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

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Linked Comment: Gjersvik. Br J Dermatol 2018; 179:247–248.

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.

DOI 10.1111/bjd.16269
The pathogenesis of psoriasis encompasses interactions between dendritic cells, T cells, keratinocytes and neutrophils. Cytokines released from these cells initiate and perpetuate the inflammation that is characteristic of psoriasis. Patients have increased risk of cardiovascular disease and diabetes, indicating systemic inflammation. This is supported by higher numbers of circulating lymphocytes and increased gene expression of transcription factors and cytokines involved in differentiation of T helper (Th)1, Th17 and Th22 cells. Furthermore, patients with psoriasis display elevated levels of inflammatory cytokines in blood.

Among the cytokines involved in psoriasis pathogenesis, tumour necrosis factor (TNF-α) is regarded as one of the most predominant. 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. TNF-α signals via TNF-receptor 1 and 2, which are expressed on all nucleated cells, and induces phosphorylation of nuclear factor kappa B (NF-kB), 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). NF-kB regulates genes involved in inflammation, cell survival and proliferation. It promotes expression of cytokines involved in the pathogenesis of psoriasis, and NFkB1 and NFkBII1 gene variants are associated with severe psoriasis.

The targets of STAT3 are mainly genes that are anti-apoptotic or pro-proliferative, or which regulate angiogenesis and cytokine production. TNF-α induces activation of STAT3 via phosphorylation of two of its residues, tyrosine (Y705) and serine (S727). STAT3 is required for the development of Th17 cells. 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. The Janus kinase–STAT pathway is considered a promising drug target in psoriasis. 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.

Fumaric acid esters, used in the treatment of psoriasis, effectively inhibit the activity of p38 MAPK, decreasing pro-inflammatory cytokine production. 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. Phosphorylation of the abovementioned intracellular epitopes can be quantified by phospho flow cytometry, potentially serving as a measure of cytokine stimulation. 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. 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. 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. This might influence biological activity, tolerability and efficacy.

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 study31 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., Dundee, Scotland) and cryopreserved in liquid nitrogen by density gradient centrifugation with Lymphoprep (Axis-Shield 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$^{-1}$.

Table 1 Characteristics of patients and healthy controls at inclusion

| Sex, n                | Originator infliximab | CT-P13           | Healthy controls |
|-----------------------|-----------------------|------------------|------------------|
| 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 | 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 | 86.83 ± 39.35 | 68.92 ± 45.75 | NA               |
| Range                 | 40–177                | 18–175           | NA               |

NA, not applicable.

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$$^{-1}$; both Life Technologies, Grand Island, NY, U.S.A.), then incubated in the dark at 4 °C. Further, cells were washed and resuspended in fluorescence activated cell sorter (FACS) buffer (PBS with 1% bovine serum albumin) before combining the eight FCB combinations. FcR blocking reagent (1 : 21) (Miltenyi Biotec, Bergisch Gladbach, Germany) was added, cells were divided into two panels followed by staining with titrated amounts of fluorochrome-conjugated antibodies for 30 min in the dark at room temperature. Cells were then washed with FACS buffer and resuspended in 200 µL FACS buffer + 2 mmol L$$^{-1}$ ethylenediaminetetraacetic 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-kB p65 (p5529, clone K10-895-12-50) and...
acquired in the intact cell gate. A representative gating strategy
porting Information). A minimum of 200 000 events was
lasers. Further specifications are given in Table S1 (see Sup-
bank40 in each stimulation condition. Basal phosphorylation
version 10.2. Cells within each subtype were analysed in Cyto-
were used for compensation, which was performed in FlowJo
conjugated anti-ERK1/2 [(pT202/pY204),20A,RUO
–
anti-p38 (pT180/pY182, clone 36/p38), Alexa Fluor 647
conjugated anti-ERK1/2 [(pT202/pY204),20A,RUO –
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 inter assay variation. All
the experiments were performed by one person within
2 weeks in July 2016 to minimize inter- and intra-assay vari-
ation 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 Sup-
porting 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 Cyto-
bank40 in each stimulation condition. Basal phosphorylation
was defined as raw median fluorescence intensity (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 deter-
mined 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 inflix-
imab 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 differ-
ces 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 anti-
drug antibodies at inclusion (originator infliximab group) and
no patients developed antidrug antibodies during the study.

| 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 | | | | | | |
| Prior use of other biological, n | | | | | | |
| Adalimumab and etanercept | 1 | 1 | 1 | – | – | – |
| Adalimumab | 1 | – | – | 0 | – | – |
| Etanercept | 2 | – | – | 7 | – | – |
| Efalizumab | 0 | – | – | 1 | – | – |

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

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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.

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

The basal phosphorylation levels in PBMCs from the psoriasis group were significantly increased in almost all analysed phospho-epitopes and immune cell subsets at inclusion compared with healthy controls, even though patients had been treated with the originator infliximab for a minimum of 18 months and had no or low-grade skin inflammation. This may indicate that systemic inflammation takes a longer time to cease than skin lesions or that signalling through the investigated pathways is maintained, at least in blood, by cytokines besides TNF-α. Basal levels of pSTAT3 (Y705) in patients with psoriasis have been reported to be similar to those observed in healthy volunteers.42 The discrepancy between these findings and ours may be explained by different methodological approaches (enzyme-linked immunosorbent assay was used in the study by Punwani et al.43) and differences in patient characteristics.

Our study would have benefited 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. 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. 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. 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 FcγRIIb. 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. To this end, assays capable of predicting which treatments are most likely to be beneficial for each individual are required, before commencing therapy. Phospho flow cytometry may be a promising tool for estimating systemic disease activity and treatment response of people with psoriasis in the future and might be helpful in the quest for new potential drug targets.

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.

Video S1 Author video.