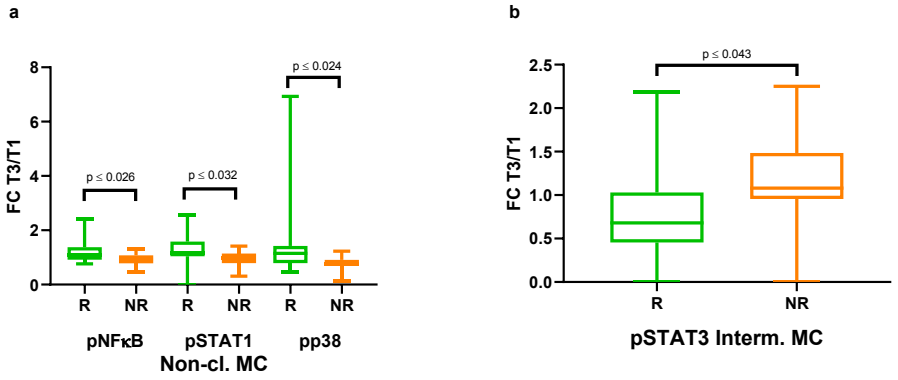


Figure S7. Change in intracellular phosphorylation of monocytes after 1 year on biological treatment for responders (R, n=15) and non-responders (NR, n=17). a: Change in phosphorylation level of NF-κB, STAT1 and p38 in non-classical monocytes (Non-cl. MC). **b:** Change in phosphorylation level of STAT3 in intermediate monocytes (Interm. MC). Box and whisker plots show quartiles with median, ends of whiskers represent min-max. T1: inclusion. T3: 1 year follow-up.

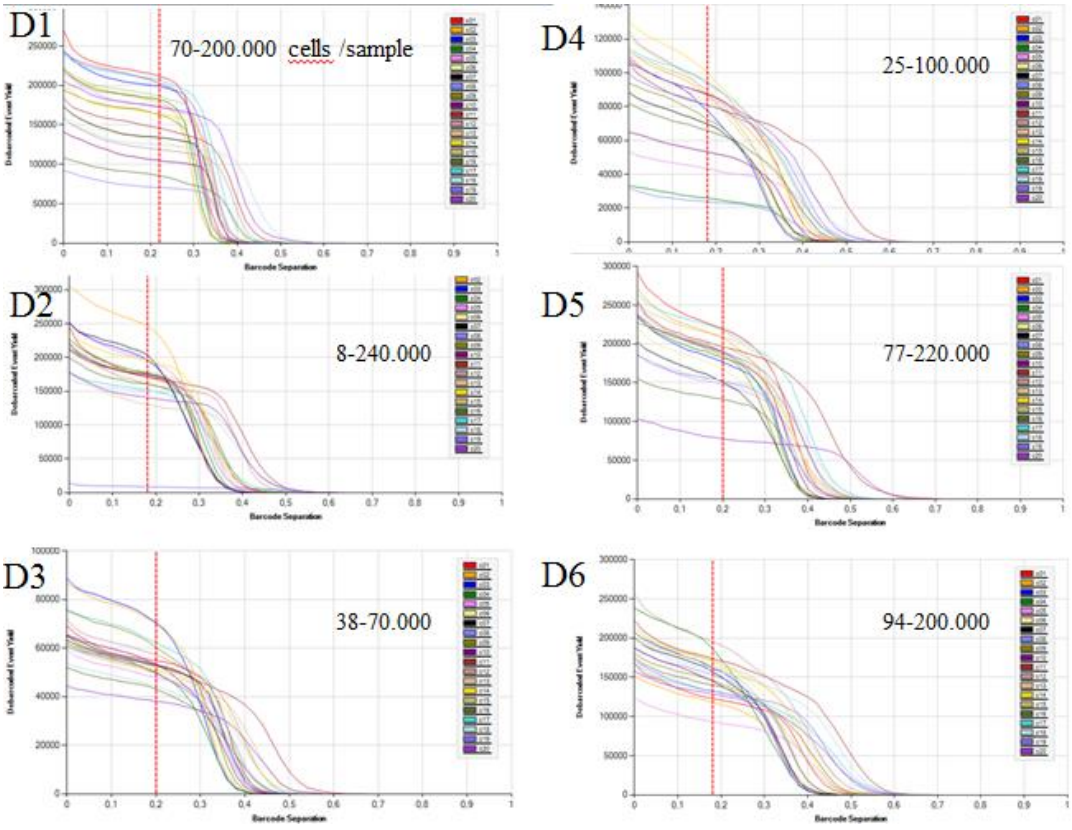


Figure S8: Debarcoded samples showing event yield for each of the 120 samples distributed on six barcodes. D1: day 1 of experiment, containing 20 samples in the first barcode. D2: day 2 of experiment, containing the next 20 samples in the second barcode. Likewise for D3, D4, D5 and D6.

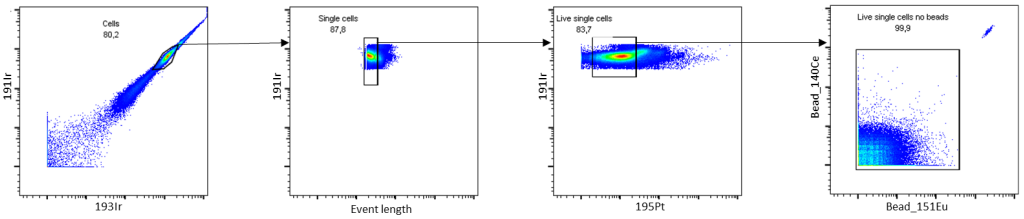


Figure S9: Example of clean-up gates made in FlowJo. The gates were tailored per file to identify live single cells 191Ir-193Ir (DNA1-DNA2); 191Ir-Event length; 191Ir-195Pt (cisplatin) and eliminate beads (140Ce-151Eu).

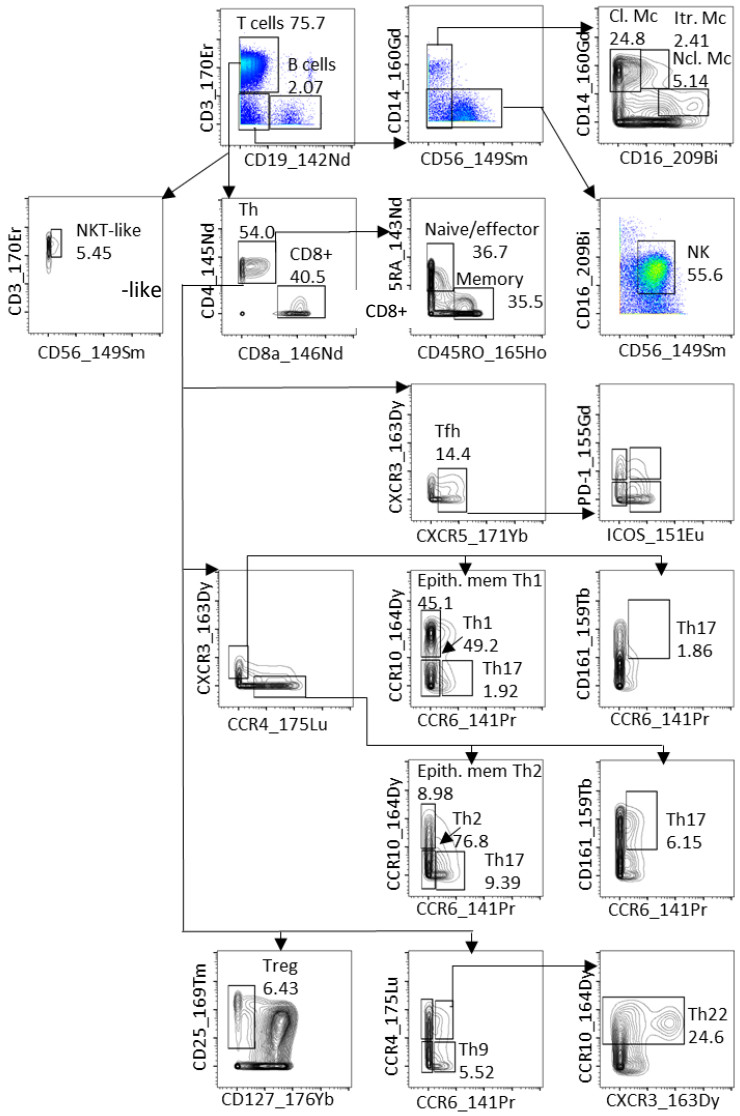


Figure S10: Representative gating strategy from FlowJo for cell populations identified by the panel consisting of 19 surface markers. Cl. Mc: classical monocytes, Itr. Mc: intermediate monocytes, Ncl. Mc: non-classical monocytes. Epith. mem.: epithelial memory cells. Tfh: T follicular helper cells.

Errata for
"Single cell signalling and immune cell profiling in psoriasis"

"Anders Krogh Aarebrot"

Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

25/1-21 _____

(date and sign. of candidate)

25/1 21 _____

(date and sign. of faculty)

Errata

Page 48-49: Duplication of "For study II, single-cell data was clustered using the *FlowSOM* R package and labeled using the Ek'Balam algorithm. Cell subset definitions followed Maecker et al., and Finak et al. **(Table 2)**, with the addition of mDCs. Cluster labeling, method implementation, and visualisation were done through the Astrolabe Cytometry Platform (Astrolabe Diagnostics, Inc.). Differential abundance analysis was done using the *edgeR* R package following the method outlined by Lun et al.. Differential expression analysis was done using the *limma* R package following the method outlined by Weber et al.. Inverse hyperbolic sine (asinh) calculations all used a cofactor of 5. Data from study 1 and 3 were transferred to the statistical package for social science (SPSS) Statistics 24 for statistical analysis. Since flow and mass cytometry generally is not normally distributed, non-parametric tests were used for comparison; Mann-Whitney U test for independent, unpaired data, and Wilcoxon signed-rank test for paired data (between inclusion and follow-up). For correlation analyses in study 3, the strength of correlations revealed by Spearman's rank order test were interpreted according to the recommendation from British Medical Journal (https://www.bmj.com/about-bmj/resources-readers/publications/statistics-square-one/11-correlation-and-regression), with rho 0.00-0.19 regarded as very weak, 0.20-0.39 as weak, 0.40-0.59 as moderate, 0.60-0.79 as strong and 0.80-1.00 as very strong correlation."
Correction: Deleted the duplicate

Page 50 table 2: Misspelling: All "CD45RO+" corrected to "CD45RA-".
Page 50 table 2: Misspelling: All "CD45RO-" corrected to "CD45RA+".

Page 148 table 4: Misspelling: All "CD45RO+" corrected to "CD45RA-".
Page 148 table 4: Misspelling: All "CD45RO-" corrected to "CD45RA+".

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