Systemic inflammation in psoriasis: Circulating immune cells and cytokines

Silje Michelsen Solberg

Thesis for the degree of Philosophiae Doctor (PhD)
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Contents

Scientific environment

This doctoral thesis was conducted at the Broegelmann Research Laboratory, Department of Clinical Science, Faculty of Medicine, University of Bergen, Norway. I have been enrolled at the Bergen Research School in Inflammation.

The flow cytometry and mass cytometry analyses were performed at the Flow Cytometry Core Facility, Department of Clinical Science, University of Bergen, Norway.

The work was carried out under the supervision of Silke Appel, Yenan T. Bryceson and Lene Frøyen Sandvik in the period 2015 to 2019.

I have been affiliated with the Department of Dermatology, Haukeland University Hospital, Norway.

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List of publications

I. *Serum cytokine measurements and biological therapy of psoriasis - Prospects for personalized treatment?*

Solberg SM, Sandvik LF, Eidsheim M, Jonsson R, Bryceson YT, Appel S.

Scandinavian Journal of Immunology. 2018 Dec; 88(6):e12725.

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

Aarebrot AK*, **Solberg SM***, Davies R, Bader LI, Holmes TD, Gavasso S, Bryceson YT, Jonsson R, Sandvik LF, Appel S.

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*contributed equally

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

Solberg SM, Aarebrot AK, Sarkar I, Petrovic A, Sandvik LF, Jonsson R, Bergum B, Bryceson YT, Appel S.

Manuscript

Summary

Psoriasis is a common, chronic inflammatory skin disease associated with arthritis and multiple comorbidities. Autoantigens in the skin elicit a response in cytotoxic T cells, leading to local inflammation and recruitment of Th1 and Th17 cells from the blood. There is a complex immunological interplay between cytokines and cells from the innate and adaptive immune system, creating self-sustaining amplification loops. Increased levels of inflammatory cytokines and cells have been detected in blood from psoriasis patients. This notion, together with mechanistic similarities in establishment of psoriatic and atherosclerotic plaques, probably contributes to the increased prevalence of cardiovascular disease in psoriasis patients, however, this link needs to be further elucidated.

No cure for psoriasis exists, and treatments aim at amelioration of symptoms. If topical treatments or UV-light are not effective enough, systemic medication including methotrexate, ciclosporin, fumarate or acitretin can be tried. Biological drugs specifically targeting the key cytokines TNF, IL-12/23 and IL-17 are available if conventional treatment is insufficient or contraindicated. However, these newer drugs are not accompanied by similarly precise laboratory analyses to aid selection of a specific drug for individual patients. As adverse events and loss of effect can be encountered, the switching from original to cheaper biosimilar drug has been controversial.

The overall aim of this thesis was to study the blood immune system in psoriasis during active inflammation and treatment with biological drugs, in the search for disease specific immune signatures and biomarkers. In Study I, Luminex® Technology was used to investigate if serum cytokine levels could reflect psoriasis activity. In Study II, we compared impact of switching from original TNF inhibitor infliximab to biosimilar CT-P13 in psoriasis patients, both evaluating clinical parameters and effect on peripheral blood cells and their intracellular signalling, measured by phosphoflow cytometry. In Study III, single cell analysis of blood immune subsets, with special emphasis on the T cell lineage and intracellular

signalling, was explored by use of mass cytometry. In all studies, clinical parameters including Psoriasis Area and Severity Index and Dermatological Life Quality Index were incorporated in analyses.

The results indicate that cytokine and single cell analysis of blood can be useful methods for describing the complex systemic immunological picture in psoriasis. In Study I, logistic regression revealed higher risk of having severe psoriasis with increased IL-17A. Increase of IL-2 positively correlated with improvement of PASI and DLQI. Moreover, increase of IL-5, IL-10, IL-12, IL-22 and GM-CSF correlated with treatment effect.

In Study II, intracellular phosphorylation levels in peripheral blood mononuclear cells were increased in psoriasis patients compared to healthy controls. This increased signalling activity decreased during continued treatment with infliximab, but did not completely normalize despite clinical remission. Switching from original to biosimilar infliximab did not affect laboratory findings, like cell abundance and phosphorylation levels, or clinical parameters.

Study III revealed that biological therapy of psoriasis facilitated a shift in the balance of Th1 and Th2 cells in blood, transition from naïve/effector to memory predominance, reduction of circulating Th17, Th22, Th9 and CD8 cells and enhancement of inhibitory PD-1 expression on T cells. In the monocyte compartment, changes in favor of reduced cardiovascular risk were observed. Intracellular phosphorylation of blood immune cells was higher in psoriasis patients compared to healthy controls and in non-responders to treatment compared to responders.

In conclusion, multiple aberrancies in circulating cells and cytokines were detected in patients with severe psoriasis, confirming that systemic inflammation is a trait of psoriasis. Further research can highlight the role of cytokines and peripheral blood mononuclear cells as potential tools for stratification of patients for personalized treatment. Optimized therapeutic strategies might alter the chronic course of psoriasis with positive implications on quality of life and long-term comorbidities.

Abbreviations

ADA	Antidrug antibodies
APC	Antigen Presenting Cell
BC	Barcode
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
CLA	Cutaneous lymphocyte-associated antigen
CRP	C Reactive Protein
CT	Computed Tomography
CTCL	Cutaneous T Cell Lymphoma
CTP-13	Infliximab biosimilar
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
CXCR3	Chemokine receptor type 3
CyA	Ciclosporin
DC	Dendritic cell
DLQI	Dermtological Life Quality Index
DM	Diabetes Mellitus
DMF	Dimethyl Fumarate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia
ERK	Extracellular signal–Regulated Kinases
FC	Fold Change
FDA	Food and Drug Administration
FlowSOM	Flow Self-Organizing Maps
GWAS	Genome-Wide Association Study
HC	Healthy Controls
HL	Hodgkin Lymphoma
HLA	Human Leukocyte Antigen
i.e.	id est
IFN	Interferon
IFX	Infliximab
IL	Interleukin
ILC	Innate Lymphoid Cell
IL-1RA	Interleukin Receptor Antagonist
JAK	Janus Kinase
KC	Keratinocyte
LFA	Lymphocyte Function-associated Antigen
LL37	Cathelicidin
MAPK	Mitogen-Activated Protein Kinase
MC	Monocyte

mDC	Myeloid Dendritic Cell
MFI	Median Fluorescence Intensity
MHC	Major Histocompability Complex
MSI	Median Signal Intensity
MST	Minimum Spanning Tree
MTX	Methotrexate
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer cell
NKT	Natural Killer T cell
NO	Nitric Oxide
PASI	Psoriasis Area and Severity Index
PBMC	Peripheral Blood Mononuclear Cell
pDC	Plasmacytoid Dendritic Cell
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PMT	Photomultiplier
PsA	Psoriasis Arthritis
PSORS	Psoriasis Susceptibility Loci
pSTAT	Phosphorylated Signal Transducer and Activator of Transcription
PUVA	Psoralen and ultraviolet A
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
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
TGF-β	Transforming Growth Factor beta
Th	Helper T cell
TL01	Narrow-band UVB
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
Trm	Tissue-resident memory cell
TYK	Tyrosine Kinase
T1, T2, T3	Timepoint 1, 2, 3
UV	Ultraviolet light

1. Introduction

1.1 Background psoriasis

1.1.1 Historical overview

The word psoriasis originates from the Greek psora– which means "itch" and –iasis which means "condition", *i.e.* “itching condition” [1,2]. The Corpus Hippocraticum aroud 300 B.C. is the first likely reference to psoriasis, and the first indisputable reference was around 50 B.C. in a medical text by A. C. Celsus [3]. Historically there has been a semantic confusion related to psoriasis and lepra [1,4]. Psoriasis vulgaris was described as a distinct entity in 1808 by R. Willan [1,5]. In 2016, the World Health Organization published a global report on psoriasis [6]. In light of the unmet needs related to treatment and management of psoriasis, more research is warranted.

1.1.2 Epidemiology

Psoriasis prevalence ranges from 0.1% to 11.4% in different ethnicities [7-9]. In children, the prevalence is 0.4-2.0% and may present in different forms and localizations than later in life [6,10]. Psoriasis can occur at any age, but the onset has two peaks, early (age 16-22), type I psoriasis, and late (age 57-66), type II psoriasis, with mean around 33 years [6,11-13]. The early form is more often severe with a positive family history compared to the later form [11]. Multiple studies have shown that psoriasis is equally prevalent in both sexes [6,14].

1.1.3 Classification

Psoriasis has been described as an organ-specific autoimmune disease that is triggered by an activated cellular immune system [15-17]. Diagnosis of psoriasis is usually made on clinical findings and biopsy is only used to exclude other diagnoses. About 70-80% have mild disease, but intensity can fluctuate depending on internal and external factors. Spontaneous remission might occur, however, recurrent plaques

often appear in previously affected sites [6,18]. At least five different presentations of psoriasis have been described **(Figure 1)** [6,19].

Psoriasis vulgaris, the chronic plaque type of psoriasis, accounts for around 90% of cases [5,6]. Typical lesions are sharply demarcated erythematous plaques covered by silvery scales (**Figure 1 A-C)** [6]. Plaques are often symmetrical and may remain localized or become generalized over time. Predilection sites are extensor surfaces on elbows and knees, peri-umbilical, peri-anal, and retro-auricular regions. In addition, 75-90% of patients have scalp involvement **(Figure 1 E)** [6,19,20]. Frequently reported symptoms are scaling (92%), itching (72%), erythema (69%), fatigue (27%), swelling (23%), burning (20%) and bleeding (20%) (27). The characteristic tendency of skin injuries to trigger psoriasis lesions is known as the Koebner Phenomenon [21]. Another characteristica, the Auspitz Sign, refers to the pinpoint bleeding that occurs when psoriasis scales are removed.

Erythrodermic psoriasis affects above 90% of the body surface and is rare, but potentially life-threatening due to widespread erythema and exudative exfoliation (**Figure 1 D)**. Triggering factors include withdrawal of systemic glucocorticosteroids, abrupt discontinuation of methotrexate, phototherapy burns or infections [19].

Inverse psoriasis occurs in flexural and intertriginous areas and is usually devoid of scales **(Figure 1 I)** [6,20].

Pustular psoriasis can appear in different forms. Generalised pustular psoriasis, von Zumbusch, is characterized by disseminated, dark erythematous patches with multiple sterile pustules **(Figure 1 J, K)** [6]. The disease is potentially life-threatening. Localized forms of pustular psoriasis include pustulosis palmoplantaris in palms and soles, and acrodermatitis continua suppurativa (of Hallopeau) affecting the tips of fingers and toes **(Figure 1 L, M)**.

Guttate (droplet) psoriasis is often preceded by a streptococcal throat infection in children or young adults. Antigenic similarities between keratinocytes and streptococcal proteins might be the cause **(Figure 1 N-Q)** [22]. Guttate psoriasis

presents as 1-2 cm large red plaques scattered over the body surface. Around 30% of children with guttate psoriasis develop plaque psoriasis later in life [23].

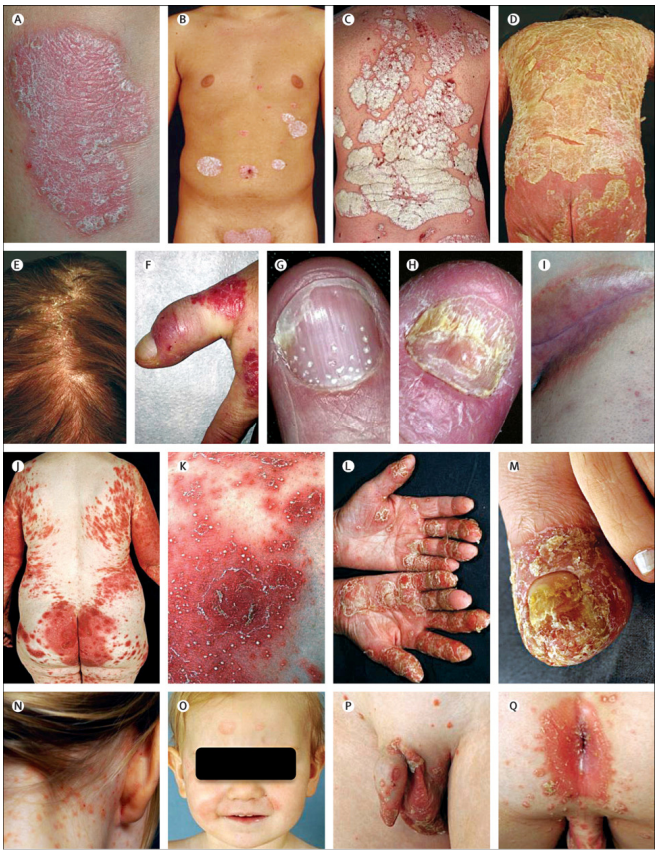

Figure 1. Clinical manifestations of psoriasis. Typical erythematous plaques with silvery scales (A) can be scattered (B, psoriasis nummularis), cover larger areas of the skin (C, psoriasis geographica) or affect the entire body surface (D, erythrodermic psoriasis). Scalp involvement might be accompanied by non-scarring alopecia (E). Psoriatic arthritis affects up to 30% of all patients (F, thumb interphalangeal joint). Nail changes are frequent and range from pitting and yellow or brown discolouration (G) to complete dystrophy (H). Psoriasis inversa occurs in intertriginous areas and is usually devoid of scales (I). Pustular psoriasis might occur in a generalised form (J, K) or localised (L, palmoplantar type and M, acrodermatitis continua suppurativa type). In children, the onset as guttate psoriasis might follow streptococcal infection of the upper respiratory tract (N) and affect any site of the body (O,P,Q).

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Psoriatic arthritis is an inflammatory, seronegative arthritis, which leads to joint deformations, bone erosion and disability in 20-30% of psoriasis patients usually debutating after several years of skin disease **(Figure 1 F)** [24-29]. Peripheral arthritis and dactylitis (profuse swelling of the fingers or toes), spondylitis and enthesitis (inflammation of the sites where tendons insert into the bone) are considered to be the most common symptoms.

Nail psoriasis occurs in 50% of patients at diagnosis with a lifetime prevalence of 80-90% [6]. Psoriatic nail changes include pitting, yellow/brown discolouration, thickening and in severe cases disabeling dystrophy **(Figure 1 G, H)** [6].

1.1.4 Assessment of disease severity

Psoriasis Area and Severity Index **(**PASI**)** is a validated tool that combines the assessment of the severity of lesions and the area affected into a single score ranging from 0 to 72 (maximal disease) [30]. Scores above 10 are usually considered severe disease, scores above 30 are rarely encountered. The body is divided into four sections were head equals 10%, arms 20%, trunk 30%, and legs 40% of a person's skin. For each section, the percent of skin involved is estimated and then transformed into a grade from 0 to 6 (0, < 10, 10–29, 30–49, 50–69, 70–89, 90–100% of involved area). Within each area, the erythema (redness), induration (thickness) and desquamation (scaling) are estimated on a scale from 0-4 (maximum). The sum of these three severity parameters is calculated for each section, multiplied by the area score for that area and by predefined weight of respective section. Response to treatment is usually presented as a percentage response rate; *e.g.* PASI75 [31,32]. Interrater and intrarater variation can affect reproducibility [31].

Dermatological Life Quality Index **(**DLQI**)** is well validated, also in Norwegian patients [33]. The DLQI consists of 10 questions concerning adult patients' perception of the impact of skin diseases on different aspects of their quality of life over the last week. Each question is scored on a four-point scale (0-3) and the sum of all questions ranges from 0-30. The higher the score, the more quality of life is impaired.

Other measures of disease severity are Body surface area (BSA), Physician's Global Assessment (PGA) and Nail Psoriasis Severity Index (NAPSI) [31]. Screening for PsA, depression and metabolic disease should be considered in selected patients [6,34,35].

1.1.5 Triggering factors

In genetically predisposed individuals, external and internal triggers can provoke psoriasis [6]. Skin injury, like mild trauma, sunburn and chemical irritants can induce psoriasis through Koebner phenomenon [6]. Infections, in particular streptococcal throat infection, can be aggravating or initiating factors [36,37]. T cells activated by streptococci migrate to the skin and cross-react with keratin self antigens presenting homology with streptococcal proteins. Tonsillectomy in patients with recurrent tonsillitis can improve the course of psoriasis [38]. Systemic drugs such as β blockers, lithium, antimalarials and non-steroidal anti-inflammatory agents, in addition to withdrawal of systemic steroids, can exacerbate psoriasis [6]. Stress, tobacco smoking and weight gain are other aggravating factors (34)[39]. Weight loss is associated with improvement of PASI in obese patients and obesity is associated with low treatment response [40-43].

1.1.6 Genetics

Genome-wide association studies (GWAS) have identified at least 12 major psoriasis susceptibility loci (PSORS) and near 40 single nucleotide polymorphisms (SNPs) associated with psoriasis [6,44,45]. These include genes related to antigen presentation, kerationocyte proliferation and regulation of the immune system [46].

The allele HLA-Cw6, psoriasis susceptibility locus 1 (PSORS1) is associated with early onset psoriasis and accounts for up to 50% of disease heritability [5,47,48]. Since PSORS1 lies in the MHC class I region, which is important for antigen

presentation to CD8+ T cells, this locus links the genetics with the autoantigens in psoriasis [17,44]. HLA-B57 is another MHC class I genotype associated with psoriasis [49].

Some of the psoriasis susceptibility loci are involved in pathways for keratinization. PSORS4 gene locus is associated with epidermal differentiation pathways S100 proteins and defensins [50].

Other psoriasis susceptibility loci correspond to genes that are associated with the adaptive and innate immune system like T and natural killer (NK) cell differentiation, proliferation and leukocyte adhesion. Genes involved in cytokine responses can have gain or loss of function, exemplified with increased activation for nuclear factor κB (NF-κB) pathway in **Figure 2** [44,46,51-57].

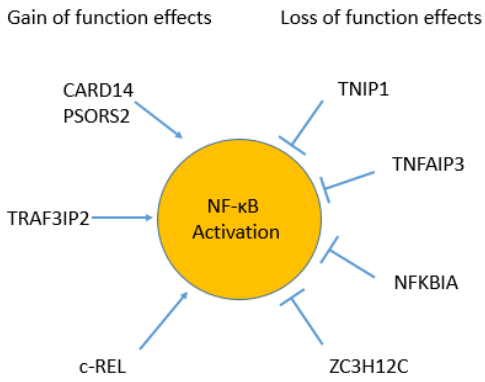

Figure 2. Genetic associations with psoriasis have been found in both components that activate and components that repress the NF-κB pathway. It is likely that gain-of-function mutations in NF-κB activating components (left) and loss-of-function mutations in NF-κB inhibitory components (right) decrease the threshold for immune activation and the subsequent onset of psoriasis.

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Genes related to interferon (IFN) and genes that encode interleukin (IL)-23A, IL-12B and IL-23R, amongst others in the IL-23/IL17 axis are associated with psoriasis [44,58-63]. Genes related to inflammasome, IL-1β, caspase, IL-22 and IL-18 are also associated with psoriasis [64-66]. Also of functional significance in psoriasis are genes involved in the JAK-STAT cascade that encode transcription factors TYK2 and STAT3 [46,49]. TYK2 is involved in signal transduction of IL-12/23 [67]. STAT3 is essential for differentiation of Th17 cells and promotes proliferation in keratinocytes **(Figure 3)** [56].

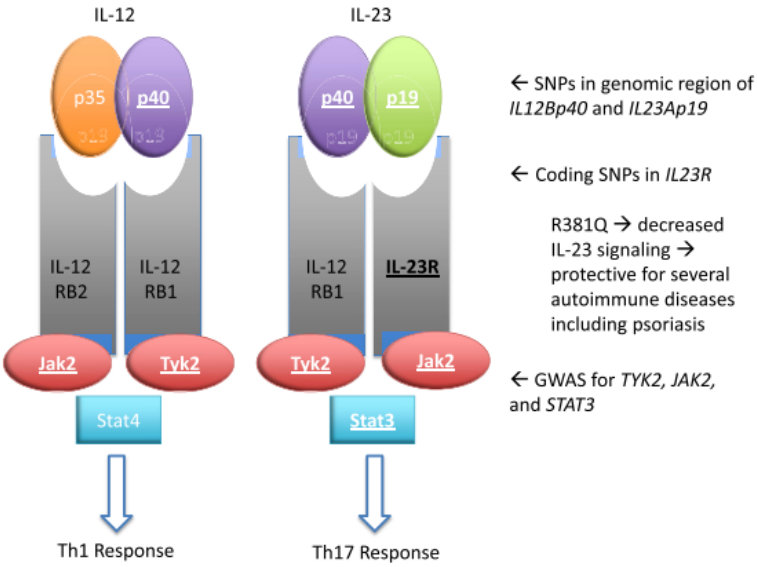

Figure 3. Genes in the IL-23 axis associated with psoriasis. IL-12 and IL-23 share a common subunit (p40) and chain in their heterodimeric receptor, IL-12RB1 andsignal though JAK-STAT signaling. IL-12 signaling activates STAT4, whereas IL-23 signaling activates STAT3. Components underlined and bolded represent the protein products of genes found to have associations with psoriasis.

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Other transcription factors of functional significance include RUNX3, involved in auto-reactive Th17 cells [68,69]. Genetic studies have confirmed that neutrophils are involved in pathogenetic mechanisms of psoriasis [70]. A recent study found new genetic pathways associated with psoriasis risk such as retinol metabolism, the transport of inorganic ions and amino acids, and post-translational protein modification [71].

PSORS1 is strongly associated with guttate psoriasis, but not with palmoplantar pustulosis [72]. Generalised pustular psoriasis has been linked to a caspase recruitment domain family member; CARD14 (gain-of-function mutation involved in inflammasome and NF-κB signalling) [6,44], and IL-36RA deficiency [49]. The IL-36-RN gene codes for an anti-inflammatory protein and a mutation leads to unopposed stimulation of NF-κB and MAPK with downstream increased production of pro-inflammatory proteins [44,73,74].

Psoriasis and PsA have some common genetic associations, including IL-23R, NK cell receptors and MHC class I alleles [16,49,50,75-77]. A psoriasis and PsA associated gene, RAPTOR, regulates the function of a key regulator of T cell function and growth [16,78].

1.1.7 Histology and inflammatory infiltrate

Psoriasis is characterized by thickening of epidermis due to tenfold increased acceleration of keratinocyte proliferation, resulting in epidermal acanthosis (thickening of viable layers), hyperkeratosis (thickening of cornified layer), and parakeratosis (reduced granular layer of the epidermis; **Figure 4**) [6,44,51]. The stratum corneum forms from incompletely differentiated keratinocytes that retain their nuclei, resulting in scaling [6,44]. Another histological feature is the epidermal elongated rete ridges that project downward and hyperplastic blood vessels that reach up in the papillary dermis, causing visible redness and punctate bleeding spots, Auspitz's sign [6,44].

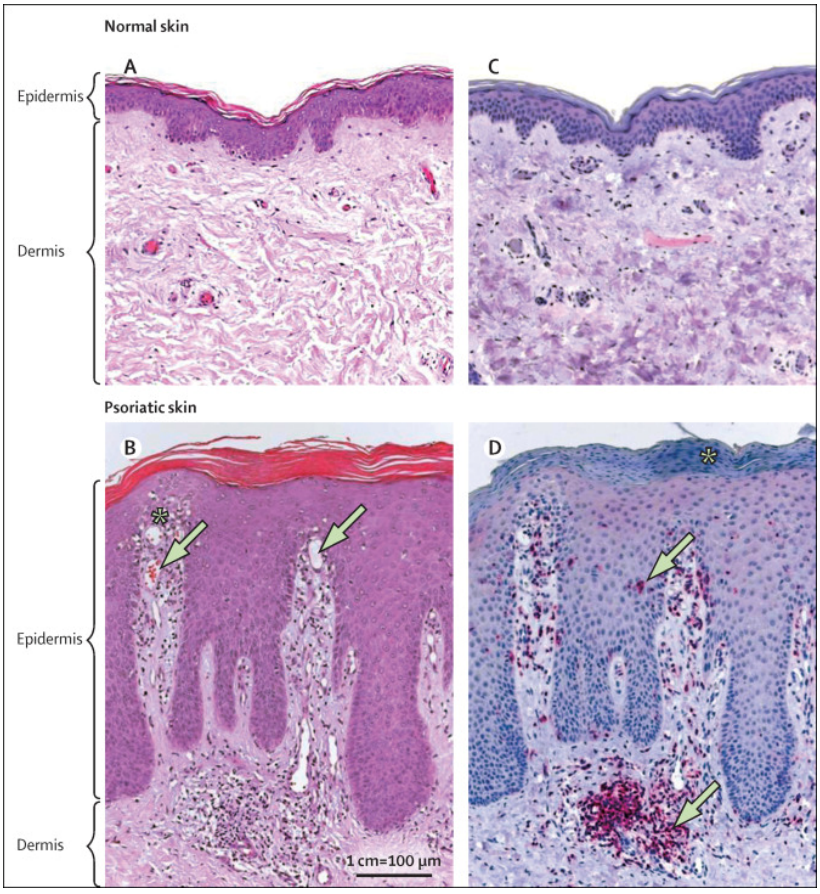

Figure 4. Histopathological features of psoriasis. Within the typical plaque, psoriatic epidermis shows marked epidermal acanthosis, hyperkeratosis, and elongation of rete ridges (A, normal skin and B, lesional psoriatic skin; stained with haematoxylin and eosin). Dilated and contorted dermal blood vessels reach into the tips of the dermal papillae (B, arrows). A mixed inflammatory infiltrate with neutrophils accumulating within the epidermis is noted (B, asterisk). By contrast with normal skin (C), immunohistochemical detection of CD3 reveals many T cells in the dermis and epidermis of lesional psoriatic skin (D, arrows). Cell nuclei present in the cornified layer of the epidermis are also characteristic for lesional psoriatic skin (D, asterisk).

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Mixed inflammatory cellular infiltrates are found in different anatomic layers of the skin, with distinct compartmentalization of T cells [6]. Epidermal T cells are heterogenous, comprised of mostly memory **CD8+ T cells** (Tc), often nearby dendritic cells (**DCs**) [79]. In addition, neutrophilic granulocytes gather within small foci in the stratum corneum (Munro's microabscesses), or in the stratum spinosum (spongiform micropustules of Kogoj) [44,49,80].

In psoriatic dermis, T helper (**Th)1 and Th17** lymphocytes predominate the inflammatory infiltrate just beneath the dermal-epidermal junction [51,81-84]. DCs are detected mainly within the upper part of the dermis together with **macrophages** [49,85]. **Innate lymphoid cells** (ILC3s), **γδ T, NK and NKT cells** in dermis also play a role in psoriasis [80,86-91].

The aggregates of mononuclear leukocytes in the dermis consist of hundreds to thousands of intermixed T cells and DCs and might function as organized lymphoid tissue that induces and perpetuates the inflammatory cascade in psoriatic plaques [44,92,93]. The skin is unquestionable a potent immunological organ as normal skin contains more than twice as many T cells as blood [94].

1.2 Immunopathogenesis and inflammation in psoriasis

In genetically susceptible individuals, psoriasis can arise as a consequence of autoantigens stimulating the innate and adaptive immune system resulting in self-amplifying inflammatory loops **(Figure 5)** [15]. The role and interconnection of the cells that participate in the different phases of psoriasis will be described in this section.

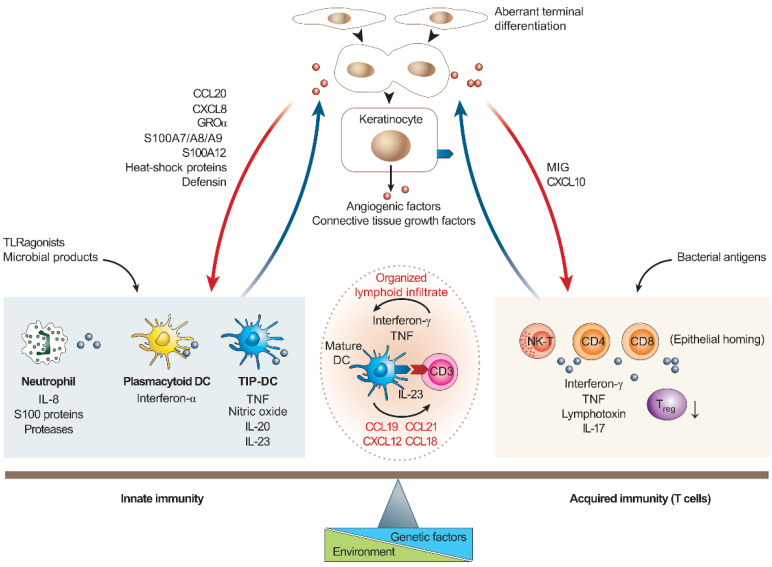

Figure 5. There is close interdependence of inflammatory infiltrate in epidermis and dermis, as well as a balance between the innate and adaptive immune systems. The genetic underpinnings of psoriasis are known to be complex, and these probably interact with environmental factors.

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Initiation of local inflammation due to autoantigens

Epidermal autoantigens LL37 (cathelicidin), keratin 17 and melanocyte-derived antigen ADAMTS-like protein 5 are increased in psoriasis and have a direct stimulatory effect on Tc, via MHC class I and KCs in predisposed individuals [17,44,49,95-97]. Other pro-inflammatory antimicrobial peptides and proteins (AMPs) like S100A7 (psoriasin), S100A15 (koebnerisin) and defensins [98,99], are also overexpressed in psoriatic lesions, act as chemoattractant for leukocytes and prime immune cells for enhanced production of proinflammatory mediators [100-102]. Thus, the interaction between KCs and DCs is crucial for initiation of psoriasis **(Figure 6)** [103].

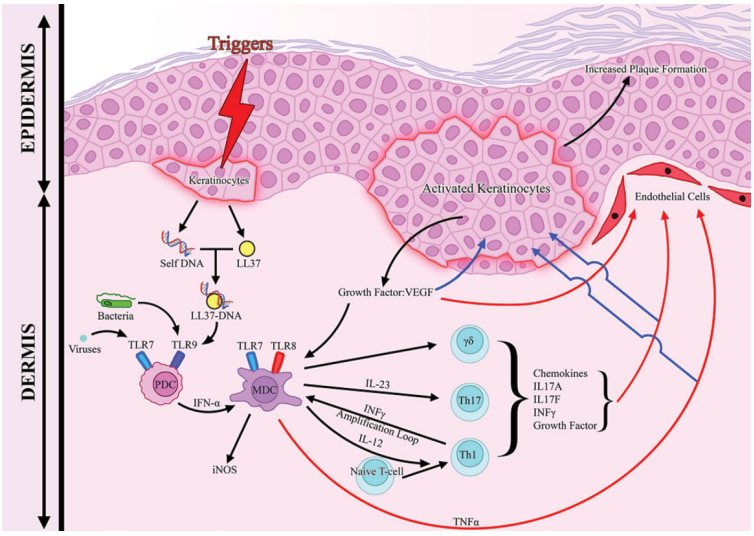

Figure 6. Summary of the main pathogenesis steps leading to psoriasis plaque formation.

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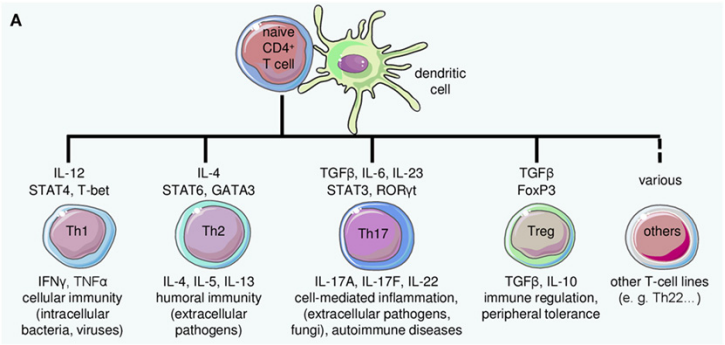
Of these AMPs, LL37 from activated KCs is hypothesized to be a main trigger [50,104,105]. Due to positive charge, LL37 can form immunostimulatory complexes with negatively charged DNA and RNA, released from neutrophil extracellular traps (NETs), enhanced in psoriasis. [49]. NETs can reduce the activation threshold of T cells and increase their response to antigens. DNA-LL37 complex is a potent trigger that stimulate toll like receptor (TLR) 9 on plasmacytoid DCs (pDCs) with subsequent production of interferon alpha (IFN-α) [44,103,106-109]. These immature pDCs migrate into the epidermis, where they recognize keratinocyte-expressed autoantigens, which might then perpetuate the pathogenic cascade [103]. Psoriatic T cells have been shown to have increased and prolonged responses to IFN-α [110]. The pDC-IFN pathway is dominant in acute forms of psoriasis such as erythrodermic psoriasis [111]. RNA bound to LL37 stimulate TLR7/8 on myeloid DCs (mDCs) to secrete TNF, IL-23 and IL-12 [112]. Activated by the AMPs, mature DCs present self-antigens and stimulate autoreactive cytotoxic T cells. Recognition of epidermal

autoantigenes by Tc1/Tc17 induces secretion of IL-17/22 that mediate the initial phase of epidermal hyperproliferation, altered differentiation and activation of KCs, which lead to progression of the inflammatory process [111,113].

In addition, mature DCs facilitate differentiation of naïve T cells into Th1, Th17 and Th22 cell subsets in the lymphnode [44,114-117]. When naïve T cells are stimulated by IL-12 and IFNγ, they will differentiate into Th1 cells (via pSTAT1/4 and T-bet) **(Figure 7 A)** [49]. Although Th17 cells are embedded in a complex regulatory network, IL-1β, IL-6 and IL-23 stimulation of naïve T cells will in general promote this cell lineage (via pSTAT3 and RORγT activation) **(Figure 7 B)** [49]. IL-6 and TNF leads to Th22 cell differentiation (via pp38 and pNF-κB), further TGFβ and IL-10 are involved in differentiation of Tregs (via pSTAT5 and FoxP3) [44,49,54,99,109,117-122]. IL-4 stimulation of naïve T cells promote Th2 differentiation (via pSTAT6 and GATA-3) [117].



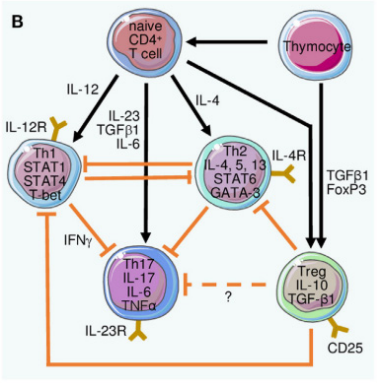


Figure 7. Factors influencing differentiation of T cells. (A) Differentiation of T cell subsets requires stimulation by DCs. Key cytokines and transcription factors are depicted above the respective T cell type, while their function is indicated below. (B) The differentiation of Th17 cells is embedded in a complex regulatory network. *Figure and text adapted from Frontiers in Immunology, The Interleukin-23/Interleukin-17 Axis Links Adaptive and Innate Immunity in Psoriasis, Schön M. P. et al, [49]. © 2018. Reprint with permission.*

Cytokines from Th1 (TNF, IFN-γ, IL-2,), Th17 (IL-17A/F, IL-22, IL-9) and Th22 (IL-22, IL-13, TNF) are crucial in the pathogenesis of psoriasis [51,117,123-126]. IL-17, IFN-γ, IL-22, and TNF cause KC proliferation and production of chemokines, cytokines, and AMPs, which act back on DCs, T cells, and neutrophils to perpetuate the cutaneous inflammatory process creating self-amplification loops [44]. IL-17 and TNF work in a synergistic manner [49,127]. IL-17A/F also act on endothelial cells, fibroblasts (increased IL-6 production), chondrocytes, synovial cells and monocytes (MC) [55,103,128]. TNF, secreted by T cells and APCs in psoriatic skin, induce adhesion molecules on vascular endothelial cells, facilitating entry of inflammatory cells to the skin [129]. In addition, neutrophils also produce pro-inflammatory cytokines (IL-17, IL-8, IL-12, IL-22 and TNF) [130,131].

Establishment of site-specific disease memory

After recognizion of autoantigens, some T cells possibly progress toward differentiation into tissue-resident memory $CD8^+$ T cells (Trm) [132,133]. Trm cells are detected also in resolved psoriasis and constitute a potential mechanism of site-specific disease memory [115,117,132,134,135]. In clinically healed psoriasis, epidermal **Tc17** and **Th22** cells are thought to form a localized disease memory [124,132,134,136]. Keratinocytes, in addition to production of innate immune mediators, may act as non-professional APCs and can induce recall immune responses in antigen-experienced Th and Tc memory T cells, leading to functional response like cytokine production or cytotoxic effect [79,137].

The establishment of plaques and maintenance of chronic inflammation

The dynamic interplay between KCs, DCs, neutrophils and T cell subsets differ in early and chronic psoriasis [44,84,138-141]. It has been hypotesized that polyclonal Th17/Tc17 cells proliferate in dermis and contribute to inflammation through IL-17A secretion [138,142]. Endothelial cells are activated in psoriasis lesions and lymphocytes,

monocytes and neutrophils can transmigrate through reactive vessels [44,143]. As a later step, polyclonal Th1/Tc1 cells (CXCR3+ T cells) from the blood are recruited, induced by chemokines (CXCL9, CXCL10, CXCL11 induced by IFNγ), amplifying the pro-inflammatory cascade [124,138,144,145]. These T cells have a complex interplay that results in chronic inflammation. It has been suggested that IFNγ from Th1 cells might program mDCs to produce CCL20, ligand of CCR6, and to secrete IL-23 [138] which favor recruitment and expansion of IL-17A producing cells amplifying inflammation **(Figure 8)** [49,79,146,147].

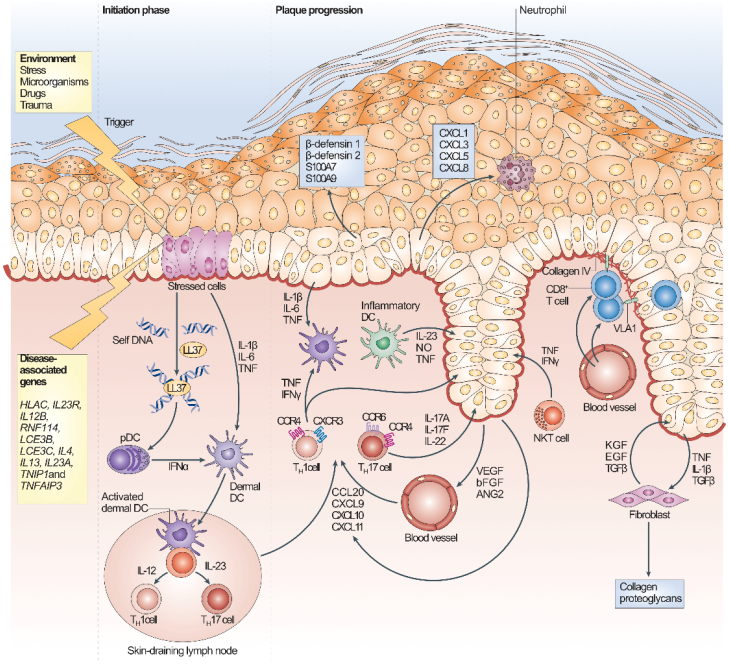


Figure 8. Environmental factors trigger psoriasis in genetically predisposed individuals. In the initiation phase, KCs release self DNA that forms complexes with LL37, and activate pDCs to produce IFNa, stimulating dermal DCs to migrate to the lymph nodes and promote differentiation into Th1 and Th17 cells that migrate via lymphatic and blood vessels into psoriatic dermis, attracted by chemokines. Th17 cells secrete IL-17A, IL-17F and IL-22, which stimulate KC proliferation and the release of AMPs.

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In addition, impaired Treg function plays a role in psoriatic inflammation [80,148]. Tregs, which usually maintain immune tolerance through release of inhibitory cytokines, induction of apoptosis, and inhibition of IL-2 secretion, are dysfunctional with reduced suppressive capacity in psoriasis [44,149,150]. Pro-inflammatory cytokines in psoriatic lesions (like IL-6 from DCs, endothelial and Th17 cells), inhibit Treg suppression which leads to increased proliferation of pathogenic T cells [80,149,151]. The balance between effector T cells and Tregs is dependent on the cytokine milieu and the priming of DCs [152,153].

Recirculation of T cells from the skin; "The psoriatic march"

A majority of patients with moderate/severe psoriasis have LL37-specific Th/Tc cells in their blood [103,154]. These autoreactive T cells and the finding of antibodies against LL37 in plasma from psoriasis patients, provide evidence of autoimmunity and systemic inflammation in psoriasis [17,155,156]. Tc cells and LL37 antibodies have also been detected in synovial fluid in PsA [155,157,158]. Recirculation of T cells from the skin to the blood has the potential to spread inflammation to distant sites and may be related to PsA and comorbidities like cardiovascular disease (CVD), a concept called the "The psoriatic march" **(Figure 9)** [44,50,138,144,159-164].

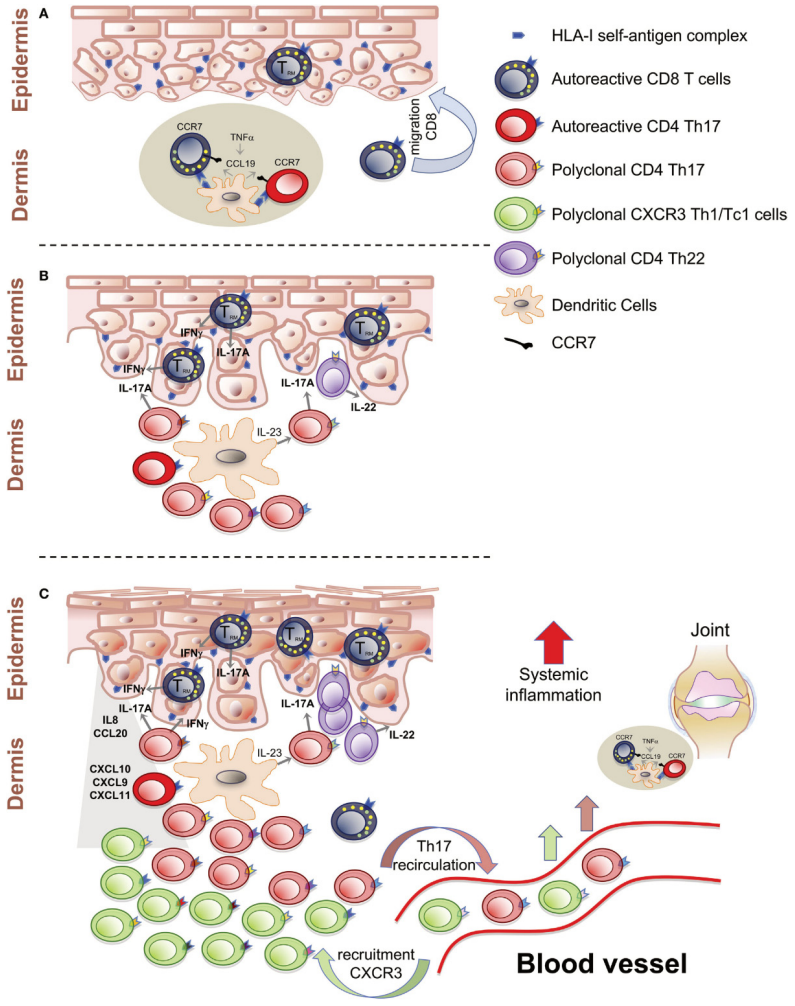

Figure 9. T cell-mediated events in the psoriatic inflammatory cascade. **(A)** Activation of autoreactive T cells by self-antigens. Establishment of $CD8^+$ T_{RM} cells as central autoimmune component of disease pathogenesis and potential mechanisms of site-specific disease memory. **(B)** Polyclonal T cell proliferation and Th17/Tc17-mediated inflammation around the IL-23/IL-17A axis. **(C)** Recruitment of Th1/Tc1 cells, from the blood. Recirculation of T cells from the skin to the blood can spread inflammation at systemic level and to distant sites. *Reprint with permission from Frontiers in Immunology, T Cell Hierarchy in the Pathogenesis of Psoriasis and Associated Cardiovascular Comorbidities, Casciano, F. et al, [138], © 2018. Text adapted.*

1.3 Risk of cardiovascular disease in psoriasis

It has been 40 years since a report showed that rates of occlusive vascular diseases were significantly higher in patients with psoriasis than controls [165]. Since then, multiple epidemiologic studies of psoriasis have confirmed the association to CVD and diseases that represent risk factors for atherosclerosis [166-176], although examples of the opposite also exist[164,177]. Meta-analyses have shown that patients with psoriasis have an increased odds ratio for myocardial infarction (OR 1.32) and stroke (OR 1.26), increased 10 year risk of coronary artery disease (28%) and stroke (12%), and the risk increases with severity and duration of psoriasis [164,167,176,178-181]. The causes of increased risk of CVD in psoriasis are complex and not fully elucidated [164,182].

An indirect link exsists because both psoriasis and CVD are associated with metabolic syndrome [169,182]. Individuals with psoriasis have increased risk of obesity (OR 1.66), hypertension (OR 1.58), diabetes mellitus (OR 1.76) and dyslipidemia (OR 1.5) [39,171,183-195]. Psoriasis severity appeares to be associated with higher prevalence of dyslipidemi [196,197]. In addition, smoking is a common risk factor for the development of both CVD and psoriasis [198].

A direct link between psoriasis and CVD is increasingly accepted as more of the immunopathogenesis of the two diseases has become elucidated [199]. The attributable risk of severe psoriasis on major CVD has been estimated to be 6% over 10 years [200]. Further, studies that quantified coronary artery calcification by CT or PET scan estimate the risk contribution from severe psoriasis to be highly significant after controlling for confounding factors [201-203].

Genes associated with psoriasis are almost completely independent from those linked with metabolic syndrome and atherosclerosis [204,205]. However, there may be some shared susceptibility loci between psoriasis and comorbidities like hypertension and diabetes mellitus [206].

Psoriasis patients may have signs of systemic inflammation. Increased abundance of Th1, Th17 and Th22 cells and elevated levels of chemokines and

cytokines have been detected [207-209]. In some patients C-reactive protein, erythrocyte sedimentation rate, platelet activation marker P-selectin and other serum biomarkers have been shown to correlate with psoriasis severity [207,210-213].

Mechanistic pathways in atherosclerosis and psoriasis have multiple similarities [20,44,212,214-218]. Studies indicate that Th1 cells, also involved in psoriasis, contribute in the formation of atherosclerotic plaques primary through IFN-γ [216,219]. Treg cells play an anti-inflammatory role in atherosclerosis and a Th17/Treg imbalance has been shown in acute coronary syndrome with decreased levels of circulating Tregs with reduced efficacy [220-223]. However, studies of Th17 lymphocytes and IL-17A in atherosclerosis are contradictory [128]. Most evidence in humans now point at a pro-atherogenic effect of IL-17A, however it might exert both anti- and pro-atherogenic effects, depending on the inflammatory context [224,225]. Balance between IL-17 and IL-10 can influence plaque stabilization [199,226,227]. IL-17A/neutrophil axis is another important link between atherogenesis and psoriasis [228]. Th17 stimulate DC to propagate the inflammatory response and increased production of angiogenic inflammatory mediators, and IL-23 drives inflammation in the aortic root through activation of T lymphocytes [138,222,229,230]. Neutrophils are important in atherosclerosis through interaction with damaged endothelium, recruitement of leukocytes, and development of foam cells driving atherosclerosis [130,164,231]. Monocytes and macrophages infiltrate psoriatic and atherosclerotic plaques [232-237]. Chronic skin inflammation accelerates macrophage cholesterol crystal formation and atherosclerosis [238].

Another pathogenetic link between psoriasis and CVD exists through insulin resistance and endothelial dysfunction [160,222,239,240]. Insulin resistance correlate with PASI score [241]. Insulin resistance and psoriasis share common inflammatory profiles through TNF, IL-6, CRP, IL-17 and IL-22 [242]. Inflammatory cytokines such as TNF induce insulin resistance in endothelial cells, leading to reduced production of vasodilating NO and thereby vascular stiffness [243,244]. Anti-inflammatory adiponect correlates with BMI and metabolic syndrome and has been shown to be reduced in psoriasis patients [245]. Insulin resistance is, however, also associated with other

adipokines, resistin and leptin, that upregulate endothelial adhesion molecules [241,246-250] [251]. Insulin resistance may lead to endothelial dysfunction and atherosclerosis **(Figure 10)** [49,222,252,253].

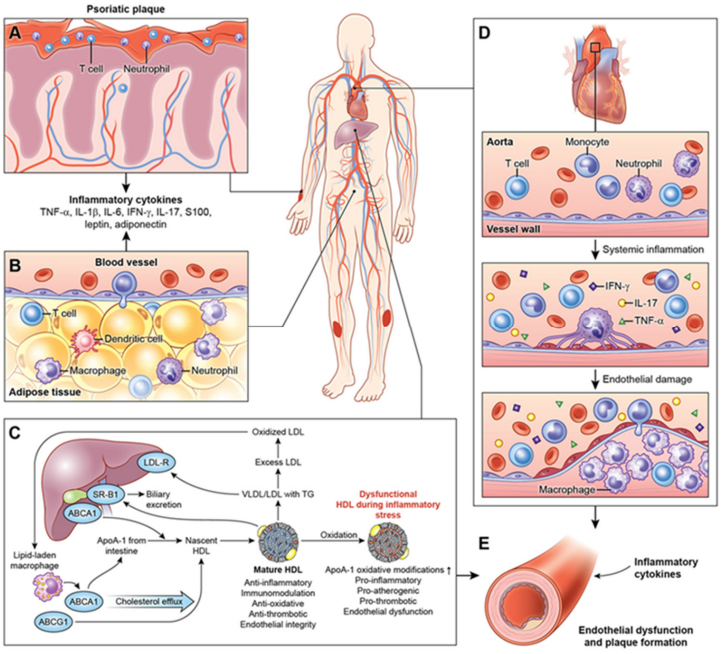

Figure 10. Increased atherosclerosis due to low-grade inflammation in psoriasis. **(A)** Psoriasis is a low-grade chronic, systemic inflammatory disease associated with increased circulating pro-inflammatory cytokines. **(B)** Adipose tissue dysfunction is characterized by pro-inflammatory cytokines and adipokines associated with endothelial dysfunction. **(C)** Psoriasis exhibits a deranged lipid profile and impaired HDL function, which in combination with chronic inflammation accelerate atherosclerotic vascular disease. **(D)** The vessel wall is infiltrated through a complex interplay of pro-inflammatory cellular components, cholesterol crystals, and various lipoproteins contributing to atherosclerosis **(E)** Psoriasis upregulate T-cell, neutrophil chemotaxis, and KC activation and endothelial dysfunction leading to increased atherosclerosis.

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1.4 Other comorbidities in psoriasis

Psoriasis can severely impact quality of life [24,254-258]. Frequency of depression is increased in psoriasis and biochemical link exists, however treatment can improve quality of life [184,259-261]. Several studies have revealed increased risk of Mb. Crohn in psoriasis [165,262,263]. Psoriasis has been shown to be an independent risk factor for non-alcoholic fatty liver disease [264]. A low increased risk of Hodgkin's lymphoma (HL), non-HL and cutaneous T cell lymphoma is debated [265,266]. In addition, some evidence of increased risk of bone and cartilage cancer exist [267]. Psoriasis patients may have an increased risk of skin cancer related to immunosuppressive treatment and light exposure [268,269].

1.5 Treatment

The need for treatment of psoriasis may vary trough life and is aimed at controlling symptoms as there is no complete cure of psoriasis. Guidelines, treatment goals and algorithms have been defined **(Figure 11)** [270-274].

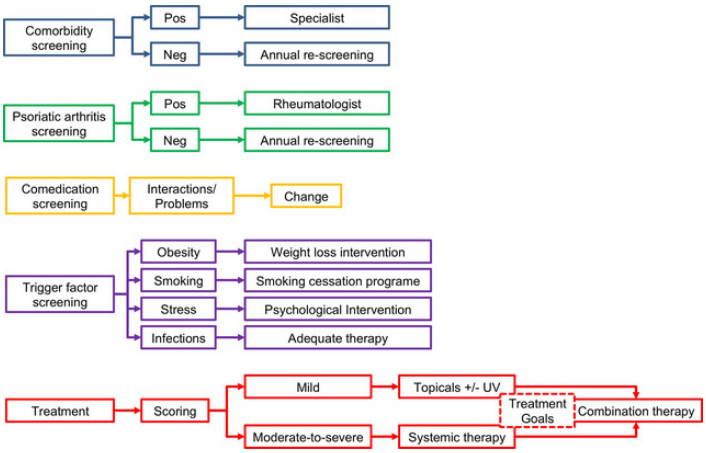

Figure 11. Concept of psoriasis management. *Reprint with permission from John Wiley and Sons, Experimental Dermatology, Psoriasis: to treat or to manage?, Mrowietz, U. et al, [274]. © 2014. Text adapted.*

1.5.1 Topical treatment

Corticosteroids, vitamin D3 analogues, and keratolytic agents (salicylic acid, urea) are used for local treatment of psoriasis and come in different combinations and formulations (creams, ointments, solutions) [275,276]. Coal tar (called Goeckerman in combination with UV-light) and tazaroten (topical retinoid) are also options. Earlier, arsenic and dithranol were used to treat psoriasis [276,277].

1.5.2 Phototherapy and photochemotherapy

Ultra violet (UV) light 311 nm (narrowband UVB) has been found to be the ideal wavelength for psoriasis treatment, and 70% of patients reache PASI75 response [6,278-281]. PUVA is the combination of UVA light (320-400 nm) with local or systemic psoralen and up to 90% achieve PASI75 response [6,282]. Grenz rays are low energy radiation in the zone between X-rays and short wavelength ultra violet radiation sometimes used for severe plaques of limited size [283].

1.5.3 Conventional systemic therapy

Methotrexate (MTX) is a folic acid antagonist that inhibits DNA synthesis, cell replication and has specific T cell suppressive effects [276]. Around 50-60% of patients experience PASI75 with MTX treatment, but it can have serious side effects that can limit use, including teratogenicity and bone marrow suppression [6,276].

Fumaric acid esters influence cytokine profile and recruitment and apoptosis of T cells [276,284-286]. 50% of patients reach PASI75, but the use is often limited by gastrointestinal side effects [6].

Retinoids are synthetic substances similar to vitamin A, not immunosuppressive, but bind to nuclear retinoid receptors, normalizing gene transcription in KCs [276,287]. Around 15% of patients treated with retinoids reach PASI75, however, systemic retinoids are especially effective in the treatment of erythrodermic and pustular

variants of psoriasis [6,276]. Side effects include teratogenicity, skin dryness and hyperlipidaemia [276].

Ciclosporine (CyA) is a macrocyclic immunosuppressant that binds immunophilin and inhibits the calcineurin phosphatase-initiated activation of T cells and has a direct effect on KCs [276,288]. 45-60% of patients treated with CyA experience PASI75 [6,276]. CyA treatment has to be carefully monitored due to side effects including nephrotoxicity [276].

1.5.4 Biological treatment

Biological drugs consist of large and highly complex molecular enteties, often designed on the basis of genetic sequences, derived from living cells cultured in a laboratory [289]. They include fusion proteins, recombinant proteins (*e.g.* cytokines, selective receptors), and monoclonal antibodies. Biological drugs are more costly than conventional therapy and given when a patient fulfills certain criteria. However, biological drugs tend to have higher efficacy and more limited side effects than conventional treatment, although risk of infections can be increased, especially tuberculosis in TNF inhibition. After patent expiry, cheaper biosimilar drugs have been launched for the TNF inhibitors, increasing the access of patients to these treatments [290]. A biosimilar drug is a copy version of an already authorized biological medicinal product with demonstrated similarity in physicochemical characteristics, efficacy and safety [289,291,292]. Due to risk of immunogenicity and relatively scarce documentation on effect and side effects of biosimilars, research in this field is required. In the studies included in this thesis, etanercept (anti-TNF), secukinumab (anti-IL-17), ustekinumab (anti-IL-12/23), original and biosimilar infliximab (anti-TNF) were investigated.

TNF inhibitors

Etanercept is a human recombinant TNF receptor p75 protein that binds to TNF and lymphotoxin, administered subcutaneously (s.c.) [293]. Etanercept breaks the self-sustaining cycle of DCs and subsequent T cell activation, and cytokine, growth factor, and chemokine production by multiple cell types [145]. Depending on dose, up to 50% of patients achieve PASI75 [276]. TNF inhibitors in general have effect also against PsA [276].

Infliximab is a chimeric monoclonal antibody, given as intravenous infusion, that binds to and neutralises the activity of TNF [294]. 80% of patients achieve PASI 75 at week 10. Immunogenicity occurs frequently, especially if comedication with MTX is not given [295,296].

Adalimumab is a fully human, anti-TNF monoclonal antibody, self-administered s.c. [297]. 54-70% of patients achieve PASI75 [6,298,299].

IL-12/23 inhibitors

Ustekinumab is an interleukin-12/23 monoclonal antibody for s.c. injection that inhibits the p40 subunit found in both IL-12 and the more pathogenically relevant IL-23 [300-303]. 70% of patients reach PASI75 and it is associated with longer drug survival than TNF inhibitors [6,304]. Patients using IL-12/23 blockers may have increased risk of infections.

Guselkumab is a monoclonal antibody that blocks the p19 subunit of IL-23, approved for s.c. injection with high efficacy in psoriasis [305,306]. 70% of patients have been reported to reach PASI90 [307].

IL-17 inhibitors

Secukinumab is a human monoclonal antibody blocking IL-17A for s.c. injection with low immunogenicity and high efficacy against psoriasis, 80% PASI75 [6,308-310]. Ixekizumab and the IL-17 receptorblocker Brodalumab have also proven to be very effective in psoriasis [311,312]. Blocking of IL-17 involves increased risk of infections and deterioration of inflammatory bowel disease.

In addition to the above mentioned biological treatments, new classes of specifically targeted, orally administered drugs with rather high costs have recently been introduced. Apremilast is a phosphodiesterase 4 inhibitor that diminishes the production of IL-23, IL-12, TNF and IFN-γ and increases IL-10 [313,314]. PASI75 is reached in 30% of patients [315]. Tofacitinib is a small molecule Janus kinase inhibitor that is given orally [316,317]. Inhibition of JAK/STAT3 signalling normalizes differentiation of and cytokine production from KCs and Th17 cells [120,318].

Treatment response and outcome in patients with psoriasis might be influenced by many aspects, both genetic and non-genetic [319]. Factors associated with low effect of biological therapy are severity, duration of psoriasis, earlier biological treatment, male gender, PsA, high age and BMI [319]. Subtypes of psoriasis might also respond differently to treatment [111]. Optimizing of treatment could maybe be aided by systemic biomarkers reflecting individual inflammatory signature.

1.5.5 Immunogenicity

Psoriasis patients responding to biological treatment may experience loss of effect, sometimes after a pause in medication or if concomitant MTX is not used [320]. Antidrug-antibodies (ADA) and decline of serum drug levels are sometimes detected, with concomitant loss of response despite dose increase and risk of allergic or infusion reactions [321-324]. Algorithms for use and interpretation of measurements of drug level and ADAs have been outlined for clinical use [323]. The different biologicals have various risk of immunogenicity [323].

1.6 Literature search

Literature studies were completed on June 21st, 2019.

2. Aims

The overall aim of this thesis was to study the systemic immune system in severe psoriasis during active inflammation and treatment with biological therapy. The specific research aim of each study were:

Study I

- To investigate if serum cytokine levels in patients with psoriasis reflect skin inflammation and thus could be used as biomarkers for evaluation of disease severity and treatment effect.

Study II

- To compare switch from original infliximab to biosimilar CT-P13 in psoriasis patients, evaluating both clinical parameters and effect on peripheral blood cells and intracellular signalling.

Study III

- To explore single cell analysis of peripheral blood mononuclear cells by mass cytometry, and search for psoriasis specific systemic immune signatures and biomarkers for treatment effect.

3. Materials and methods

3.1 Materials

3.1.1 Study population

The biobank used for these studies was initiated and organized by Silje M. Solberg (SMS). 101 patients diagnosed with psoriasis and found egliable for biological treatment at the Department of Dermatology, Haukeland University Hospital, Bergen, Norway were included from April 2015 to September 2018. Inclusion criteria were age >18 years, moderate-severe psoriasis and prescription of biological treatment. SMS kept track of patients from regular hospital visits, with help from Lene F. Sandvik. Around half of the cohort gave the first blood sample prior to starting current biological drug and the other half were already on biological treatment when they were included in the biobank. Samples from healthy controls (HC) without psoriasis were collected from the Blood bank at the Haukeland University Hospital, Bergen, Norway spread through the year. In addition, healthy volunteers in the lab, without psoriasis, gave blood at three timepoints as longituidal controls. All patients and controls signed written informed consent.

3.1.2 Sample collection, handling and storage

The blood samples were collected at the laboratory of the Dept. of Dermatology, HUS, and further processing and storing were done at the Broegelmann Research Laboratory, UIB, in general by one dedicated technician (M. Eidsheim) or SMS. Patients gave blood at inclusion, after approximately 4 and 12 months (daytime). Variation in sampling processing (*e.g.* time on bench, temperature etc.) was keept to a minimum with few and dedicated persons involved.

The biobank included serum with and without clotactivator, PBMCs and plasma from Li-Heparin tubes. Cryopreservation was used for storage since samples collected over time were to be analysed together.

Serum tubes were left 30-60 min on bench, centrifuged, divided in cryotubes (0.5-1.5ml) and stored at -70 °C. Li-Heparin tubes were turned gently x 10 and transported directly to the cell lab for immediate density gradient centrifugation. Thereafter, plasma was collected, aliquoted in 1ml and frozen at -70 °C before isolation of PBMCs. Cells were frozen in a chemically defined freeze medium, Profreeze™ CDM (47.5%), mixed with serum free cell media X vivo-20™ (50%) and cryoprotectant dimethyl sulfoxide (DMSO) (7.5%). PBMC samples were then put in a CoolCell® freezing chamber (consistent and reproducible -1°C/min cell freezing rate [325]) at -70 °C overnight and then moved to -150 °C for long time storage. Controlled temperature reduction and the use of DMSO minimize damage to the cells by increasing permeability and disrupting the formation of ice crystals [326].

3.1.3 Data collection and storage

DLQI was filed out by the patients, however clinical evaluation including PASI was accomplished by doctors as part of scheduled hospital visits. Since PASI estimation can be prone to interindividual varability, one patient was in general evaluated by the same doctor at all visits. Information about sex, BMI and age was registered. Collected data were then stored «non-identifiable» on the «Forskningsserver» from Helse Vest with oportunity for identification of patients through a «digital key», as required by regional ethics committee.

3.2 Methods

3.2.1 Luminex® Technology

The Luminex® Technology allows for simultaneously measurement of up to 80 different analytes from a single well on the microplate, using very small sample volumes. This bead-based multiplex immunoassay is a multistep procedure, illustrated in **Figure 12** [327].

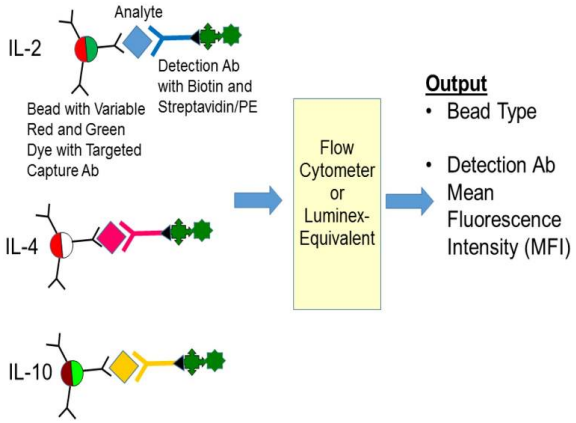

Figure 12. Overview of bead-based immunoassays. Different color-coded beads with dyes that fluoresce either red or green are used. The instrument measures the bead color intensity and the mean fluorescence intensity of the labeled detection antibody which is typically labeled with a streptavidin/phycoerythrin (PE) conjugate.

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Beads are pre-labeled with red and infrared dye in different concentrations to ensure detection of distinct beads coated with antibody against one type of cytokine. Cytokine bound to the cytokine-specific antibody of that bead is detected by a biotinylated secondary antibody labeled with a fluorochrome phycoerythrin (PE) attached via streptavidin [328]. The Luminex 200™ machin has two lasers. The 532 nm green laser is for excitation of PE and fluorescence intensity indicates amount of cytokine. The 635 nm red laser is for detection of type of bead. We used a 96 well plate, where the first 16 wells were used for standards and the remaining 80 wells for patient samples. We included 40 patients with severe psoriasis and analysed samples collected before and 4 months after starting on biological therapy, capturing all 25 cytokines from one sample at a time.

Cytokines were measured by Luminex 200™ by using a ProcartaPlex™ Human Cytokine Panel 1B 25plex kit (EPX250-12166-901) (Invitrogen Thermo Fisher, MA USA), designed to detect granulocyte-macrophage colony-stimulating

factor (GM-CSF), IFN-α (2 a,b,c), IFN-γ, TNF, lymphotoxin, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27 and IL-31.

3.2.2 Phosphoflow cytometry

Flow cytometry is a laser-based technology capable of performing multiple quantitative measurements on a particle or cell in a fluid. Cells are hydrodynamically focused to separate from each other and the fluid stream passes through one or more lasers [329]. Fluorochrome coupled monoclonal antibodies are excited by a specific wavelength of laserlight. The emitted light from the fluorochrome is of specific wavelength and can be discriminated by optical filters before detection. Fluorescent and scattering light are detected by photomultiplier tubes. This signal is amplified and converted to a voltage pulse and digital value allowing identification of individual cells and their characteristics **(Figure 13)** [330]. Flow cytometry can be used to measure relative cellular abundances of cell subtypes in peripheral blood [331].

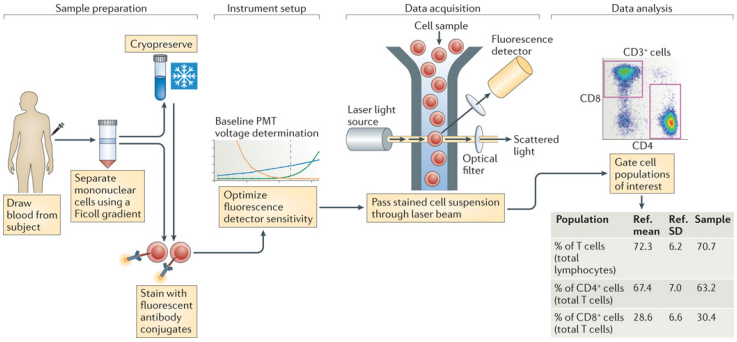

Figure 13. A typical flow cytometry experiment. Sample preparation from blood often involves Ficoll gradient separation of PBMCs, sometimes cryopreservation, before staining with fluorescent antibody conjugates. Instrument setup involves setting voltage gains for the photomultiplier tubes (PMTs) to achieve optimal sensitivity. Data acquisition involves passing the stained cells through a laser beam and recording the fluorescence emission from the bound antibody conjugates. This is followed by data analysis, in which cell populations of interest are defined and reported on. Ref., reference; SD, standard deviation. *Reprint with permision from Springer Nature, Nature Reviews Immunology, Standardizing immunophenotyping for the Human Immunology Project, Maecker, H.T. et al, [330]. © 2012. Text adapted.*

Phosphoflow cytometry combines identification of individual cells and sub-types by surface markers, with the evaluation of intracellular signalling pathways **(Figure 14)** [332,333]. This methodology has been used to investigate different conditions, including hematopoietic malignancies and autoimmune diseases, and responses to treatment, *e.g.* intracellular phosphorylation in T cell subsets in rheumatoid arthritis after biological treatment [334-336].

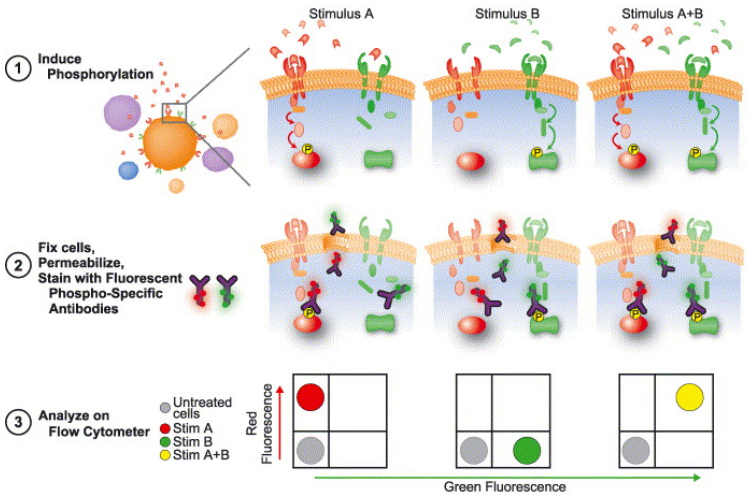

Figure 14. General phospho-protein staining technique for flow cytometry. (Step 1) A heterogeneous sample of cells is treated with two different stimuli, A and B (i.e., cytokines), to induce distinct signaling cascades and phosphorylation of two target proteins. A third sample is treated with both stimuli simultaneously to induce phosphorylation of both proteins of interest. (Step 2) The cells are then fixed, permeabilized, and stained with fluorophore-conjugated phospho-specific antibodies to the phosphorylated forms of the two proteins. (Step 3) The cells are analyzed on a flow cytometer with two or more fluorescence channels. *Reprint with permission from Elsevier, Clinical Immunology, Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical application, Krutzik, [333]. © 2004. Text adapted.*

By phosphoflow cytometry we investigated abundance and intracellular phoshphorylation of PBMC subpopulations from psoriasis patients and healthy controls. Samples from patients and healthy controls were stimulated with TNF in X vivo-20™ in vitro in sterile conditions after a 2 hour resting period after thawing [337]. Following fixation (PFA) and permeabilization (methanol), PBMCs were stained according to a 2 x 4 barcoding (BC) grid using 4 levels of pacific orange and 2 concentrations of pacific blue succinimidyl ester dyes before combing the 8 samples together, adapted from Krutzik et al, prior to acquisition on a LSRI Fortessa flow cytometer with BDFACSDiVaTM Software (both BD Biosciences) [338].

3.2.3 Mass cytometry

Mass cytometry is a relatively new technological platform, described in 2009, that couples flow cytometry with mass spectrometry [339,340]. Mass cytometry has expanded the number of detectable targets to >40 on the single cell level [341,342].

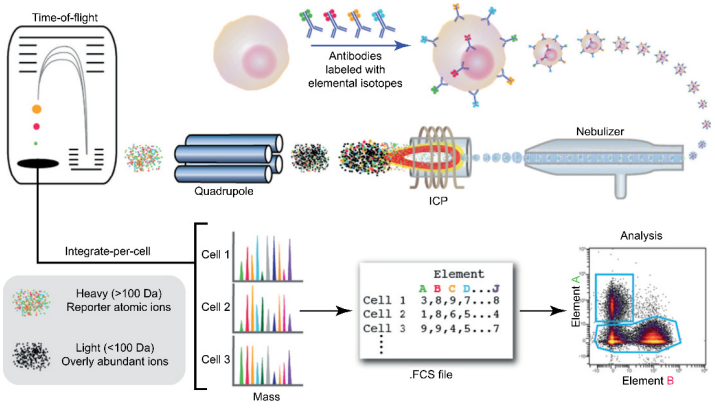

Figure 15. Schematic of mass cytometry analysis of cellular markers. An antibody tagged with a specific element binds to the cellular epitope. The cell is vaporized in the nebulization, atomized, ionized and the elemental composition of remaining heavy elements is determined. Signals corresponding to each elemental tag represent the presence of the respective marker and analyzed using conventional cytometry platforms. *Reprint with permission from Trends in immunology, A deep profiler's guide to cytometry, Bendall, S.C. et al, [344]. © 2012. Text adapted.*

Metal tagged antibodies serve as surrogate markers for extra- and intracellular epitopes [341]. Stained cells in suspension travel through a nebulizer to create a mist of droplets with single cells that pass through a spray chamber and injector, where the water evaporates **(Figure 15)** [343,344]. Thereafter, cells enter the argon plasma, where they are completely atomized and ionized [343]. The resulting ion cloud is then passed through a quadrupole, removing the lighter elements of biological material, retaining only the rare-earth metal isotopes. This cloud is sampled (termed a push) in the channels of the detector. The velocity of lighter ions is higher and they reach the detector first, followed by heavier (and slower) ions, in the sequence of increasing ion mass [339,343]. Daily tuning is important for accurate results, as well as monitoring of pressure and signal drift [345,346]. All data in these pushes are integrated over time and subsequently recorded as dual counts (of atoms) for each channel and recorded in the .fsc format **(Figure 16)** [343,347].

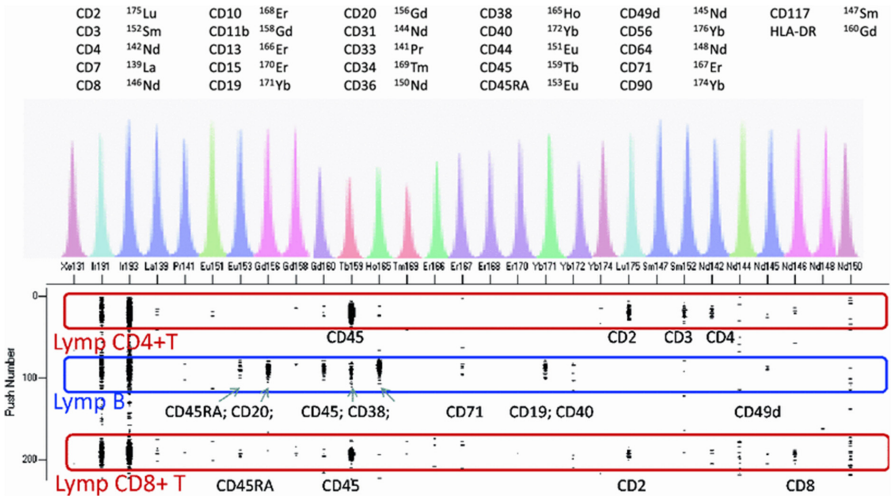

Figure 16. Computer screen shot during mass cytometric analysis of PBMCs stained with a panel consisting of 27 antibodies. Each antibody was labeled with a different stable isotope (given in the table at the top of the figure: the antigen is indicated, followed by the isotope tag). *Reprint with permission from Springer Nature, Cancer Immunology, Immunotherapy, An introduction to mass cytometry: fundamentals and applications, Tanner S.D. et al, [343]. © 2013. Text adapted.*

Isotopes with different atomic masses can be identified by the detector with minimal signal spillover between «mass channels». Additionally, the limitation of spectral overlap is abolished by use of heavy metal-tagged antibodies [348]. In addition to cell surface markers, Cisplatin (195Pt) is used as dead cell marker and iridium as cell-identifier (cross-links DNA) [343,349,350]. Other cell features can be investigated with mass cytometry, like phosphoepitopes in signalling pathways, cytokines, cell cycle markers and RNA [351,352]. Multiplexing of samples by BC of cells has been adapted to mass cytometry by the use of unique combinations of palladium isotopes for labeling of fixed and permeabilized cells that can be de-convoluted after acquisition [353,354]. In mass cytometry, investigation of a high number of markers with single cell resolution yields multi dimensional data with minimal experimental artefacts [342]. Mass cytometry has proven to be a valuable tool for discription of immune cell subsets and signatures in autoimmune diseases and cancer [355-362]. By mass cytometry we investigated PBMCs from 32 patients with severe psoriasis before, 4 and 12 months after initiation of biological therapy. Samples were acquired on a Helios™ Mass Cytometer with WB injector in the Flow Cytometry Core Facility, UIB.

3.2.4 Data processing and statistical analysis

Initial visualization and processing of single cell data was done in FlowJo (Tree Star). For flow cytometry experiment, compensation was done with beads and barcoded samples were deconvoluted by gating on relevant combinations of barcoding dyes. Identification of immune cell populations was based on light scatter properties or relative expression of CD markers and manual gates were tailored to identify PBMC subpopulations for each patient. Further analysis of patient data were based on median fluorescence intensity (MFI) in Cytobank Cellmass v7 [363].

In the mass cytometry study, normalization to beads followed by deconvolution of barcodes were done by use of Fluidigm software. Clean-up of data removing beads, doublets and cell debris was done in FlowJo by manually tailoring

gates, before these new .fsc files were imported to Cytobank for further data analysis of cell subsets and phosphomarkers.

Because of the complexity of mass cytometry data, dedicated computational tools for dimensionality reduction and cell clustering have been developed [364].

Dimensionality reduction algorithms aim to retain the single cell resolution by projecting the high dimensional space to lower dimensionality that can be more easily interpreted. The t-distributed stochastic neighbour embedding (t-SNE) algorithm initially measures the pairwise distances between all cells in the high-dimensional space to generalte a matrix, allowing for cell to be distributed in a two-dimensional dot plot as a function of t-SNE vectors (dimensions) [365,366]. By iteratively adjusting the position of cells, the algorithm minimizes discrepancy between high and low dimensions. In this way, expression of cell markers can be visualize in a ViSNE plot to identify immune subsets with single cell resolution.

Clustering algorithms segregate similar cells into groups that can be evaluated as one entity (loosing singel cell resolution) in a two dimensional plot. The clustering is un-supervised to achieve reproducible and unibiased assignment. However, number of desired clusters must be determined by the analyser. FlowSOM is an algorithm which uses Self-Organizing Maps (SOMs) that can reveal how all markers are behaving on all cells. It clusters cells based on chosen markers, generates a SOM of clusters, produces a Minimum Spanning Tree (MST) of the clusters, and assigns each cluster to a metacluster/population.

Data from all three studies were transferred to the statistical package for social science (SPSS) Statistics 24 for statistical analysis. Since cytokine, phosphoflow and mass cytometry data in general were not normally distributed, non-parametric tests were used for comparison; Mann-Whitney U test for independent, unpaired data (group-to-group comparison), and Wilcoxon signed-rank test for paired data (between inclusion and follow-up).

For correlation analyses in paper I and III, the strength of correlations revealed by Spearman's rank order test were interpreted according to the recommendation from British Journal of Medicine (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.

Linear regression was used in paper I to explore if different cytokines could predict disease severity. To evaluate the effect of change in serum cytokines on improvement of PASI [272], logistic regression was applied.

To overcome intra-assay differences, fold changes (FC) of MFI and median signal intensities (MSI) for samples at inclusion relative to corresponding internal control (IC) was used in study II and III. To compare individual variation over time, FC of follow-up samples relative to corresponding inclusion value (T2/T1 or T3/T1) were used in all three studies. A p-value ≤ 0.05 was considered statistically significant.

In all three studies, a large number of features from relatively small patient cohorts were analysed with the risk of false positive results due to multiple comparisons [367-369]. Since studies presented here were hypothesis-generating, no correction for multiple comparison or power calculations were done a priori. These studies aimed to assess clinically relevant differences in cytokines and PBMCs and thus should show statistical significance despite low power.

Figures were made in GraphPad Prism v8.0 and Cytobank Cellmass v7.

3.3 Legal and ethical aspects

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 or presentation of results.

4. Results

4.1 Study I

Serum cytokine measurements and biological therapy of psoriasis - Prospects for personalized treatment?

Since pro-inflammatory cytokines are a prominent feature in psoriasis pathogenesis, we measured serum cytokines in patients before and 4 months after initiation of biological therapy. Change of PASI correlated positively with fold change (follow-up/inclusion) of IL-2 and IL-12. Change in DLQI correlated with fold change of IL-2. An increase of IL-10, IL-5 and IL-15 at follow-up gave higher chance of achieving PASI90. Logistic regression revealed increasing risk of having severe psoriasis with increase of IL-17A, corrected for other pro-inflammatory cytokines.

By linear regression, we investigated impact of cytokines upstream of T cells in psoriasis pathogenesis and found that variation in TNF seemed to be influence by IL-1β, variation in IL-2 influenced by TNF, while variation in IL-17 and IFN-γ influenced by IL-1β, IFN-α and TNF. BMI was correlated to inclusion values of TNF, IL-22 and IL-1RA.

4.2 Study II

Psoriasis patients on infliximab have increased phosphorylation of intracellular signalling molecules in peripheral blood cells.

Phosphoflow cytometry was used to evaluate PBMCs from psoriasis patients on infliximab who either continued or switched to biosimilar IFX. 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 analyzed, despite the fact that all patients were in clinical remission on treatment with originator IFX. Twelve months later, patients still displayed significantly higher basal

phosphorylation levels than HC, but the number of epitopes with significant differences was decreased.

After TNF stimulation of PBMCs, the level of phosphoylation relative to basal level was significantly lower for pSTAT3 in monocytes, T-, B- and NK cells, pERK in T cells and pNF-κB in NK cells from patients at inclusion compared to HC. After 12 months, only fold change of pNF-κB in monocytes and NK cells in addition to pSTAT3 in monocytes were decreased in patients compared to HC.

Comparing patients continuing on originator IFX to patients who switched to CT-P13, no difference in basal phosphorylation of NF-κB, ERK, p38 and STAT3 was detected. However, fold change of pSTAT3 in B cells was increased in patients on biosimilar relative to originator IFX after 3 months, but not 12 months after switch.

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 by mass cytometry at inclusion, 4 and 12 months after initiation of infliximab, etanercept, ustekinumab or secukinumab. Mass cytometry analysis detected a shift in the Th1/Th2 balance of circulating CD4 population in psoriasis patients after receiving biological therapy. At inclusion, patients had lower abundance of Th2 cells and a tendency of higher abundance of Th1 cells compared to HC and follow-up. Further, patients had higher abundance of Th22 and Th9 at inclusion compared to 1 year follow-up. Contrary, Tregs had a tendency of lower abundance in patients at inclusion compared to HC, but after 1 year the level was more similar. There was a shift from naïve/effector (CD45RA+) to memory (CD45RO+) predominance in the CD4 population, specifically Tregs, from patients during biological treatment. Also of interest, patients had higher abundance of epithelial homing B and memory Tc cells (CCR10+) at inclusion than HC.

PD-1 has an inhibitory function on immune cell activation. PD-1 expression on Th2 cells was higher and on CD8 cells lower in patients with active disease than in HC. After 1 year, the expression of PD-1 on CD4 cells and Tregs in patients was increased. Responders had higher increase of inhibitory PD-1 on CD4 cells after 4 months and on NK cells after 1 year than non-responders**.**

Abundance of classical and intermediate MC and basal phosphorylation of STAT1 and p38 in classical MC were reduced after 4 months of biological treatment. After 1 year, responders had higher increase of intracellular phosphorylation (pNF-κB, pSTAT1 and pp38) in non-classical MC compared to non-responders.

Patients with severe psoriasis had elevated intracellular phosphorylation in PBMCs that decreased, but not necessarily normalized, with biological treatment. At inclusion, patients had increased levels of pp38 in CD4 cells and Tregs, pSTAT1 in classical MC and pERK in CD4 cells compared to HC**,** which was still higher after 1 year. However, phosphorylation of pSTAT1 in Th17 cells from patients was decreased after 1 year.

In responders, PASI improvement negatively correlated with FC of Th17 after 4 months and with FC of CD8 after 1 year. Of predictive value for later response to treatment, was the notion that responders had relatively more CD45RO+ Tregs compared to CD45RA+ at inclusion than non-responders. Intracellular phosphorylation also had predictive value regarding later treatment response. At inclusion, non-responders had higher pSTAT1 in Th17 cells, pp38 in classical MC, in addition to pp38 and pNF-κB in intermediate MC than responders.

5. Discussion

5.1 Novelty

The last two decades have provided new knowledge about immunopathogenesis in psoriasis, resulting in targeted therapies directed against immunological aberrancies. However, clinicians do not tailor treatment individually according to this, but choose systemic medication based on stepwise algorithms, empiri and cost. In addition, knowledge about mechanistic association between psoriasis and comorbidities like CVD has been scarce until now, resulting in little attention and few consequences with respect to follow-up and treatment. Optimizing treatment can benefit patients both short-term, through reduced skin inflammation and improved qulity of life with positive implications on relations and career, but also long-term by directing individuals to a healthier immunological trajectory with potentially lower risk of comorbidities later in life.

Luminex® Technology has earlier been used for detection of cytokines as biomarkers in patients with psoriasis and PsA [208,209]. However, the majority of papers on cytokines in psoriasis focus on comparison to healthy controls with only a few reports investigating effect of treatment and individual variation over time [370]. Thus, our investigations of cytokines in serum from severe psoriasis patients during treatment with biological drugs represent a new approach.

Flow cytometry is a well established and validated method for investigation of blood cells and the addition of intracellular targets increases the possibility for more functional analyses. In psoriasis, flow cytometry has been used to investigate abundances of *e.g.* T cell subsets in patients on TNF inhibitors and to evaluate phosphorylation of T cell subsets [67,148,158,207,371-373]. Since biosimilars raise questions regarding efficacy, safety and immunogenicity, we wanted to investigate the effect of switching from original to biosimilar IFX in PBMC subsets from psoriasis patients.

Mass cytometry has been used for biomarker discovery related to treatment of rheumatic diseases and recently this technology was applied on a limited number of

psoriasis patients [356,374]. We explored mass cytometry in a longitudinal comparative study to comprehensively characterize PBMCs with special emphasis on T cell subsets and intracellular phosphorylation in psoriasis patients before and after treatment with biologicals. Since psoriasis is a complex disease, a simultaneous and broad description of interacting PBMC subpopulations was desirable.

5.2 Methodological considerations

5.2.1 Cytokine analysis

Cytokine level measured can be influenced by many factors. When processing samples for storage, time duration until cryopreservation is important and it is recommended to centrifuge within 2-4 hours [375]. Storage duration and temperature can influence final measurements [376,377]. Collection media (serum, plasma) can impact on detection of cytokines, as can anticoagulant (heparin, EDTA, citrat) in plasma tubes [378,379]. Some recommendations favour plasma because coagulation might impact on cytokine release from cells [375]. In spite of this, we chose serum with no additive because it was not recommended to use plasma anticoagulated with more than 10 IU/ml of heparin and our Li-Hep tubes had 17 IU/ml heparin. Serum samples were thawed slowly on ice, overnight in 4 °C, to maintain protein stability and avoid enzyme activation. Matrix effect denotes inhibition of readout due to specific or non-specific factors and varies by both cytokine and donor [380]. Serum tend to cause greater inhibition than plasma, however, serum measurements of some cytokines can yield higher levels than plasma due to the coagulation process.

Biological variability of cytokines is related to both intra- (like circadian rhythm, infections etc.) and inter-individual factors [381,382]. If samples are analysed in a random order on the plate, intra-assay variability related to technical procedure can be decreased. Based on earlier experience with the kit, we did some minor adjustments to the manufacturer's instructions including an extra dilution of standard

in the lowest range to be able to discriminate levels in the lowest end of the scale. In addition, we optimized incubation of beads in serum and detection antibody.

Missing values were denoted as 0.0001 when comparing absolute numbers of MFI, but excluded in analyses of fold changes. When conducting non-parametric analyses, setting non-detected values to close to 0 will have no effect on the results, as the rank-sum test was used and therefore these values will be ranked below the others. Setting missing values close to 0 would have greater impact on the results when executing logistic regression and correlations, and hence it will be a matter of debate which values were most appropriate to use. Values above the detection range were a minor problem.

Measurement of cytokine levels will be influenced by the sensitivity of methodology used, *e.g.* electrochemiluminescence and enzyme-linked immunosorbent assay (ELISA) may yield different results than Luminex® Technology [383-386]. Different approaches for cytokine investigations exsist, *e.g.* analysis of supernatant by enzyme immune assay after stimulation of PBMCs or intracellular staining after brefeldin A [387]. Thus, many factors complicate directe comparison of cytokine levels from different studies.

5.2.2 Advantages and drawbacks of flow and mass cytometry

Both single cell analysis methods, flow and mass cytometry have different advantages and drawbacks [348]. The greates advantage of mass cytometry is the high number of markers that can be investigated at single cell level. For flow cytometry, panels encompassing near to 17 markers are seldom [388]. However, detection of > 40 targets using a single panel is possible with mass cytometry enabeling broad characterization of the immune system [342].

Other advantages of mass cytometry include low level of noise, *e.g.* signal spillover compared to flow cytometry, which deals with autofluorescense and spectral overlap [340,348]. The difference in signal intensity of each metal isotope is fairly equal

in mass cytometry (3-4 fold difference) while fluorophores in flow cytometry have a larger range of brightness (50-fold) [341,344]. For flow cytometry, this can complicate design of antibody pannels, but also be useful for detection of low abundance antigens, compared to mass cytometry.

One disadvantage of mass cytometry is that biological material vanishes before analysis, precluding the ability to sort cells after analysis. Further, detection limit of molecules per cell is 40 for the best fluorochromes, while 400-500 for mass cytometry [343,348]. The acquisition rate is lower in mass cytometry (400 events/sec.) compared to flow cytometry (several thousands of events/sec.) [344,345]. At the moment, availability of mass cytometers is considerably lower and the cost higher than for flow cytometers.

5.2.3 Phosphoflow cytometry: special considerations

Panel design. Antibodies for detection of surface molecules were selected based on their ability to differentiate PBMC subsets [389]. Phosphorylation potential of immunologically relevant signaling pathways after TNF stimulation was considered when chosing antibodies for intracellular epitopes. Choice of fluorophores was a compromise between wavelength of available lasers, filters on the flow cytometer (ideally bright fluorophores used for rare antigens and dim for common antigens), simultaneously as minimizing spillover into important and sensitive channels (low abundance markers and phospho-antigens) reducing the need for compensation.

Flow cytometric controls. 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, sensitivity) [390]. In addition, we used single fluorescent stained compensation beads for the measurement and removal of fluorescent spillover.

5.2.4 **Mass cytometry: special considerations**

Panel design with mass tagged antibodies is easier than for flow cytometry, but spillover from channel +/-1 or +16 mass, which corresponds to oxidated metals, should be considered [391]. In addition, purity of isotopes, abundance of marker and sensitivity of different channels of the machine can influence signal detection [391]. A general ruel is to asign low abundance markers to sensitive metals/channels and avoid spillover from abundant into low abundant neighboring channel. DVS panel designer is a helpful tool that can calculate spillover and marker tolerance (for spillover) for metal tagged antibodies assigned to different sensitivity areas of the machine. When designing the panel, we included antibodies for detection of PBMC subpopulations and for different T cell subsets, in addition to co-inhibitory/stimulatory, naive/effector-memory and skin-homing markers [330,331,350,392,393] **(Figure 17**, **Table 1)**. In addition, phosphoepitopes were selected based on relevant signalling pathways in different celltypes of interest.

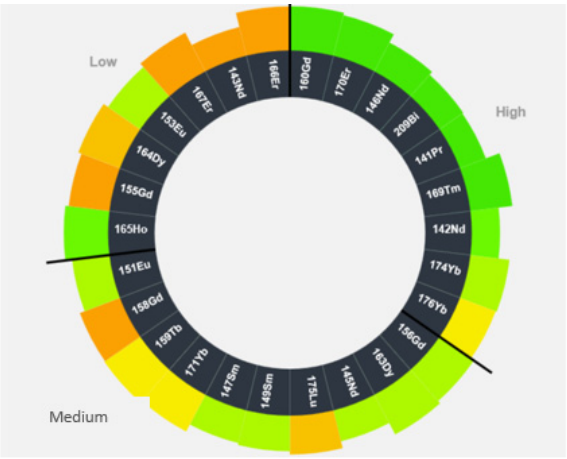

Figure 17. DVS panel designer. Each channel tile is arrayed counterclockwise on the wheel in order of ascending tolerance values: low, medium, or high. The channel tile height is proportional to the sensitivity area of the machine (optimal delivery of metals in 153-176). The tile is heat-mapped (green-orange) to indicate the signal overlap into the channel (% of tolerance value for the target in that channel). *Printed with permission from Fluidigm.*

Table 1. List of epitopes and metal conjugated to antibody for Study III.

Marker	Metal		
CCR6	141Pr	CD14	160Gd
CD19	142Nd	CXCR3	163Dy
CD45RA	143Nd	CCR10	164Dy
CD4	145Nd	CD45RO	165Ho
CD8a	146Nd	p-NFkB	166Er
pSTAT5(Y694)	147Sm	pERK	167Er
CD56	149Sm	CD25	169Tm
ICOS	151Eu	CD3	170Er
pSTAT1(Y701)	153Eu	CXCR5	171Yb
PD-1	155Gd	pSTAT4(Y693)	174Yb
p-p38	156Gd	CCR4	175Lu
pSTAT3(Y705)	158Gd	CD127	176Yb
CD161	159Tb	CD16	209Bi

Some epitopes were sensitive to fixation/permeabilization (CXCR3, CCR4, CCR6 and CD127), and these were added to live cells before barcoding [346]. As illustrated, a few markers in our panel were prone to some signal overlap. Much effort was put in to optimizing the panel, but due to availability of antibodies, some compremises had to be done. CCR4 (175Lu) and pSTAT3 (158Gd) had medium tolerance, were in the medium sensitivity area of the machine and had medium overlap. PD-1 (155Gd), CCR10 (164Dy), pERK (167Er), CD45RA (143Nd) and pNF-kB (166Er) all have low tolerance for spillover, however, except for CD45RA these markers were assigned to relatively sensitive channels and we did not experience any obvious problems with CD45RA. We did in general detect low levels of basal intracellular phosphorylation, and therefore spillover into these mentioned channels can have occluded small differences.

Contamination/Background noise. Rare-earth metal isotopes should not occur in biological material, thus making the endogenous cellular background zero. Some

environmental sources exist, *i.e*. iodine (127I), tin (120Sn) and lead (209Pb), and these can be found in insufficiently purified water but are outside the analytical window (141Pr to 176Yb, 89Y and 209Bi) [391]. However, Barium (137-138 Ba) from soaps and gloves can contaminate samples and interfere with nearby channels. In addition, Ba contribute to accelerated detector aging.

5.2.5 Common considerations for single cell analyses

Selection of phosphoepitiopes. Phosphoepitopes in study II and III were selected based on relevant signalling pathways in PBMC subsets. IL-12 phosphorylates STAT4 (involving pSTAT1/3/5) promoting differentiation of Th1 cells, IL-23 phosphorylates STAT3 (and NF-κB) leading to Th17 differentiation, and TGF-β induces phosphorylation of STAT5, promoting differentiation of Tregs (Figure 7) (44, 49, 119, 120). In addition, TNF stimulation leads to phoshorylation of p38 and NF-κB, and IL-17 stimulation leads to phosphorylation of p38, ERK, and NF-κB in different cells. Other upstream activators may well be IFNs for STAT1, and IL-1 for NF-κB, but more recently discovered cytokines such as IL-20 and IL-22 also have the ability to activate STAT and NF-κB pathways (402, 403). Based on this, phosphorylation of p38, ERK, NF-κB and different STATs were selected for analyses.

Antibody titration. Antibodies were titrated to find optimal staining for surface and phospho-antigens for that specific protocol, aiming at clear separation between positive and negative cells and, at the same time, minimizing spillover [346].

Fixation and permeabilization. Signal transduction and transcripton of genes in cells are dependent on intracellular phosphorylation of proteins, and measurement of such phosphoepitopes can therefore describe activity in the cell [332]. Cells must be fixed to stabilize/cross-link the phosphoproteins and then permeabilized to allow for entry of the phospho-specific antibodies [332]. Both extra- and intracellular epitopes and antibodies can be sensitive to fixation and permeabilization reagents, so testing of antibody performance for individual protocols is important [394].

Barcoding; advantages and obstacles. Barcoding allows for antibody staining and data acquisition of multiple samples together, reducing antibody consumption and variation related to experimental conditions [353,395]. The different samples are separated later based on their signal intensity of the barcoding dyes. If sensitive epitopes that are affected by fixation/permeabilization are included in the panel, BC of live cells is an option. This is possible in mass cytometry through labeling with antibodies conjugated to combinations of CD45 isotopes or by targeting MHC class I complex (beta-2-microglobulin) and a broadly expressed sodium-potassium ATPase-subunit [395-398]. Acquisition of multiple BC samples increases running time (which is higher in mass than flow cytometry) and a temporal stable mass cytometer and cellular staining integrety during long acquisitions are important [353,354,399]. Adjustments related to detector sensitivity of the mass cytometer can be accomplished by use of normalization beads that contain a blend of different metal salts (140/142Ce, 151/153Eu, 165Ho, 175/176Lu) that are mixed into the cell suspension before acquisition [399].

Internal controls. Cryopreserved PBMCs from a single donor was processed with each experimental run as an internal control for monitoration and adjustment of inter-assay variation in Study II and III.

Effect of freezing and thawing on cells. Due to collection of many patient samples and the wish to stain and acquire samples together by use of barcoding, PBMCs had to be frozen in liquid nitrogen, as earlier described. The chemicals and stress on cells by this process might impact on epitopes and signalling detected. Thawing of PBMCs was accomplished rapidly (37 °C) (to avoid osmotic stress and recrystallization) according to recommendations in the literature [325]. A resting period of 2 hours was included after thawing, to let cells recover [337,400,401]. For the mass cytometry study, samples were refrozen after fixation and barcoding and later thawed for further staining. However, this should have low impact on final results, according to the literature [346,394,402,403].

5.3 Biological and clinical implications of the results

Psoriasis is a chronic autoimmune skin disease that involves a complex interplay between many cell types and cytokines creating self-perpetuating amplification loops [44]. The literature provides convincing support for systemic inflammation and increased risk of comorbidities like CVD in psoriasis [164,404]. The era of biological therapy has provided new and effective treatments of immunologically mediated diseases like psoriasis. In our studies, the clinical effect of all four biological drugs, measured by disease severity (PASI) and life quality (DLQI), was excellent. Biological interpretations of findings related to immune cells and cytokines in addition to clinical implications, will be described in the following.

5.3.1 Abberant abundance of PBMCs in psoriasis patients improves with treatment

Circulating immune cells can travel from bone marrow to lymphnodes or distant sites of infection or inflammation, but also recirculate back to blood from peripheral organs, like skin [117,161]. In psoriasis, recirculation of T cells is hypothesized to play a role in amplification of cutaneous manifestations and in the development of comorbidities [124].

By mass cytometry we detected a shift in the Th1-Th2 cell balance in the blood from psoriasis patients during successful treatment, which is in agreement with earlier reports [52,55,159,336,372]. During active disease, increased amounts of Th1 cells have been detected in blood from psoriatic patients and it is known that these CXCR3+CD4+ cells are recruited from blood to skin in psoriasis [138,405]. This is underlined by the fact that blocking of T cell invasion from blood to skin ameliorates psoriasis, supporting importance of recruitment of circulating T cells in psoriasis pathogenesis [406]. On the contrary, others have found circulating Th1 subsets to be reduced in active psoriasis, but they used slightly different markers, and the cohorts differed in disease activity and size [159,407]. During follow-up, we detected a tendency of the Th1 fraction (CXCR3+ CD4 cells) to decrease in patients on biological treatment and a significant

increase in the Th2 fraction (CCR4+ CD4 cells), also in agreement with the literature [358,372].

The pathogenic subsets Th17, Th22 and Th9 have been shown to be associated with active psoriasis in patients [124]. In addition to raised blood levels of Th22 cells being associated with psoriasis, it has been proposed that Th22 together with Tc17 cells constitute a repository of disease memory in skin in recurrent psoriasis [132,207,408]. As such, these cell subsets can constitute interesting treatment targets to avoid chronicity of the disease.

Patients with active psoriasis in our study displayed reduced levels of Tregs, which normalized with treatment. In psoriasis, Tregs are deficient in suppressor activity and relatively decreased compared to T effector cells in the skin, combined leading to insufficient peripheral tolerance against autoreactive T cells [149]. In addition, propensity for differentiation into IL-17 producing Tregs probably contribute to chronic inflammation [149,152,409]. Others have also detected downregulation of Tregs in blood from psoriasis patients and upregulation of Treg subsets with biological treatment has been shown [372]. Interestingly, Tregs are also known to be decreased and dysfunctional in coronary artery disease [222].

In light of recently discovered antibodies against the autoantigen LL37, the mass cytometry detection of elevated levels of skin-homing B cells (CCR10+) at inclusion compared to HC is highly relevant [118,155]. It is tempting to speculate if these cells are involved in an autoimmune response against skin autoantigens, which in that case could constitute a new treatment target.

Increased levels of CD8 cells in blood from patients with active psoriasis has been detected by others [374,410]. We found that reduction of circulating CD8 cells during biological treatment correlated with improvement of skin disease. Of special interest in the CD8 subset is the Tc17 phenotype (CD8+CCR6+CD161+) that probably is involved in the early stages of psoriasis with detection of autoantigens and production of IL-17, and as such is a potential treatment target [411-413]. We detected a circulating Tc17 population, but differences between groups did not reach

statistical significance [138,411]. Another interesting population that we detected in the CD8 subset, the CD8+CCR4+ memory cells, have also been shown by others to be elevated in psoriasis [374]. It has been postulated that in chronically inflammed tissue, a fraction of resident CD8 memory cells can be released to the circulation, contributing to systemic inflammation associated with severe psoriasis [414].

Activation of T cells requires interaction between T cell receptor and major histocompatibility complex (MHC) on APCs, which have strong genetic association to psoriasis [44]. In addition, co-stimulatory or co-inhibitory signals are required. Of these, PD-1 plays a role in normal immune response silencing and the power of this mechanism has been shown with the success of check-point inhibitors in cancer treatment [415]. In psoriasis there might be a failure of the feedback mechanism which normally would prevent the immune overstimulation, as downregulation of PD-1 on immune cells has been observed. It has been postulated that impaired interaction of PD-1 on T cells with its ligand on APCs can be responsible for an up-regulated immune response in psoriasis [416]. Interestingly, it has been shown that blockade of PD-1 augments Th1 and Th17 responses but suppresses Th2 responses [417]. In line with this, the psoriasis patients in Study III had lower expression of PD-1 on CD8 cells at inclusion than HC, however, the opposite was detected for PD-1 expression on Th2 cells, which is in line with earlier studies [416]. After 1 year with biological therapy, the expression of PD-1 on CD4 cells and Tregs was increased. Further reinforcing the evidence for involvement of PD-1/PD-L1 interaction in psoriasis pathogenesis, responders had significantly larger FC of PD-1 on CD4 and NK cells at follow-up than non-responders. If this potent check-point mechanism can be of use in immune profiling or as a treatment target in psoriasis is too early to say.

Intriguingly, a shift in favor of increased memory cells compared to naïve/effector CD4 cells, and more specifically Tregs, was detected, in congruence with the findings of others [159]. Interestingly, responders had relatively more memory Tregs than non-responders at inclusion, indicating that prospectively stratifying of response to future treatment might be possible.

5.3.2 Psoriasis patients have increased intracellular phosphorylation of blood immune cells

Patients in Study III had higher levels of intracellular phosphorylation at inclusion compared to healthy controls in CD4 cells (pERK, pp38), Tregs (pp38) and classical monocytes (pSTAT1), all important in the immunopathogenesis. Guo et al. also found increased phosphorylation in CD4 cells from psoriasis patients and earlier studies of inflammatory disorders have shown that activation levels in PBMCs decreases with treatment [356,372,374,418]. In addition, we detected by mass cytometry that intracellular phosphorylation was still higher in CD4 cells at follow-up, but with a decreasing trend.

In the Study II we found increased basal phosphorylation in almost all immune cell subsets analysed from patients compared to HC, even though patients had been treated with original IFX for a minimum of 18 months and were in remission. This might indicate that systemic inflammation takes longer time to cease than skin lesions, which might be a possible link to comorbidities. The published results from this study was commented in British Journal of Dermatology [419]. No correlation between intracellular phosphorylation and trough level (drug measured in blood) nor the length of IFX treatment prior to inclusion were detected.

One reason for this elevated intracellular phosphorylation in PBMCs can be stimulation from inflammatory cells and cytokines other than those targeted by the biological drug [15,356]. In addition, constitutive activation of intracellular signaling pathways due to genetic susceptibility loci, like mutations in genes encoding NF-κB and MAPK or "the breaks" of NF-κB might explain increased phosphorylation despite years on treatment [54]. If so, therapies targeted against this increased intracellular activity could be of interest, but would have to overcome obstacles related to adverse events as these signaling cascades are involved in general cell processes. This reduction, but not complete normalization, underlines the chronicity of psoriasis and the need for long-term targeted treatment [49,132]. Systemic inflammation promotes cardiovascular disease. Reduction of systemic inflammation might have positive implications on comorbidities of psoriasis.

5.3.3 Prediction of severity of psoriasis and response to biological treatment by immune profiling

In addition to the immune signatures so fare described, some predictive potential for disease severity and treatment effect was detected.

IL-2 at follow-up positively correlated with PASI and DLQI, which might reflect its role as facilitator of differentiation of immature T cells into regulatory T cells and antigen exposed T cells into effector and memory cells [370]. IL-17A is a key cytokine in psoriasis pathogenesis [420]. We found that a level of serum IL-17A was associated with increased severity of psoriasis.

IL-12 from DCs favour differentiation of naïve T cells towards Th1 lineage. Increase of IL-12p70 was positively associated with improvement of psoriasis. This finding is therefore somewhat surprising, although Th1 cells role in psoriasis is less prominent than that of Th17 cells. This finding of increased IL-12 might be due to low number of samples with detectable cytokine or that this cytokine mostly exerts its effect locally and that blood level does not reflect DCs priming of naïve T cells. Reports on serum level of IL-12 in psoriasis are conflicting, possibly because of different methodologies used and detection of different subunits [383,384]. However, with support in the literature, our mass cytometry results indicated a reduction of circulating Th1 cells with treatment [207]. The Th2 cell-associated IL-5 correlated positively with a good clinical response, possibly reflecting restorement of the Th1-Th2 balance, confirmed by the mass cytometry experiment.

The anti-inflammatory cytokine IL-10 executes important regulatory functions, and in psoriasis, different genetic variants and reduced levels of IL-10 have been reported [384,421,422]. Our cytokine analysis showed that increased level of IL-10 at follow-up was associated with good treatment response. IL-10 can be produced by monocytes upon PD-1 triggering [423]. In line with this, the responders in the mass cytometry study had an upregulation of PD-1on non-classical MCs at follow-up.

At inclusion, patients that later turned out to have low treatment response had higher intracellular phosphorylation in Th17 cells and in classical and intermediate

MCs than responders, indicating that early stratification based on immune-profile may predict future treatment response. Larger studies are needed to confirm if intracellular phosphorylation of PBMCs have a predictive value in terms of response to different treatments.

Decreased levels of circulating NK and NKT cells have been reported in psoriasis [424-426]. Responders had higher increase in number of NK and NKT cells at follow-up than non-responders and for NKT cells this increase correlated with PASI improvement. By mass cytometry, we also detected higher expression of epithelial-homing CCR10 on CD4, Th2, Th17, CD8, B and NK cells in responders compared to non-responders at follow-up. Possible explanations for this can be sustained production of skin-homing PBMCs despite diminished expression of ligands in the skin or efflux from healed skin [159,414].

5.3.4 Implications of findings in relation to comorbidities

Since psoriasis is a systemic immune-mediated disease with mechanistic similarities to CVD, the question whether biological treatments can lower risk of comorbidities has arosen [160,427].

Inflammation is central in CVD and anti-inflammatory therapies such as aspirin, colchicine and canakinumab lower CVD risk [164,428-430]. In addition, observational studies have reported reduction of myocardial infarction in patients treated with MTX and TNF inhibitors, while results for IL-12/23 and IL-17 remains to be clarified [431-433]. Register data are conflicting regarding impact of biologicals on CVD risk and systematic reviews the latest years have stated that data for psoriasis were insufficient to reach definitive conclusions due to small sample size and short duration of follow-up [222,431,434-438]. Studies on other inflammatory diseases like rheumatoid arthritis have indicated that systemic treatment impact positively on lowering CVD risk [439].

Imaging studies using PET-CT are mostly in favour of decreased vascular inflammation in the asscending aorta and carotid arteries in psoriasis patients treated with biologicals [440-442]. Carotid intima/media thickness has been shown to decline with systemic treatment of moderate to severe psoriasis [443].

Psoriasis and atherosclerosis have overlapping pathogenic mechanisms in terms of inflammatory cytokines and cells. Patients with acute coronary syndrome have increased levels of Th17-related cytokines and decreased levels of Treg mediators demonstrating a cytokine and cellular milieu reminiscent of psoriasis [404]. Th1 and Th17 cells produce inflammatory mediators attracting MCs, neutrophils and Tc cells [222]. Monocyte recruitment to vessel wall is an early event in atherosclerosis and these cells can be polarized toward pro-atherosclerotic phenotype by skin-inflammation in psoriasis [218,238]. Interestingly, elevated levels of intermediate MCs have been shown to be associated with CVD in multiple studies [232,235]. We detected higher levels of intermediate MCs in blood from psoriasis patients at inclusion compared to healthy controls, and the level decreased during the first 4 months of treatment. Further, we detected higher phosphorylation in intermediate MCs of non-responders compared to responders at inclusion, which could indicate a larger risk of CVD [233].

Classical MCs are also associated with atherosclerosis. Classical MCs from patients at inclusion had lower abundance in blood with higher intracellular phosphorylation than healthy controls. One hypotezis can be that monocytes were recruited to places of inflammation, like vessel wall, in patients with active psoriatic disease [444]. After 1 year, the abundance of classical monocytes was equal to HC.

Contrary, non-classical MCs have been postulated to exert an atheroprotective effect [444]. Responders had higher intracellular phosphorylation in non-classical MCs after 1 year than non-responders. The distribution of subtypes in the monocyte compartment may indicate that systemic treatment of psoriasis may lower CVD risk.

Biological treatment reduces inflammatory cells and cytokines in the blood, also supported by our findings of normalization of Th1-Th2 balance and abundance

of Th17, Th22, Th9, Tc cells and Tregs with treatment [158,372]. Some epidemiologic studies point at lower CVD risk in psoriasis patients on systemic treatment opposed to other treatments [435]. In addition, it has been demonstrated that successful continuous systemic treatment of psoriasis reduces insulin resistance and ameliorates biomarkers for cardiovascular risk, including cytokines, adipokines and endothelial cell dysfunction **(Figure 18)** [164,385,445-448].

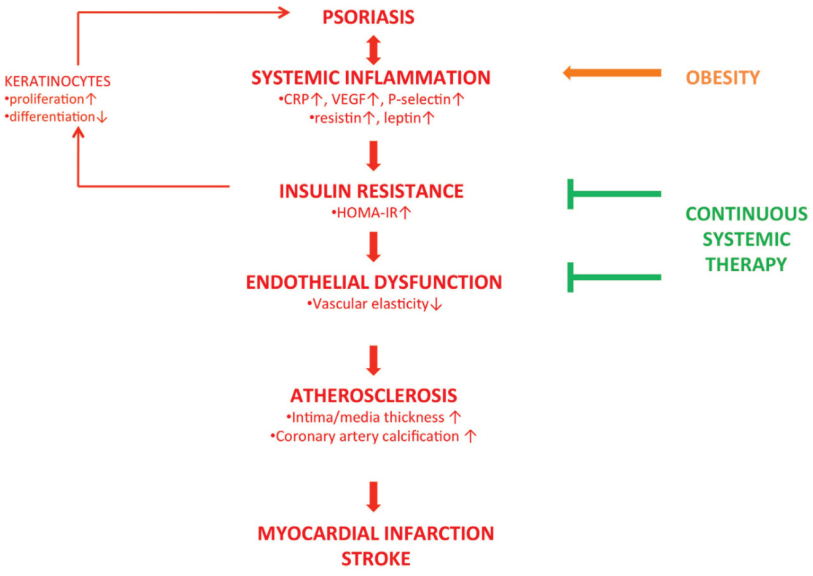

Figure 18. The concept of the "psoriatic march." This hypothesis suggests that psoriasis is a systemic inflammatory condition. Functional consequences are insulin resistance, and endothelial dysfunction, resulting in increased vascular stiffness. This provides the basis for atherosclerosis (red, bold). Insulin resistance has been shown to alter epidermal homeostasis (red, fine). Obesity, causing a state of systemic inflammation is a known risk factor for psoriasis (orange, bold). Systemic anti-inflammatory therapy may reduce the patients' cardiovascular risk (green).

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In light of this, our results from cytokine and single cell analysis might indicate that use of systemic rather than local treatment in psoriasis patients at risk of cardiovascular disease might be beneficial.

5.3.5 Biosimilar infliximab equals original in treatment of psoriasis

Phosphoflow cytometry and clinical examination in Study II did not uncover clinical differences between psoriasis patients continuing treatment with original infliximab compared to those who switched. The NOR-SWITCH trial also showed that switching from infliximab originator to CT-P13 was not inferior to continued treatment with infliximab originator [449]. Later, similar discontinuation risk has been found for original and biosimilar infliximab and etanercept [450].

We noted a tendency for intracellular phosphorylation to be decreased in more immune cell subsets and epitopes in the CT-P13 group compared to originator IFX group at follow-up, but this must be interpreted with caution, as sample size was limited.

A modest increase of pSTAT3 in B cells from patients who switched to biosimilar IFX was detected after 3 months compared to those who continued on originator IFX. However, we did not detect anti-drug antibodies [418]. As patients remained in remission, it is not likely that this increase of B cell activation was related to renewed response towards autoantigens either. The transient raise in B cell activity might therefore represent coincidence due to low sample size or multiple comparison, and no difference in B cell activity was detected after 12 months. During the 12 months follow-up, patients on original and biosimilar IFX were doing equally well, so switching did not result in loss of response or adverse events in this group.

5.4 Limitations of the study

In all three studies, sample size was a compromise between chance of detecting differences between groups and feasibility (use of relatively expencive and work laborious methods). In Study II and III patients were subdivided in 4 treatment groups, mainly due to eligible patients in the biobank, but also to have the opportunity for investigation of different therapeutic strategies. As all biologicals were highly effective, clinical and biochemical differences of significance between

groups were scarce and the cohort therefore analysed as one group. The approach of including all PBMC subsets in the FlowSOM analysis might have failed to identify small subpopulations and a relatively large population of CD4 cells were not identifiable as any of the T cell subsets with certainty. To detect differences in cytokines, intracellular signalling and PBMC subsets between groups of psoriasis patients receiving different treatments, larger sample size is necessary.

Since blood samples were collected during rutine controls with no extra visits for the patient, and we wanted to analyse them simultaneously, cryopreservation/freezing of samples was required. This could possibly impact on cytokines, cell recovery and intracellular signalling. However, the same procedure was used for all patient and control samples, which in that case would be a systematic error. Although many patient samples were cryopreserved a few months longer than HC samples, there was broad inter-individual variation regarding duration of storage in liquid nitrogen without having an obvious effect on phosphorylation levels.

A limitation of Study I was low sensitivity of the cytokine assay. Some cytokines were detected in most serum samples (*i.e.* IL-22, IL-1RA, IL-7, IL-18 and IFN-γ), while others (*i.e.* GM-CSF, IL-2 and IL-21) were detected in around ¼ of the samples and the remaining cytokines were detected in less than 25 of the 80 samples. Other techniques or kits might be more sensitive with regards to detection of cytokines in blood. Serum measurements might not be a sensitive enough method for investigation of cytokines that exert their effect mostly in local tissue with predominant paracrine and autocrine release. Other tissues, like skin, synovia or lymph glands might be more informative. Since the levels of cytokines detected were low, changes between groups and timepoints were difficult to uncover.

Concomitant medication might have impacted on levels of blood cells and cytokines. Some of the patients were already on methotrexate at inclusion or were prescribed MTX after starting on TNF inhibitor, but concomitant use did not seem to have a major impact on their cytokine levels. Samples from patients on IFX were

taken just before next infusion, and many of samples from patients on biological agents for s.c. injection were taken just prior to next injection, with some variation.

Healthy controls were not included in Study I, as the aim was to evaluate intra-individual changes of cytokine levels in the light of disease severity and biological treatment. In Study II, healthy controls with one timepoint were included, but not pre-treatment samples from patients. However, in Study III we included pre-treatment samples and matched healthy controls, some of which had multiple timepoints.

Adipokines, CRP, SR, S100-proteins and clinical examination to discover metabolic syndrome could have added extra value to the studies. Further optimization of panels for flow and mass cytometry could be possible, *e.g.* with self-conjugation of desired metal to specific antibodies. For Study III, we could have included more markers in the panel, like CD45 (lymphocytes), CCR7 (central versus effector memory, naïve versus terminal effector), HLA-DR (activated cells), CD69- and 103+ cells (recirculating memory T cells) and DC markers. In Study III antibodies for CXCR3, CCR4, CCR6 and CD127 were added to live cells before BC because they were sensitive to fixation, introducing risk of intersample variability of staining. In addition, Barcode Perm Buffer likely interfered with detection of chemokine reseptors as some were increased (CCR10 and CD161) compared to expression in test runs without BC. Live barcoding might interfere less with sensitive epitopes and is probably better for detection of chemokine receptors. However, live staining introduces the possibility of initiation of phosphorylation cascades inside cells by antibodies binding to receptors. The basal phosphorylation was in general low and pSTAT3/4/5 barely detected despite reports in the literature [358,374]. Basal levels might have been below detection limits, and differences between groups might have been abolished by spillover from nearby channels. Measurement of phosphorylation levels after cytokine stimulation was not possible due to live staining of some markers.

6. Conclusions

The three studies included in this thesis identify blood cytokines and single cell analyses as useful methods for describing the complex immunological interplay in psoriasis. Also highlighted by these studies is the fact that psoriasis patients have systemic inflammation potentially associated with comorbidities. Patient stratification based on immune-signatures, may enable personalized treatment that have the potential to alter the chronic course of psoriasis with positive implications on long-term comorbidities.

6.1 Study I

Increase of serum IL-2 is associated with both improvement of disease severity and quality of life in psoriasis. Serum level of IL-17A is associated with disease severity. In addition, in this study increase of IL-5, IL-10, IL-12, IL-22 and GM-CSF levels correlate with clinical response to treatment.

6.2 Study II

Psoriasis patients have higher phosphorylation levels in PBMCs than healthy controls. This increased intracellular signalling does not completely normalize with anti-TNF treatment. Switching from original to biosimilar infliximab does not affect biochemical or clinical parameters.

6.3 Study III

Biological therapy facilitate shift in Th1-Th2 balance, transition from naïve/effector to memory predominance, reduce circulating Th17 and CD8 cells and increase PD-1 expression on T cells. In addition, efflux of epithelial-homing lymphocytes from skin is likely. In the monocyte compartment, changes in favor of reduced CVD risk were observed. Results show that intracellular phosphorylation of PBMCs is higher in

psoriasis patients than healthy controls and in non-responders than responders to treatment.

7. Future perspectives

The skin is an easily accessible organ and it has therefore been possible to study its cellular and genomic features in detail [15]. Despite this, unanswered questions in the psoriasis pathogenesis still remain, including the mechanisms involved in the initiation, the characterization of the autoimmune and autoinflammatory responses, as well as the link to extra-cutaneous manifestations [451-453]. In addition, therapeutic strategies can be optimized. Research during the two last decades has resulted in novel treatment concepts, however, selection of patients for these specific immune-modulatory treatments, based on the immunological aberrancy and has not been implemented. Laboratory tests to evaluate severity and treatment effect are also lacking [454].

Stratification of patients and optimizing treatment

PBMCs and cytokines are promising biomarkers for estimating systemic inflammation in psoriasis and treatment response, and they constitute potential new drug-targets [356,455]. Cell analysis by mass cytometry is particularly advantageous in dermatological research since this methodology can be applied on both blood and tissue [456,457]. Imaging Mass Cytometry enables visualization of many markers simultaneously and therefore can reveal more of the interplay among the many cell subsets involved in psoriasis plaques. For clinical analyses of PBMCs, flow cytometry may be more feasible than mass cytometry. Another potential mechanism for evaluating response to treatment, can be transcriptome analyses; mRNA expression of cytokines [458]. Th1, Th2 and Th17 chemokins in serum have also been found to be potential biomarkers in psoriasis [208]. Other soluble biomarkers of interest are S-100 proteins (like psoriasin), immune receptors and e-selectin [208,213,459-461]. MicroRNAs (miRNAs) from PBMCs have also shown promissing results as psoriasis biomarkers [462].

Theranostics refers to the combination of diagnostics and therapy, like monitoring of functional drug levels and neutralizing ADA in the circulation [463,464]. Objective measurements rather than empirical dose-escalation may provide more cost-effective treatment strategies, tailored according to individual requirements [464]. As immunological infiltrate may vary in acute versus chronic psoriasis and in different subtypes of psoriasis (plaque versus pustular), therapeutic choice adapted to these concerns also makes sence [465].

Pharmacogenetic studies identify variations in the genome that can be predictive for treatment response or adverse effects of a given drug [431,466]. Genetic polymorphism might predict response to and side-effects from methotrexate and retinoids, in addition to biological treatment of psoriasis (etanercept, ustekinumab and TNF inhibitors) [111,276,466-471].

Holistic approach; comorbidities and quality of life

Psoriasis is currently an incurable disease, therefore follow-up with long-term safe treatments with concern for quality-of-life is important [199,276]. In Germany significant improvements on both the clinical level and quality of life, including indirect costs and days of work lost, have been achieved by implementing a national program on psoriasis care [472].

Identification of comorbidities, including PsA, depression and metabolic syndrome in psoriasis patients, should gain increased attention among dermatologists [473,474]. In patients with severe and longstanding psoriasis, screening for metabolic and cardiovascular disease should include blood pressure, BMI, waist circumference, fasting blood glucose, transaminases and lipids [6,475]. In most cases, however, patients should be referred for specialist management if other comorbidities are detected [164,268,476]. Biological treatment reduces systemic inflammation opposed to local treatment, a notion that should be taken into account when choosing treatment for psoriasis patients with metabolic syndrome [213].

Emerging therapies

Future research will hopefully enlighten more of the psoriasis puzzle and enable us to apply new knowledge for precision medicine and development of improved therapeutic strategies. A large number of new anti-psoriatic drugs are currently being developed [111,477]. New treatments should be safe with low risk of infections, malignancies and development of auto-immune diseases [111]. Potential research areas and treatment targets can be cytokines, receptors, signalling pathways, transcription of genes, cell interaction, cell recruitment etc. Among cytokines and their receptors, other members of the IL-17 family, IL-22, IL-6, IL-9, IL-36, IL-1, IFN-α and IFN-γ are candidates [111,123,199]. One recent advance in the field of therapeutic modalities, has been the design of multispecific antibodies [111]. Bispecific antibodies that block both TNF and IL-17 have been developed and trispecific antibodies are on their way.

Since biological drugs are costly, require injections, and some patients experience tacaphylaxis, the development of orally available, small-molecule inhibitors is desirable. Directly attacking intracellular signalling pathways can inhibit stimulation from diverse extracellular sources and could therefore ameliorate different immune axes. The selective JAK inhibitors can be given as oral treatment, but it remains to be seen whether the potential risks of infections with this treatment will limit the broad systemic use of JAK inhibitors [478]. Topical formulas of JAK inhibitors are in development [479]. Another target is mTORCI, which, when activated by IL-17A or TNF results in epidermal hyperproliferation [480].

Given the importance of IL-23/IL-17 axis in psoriasis and the expression of the transcription factor RORγt in Th17 cells, blockade of RORγt with orally administered drugs is also aimed at [481,482]. The development of other inhibitory technologies such as siRNA, antisense nucleotides, aptamers (oligonucleotide or peptide molecules that bind to a specific target molecule) are possible scenarios for the future [111].

Tregs are potential treatment targets for both psoriasis and atherosclerosis [222]. Reversing dysfunction, augmenting activity and amplification are possible ways for modulation of Tregs [483]. Curative and preventive strategies will have to eliminate or

dampen pathogenic autoimmune T cells. This could potentially be achieved via tolerogenic vaccines that induce autoantigen-specific Tregs to control activation of pathogenic cells in psoriasis [111]. Restorement of immune regulation or tolerance and prevention of specific antigen triggers, *e.g.* by blocking antigen presentation by APCs to effector cells, targeting LL37 autoantibody production by B cells or preventing differentiation and maintenance of resident memory T cells, could result in treatments without long-term immune suppression [44,484].

Genetic variants that predispose for or protect from psoriasis have been identified [67]. Persons at risk of developing severe psoriasis could potentially be identified early through combination of several gene varaiants, allowing for calculation of a predictive genetic risk score [77,111,485,486]. If "early intervention" that blocks inflammatory mediators in the initial disease stages can prevent the psoriatic march remains for the future to reveal [487].

Immunological and genetic profiling applied in a systematic way, are promising avenues for optimization of personalized treatment in psoriasis, which might lead to a healtier trajectory with improved quality of life and reduced risk of comorbidities.

8. References

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HUMAN IMMUNOLOGY

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Serum cytokine measurements and biological therapy of psoriasis – Prospects for personalized treatment?

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Abstract

Psoriasis is an immune-mediated disease where the IL-23/Th17 axis as well as TNF comprise main targets of biological therapy. Immune profiling has so far not been embraced as a clinical tool. We aimed to investigate relationships between individual serum cytokine levels in 40 psoriasis patients before and after receiving biological therapy and Psoriasis Area and Severity Index (PASI) and Dermatological Life Quality Index (DLQI). Serum concentration of 25 cytokines was determined by Luminex technology. Mean PASI and DLQI decreased by 71% and 65%, respectively. Increase of IL-2 positively correlated with improvement of PASI and DLQI. Moreover, increase of IL-5, IL-10, IL-12, IL-22 and GM-CSF correlated with treatment effect. Notably, logistic regression revealed four times higher risk of having severe psoriasis when IL-17A increased by 1 pg/mL (OR: 4.06, $P < 0.05$). Selected serum cytokines might constitute useful biomarkers for monitoring disease activity and optimizing therapeutic strategies in psoriasis patients.

1 | INTRODUCTION

Cytokine targeted therapy is a rapidly evolving field of treatment for many immune-mediated diseases. However, mapping of an individual's cytokine profile before initiating biological treatment is not in routine clinical use. The decisions of which drugs to choose are often based on economical or practical reasons and preferences.

Psoriasis is a chronic inflammatory immune-mediated skin disease associated with increased risk of arthritis, cardiovascular disease, obesity and diabetes.[1,2] Living with severe skin disease and comorbidities can impact quality of life. Physical trauma, certain infections, stress or medications can trigger and subsequently maintain an eruption of psoriasis in genetically predisposed individuals.[3] Skin damage may induce keratinocyte-derived antimicrobial peptides, which have chemoattractant and immunomodulatory

effects on dendritic cells (DCs) and T cells.[4] Macrophages located along the basement membrane exhibit a dual role as antigen presenting cells and producers of cytokines, for example, TNF.[5] Plasmacytoid DCs (pDCs) produce IFN-α and are upregulated in early psoriasis plaques.[6,7] Furthermore, myeloid DCs (mDCs) are markedly increased in psoriatic skin and can release nitric oxide, a vasodilating agent affecting skin vasculature. More importantly, when mDCs are stimulated by IFN-α, TNF-α, IL-1 β and IL-6, they secrete IL-12 and IL-23. These cytokines are instrumental in the activation and differentiation of naïve T cells into T helper cells (Th)1 and Th17 cells, respectively, in the draining lymphnode.[8,9] Th1 cells in turn secrete TNF, IFN-γ and IL-2, while IL-17A, IL-17F and IL-22 are produced by Th17 and Th22 cells.[10] In addition, chemotactic cytokines, (ie, chemokines, eg, CXCL8, CXCL9, CXCL10, CXCL11 and CCL20 [11]) and S100 proteins from keratinocytes also take part in the pathogenesis, together with complementary TNF and IFN-α from natural killer T (NKT) cells.[12,13] The inflammatory cytokines IL-22 (from Th cells) and IL-20 (from keratinocytes, DC and macrophages) are increased in psoriatic skin lesions and induce keratinocyte hyper-proliferation.[14] A vicious cycle can ensue where inflammatory cytokines from activated keratinocytes act on innate and adaptive immune cells and sustain the inflammatory cascade.[3] Neutrophils gathered in epidermal microabscesses contain IL-17A and are a characteristic feature of psoriasis. It has been shown that they are numerically dominant in the skin compared to IL-17A-containing T cells in active psoriasis.[15] From the complex interplay between immune cells and cytokines in the pathogenesis of psoriasis, TNF-α, IL-12/23 and IL-17 stand out as key cytokines and serve as targets for biological drugs.[9]

The aim of this study was to investigate putative relationships between serum cytokines in psoriasis patients before and after initiating biological (anti-cytokine) therapy, with focus on intra-individual changes, and relate this to disease severity and quality of life. In this way, we wanted to explore whether cytokine measurements could be of value in a clinical setting for evaluation of disease activity and treatment effects, thereby enabling individually tailored therapy.

2 | MATERIALS AND METHODS

Serum samples were collected at the Department of Dermatology, Haukeland University Hospital from April 2015 until August 2017. Inclusion criteria were severe psoriasis, age above 18 years and need of biological treatment. A total of 40 psoriasis vulgaris patients were included, with 10 consecutive patients each prescribed infliximab (anti-TNF-α antibody), ustekinumab (anti-IL12/23 antibody), secukinumab (anti-IL17A antibody) or etanercept (TNF-receptor blocker). The patients' characteristics are displayed in Table 1 and previous and co-medication in Table S1. All patients were naïve to the biological drug they were prescribed at inclusion, and blood was collected prior to initiation of this treatment but after the recommended washout period of any previous biological drug. At the first time point, patients had active psoriasis with extensive skin inflammation. The follow-up sample was taken after approximately 16 weeks, preferably before a scheduled infusion or injection. Blood was drawn in the morning in 5 mL serum tubes with no additive (BD Vacutainer 367614). The tubes were carefully inverted six times and rested in room temperature (RT) for 60 minutes for coagulation before centrifugation at 2000× *g* for 10 minutes (RT). Serum was aliquoted and stored at −70°C until analysis. Psoriasis Area and Severity Index and body mass index (BMI) were measured by the treating dermatologist, and Dermatological Life Quality Index questionnaire was completed on the same day. Patients gave written informed consent (Regional ethical committee approvals 2014/1373 and 2014/1489).

2.1 | Cytokine analysis

Cytokines were measured using a ProcartaPlex™ Human Cytokine Panel 1B 25plex (EPX250-12166-901) kit (Invitrogen Thermo Fisher, MA) according to the manufacturer's instructions with the following adjustments: (a) one additional standard was included in the four-fold serial dilution, making the standard range from 1:4 to 1:16.384; (b) the total volume of diluted beads were transferred to a 96-well plate with equal volume in each well; (c) sample incubation was performed for 20 hours at 6°C with dark lid and gentle agitation (450 rpm); (d) detection antibody and streptavidin-PE were incubated for 60 minutes; (e) beads were resuspended in 90 μL reading buffer on a plate shaker with a dark lid for 10 minutes before data were acquired on a Luminex® 100/200™, counting 2500 beads per well. The five-parameter logistic algorithm (weighted by 1/y, (V2.4)) and raw median fluorescence intensity values were used for the creation of standard curves.

2.2 | Statistics

The statistical package for social science (SPSS) Statistics 24 (IBM, Armonk, NY, USA) was used for data analysis. Wilcoxon signed rank test for paired samples was applied to investigate the differences in parameters between inclusion and follow-up. The strength of correlations, revealed by Spearman's rank-order test, were interpreted according to the recommendation from British Journal of Medicine (https://www.bmj.com/about-bmj/resources-readers/publica

TABLE 1 Characteristics of all patients (n = 40) and the four treatment subgroups (n = 10 in each) at inclusion and follow-up, approximately 16 wk after starting biological treatment

Group	Sex (M/F)	Age	BMI	PASI inclusion	PASI follow-up	% PASI change	DLQI inclusion	DLQI follow-up	% DLQI change
All	28/12	42.08 (14.94)	29.96 (5.37)	9.76 (6.28)	2.65 (2.83)	71.03 (24.47)	14.68 (6.94)	3.98 (3.61)	64.87 (40.12)
Infliximab	5/5	41.40 (16.56)	32.07 (6.40)	10.16 (3.91)	2.02 (1.70)	80.16 (17.31)	16.20 (6.37)	2.20 (2.30)	81.81 (26.21)
Ustekinumab	6/4	38.20 (16.48)	27.33 (2.52)	10.11 (9.09)	3.58 (4.72)	66.17 (30.53)	11.80 (6.75)	4.13 (2.96)	37.22 (61.80)
Secukinumab	9/1	50.40 (9.96)	32.55 (4.21)	9.36 (6.79)	1.87 (1.38)	75.31 (18.45)	13.00 (8.01)	4.40 (4.65)	73.10 (23.12)
Etanercept	8/2	38.30 (14.55)	27.87 (5.91)	9.42 (5.13)	3.14 (2.28)	62.48 (28.31)	17.70 (5.77)	5.20 (3.93)	67.36 (26.14)

BMI, body mass index; DLQI, Dermatological Life Quality Index; PASI, Psoriasis Area and Severity Index.
Values are listed as mean (SD).

tions/statistics-square-one/11-correlation-and-regression), with ρ: 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. Linear regression was used to explore whether the different cytokines could predict disease severity. To evaluate the effect of cytokines on 50%, 75% or 90% improvement of PASI (PASI50, PASI75, PASI90), logistic regression was applied. Δ value was calculated by substracting cytokine value at follow-up from value at inclusion. Fold change (FC) was calculated by dividing cytokine value at follow-up by value at inclusion. Analyses were done on the whole patient group, adjusted for age, gender, BMI and one outlier for IFN-γ, IL-1β, IL-18 and IL-21. A P-value ≤0.05 was considered statistically significant. As the analyses were exploratory, no correction for multiple comparisons was made. Figures were made in GraphPad Prism 7.

3 | RESULTS

3.1 | Disease severity and treatment effect

The clinical measures PASI and DLQI both decreased during treatment, with 71% and 65% reduction of mean values, respectively, for the whole group. 35 of 40 patients achieved at least PASI50, 20 patients PASI75 and eight patients reached PASI90 response. Patients receiving infliximab or secukinumab had the largest improvement of clinical parameters (PASI 80.2% and 75.3%, DLQI 81.8% and 73.1%, respectively), while the amelioration for patients on etanercept and ustekinumab was slightly inferior (PASI 62.5% and 66.2%, DLQI 67.4% and 37.2%, respectively) (Table 1, Figure 1). Treatment effect of etanercept can increase for up to 24 weeks[16] implying that further improvement in this group could be anticipated.

3.2 | Cytokine levels at inclusion and follow-up

Further analyses encompassed serum levels of cytokines before and approximately 16 weeks after introduction of biological therapy, their interrelationship and fold changes (FC). Serum cytokine levels at inclusion and follow-up are displayed in Figure 2. Some cytokines were detected in most serum samples (ie, IL-22, IL-1RA, IL-7, IL-18 and IFN-γ), while others (ie, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2 and IL-21) were detected in around ¼ of the samples, and the remaining cytokines were detected in less than 25 of the 80 samples. Three cytokines (ie, IL-23, IL-31 and lymphotoxin) were not detected by this assay. Wilcoxon signed rank test revealed significant increases in cytokine level at follow-up for IL-5 ($P < 0.05$) and IL-15 ($P < 0.05$), but only a few patients contributed to this increase. Concomitant use of methotrexate did not seem to have a major impact on cytokine levels (Figure S1). Subgroup analysis of cytokines was not robust due to limited number of patients included. The mean TNF, IL-12 and IL-17A at inclusion and follow-up for the four treatment groups are displayed in Figure S2 but should be interpreted with caution due to low number of values for each condition.

3.3 | Cytokines as potential biomarkers in psoriasis

A logistic regression model with pro-inflammatory markers (IL-1α/β, IL-6, IL-12, IL-17A, IL-18, IL-22, TNF-α, IFN-γ and IFN-α) revealed four times increased risk of having severe psoriasis, PASI > 10, with increases of IL-17A by 1 pg/mL (Odds Ratio (OR): 4.06, 95% CI: 1.01-16.38, $P < 0.05$).

Spearman correlation revealed that percentage change of PASI correlated very strongly with FC of IL-2 and IL-12, while moderately with IL-5 (Table S2). Change in DLQI was very strongly correlated to FC of IL-2 (ρ: 0.82, $P < 0.05$). Logistic regression detected a reduced chance of achieving remission (PASI90) with decline in cytokine level at follow-up for ΔIL-12 (OR: 0.03), ΔIL-5 (OR: 0.66), ΔGM-CSF (OR: 0.59) and ΔIL-22 (OR: 0.88). On the other hand, an increase of IL-10 at follow-up gave higher chance of achieving PASI90 (OR: 1.26) (Figure S3).

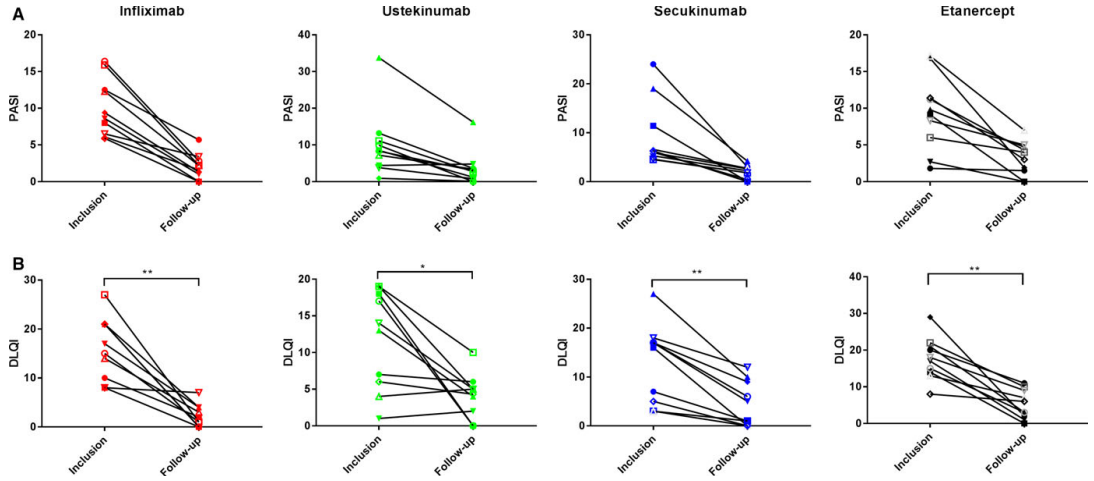

FIGURE 1 Clinical parameters at inclusion and after approximately 16 wk for the four treatment groups (10 patients in each). A, Change in Psoriasis Area and Severity Index from inclusion to follow-up. B, Change in Dermatological Life Quality Index from inclusion to follow-up. Infliximab: red, ustekinumab: green, secukinumab: blue, etanercept: black. Wilcoxon signed rank test. * = $P \leq 0.05$, ** = $P \leq 0.01$

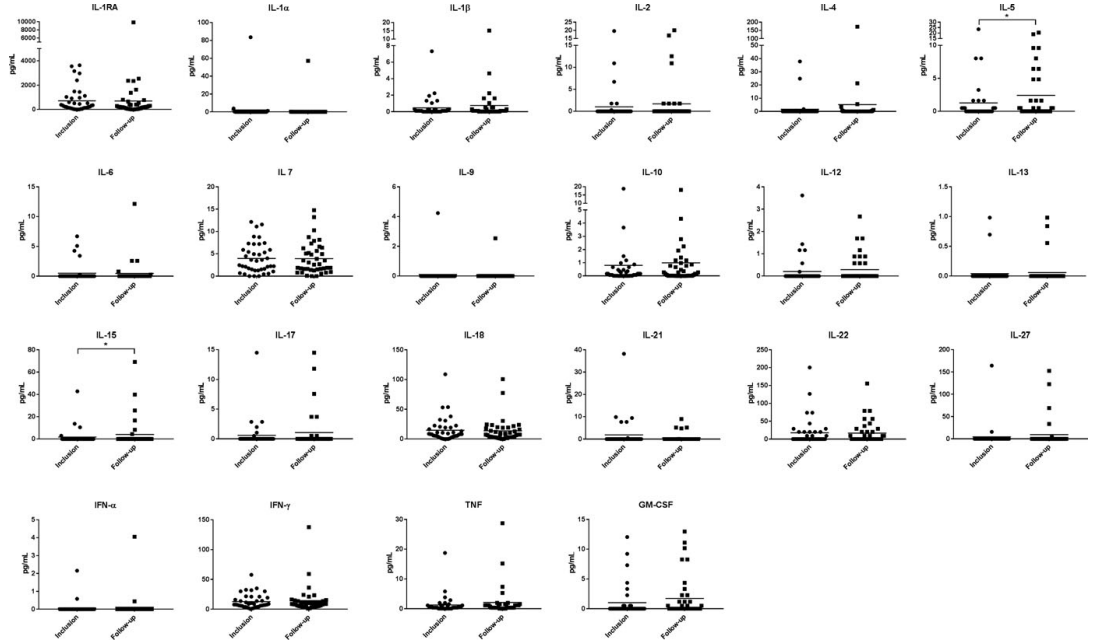

FIGURE 2 Cytokine values at inclusion and follow-up for all patients. One outlier was removed for IL-21. Wilcoxon signed rank test. * = $P \leq 0.05$, ** = $P \leq 0.01$

Body mass index was correlated to inclusion values of TNF-α (ρ: 0.38, $P < 0.05$), IL-22 (ρ: 0.38, $P < 0.05$) and IL-1 RA(ρ: 0.44, $P < 0.001$).

3.4 | Relationship between different cytokines

In order to investigate putative relationships between pro- and anti-inflammatory cytokines, Spearman correlation was

performed. Inclusion values of cytokines showed very strong correlation between pro-inflammatory IFN-γ and IL-18. In addition, IL-17A and IL-15 had very strong correlations with IL-2 (Table S3). FC of pro-inflammatory cytokines revealed very strong correlations between IFN-γ/IL-18, IL-4/TNF-α and IL-5 with IL-4, IL-10, IL-22 and TNF (Table S4).

To shed light on the pathogenic mechanisms in psoriasis, linear regression was used to investigate whether inclusion levels of cytokines from Th1 cells (TNF, IFN-γ and IL-2) and Th17 (IL-17) could be predicted by levels of cytokines that are considered to be upstream in the cytokine cascades (Table S5). The model included IL-1β, IL-6, IL-12, TNF and IFN-α as independent variables. Variation in TNF could to a strong degree be explained by this model ($R^2 = 0.97$) with significant influence from IL-1β. Less of the variation of IFN-γ could be explained ($R^2 = 0.69$), with IL-1β, IFN-α and TNF contributing to different degrees. For IL-2, the model could explain much of the variation ($R^2 = 0.89$) with TNF as the only significant contributor. The same model could also predict level of IL-17A ($R^2 = 0.97$) with significant contributions from IL-1β, TNF and IFN-α.

4 | DISCUSSION

Finding the most effective treatment for patients with immune-mediated diseases can be challenging and often entails multiple treatment attempts resulting in prolonged suffering and unnecessary medication costs. From a clinical perspective, it is often observed that some patients with severe psoriasis benefit from a TNF inhibitor and others from drugs targeting the IL23/Th17 axis. The effect of certain drugs can decline with time, possibly reflecting a shift in the underlying immune profile or immunogenicity. The majority of papers investigating cytokine levels in psoriasis focus on patients as a group with comparison to healthy controls. Only a few reports investigate treatment effects on cytokine levels[17] and the intra-individual variability over time. This paper is, however, focused on cytokine levels in individuals over time, in the light of disease activity and treatment effect. The expanding knowledge regarding immune-mediated diseases makes the search for new biomarkers highly relevant.

A recent meta-analysis concluded that levels of TNF-α, IFN-γ, IL-2, IL-6, IL-8, IL-18, IL-22 were higher in psoriasis patients than in healthy controls, but this was not the case for IL-1β, IL-4, IL-10, IL-12, IL-17, IL-21, IL-23.[18] Regarding disease severity, PASI has been found to be correlated with IL-6, IL-8, IL-12, IL-17, IL-18, TNF-α and IFN-γ in some studies, but not in others.[12,19-27] Differences in sample collection, storage and assessment methodologies may influence in part explain conflicting results.

It is well established that IL-17A is a key cytokine in psoriasis pathogenesis.[2] Pharmaceuticals targeting the IL-23/Th17 axis are highly effective.[28] Congruently, we found that an increase of serum IL-17A by 1 pg/mL was associated with four times increased risk of severe psoriasis, PASI > 10. Furthermore, the pro-inflammatory cytokines IL-1β, IL-6, IL-12, IL-23, TNF and IFN-α induce, at different steps, the differentiation of Th1 and Th17 cells.[3] Our linear regression analyses indicated that the levels of TNF, IFN-γ and IL-2, and IL-17A could be predicted from the level of these mentioned cytokines upstream in the pathogenesis.

The interaction between T cells and IL-2 is important for both tolerance and immunity. We found that improvement of PASI positively correlated with a fold change of IL-2. This is supported by a recent study which found that IL-2 was negatively correlated with active psoriasis.[17] Our data also revealed a correlation between IL-2 and IL-17A at inclusion, which reflect that both the Th1 and Th17 pathways are activated. One role of IL-2 in psoriasis may be differentiation of immature T cells into regulatory T cells. Further, IL-2 also facilitate the differentiation of antigen exposed T cells into effector and memory cells. In our cohort, levels of IL-2 correlated with improvement of quality of life. However, the role of IL-2 in depression is currently debated.[29,30]

IL-12 and IL-23 are instrumental in the differentiation of naïve T cells into Th1 and Th17 cells, respectively, and they share a common subunit, p40 (targeted by ustekinumab). The active IL-12 is the heterodimeric p70, comprised of p40 and the unique subunit p35. Surprisingly, decrease of IL-12p70 at follow-up was associated with reduced chance of achieving PASI90 and FC of IL-12p70 positively correlated with PASI improvement. The literature provides reports on both elevated (IL-12p40)[12] and reduced (subunit not specified)[24] levels of IL-12 compared to healthy controls. Different methodologies and detection of distinct subunits of IL-12 might explain these discrepancies.

Noteworthy, the Th2 associated IL-5 correlated positively with a good clinical response, possibly reflecting restitution of the Th1/Th2 balance. A decrease in the immune regulatory cytokine IL-22 was associated with lower chance of achieving PASI90. IL-22 has been shown to correlate with psoriasis, but can have diverse biological activity resulting in protective or pathogenic effects.[31] Further, reduction of GM-CSF during treatment was also associated with poor clinical response. GM-CSF has the ability to inhibit neutrophil migration and induce anti-apoptotic signalling pathways, both of which might be of importance in psoriasis. These functions can be influenced by IL-17.[32]

The anti-inflammatory cytokine IL-10 executes important regulatory functions and can be involved in autoimmune diseases.[33] In psoriasis, genetic variants and reduced levels of IL-10 have been reported.[24,34] To this end, our data show that increased levels of IL-10 at follow-

up was associated with good treatment response (PASI90). Even though low-dose IL-10 administration can be beneficial in psoriasis, clinical use has been halted because of adverse events.[35]

Psoriasis is associated with adiposity, and the patients in our study had high BMI.[36] Adipose tissue is one source of TNF, and we found a correlation between BMI and TNF, in congruence with reports in obese individuals.[37] Moreover, TNF inhibition is associated with weight gain, while drugs targeting the IL-23/Th17 axis are not.[38] In addition, high BMI has been associated with poor treatment response.[39] Further, we found that IL-22 and IL-1RA correlated with BMI. It was recently discovered that IL-22 is associated with obesity and type 2 diabetes.[40] Variants in the gene encoding IL-1RA have also been linked to adiposity.[41]

The clinical effect of all four biological drugs in this study, measured by PASI and DLQI, was excellent. Since the levels of cytokines detected were low, changes at follow-up were difficult to uncover. We were not able to show decrease of TNF in patients receiving anti-TNF treatment or reduction of IL-12 or IL-17 in patients receiving ustekinumab or secukinumab, respectively.

Limitations of the study are low patient numbers for subgroup analyses and detection mode of cytokines by the assay. Serum measurements might not be the most sensitive way to monitor levels of cytokines that exert their effect mostly in local tissue. Some of the patients were already on methotrexate at inclusion or were prescribed the drug after starting on infliximab, but concomitant use did not seem to have a major impact on their cytokine levels. Healthy age-matched controls were not included in this study, as the aim was to evaluate intra-individual changes of cytokine levels in the light of disease severity and biological treatment.

Treatment of inflammatory diseases has improved tremendously over the last two decades. Still, clinicians often struggle to optimize individualized therapy of psoriasis vulgaris. There is currently no laboratory test to evaluate severity of or treatment effect in psoriasis, but inflammation markers (CRP, SR), trough levels and anti-drug antibodies are used to some extent.[42] Our results indicate that increase of serum IL-2 is associated with both improvement of disease severity and quality of life and confirm that the serum level of IL-17A is associated with disease severity. In addition, the data unveiled that increases in IL-5, IL-10, IL-12, IL-22 and GM-CSF levels correlated with clinical response to treatment. Future studies using more sensitive methodologies, including patient groups where treatment choice is based on individual immune profile, might shed light on the usefulness of serum cytokines as biomarkers in treatment algorithms aiming at optimizing therapeutic strategies in psoriasis patients.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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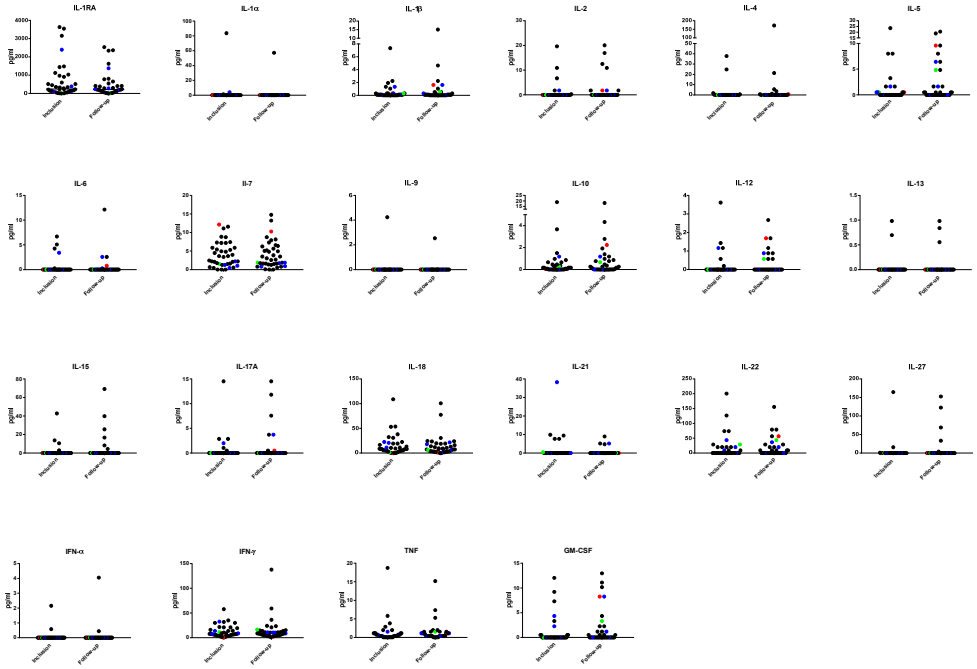
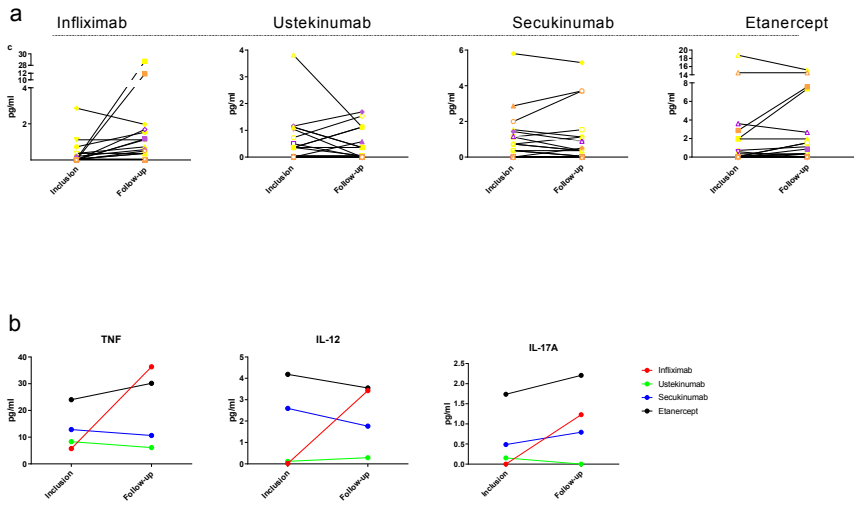
Suppl. table 1. Characteristics of all 40 patients in the four treatment subgroups (n= 10 in each).

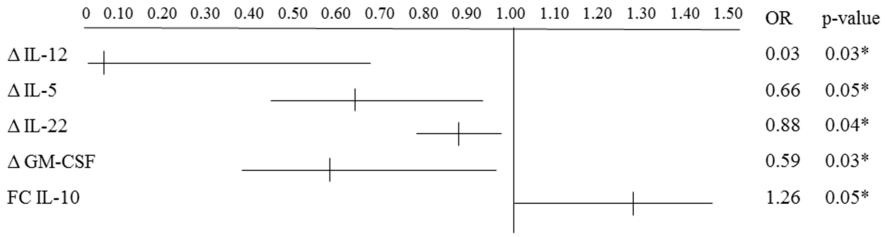
Group	Mean number of previous biological drugs	Use of methotrexate at inclusion	Concomitant methotrexate during follow-up
Infliximab	0.4	1	10
Ustekinumab	0.9	1	1
Secukinumab	1.9	4	4
Etanercept	0	0	0



Suppl. fig. 1. Cytokine levels in psoriasis patients taking concomitant methotrexate compared to those who did not take methotrexate. Patients using methotrexate at inclusion or follow-up are marked with colors. Infliximab: red (1), ustekinumab: green (1), secukinumab: blue (4). Patients not using methotrexate are black. One outlier was removed for IL-21.



Suppl. fig. 2: Level of the cytokines targeted by treatment (TNF, IL-12 and IL-17A) at inclusion and follow-up. a: Intra-individual change in TNF, IL-12 and IL-17A from inclusion to follow-up for the four treatment groups. One type of symbol is used for the same patient in each group displaying TNF: yellow; IL-12: purple; IL-17A: orange. **b:** Mean level of TNF, IL-12 and IL-17A displayed for the four treatment groups (n=10 in each group). Infliximab: red, ustekinumab: green, secukinumab: blue, etanercept: black.



Suppl. fig 3: Odds ratio (OR) of achieving PASI 90 for Δ IL-12, Δ IL-5, Δ IL-22, Δ GM-CSF and FC IL-10. Δ: cytokine value at inclusion minus value at follow-up. FC: cytokine value at follow-up divided by value at inclusion. Logistic regression, with 95% confidence interval of OR. *= $p \leq 0.05$

Suppl. table 2. Spearman`s rank correlation coefficients between cytokine fold changes and percentage improvement of PASI in addition to categories of PASI improvement.

	PASI improvement (%)	PASI improvement category
FC IL-2	.820*	.718
FC IL-12	.657	.845*
FC IL-5	.536*	.412

* = $p \leq 0.05$

PASI improvement category includes the following categories: 1: <50, 2: 50.00-74.99, 3: 75.00-89.99, 4: 90.00-99.99, 5: 100

Suppl. table 3. Spearman`s rank correlation coefficients between cytokines at inclusion. Cytokines with very strong (0.80-1.00) correlations are included in the table.

Spearman`s rho	IFN-γ	IL-18	IL-2	IL-17A	IL-15
IFN-γ	1.000	.809**	.104	.134	.063
IL-18		1.000	-.105	-.001	-.090
IL-2			1.000	.838**	.833**
IL-17A				1.000	.630**
IL-15					1.000

* = p ≤ 0.05
** =p ≤ 0.01

Suppl. table 4. Spearman`s rank correlation coefficients between fold change of cytokines with very strong (0.80-1.00) correlations.

Spearman`s rho	IFN-γ	IL-18	IL-4	IL-5	TNF-α	IL-10	IL-22
IFN-γ	1.000	.861**	.220	.744**	.301	.582**	.190
IL-18		1.000	.331	.674**	.237	.429*	.438
IL-4			1.000	.812**	.854**	.000	.112
IL-5				1.000	.869**	.809**	.850**
TNF-α					1.000	.283	.136
IL-10						1.000	.642
IL-22							1.000

* = p ≤ 0.05
** =p ≤ 0.01

Suppl. table 5. Linear regression of IL-17A, TNF, IFN-γ and IL-2 with cytokines situated upstream in the psoriasis pathogenesis (IL-1β, IL-6, IL-12, IFN-α and TNF) as independent variables.

	TNF			**IFN-γ**			**IL-2**			**IL-17A**		
	B	95% C.I.	Sig (p)	B	95% C.I.	Sig. (p)	B	95% C.I.	Sig. (p)	B	95% C.I.	Sig. (p)
IL-1β	2.13	1.32, 2.93	.001***	-23.34	-36.45, -10.24	.001***	1.03	-0.91, 2.97	.288	1.12	0.28 , 1.96	.010**
IL-6	0.09	-0.10, 0.28	.334	-0.80	-3.14, 1.54	.492	-0.30	-0.65, 0.04	.085	-0.03	-0.18, 0.12	.715
IL-12	0.64	-0.77, 2.06	.364	4.38	-12.88, 21.64	.609	-0.46	-3.02, 2.09	.716	-0.46	-1.56, 0.65	.407
IFN-α	-0.97	-2.03, 0.09	.072	23.83	10.45, 37.21	.001***	-1.72	-3.70, 0.26	.086	-1.18	-2.03, -0.32	.008**
TNF				8.99	4.86, 13.13	.001 ***	0.92	0.31, 1.53	.004**	0.45	0.28, 1.96	.002**

*= ≤ 0.05
**= ≤ 0.01
***= ≤ 0.001
B= unstandardized coefficient of the regression equation
C.I.= confidence interval

II

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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.

A.K.A. and S.M.S. contributed equally to this work.

The laboratory work was performed at the Broegelmann Research Laboratory in Bergen, Norway.

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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

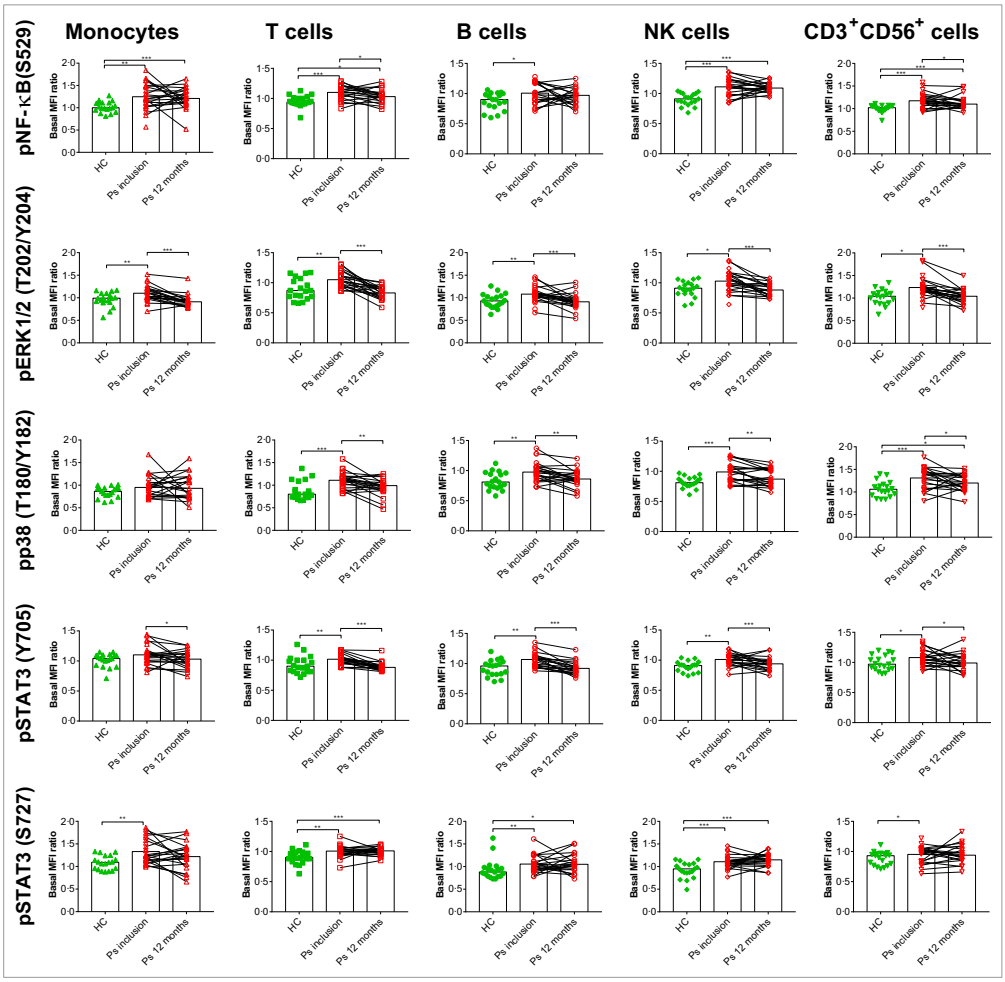

Fig 1. Phosphorylation of intracellular epitopes in unstimulated peripheral blood mononuclear cells from the psoriasis and healthy control groups at inclusion and after 12 months. Basal median fluorescence intensity (MFI) is shown as a ratio of raw MFI of unstimulated cells relative to raw MFI of unstimulated 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).

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 origina-

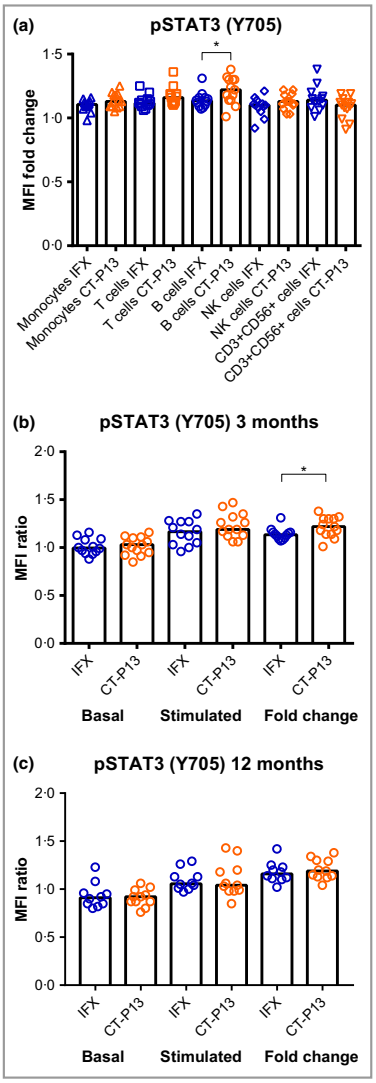

Fig 2. Significant difference in phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Y705) in B cells between patients on infliximab and CT-P13 3 months after switch. (a) Fold change of pSTAT3 (Y705) in immune cell subsets 3 months after inclusion. (b) Basal median fluorescence 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 *$P \leq 0{\cdot}05$

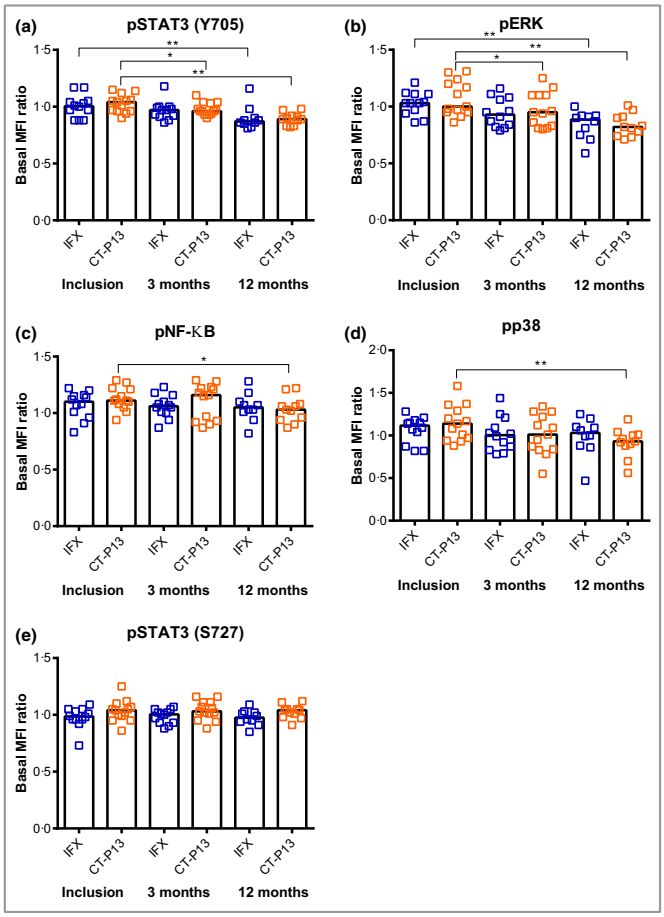

Fig 3. Basal phosphorylation over time for both patient groups, exemplified with T cells. (a) Phosphorylated signal transducer and activator of transcription 3 (pSTAT3, Y705); (b) phosphorylated extracellular signal-regulated kinase (pERK); (c) phosphorylated nuclear factor kappa B (pNF-κB); (d) phosphorylated 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.

tor 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.

Acknowledgments

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.

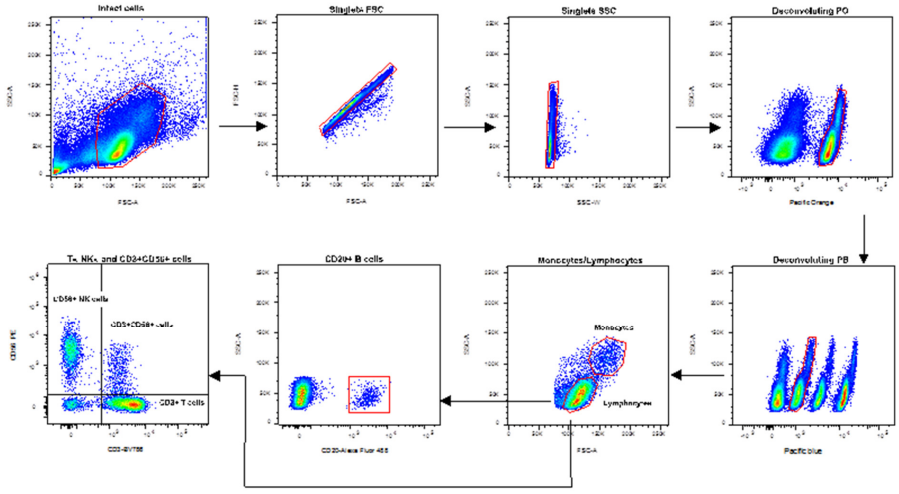

Figure S1: Representative gating strategy used for the flow cytometry data. Intact cells were gated based on forward scatter area (FSC-A) and side scatter area (SSC-A) followed by single cell gating. The different samples were then identified through the intensities of their pacific blue (PB) and pacific 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.

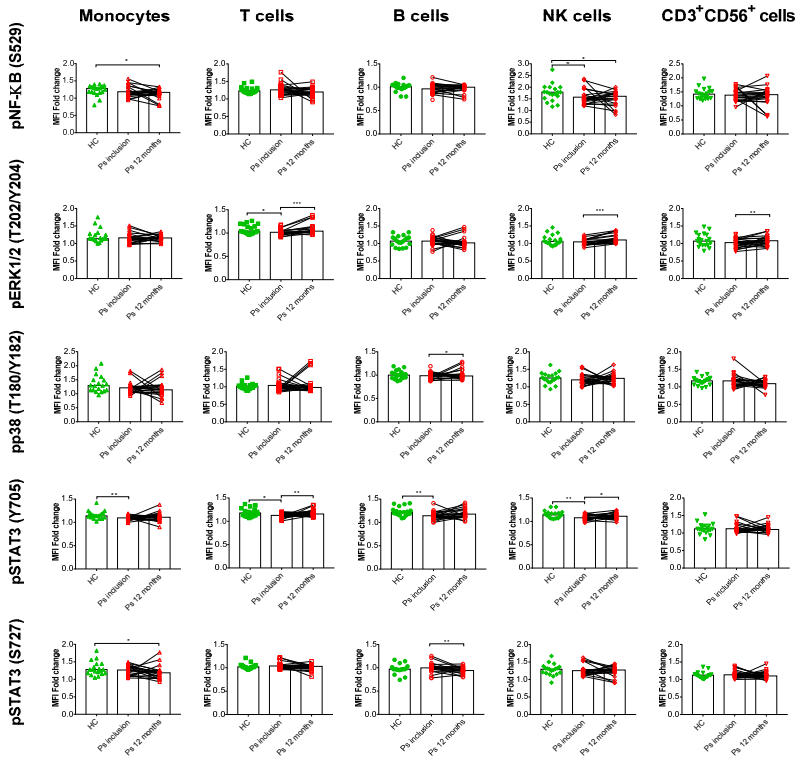

Figure S2: Fold change of intracellular epitopes in PBMC from healthy controls and psoriasis patients at inclusion and after 12 months. Fold change was defined as raw MFI of measured phosphoprotein in TNF stimulated cell subset relative to raw MFI of corresponding phosphoprotein in unstimulated cell 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. * = $P \leq 0.05$, **=$P \leq 0.01$

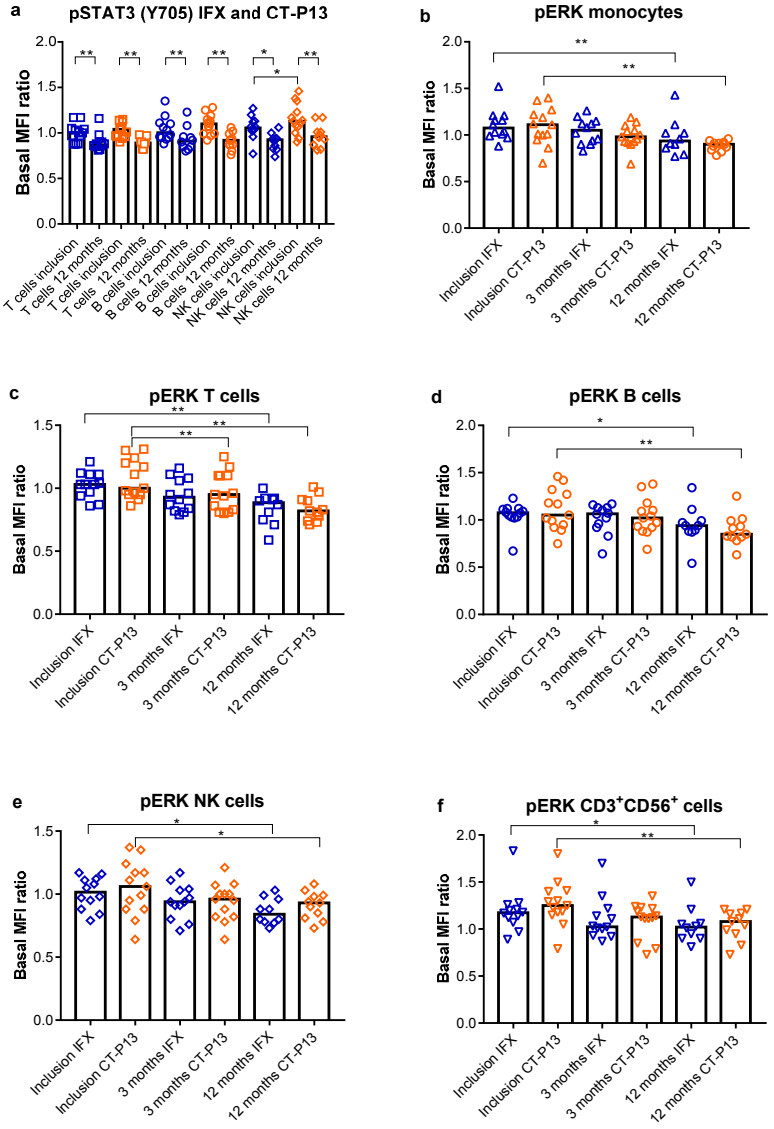

Figure S3: Significant changes in basal phosphorylation of intracellular epitopes with time in both treatment groups. a: pSTAT3 (Y705) in T, B and NK cells at inclusion and after 12 months. b-f: pERK in immune cell subsets at inclusion, after 3 and 12 months follow-up. b: monocytes, c: T cells, d: B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

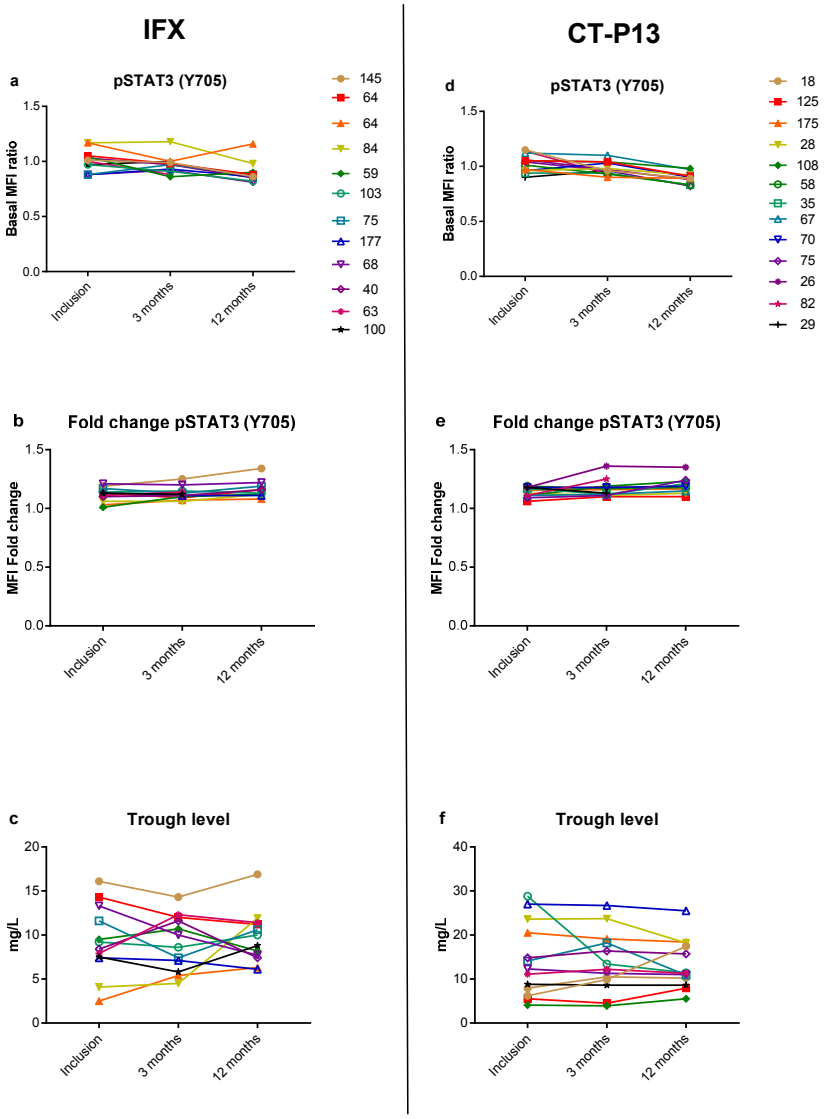

Figure S4: Basal MFI ratio and fold change for STAT3 (Y705) in T cells from originator infliximab (IFX) and CT-P13 patients compared to duration of treatment before inclusion and trough level. a and d: Basal MFI and duration of IFX treatment before inclusion (months; shown at the right side of graph); 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

III

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

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Key Points

Question: Can PBMC subset analysis utilizing mass cytometry be used to determine disease activity in psoriasis patients and predict responders and non-responders?

Findings: In this longitudinal comparative study with 32 psoriasis patients and 10 healthy controls, number of circulating Th17, Th22, Th9 and cytotoxic T cells were increased in severe psoriasis. Treatment with biologics resulted in changes in T cell subsets and monocyte compartment. Responders had relatively more memory than naive/effector Tregs compared to non-responders.

Meaning: These changes indicated amelioration of systemic inflammation, with favorable implications on psoriatic comorbidities. Prospective stratification of patients regarding future treatment response might be possible.

ABSTRACT

Importance.

Psoriasis is an immune-mediated skin disease with different phenotypes, initiated by autoantigens in susceptible individuals and accompanied by systemic inflammation. Dendritic cells, Tc and Th subsets are involved in plaque formation, creating self-amplification loops. Inflammatory cells and cytokines can recirculate, possibly contributing to comorbidities. Biological therapy is effective, but not prescribed based on individual immune signature.

Objective.

The aim of this study was to characterize PBMC subsets in the search for immune signatures and biomarkers related to psoriasis severity and treatment effect.

Design.

This was a longitudinal comparative study using 26-parameter mass cytometry to analyze cryopreserved peripheral blood mononuclear cells (PBMCs), regarding cell subsets and phosphorylation state.

Setting: population-based

Participants

PBMCs from 32 patients with severe psoriasis vulgaris before and after initiation of biological therapy (etanercept, infliximab, ustekinumab and secukinumab) were included.

Results.

A shift in Th1-Th2 balance and transition from naïve/effector to memory predominance was detected in patients after initiation of biological therapy. Abundance of circulating Th17, Th22, Th9 and cytotoxic T (Tc) cells was increased in severe psoriasis but normalized with

treatment. Intracellular pp38 and pERK in Th cells were associated with disease severity. PD-1 expression on Tc cells was reduced at inclusion but increased for Treg and Th cells during treatment. Systemic treatment of psoriasis reduced number of classical monocytes and their STAT1 phosphorylation.

Conclusions and Relevance.

Mass cytometry identified aberrancies in abundance and intracellular phosphorylation of PBMC subsets in psoriasis patients. Improvement was observed with biological treatment, but intracellular phosphorylation did not completely normalize, which might reflect constitutively activated pathways. In addition, biological treatment induced memory cells and restored inhibitory function of T cells. Together with reduction of proatherogenic profile in monocytes, these changes ameliorate systemic inflammation, possibly with favorable implications on psoriatic comorbidities.

INTRODUCTION

The recent years' advances in the characterization of psoriasis immuno-pathogenesis have revealed that the complexity of this systemic inflammatory disease reaches beyond chronic skin lesions [1-5]. Crosstalk between components from innate and adaptive immune system leads to self-sustaining inflammatory loops with the TNF/IL-23/IL-17 axis playing a central role [6,7]. Participants in these cycles are keratinocytes, dendritic cells, T cells and neutrophils, which undergo activation, amplification, dysregulation and decreased suppression at different levels [8,9]. Furthermore, psoriasis pathogenesis has an autoimmune component mediated by autoreactive tissue-resident CD8 cells, and autoantibodies against LL37 were recently discovered [7,10,11]. The vast majority of T cells in psoriatic skin are polyclonal CD4+ T cells, which are capable of plasticity and pathogenic polarization, with recirculating potential [7]. Th1 cells are involved in the initiation of psoriasis, while Th17 and Th22 cells predominate in later chronic phases [6]. Comorbidities like cardiovascular disease (CVD), metabolic syndrome, Mb. Crohn and depression share susceptibility genes with psoriasis, and mechanistic links through systemic inflammation exist [3,4,12-14].

Cytokine production and transcription factors can define immune cell subsets of the blood. However, surface markers, including chemokine receptors, can also be used for cell characterization [15-18]. Flow cytometry is traditionally applied for immuno-phenotyping of peripheral blood mononuclear cells (PBMCs), but a limited number of markers can be used at a time partly because of overlapping fluorescence spectra [16]. As an increasing number of defining molecules are used to describe cell subtypes, a need for technologies that can characterize multiple epitopes simultaneously has arisen. Mass cytometry has expanded the number of detectable targets to 40 at a single cell level [19,20]. Furthermore, the limitation of spectral overlap is almost abolished by use of heavy metal-tagged antibodies [21]. Extra- and intracellular molecules are labeled before single cells are passed through argon plasma, which

evaporates all biological material, leaving only ions to be identified depending on their time of flight as a function of atomic mass [19]. Mass cytometry has facilitated broader characterization of immune cells in health and disease, and through biomarker discovery given new insights to immunopathology in both cancer and inflammatory diseases [22-27]. This methodology may be particularly advantageous in dermatological research since mass cytometry can be applied on both blood and tissue [28-30].

Patient stratification based on immune-profiling may be useful in the clinic for individualizing therapeutic strategies [31,32]. Cytokines and immune cells in blood are possible biomarkers for psoriasis severity [33]. Targeting systemic inflammation might alter the course of chronic psoriasis and have positive implications on comorbidities like CVD. The purpose of this study was to extensively characterize PBMC subsets by use of mass cytometry, in the search for immune signatures and systemic biomarkers related to psoriasis severity and treatment effect.

MATERIAL AND METHODS

Characteristics of patients and healthy controls

32 patients with severe psoriasis vulgaris were included from the Department of Dermatology, Haukeland University Hospital, Norway. They were prescribed infliximab (anti-TNF-α antibody, n=8), ustekinumab (anti-IL12/23 antibody, n=8), secukinumab (anti-IL17A antibody, n=8) or etanercept (TNF-receptor blocker, n=8). Blood samples and clinical data were collected at inclusion, before initiation of biological therapy, and after 4 (range 2-9) and 12 months (8-20) follow-up.

10 age, sex and body mass index (BMI) matched healthy controls without psoriasis (HC) were included. Of these, four volunteers gave blood at three time points. The rest were collected from the Blood bank at the Haukeland University Hospital. The study was approved by the regional ethics committee (approvals 2014/1373 and 2014/1489). Written informed consent was obtained from patients and HC before entering the study. Demographic characteristics are summarized in **Table 1**.

Blood sampling and set-up

Blood was collected in Lithium-heparin tubes (Becton Dickinson Ltd., UK) and PBMCs were cryopreserved in liquid nitrogen following density gradient centrifugation, as previously described [34]. The study encompassed 120 samples (96 patient samples, 18 HC samples and 6 internal controls (IC)), divided in six 20-plex barcodes (BC). One PBMC sample from the same donor (buffy coat) was included in every BC and used as IC. Each BC was a mixture of patients and HC, but included all the time points from the same individual.

Sample preparation and viability staining

Cells were thawed in a water bath (37°C) and washed in 9 ml serum-free medium X-VIVO-20™ (Lonza, Switzerland) (37°C), containing 250 U endonuclease (Pierce™ Universal Nuclease for Cell Lysis; Thermo Fisher Scientific, MA, USA), followed by resuspension in X-VIVO-20™ medium at room temperature (RT) before 120 min incubation in an incubator (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 without additives (Lonza, Switzerland) before 5 min incubation, 37°C, with live/dead marker Cell-ID™ Cisplatin (Fluidigm, California, USA, [35]) 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 washing 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) in a total volume of 50 μl per well and incubated 30 min on ice with 50 μl per well of Antibody Mix 1 **(Suppl. Table S1)**. Cells were washed twice with CSB followed by 10 min fixation with 1ml Fix I Buffer from Fluidigm (RT). Barcoding was conducted in accordance with Fluidigm protocol for Cell-ID 20-Plex Pd Barcoding kit. After that, the 20 samples were combined in a 5 ml Polystyrene Round-bottom Tube (BD Biosciences, MA, USA), and cells were counted.

A mix of 9 million barcoded cells was either further processed the same day or frozen down in 90% Fetal Bovin Serum Gold, (PAA Laboratories, Austria) with 10% dimethyl sulphoxide (DMSO) (Merck, Germany) in -70°C, as recommended [36]. The frozen samples

were thawed on ice after 6 weeks, diluted in 3 ml CSB with 250 U endonuclease. Further processing was the same for all the six barcodes.

For additional surface staining, the 9 million barcoded cells were washed and resuspended in 150 µl CSB followed by incubation with 150 µl Antibody Mix 2 **(Suppl. Table S1)** 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, volume was adjusted to 150 µl with CSB after washes, followed by incubation with 150 µl Antibody Mix 3 **(Suppl. Table S1)** 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 then 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 a 1:10 concentration of EQ™ Four Element Calibration Beads [37]. Samples were then 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) at a speed of 300-500 events per second. The resulting FCS files were normalized to EQ beads (140Ce, 151Eu, 153Eu, 165Ho, 175Lu), concatenated per barcode and debarcoded (Fluidigm softwear) **(Suppl. Fig. S1**).

Gating, clustering and dimensionality reduction

FlowJo 10.2 was used to check each antibody conjugated metal against time. In addition, clean-up gates for live single cells and elimination of beads were drawn prior to further analysis **(Suppl. Fig. S2**). The consistency of staining with the 26 marker panel for each of the six barcodes is displayed in **Suppl. Fig. S3** as viSNE plots (dimensionality reduction method) [38].

Defining markers used for identification of PBMC subpopulations are listed in **Table 2** [15-18]. Manual gates were tailored per file and population according to gating strategy shown in **Suppl. Fig. S4**. In addition, FlowSOM analysis including 20.000 events (randomly subsampled) from each sample, was conducted [39]. Adjusted meta-clusters were tailored from the resulting 100 FlowSOM clusters by use of Star plots and Minimum Spanning Tree (MST), identifying defining markers for each population **(Fig. 1).** Event counts and surface marker expression of PBMC subsets are displayed in **Suppl. Table S4** and **Suppl. Fig. S5.**

Statistical analyses

Statistical analyses were conducted in SPSS version 24. Wilcoxon test was used for investigation of individual patients at different time points. Mann-Whitney *U* test was applied for comparison of patients with HC, or non-responders (< 75 % improvement) with responders (≥ 75% improvement: PASI 75). Fold changes (FC) of MSI (Median Signal Intensity) 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 (T2/T1 or T3/T1) 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 to generate the figures [40].

RESULTS

Psoriasis and life quality improved with biological treatment

At inclusion all patients had severe psoriasis with impact on quality of life. Psoriasis Area and Severity Index (PASI) and Dermatological Life Quality Index (DLQI) were significantly improved at follow-up **(Table 3)**. However, 15 of 32 patients reached PASI75 and were denoted as responders, while the 17 non-responders improved moderately, but did not reach PASI75 **(Suppl. Table S2).**

Pathogenic PBMC subsets normalize with biological treatment

Abundance of Th2 cells was significantly lower at inclusion than at follow-up, and Th1 cells tended to decline to level of HC during treatment **(Fig. 2 a-b).** Abundance of Th22 and Th9 was higher in patients at inclusion compared to those at 1 year follow-up **(Fig. 2 c-d)**. At inclusion, abundance of CD4 cells expressing CD45RA was significantly higher, and CD4 cell expressing CD45RO significantly lower compared to both follow-up time points (**Fig. 2 e-f)**. Further, patients at inclusion had higher abundance of memory Tc cells (CCR4+) than HC **(Suppl. Fig. S6)**. Regression analyses after clustering (CITRUS [41]), indicated that the most prominent stratifying cell populations to differentiate psoriasis patients from HC belonged to the CD8 population (data not shown). However, these analyses did not give significant results for the different treatment groups.

Patients tended to have lower abundance of Tregs than healthy controls at inclusion and 4 months, however, after 1 year it was more similar to HC (**Fig. 2 g)**. Two different clusters of Tregs were identified; CD45RA+ $CCR4^{low}$ and CD45RO+ $CCR4^{high}$ **(Suppl. Table S3)**. At inclusion, Treg CD45RA expression was higher and CD45RO expression lower than at follow-up **(Fig. 2 h).**

Abundance of classical and intermediate monocytes (MC) decreased during the first 4 months of treatment. Both reached similar levels as HC after 1 year **(Fig. 2 i-j)**.

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

At inclusion, PD-1 expression on Th2 cells was higher and on CD8 cells lower in patients compared to HC **(Suppl. Fig. S7 a)**. After 1 year, the expression of PD-1 on CD4 cells and Tregs in patients had increased **(Suppl. Fig. S7 b)**.

Intracellular phosphorylation of PBMCs improved, but did not completely normalize, during biological treatment

At inclusion patients had higher pp38 in CD4 cells, Th2 and Tregs, pERK in CD4 and pSTAT1 in classical MC than HC **(Fig. 3 a-d)**. The phosphorylation was still higher at both follow-up time points for Th2 cells and after 1 year for pERK in CD4 cells.

Basal intracellular phosphorylation of STAT1 and p38 decreased in classical MC from patients during the first 4 months of treatment (**Suppl. Fig. S8 a)**. The level of pSTAT1 was reduced in Th17 cells after 1 year, and pNF-κB in NKT cells increased during the first year of treatment **(Suppl. Fig. S8 b)**. Increased intracellular phosphorylation is exemplified with one HC and one patient at inclusion in **Suppl. Fig. S9.**

Stratification of responders and non-responders based on PBMC features

In responders, there was a negative correlation between PASI improvement and fold change (FC) of Th17 abundance 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).

A positive correlation between PASI improvement and FC of NKT cells after 4 months was detected in the patient population (T2/T1) ($p \leq 0.023$, rho 0.40) (data not shown).

Non-responders had decrease of NK and NKT cells after 4 months relative to inclusion **(Fig. 4 a)**. Further, CD45RA/RO ratio in Tregs at inclusion was lower in those that turned out to have good treatment response compared with those who did not reach PASI75 **(Fig. 4 b)**.

Responders had higher FC of PD-1 on CD4 cells after 4 months and on NK cells after 1 year **(Suppl. Fig. S10).**

The expression of the epithelial-homing marker CCR10 on B cells was higher in patients than HC at inclusion **(Fig. 4 c).** Interestingly, responders had higher FC of epithelial-homing CCR10 on CD4-, CD8- and B cells after 4 months and on Th17 cells and NK cells after 1 year with treatment compared to non-responders **(Fig. 4 d)**.

At inclusion non-responders had higher pSTAT1 in Th17 cells, pp38 in classical MC, and pp38 and pNF-κB in intermediate MC than responders **(Fig. 4 e)**. Responders had higher FC of pNF-κB, pSTAT1 and pp38 in non-classical MC compared to non-responders after 1 year **(Suppl. Fig. S11 a).** On the other hand, non-responders had larger FC of pSTAT3 after1 year in intermediate MC **(Suppl. Fig. S11 b).**

DISCUSSION

By using mass cytometry in this longitudinal comparative study, we get a novel, in-depth characterization of blood immune signatures in psoriasis patients on biological treatment. A relative increase in the Th2 fraction and a tendency of reduction in the Th1 fraction were detected during follow-up. This shift in the Th1-Th2 balance is in agreement with earlier reports [4,42,43]. During active psoriasis, elevated amounts of Th1 cells in blood are found and these can be recruited to the skin [7,44,45].

Reduction in Th17 and CD8 subsets, two main players in the pathogenesis of psoriasis, correlated with improvement of skin disease. Increased level of CD8 in active psoriasis has been shown earlier, including memory CD8 cells (CCR4+) [22,46,47], which we found to be elevated in psoriasis patients compared to HC. It has been postulated that in chronically inflamed tissue, a fraction of resident CD8 memory cells can be released into the circulation, contributing to systemic inflammation associated with severe psoriasis [48]. Although the Tc17 phenotype (CD8+CCR6+CD161+) is of pathogenic interest in psoriasis [7,49-51], we did not detect significant differences between groups for this subset.

Elevated levels of circulating Th22 and Th9 were associated with active disease. Raised blood levels of Th22 has earlier been detected in psoriasis, and Th22 is proposed, together with Tc17 cells, to constitute disease memory in recurrent psoriatic plaques [8,52,53]. Th9 is thought to exert a role in the induction and maintenance of cutaneous inflammation [54].

In psoriasis, Tregs have deficient suppressor activity and are relatively decreased, leading to insufficient peripheral tolerance against autoreactive T cells [55]. In addition, differentiation into IL-17 producing Tregs probably contribute to chronic inflammation [55-57]. At inclusion, patients had lower levels of Tregs than HC, however, after 1 year with biological treatment, the level was normalized, in accordance with findings of others [42]. In coronary artery disease, where Tregs also are known to be decreased and dysfunctional [13].

A shift from naïve/effector to memory predominance for CD4 cells was detected, in concordance with the findings of others [43]. Interestingly, responders had relatively more memory than naive/effector Tregs compared to non-responders, reinforcing that prospective stratification of patients regarding future treatment response might be possible.

Activation of T cells requires interaction between T cell receptor and major histocompatibility complex on antigen presenting cells, in addition to co-stimulatory or co-inhibitory signals. PD-1 that normally plays a role in immune response silencing, has in psoriasis been shown to be downregulated, resulting in immune overstimulation [58,59]. Blockade of PD-1 augments Th1 and Th17 responses, but suppresses Th2 responses [60]. Patients had reduced expression of PD-1 on CD8 cells at inclusion, however, the opposite was detected for Th2 cells. After 1 year with biological therapy, the expression of PD-1 on CD4 cells and Tregs was increased. Further, responders had significantly larger increase of PD-1 on CD4 and NK cells at follow-up than non-responders.

NK cells have increased cytotoxic potential in psoriasis and NKT cells are potent IL-17 and IFN-γ producers [5,61]. Decreased levels of circulating NK and NKT cells have been reported in psoriasis [62-64]. Responders had higher increase in number of NK and NKT cells at follow-up than non-responders and the change of NKT cells correlated with PASI improvement, which could indicate efflux from healed skin.

In light of the recently discovered autoantibodies against LL37, a main trigger in psoriasis, the higher expression of CCR10 on B cells in patients with severe psoriasis is especially interesting [10,65]. It is tempting to speculate if these epithelial directed B cells reflect an autoimmune response. We detected higher expression of epithelial-homing factor CCR10 on CD4-, Th2, Th17, CD8-, B- and NK cells in responders compared to non-responders at follow-up, which might reflect sustained production of skin-homing PBMCs despite diminished expression of ligands in the skin or efflux from healed skin [43,48].

The immuno-pathogenesis of psoriasis consists of a complex interplay between cells and cytokines creating inflammatory amplification loops [6,7,12,61,66]. Upon cell activation, intracellular signalling molecules will be phosphorylated before the signal reaches *e.g.* the nucleus [6,67]. We found that psoriasis patients with severe disease had higher levels of intracellular phosphorylation compared to healthy controls in CD4 (p38, ERK), Th2 and Tregs (p38), in addition to classical monocytes (pSTAT1). Further, intracellular phosphorylation was still higher in CD4 and Th2 cells after several months on biological treatment, but with a decreasing trend. This reduction, but not complete normalization, might be related to genetic susceptibility associated with signalling pathways in psoriasis, underlining the chronicity of psoriasis and need for long-term treatment [6,53,68,69]. Recently, Guo et al. found increased phosphorylation in CD4 cells in a limited number of psoriasis patients, by use of mass cytometry [46]. Earlier studies of inflammatory diseases using other techniques have shown that activation levels in PBMCs decrease with treatment [22,42,70]. Interestingly, a recent study has shown increased ROS production in activated memory CD4+ T cells [71]. Further, our patients had a significant reduction of intracellular phosphorylation in classical monocytes after 4 months and in Th17 after 1 year. We also detected higher intracellular phosphorylation in Th17 cells, classical and intermediate monocytes from non-responders than responders at inclusion, indicating that early stratification based on immune-profile may predict future treatment response.

Cardiovascular risk is linked to psoriatic disease by mechanistic pathways [13,66,72]. It is hypothesized that recirculation of T cells contribute to amplification of inflammation and comorbidities associated with psoriasis [54,73]. For instance, Th1 and Th17 produce inflammatory mediators attracting monocytes, neutrophils and CD8+ T cells [13]. Monocyte recruitment to vessel walls is an early event in atherosclerosis and these cells can be polarized towards pro-atherosclerotic phenotype by skin-inflammation in psoriasis [74,75]. Interestingly,

elevated levels of classical and intermediate monocytes have been shown to be associated with CVD [76,77]. 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, maybe because they were recruited to places of inflammation, like vessel walls [78]. After 1 year, the abundance of classical monocytes was normalized to HC. Patients at inclusion had elevated levels of intermediate monocytes, which decreased during the first 4 months of treatment. Further, intracellular phosphorylation in intermediate monocytes was higher in non-responders than responders at inclusion. Non-classical MC, however, have been postulated to exert an atheroprotective effect [78]. Responders had higher intracellular phosphorylation in non-classical MC after 1 year than non-responders. The distribution of subtypes in the monocyte compartment may indicate that systemic treatment of psoriasis may lower CVD risk [79].

Although this study is unique in exploring a large quantity of PBMC subsets simultaneously in psoriasis, it could have been possible to include even more markers in the panel. Since CXCR3, CCR4, CCR6 and CD127 are sensitive to fixation, these antibodies were added to live cells before barcoding, potentially introducing inter-sample staining variability [36,80,81]. Some authors also recommend staining with CD161 before fixation [36,80]. However, barcoding reduces staining variability, saves time and reagents [82-85]. Live barcoding might interfere less with sensitive epitopes [25,86]. The basal phosphorylation was in general lower than expected and pSTAT3/4/5 barely detected. Future experiments could include cytokine stimulation of PBMCs. Although the largest mass cytometry study on psoriasis so far, the number of included patients was relatively limited. In addition, some patients were using MTX as co-medication. Those that were not naïve to biological treatment underwent the recommended wash-out period before inclusion.

Conclusions

Through mass cytometry analysis of immune cell lineages in blood and their intracellular phosphorylation, we have given a comprehensive description of systemic disease characteristics and possible biomarkers of treatment effect in psoriasis. A shift in Th1-Th2 and naïve/effector-memory balance during treatment fit well with psoriasis immuno-pathogenesis. Circulating CD8-, Th17, Th9 and Th22 cells were found to correlate with disease activity. Expression of PD-1 on lymphocytes might be involved in psoriasis pathogenesis and could potentially constitute a new treatment target. Findings in the monocyte compartment are possibly linked to development of cardiovascular disease in psoriasis. Further, intracellular phosphorylation of PBMCs was higher in psoriasis patients than HC and in non-responders relative to responders. Hopefully, future research will provide promising avenues for patient stratification based on immune-profiling, enabling personalized treatment that can alter the chronic course of psoriasis with positive implications on long-term comorbidities.

Author contributions

Planning of study: SMS, SA, YTB, AKA, RJ. Collection of samples: SMS, LFS. Experimental work: SMS, IS, AP. Acquisition on mass cytometer: BB. Data analysis: SMS. Writing of manuscript SMS, SA, YTB. Preparation of figures: SMS. All authors revised the manuscript and approved its final version.

Conflict of interest

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. 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.

Figure 2. Significant differences in abundance of PBMC subpopulations between healthy controls and psoriasis patients at inclusion and follow-up. Displayed is percentage of CD4 cells **a:** Th2, **b:** Th1, **c:** Th22, **d:** Th9, **e:** CD45RA expression, **f:** CD45RO expression and **g:** Tregs. **h:** Treg expression of CD45RA and CD45RO. **i:** classical and **j:** intermediate monocytes (MC) out of 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 3. Comparison of intracellular phosphorylation in PBMCs from healthy controls (HC) and psoriasis patients (PS). 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.

Fig. 4. Comparison of different features detected by mass cytometry in responders (R) versus non-responders (NR). a: Fold change (FC) of NK and NKT cell abundance after 4 months; **b:** Treg CD45RA/RO inclusion ratio for healthy controls (HC), R and NR; **c:** expression of CCR10 on B cells at inclusion for HC and patients, **d:** FC of CCR10 in PBMCs during follow-up; **e:** Intracellular phosphorylation at inclusion. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. Psoriasis patients at inclusion: T1, after 4 months on biological therapy: T2 and after 1 year: T3

Table 1. Characteristics of patients and controls at inclusion: sex, age, weight, body mass index (BMI), psoriatic arthritis (PsA) and methotrexate (MTX) use; values are listed as min-max and mean.

	Patients	**Controls**
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 (12mg/week)	0/10

Table 2. Defining markers for identification of PBMC subpopulations.

Population	Defining markers
T cells	CD3+CD19-
CD4	CD3+CD19-CD4+CD8-
CD8	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 3. PASI and DLQI at inclusion, 4 and 12 months after starting biological treatment, mean ± S.E.

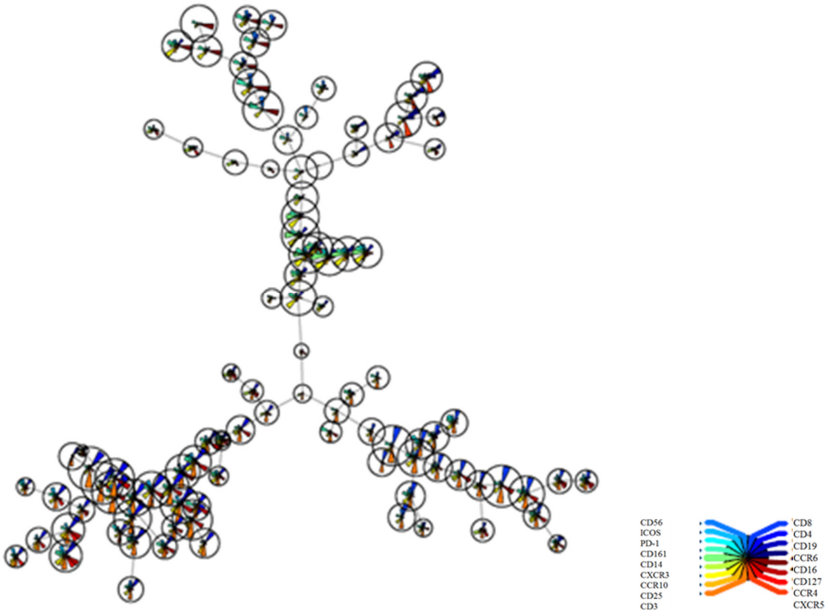
	PASI incl.		PASI 4M		PASI 12M		DLQI incl.		DLQI 4M		DLQI 12M	
Infliximab	11.7	±1.1	2.2	±0.6	1.4	±0.5	15.3	±2.3	1.5	±0.5	1.9	±0.5
Ustekinumab	11.1	±3.4	4.3	±1.8	2.9	±1.1	10.0	±2.2	3.1	±0.8	2.5	±0.8
Secukinumab	8.0	±1.8	1.9	±0.5	1.9	±0.4	13.3	±3.1	4.6	±1.8	4.1	±2.0
Etanercept	9.5	±1.5	3.5	±0.8	2.9	±0.7	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

*= $p \leq 0.001$

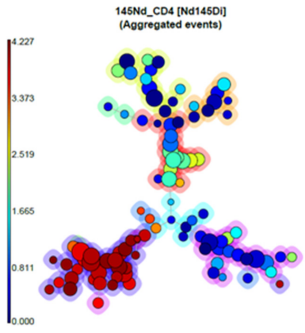
4M= 4 months, 12M= 12months

FIGURES

a



b



c

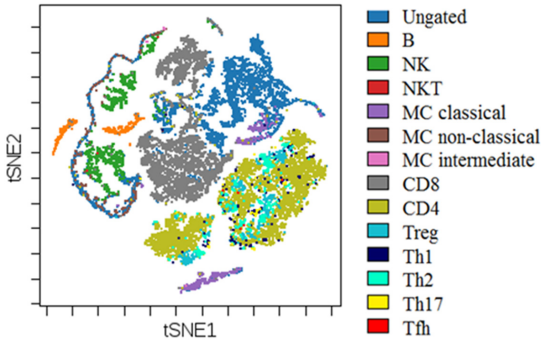


Figure 1

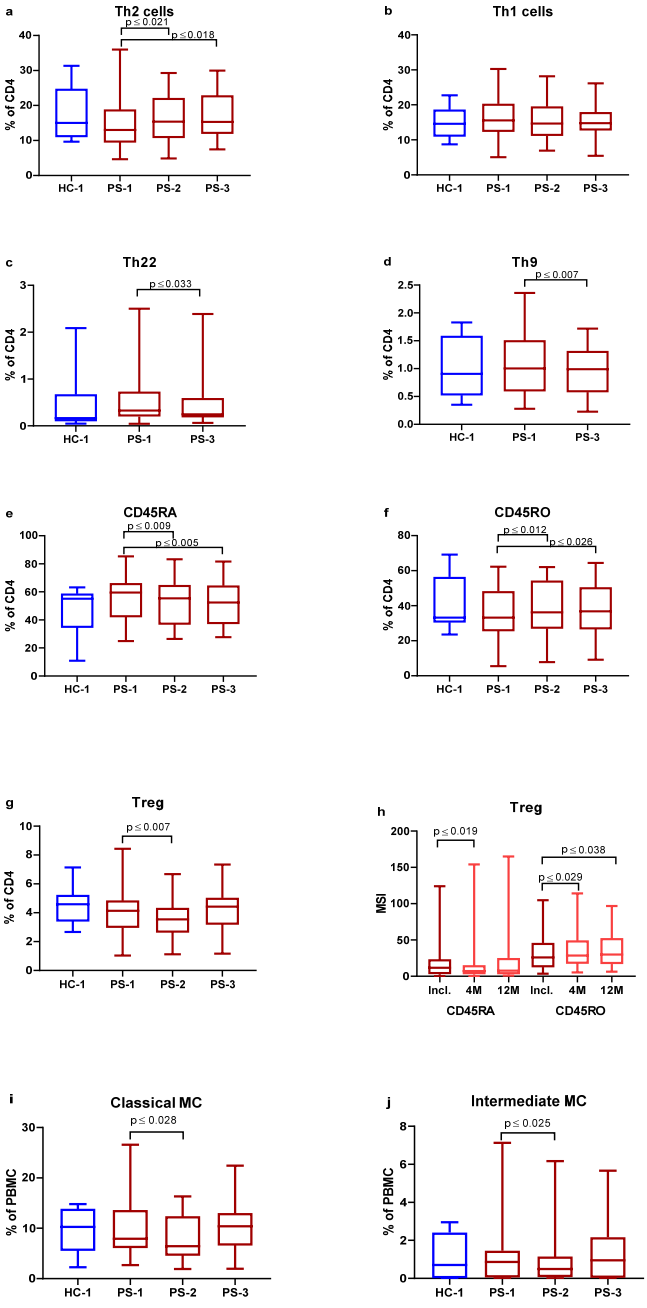

a
Th2 cells
p ≤ 0.021
p ≤ 0.018
% of CD4
HC-1
PS-1
PS-2
PS-3
b
Th1 cells
c
Th22
p ≤ 0.033
d
Th9
p ≤ 0.007
e
CD45RA
p ≤ 0.009
p ≤ 0.005
f
CD45RO
p ≤ 0.012
p ≤ 0.026
g
Treg
h
Treg
p ≤ 0.019
p ≤ 0.038
p ≤ 0.029
MSI
Incl.
4M
12M
CD45RA
CD45RO
i
Classical MC
p ≤ 0.028
% of PBMC
j
Intermediate MC
p ≤ 0.025

Figure 2

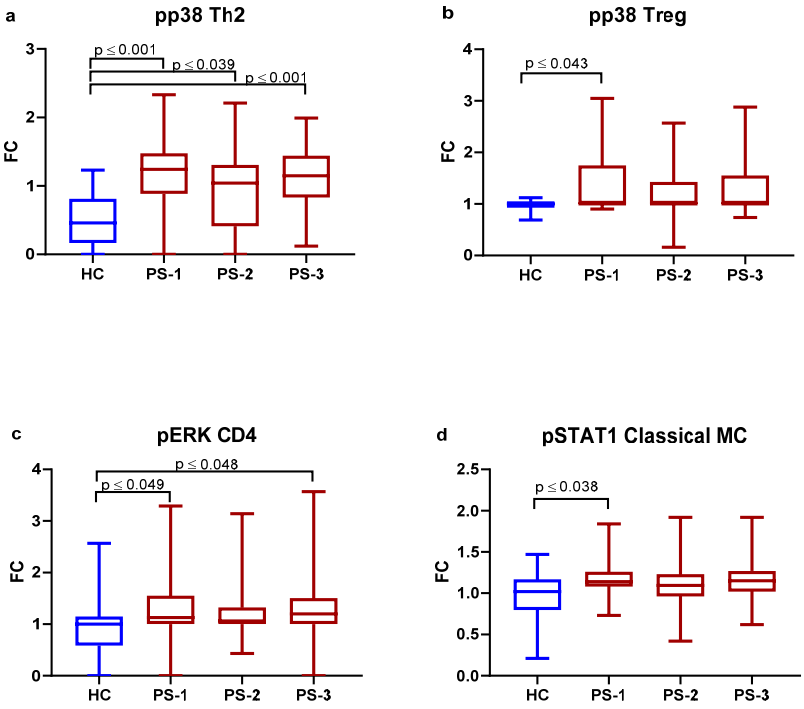

a
pp38 Th2
p ≤ 0.001
p ≤ 0.039
p ≤ 0.001
FC
HC
PS-1
PS-2
PS-3
b
pp38 Treg
p ≤ 0.043
FC
HC
PS-1
PS-2
PS-3
c
pERK CD4
p ≤ 0.048
p ≤ 0.049
FC
HC
PS-1
PS-2
PS-3
d
pSTAT1 Classical MC
p ≤ 0.038
FC
HC
PS-1
PS-2
PS-3

Figure 3

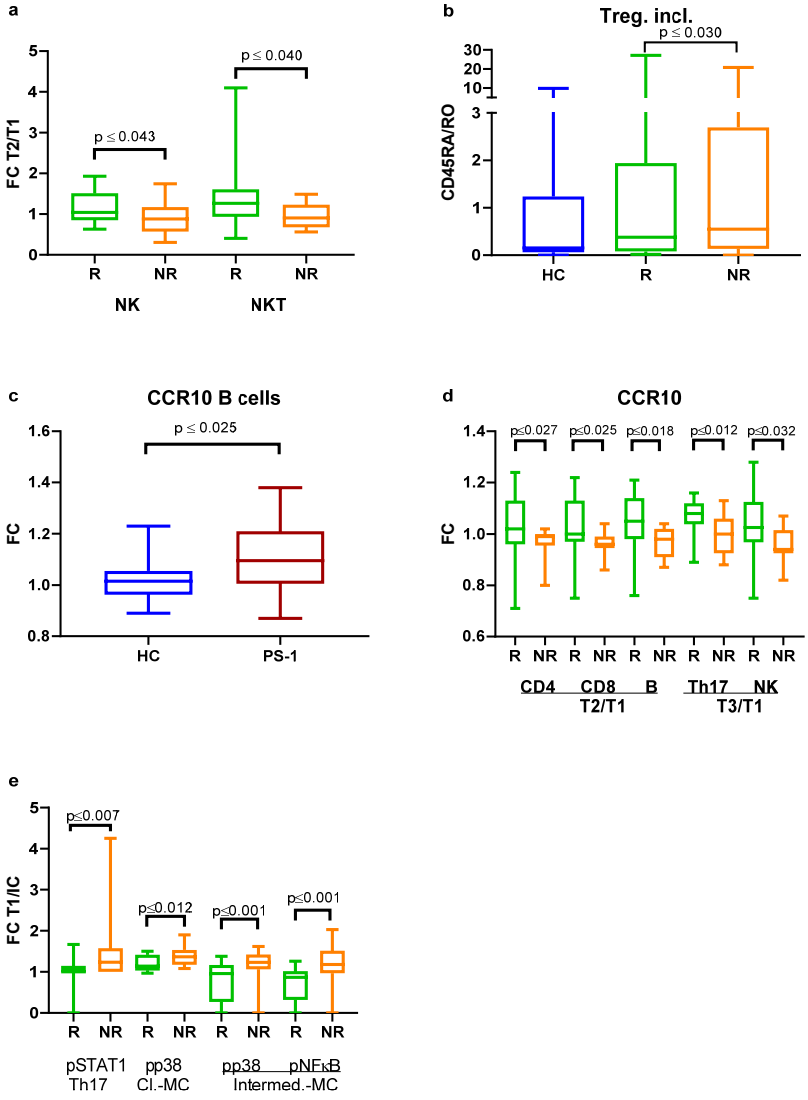

a
FC T2/T1
p ≤ 0.043
p ≤ 0.040
R
NR
R
NR
NK
NKT
b
Treg. incl.
p ≤ 0.030
CD45RA/RO
HC
R
NR
c
CCR10 B cells
p ≤ 0.025
FC
HC
PS-1
d
CCR10
p≤0.027
p≤0.025
p≤0.018
p≤0.012
p≤0.032
FC
CD4
CD8
B
Th17
NK
T2/T1
T3/T1
e
FC T1/IC
p≤0.007
p≤0.012
p≤0.001
p≤0.001
pSTAT1
Th17
pp38
Cl.-MC
pp38
Intermed.-MC
pNFκB

Figure 4

SUPPLEMENTARY TABLES AND FIGURES

Suppl. Table S1. List of antibody target, clone and conjugated metal.

	Antibody	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	p-NFkB	K10x	166Er
	p-p38	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

Suppl. Table S2. Improvement of PASI relative to inclusion value for non-responders (NR) and responders (R) after 4 and 12 months.

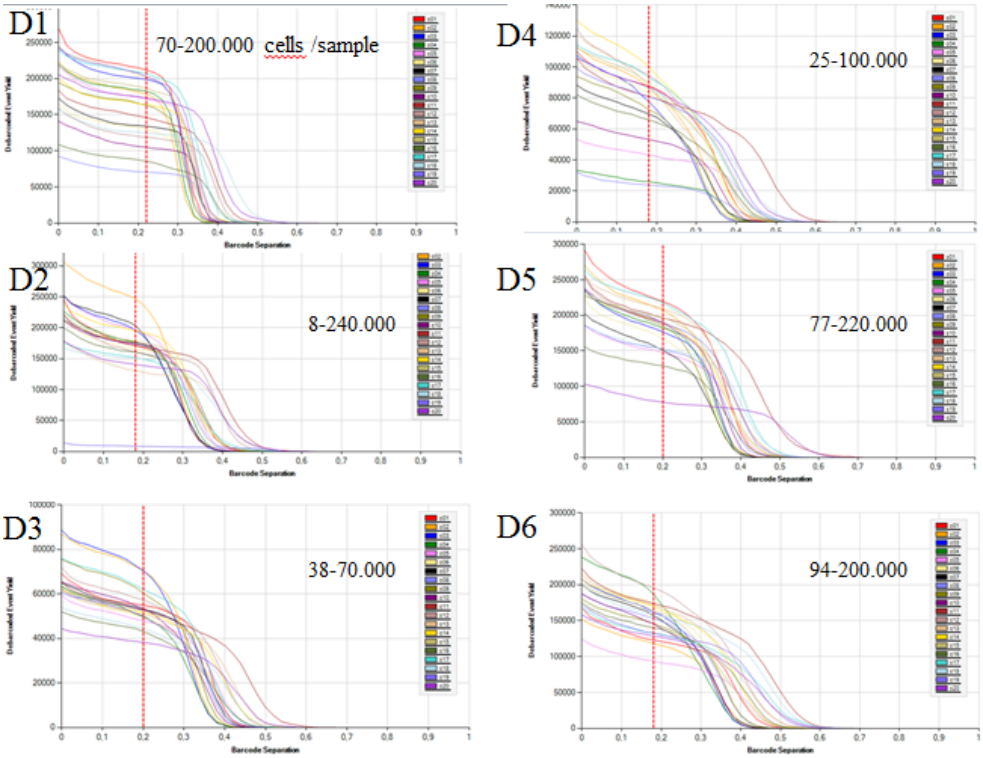
		% improvement from inclusion	Min-max
4 months	NR	52	43-62
	R	90	84-95
12 months	NR	57	46-67
	R	92	89-96

Suppl.Table S3. Marker expression on the two Treg clusters. Mean abundance relative to total PBMCs (SD) and Median Signal Intensity of selected markers are shown.

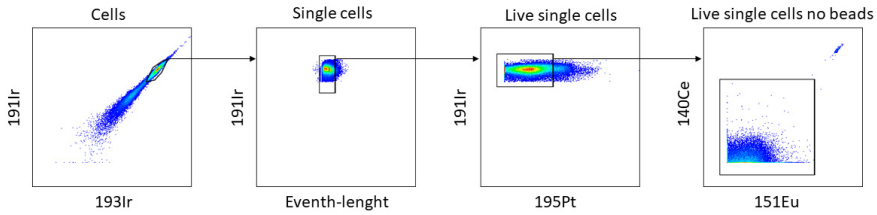
	Abundance	CD3	CD4	CD8	CD14	CD19	CD25	CD127	CCR4	CD45RA	CD45RO
Naive/effector Tregs	1.01 (0.70)	396.12	111.15	4.50	3.41	0.42	58.03	2.59	0.11	50.45	12.20
Memory Tregs	0.71 (0.39)	308.63	110.97	4.65	3.55	0.17	62.29	1.05	33.40	3.97	97.85

Suppl. Table S4: Event counts for PBMC subsets from FlowSOM analysis; mean of 119 samples and S.E. of mean.

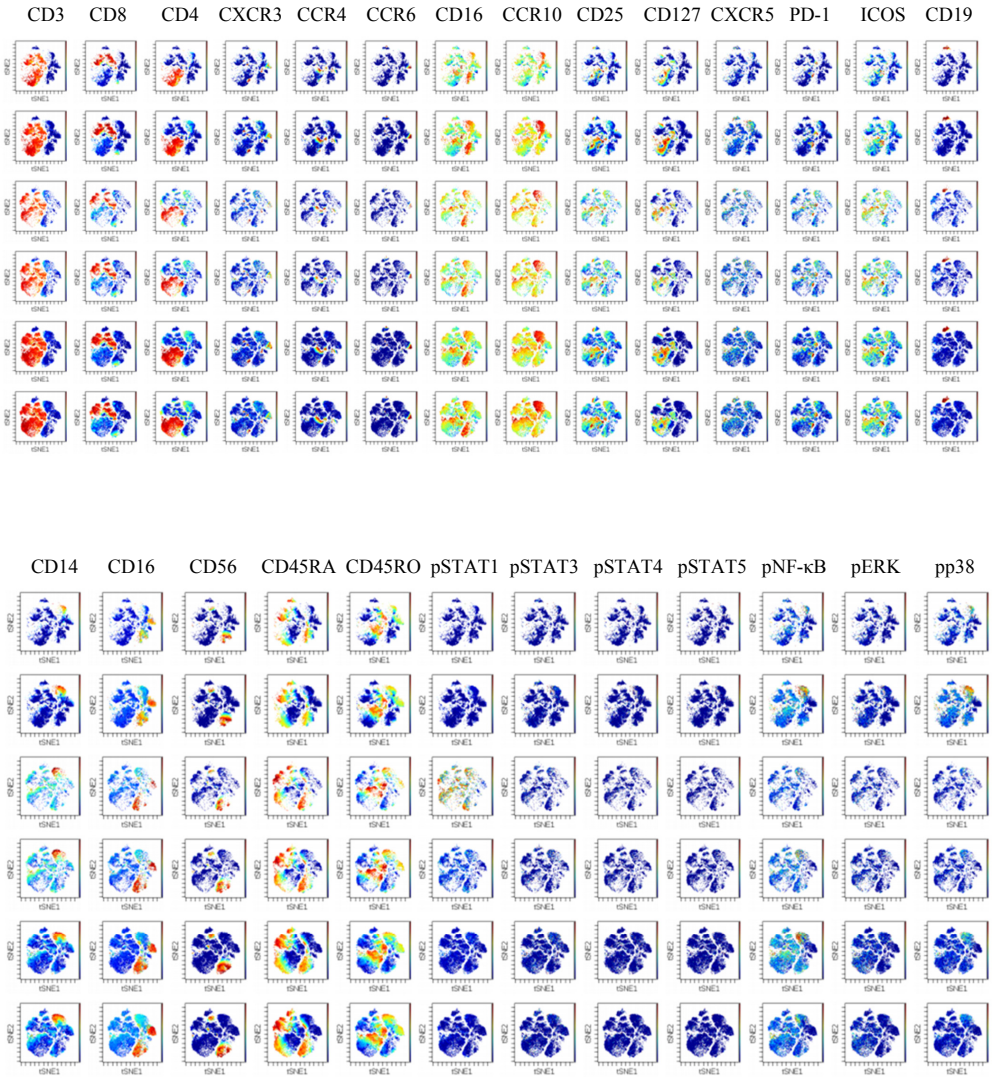
	CD4	CD8	Th1	Th2	Th17	Treg	Tfh	B	NK	NKT	Cl. MC	Non-cl. MC	Intermed. MC
Mean	7149	3930	472	1002	180	343	299	1293	1257	639	1819	443	210
S.E.	210	177	31	55	15	19	19	58	59	98	90	26	24



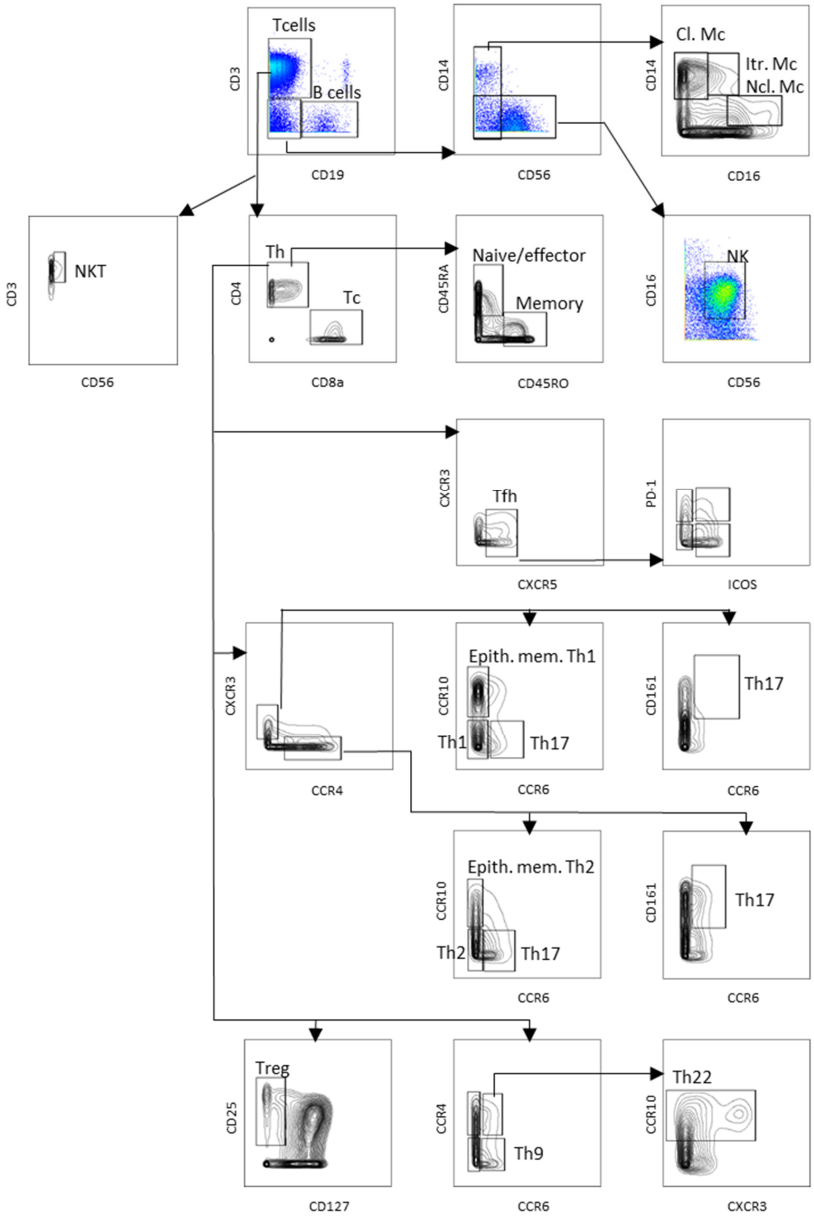
Suppl. Fig. S1: Debarcoded samples showing event yield for each of the 120 samples distributed on six barcodes. D1: day 1 of experiment, containing 20 samples in the first barcode. D2: day 2 of experiment, containing the next 20 samples in the second barcode. Likewise for D3, D4, D5 and D6: they all contain 20 samples in one barcode.



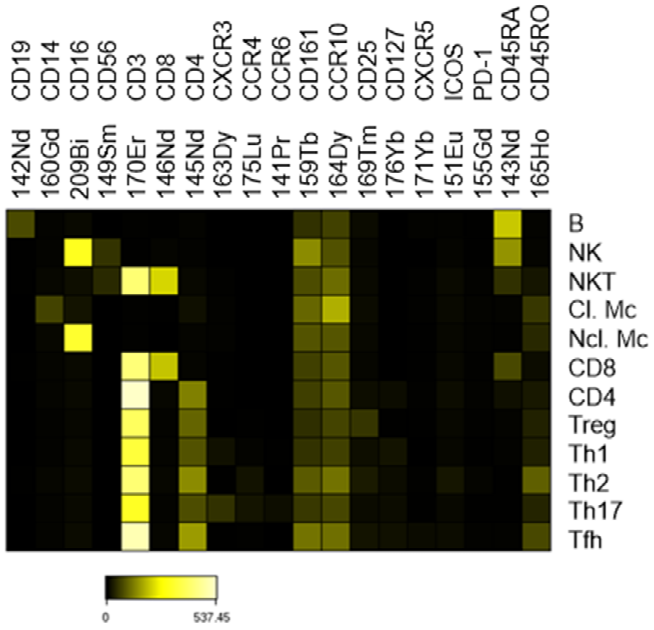
Suppl. Fig. S2: Example of clean-up gates made in FlowJo. The gates were tailored per file to identify live single cells 191Ir-193Ir (DNA1-DNA2); 191Ir-Event length; 191Ir-195Pt (cisplatin) and eliminate beads (140Ce-151Eu).



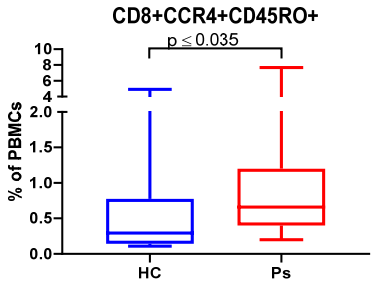
Suppl. Fig. S3. ViSNE plots for all markers in PBMCs displayed for the six internal controls included; one in each barcode.



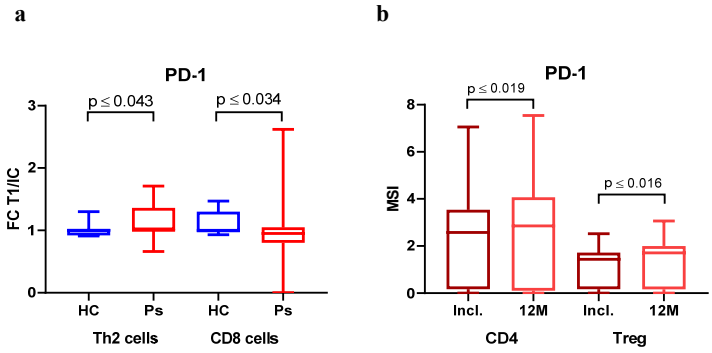
Suppl. Fig. S4: Representative gating strategy from FlowJo for cell populations identified by the panel consisting of 19 surface markers. Cl. Mc: classical monocytes, Itr. Mc: intermediate monocytes, Ncl. Mc: non-classical monocytes. Epith. mem.: epithelial memory cells. Tfh: T follicular helper cells.



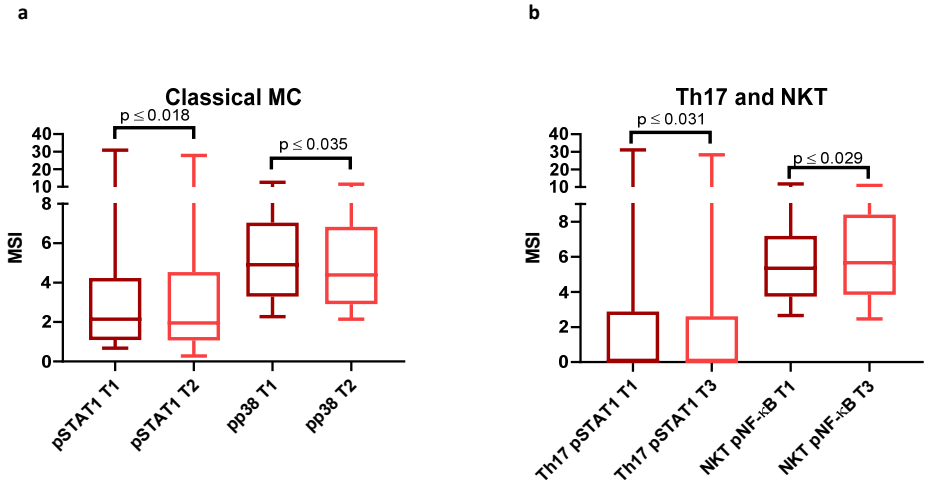
Suppl. Fig. S5: Heatmap showing expression of surface markers in subpopulations, exemplified with the internal control. Cl. Mc: classical monocytes. Ncl. Mc: non-classical monocytes. Tfh: T follicular helper cells.



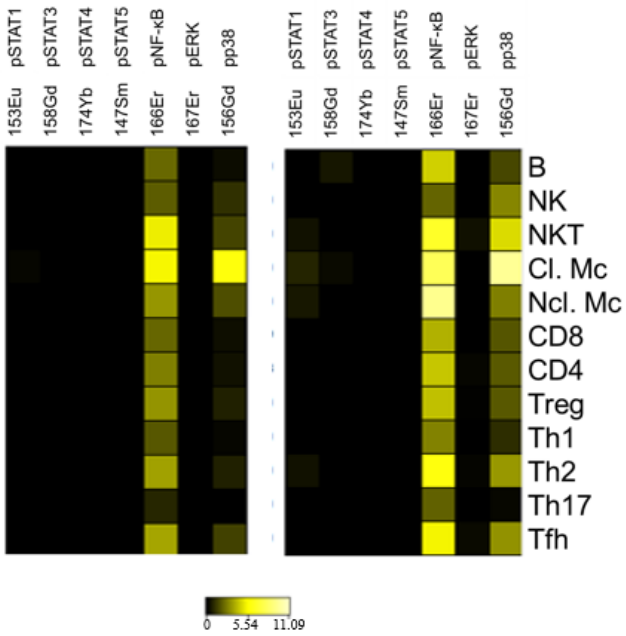
Suppl. Fig. S6. Abundance of memory CD8 (CCR4+) cells in healthy controls (HC) and psoriasis patients (Ps) at inclusion. Box and whisker plots show quartiles with median, ends of whiskers represent min-max.



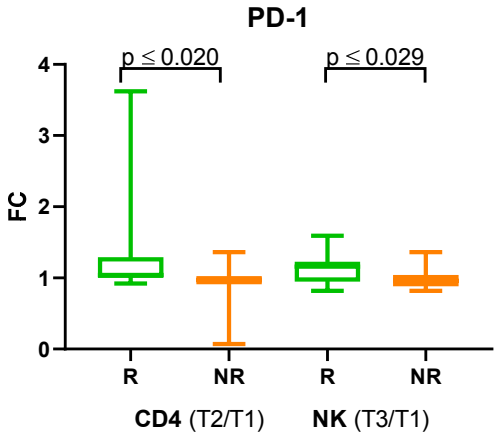
Suppl. Fig. S7. PD-1 expression. a: Th2 and CD8 cells at inclusion, b: CD4 cells and Tregs at inclusion and after 1 year of treatment. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. HC: healthy controls. Ps: psoriasis patients. 12M: 12 months follow-up. FC T1/IC: fold change timepoint 1/internal control. MSI: median signal intensity.



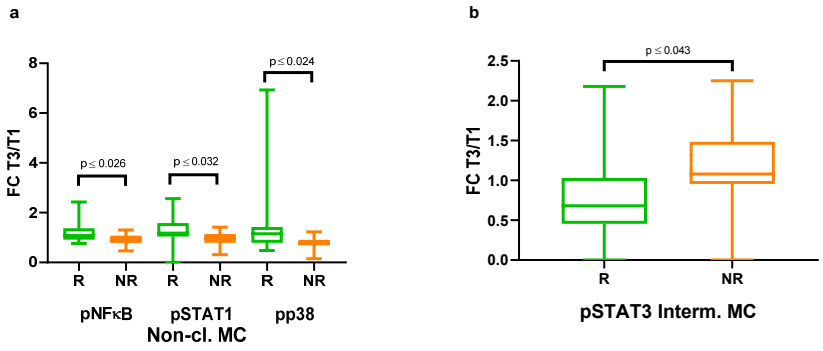
Suppl. Fig. S8. Intracellular phosphorylation during follow-up of patients; a: classical monocytes (MC), **b:** Th17 and NKT cells. Box and whisker plots show quartiles with median, ends of whiskers represent min-max. T1: inclusion. T2: 4 months follow-up. T3: 1 year follow-up. MSI: median signal intensity.



Suppl. Fig. S9. 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.



Suppl. Fig. S10. Change in PD-1 expression for responders (R) and non-responders (NR), fold change after 4 months for CD4 cells and fold change after 1 year for NK cells. 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.



Suppl. Fig. S11. Differences in intracellular phosphorylation after 1 year follow-up between responders (R) and non-responders (NR). a: non-classical monocytes (Non-cl. MC), **b:** intermediate monocytes (interm. MC). Box and whisker plots show quartiles with median, ends of whiskers represent min-max.

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