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The Role of Th17/Tc17 Peripheral Blood T cells in Psoriasis and Their Positive Therapeutic Response

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Abstract

It is known that NB-UVB therapy can suppress a broad range of immune cells, but the additional effect of bathing in geothermal seawater still remains unclear. To study the influence of treatment on the expression of circulating immune cells contributing to the pathogenesis of psoriasis, six patients with psoriasis were treated with bathing in geothermal seawater two times daily combined with NB-UVB five times/week for 2 weeks and six patients were treated with NB-UVB therapy three times/week for 8 weeks. Disease severity (Psoriasis Area and Severity Index, PASI), chemokines, inflammatory cytokines, T cells and Toll-like receptors in the blood and skin samples were evaluated on enrolment (W0) and at 1 (W1), 3 (W3) and 8 (W8) weeks. Compared with healthy controls, psoriasis patients with active disease had significantly higher proportion of peripheral CLA+ T cells expressing CCR10 and CD103 and T cells with both Th1/Tc1 (CD4+/CD8+ IFN-γ+ or TNF-α+ cells) and Th17/Tc17 (CD4+CD45R0+IL-23R+, CD4+/CD8+ IL-17A+ or IL-22+ cells) phenotypes. Both treatments gave a significant clinical effect; however, bathing in geothermal seawater combined with NB-UVB therapy was more effective than NB-UVB therapy alone. This clinical improvement was reflected by a reduction in circulating CLA+ peripheral blood T cells and by a decreased Th1/Th17 and Tc1/Tc17 inflammatory response. These findings suggest that the inflammatory response in psoriasis is predominantly driven by both CD4+ and CD8+ skin-homing tissue retaining T cells of the Th17/Tc17 lineages.

Introduction

Bathing in geothermal seawater from the Blue Lagoon (BL) in Iceland has been reported to have a beneficial effect on psoriasis [1, 2]. Additional treatment with narrow-band ultraviolet (NB-UVB) phototherapy further increases the efficacy of the treatment [3–5]. The BL contains geothermal seawater originating from underground reservoirs filled with a mixture of fresh water and seawater. Sampling from the lagoon shows that no pathogenic bacteria thrive in this ecosystem [6]. The fluid in the lagoon has a high level of silica but is moderate in temperature (37 °C) and salinity (2.7%) [7]. Recent data indicate that both the silica mud and two microalgae species growing in the BL promote the integrity of the skin barrier and delay extrinsic skin ageing, thus indicating a biological activity in the lagoon [8].

Psoriasis is mediated by T cells that trigger keratinocytes to hyperproliferate and perpetuate the disease [9]. T helper (h)17 and Th1 cells and the cytokines produced by these cells are found in increased levels within psoriasis plaques [10] as well as in the circulation [11] and are thought to have an important role in psoriatic inflammation. The relationship between Th1 and Th17 cells is still unclear. The tissue-specific localization of T cells is thought to be guided by the skin-homing molecules such as cutaneous lymphocyte-associated antigen (CLA), various chemoattractants and their receptors, including chemokine receptors 4 (CCR4) and 10 (CCR10) [12]. In addition, adhesion molecules are thought to mediate T cell migration and retention in cutaneous tissue, such as the αE (CD103) β7 integrin that is overexpressed in psoriasis skin [13].

The main objective of this study was to evaluate the immunological therapeutic effect of two treatment protocols on psoriasis, focusing on the main inflammatory cytokines and effector T cell phenotypes known to be important for skin homing and tissue retention, thus potentially providing new insight into the immunopathogenesis of psoriasis. Our results confirm the role of Th1 and Th17 effector T cells in psoriasis. It also provides insight

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into the role of $CD8^+$ T cell secreting IFN-γ (Tc1) and IL-17 (Tc17) and $CLA^+/CD103^+$ effector T cells in its immunopathology.

Materials and methods

Patients and inclusion criteria. The Icelandic National Bioethics Committee (Nr. 08-010-S1) and the Icelandic Data Protection Authority approved the study. After providing informed consent, twelve patients with plaque psoriasis entered the study. They were assessed at baseline (W0), one (W1), three (W3) and eight (W8) weeks after starting treatment. Disease severity was assessed by the same physician (J.H.E.) at each time point with Psoriasis Area and Severity Index (PASI) [14] score and photographic documentation, and punch biopsies and blood samples were obtained.

Eligible patients were recruited to the study from January to May 2008. They were referred by dermatologists, and they were randomly assigned to two treatment groups. Patients were excluded if they had other forms of psoriasis, had other skin diseases or had received systemic psoriasis therapy, phototherapy or topical treatment within the previous 4 weeks. Of the 12 patients enrolled, six received inpatient treatment at the BL clinic for two weeks and 6 were treated with NB-UVB therapy three times weekly for 8 weeks. Psoriasis treatment at the BL clinic included bathing in geothermal seawater twice daily for at least 1 h combined with NB-UVB therapy 5 days per week for 2 weeks. After treatment at the BL clinic, patients used moisturizing creams for 6 weeks. The same type of Waldmann 7000 UVB cabins (Philips TL 100W/01, Philips, Villingen-Schwenningen, Germany) were used at the outpatient dermatology clinic at Landspitali University Hospital in Reykjavik and at the BL clinic. The same UVB treatment protocol was used for all patients based on skin type, with initial doses of 130–400 mJ/cm² with subsequent increases of 15–65 mJ/cm² after each treatment session [15]. Both groups were advised to use moisturizing creams daily. Patients who received combination treatment and NB-UVB therapy alone were comparable regarding age (mean: 36.7 years [range: 19–57] versus 33.7 years [range: 27–42]; $P = 0.41$), gender (five women/one man and five women/one man) and Psoriasis Area and Severity Index (PASI) [14] (18.2 [range: 7.8–32.2) versus 12.3 [range: 8.2–15.1]; $P = 0.19$). The only difference was that patients receiving combination treatment had a longer duration of the disease compared with patients receiving NB-UVB therapy (mean: 22.3 years [range: 6–36] versus 12.3 years [range: 5–23]; $P = 0.036$).

The control group consisted of 3 anonymous healthy blood donors from the Landspitali University Hospital (Reykjavik, Iceland) blood bank.

Cell preparation, stimulation and flow cytometry analysis. Heparinized peripheral venous blood was collected at each time point, and peripheral blood mononuclear cells (PBMC) were obtained by gradient centrifugation with Ficoll-Paque PLUS (Healthcare, Uppsala, Sweden), collected at the interface and washed with HBSS medium (Gibco, Carlsbad, CA, USA) prior to staining with such as anti-human CD3, CD4, CLA, CD103 (all from Biolegend, San Diego, CA, USA), CD8, CD45R0, CD54, CCR4 (all from BD Biosciences, San Jose, CA, USA), IL-23R and CCR10 (both from R&D Systems, Abingdon, UK) monoclonal antibodies (mAbs) for T cell analysis and CD14, CD11c, TLR2 (Biolegend) and TLR6 (HyCult Biotechnology, Uden, The Netherlands) mAbs for monocyte analysis.

The PBMC (1.0×10^6 cells/ml) were cultured for 16 h in RPMI 1640 medium with penicillin–streptomycin (100 IU/ml and 0.1 mg/ml) (Gibco), in the presence of anti-CD3 (5 μg/ml), anti-CD28 (5.0 μg/ml) mAbs (Biolegend) and brefeldin A (3.0 μg/ml) (eBioscience, San Diego, CA, USA) at 37 °C. The T cells were first stained for CD4 and CD8, then fixed and permeabilized and stained intracellularly with anti-human tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-17A (all from Biolegend) and IL-22 (R&D Systems) mAbs. The cells were washed with phosphate-buffered saline (PBS) prior to fluorescence-activated cell sorting (FACS) analysis.

Serum cytokine measurements. Serum samples were collected at each time point and frozen at −70 °C until used. At the end of the study period, the levels of IL-22, IL-17, IL-23, CCL20, IL-1β and TNF-α were determined by enzyme-linked immunosorbent assays (ELISAs), using commercially available kits (R&D Systems), according to the manufacturer's instructions.

Skin biopsies. A 3-mm punch biopsy was taken from the arm of each patient at every evaluation. The biopsy was taken from the edge of the thickest lesion on the forearm, then fixed in formaldehyde and stained using HE for histologic evaluation. Trozak's histological grading score [16] was used to evaluate the severity of the disease. The individual parameters were scored from 1 to 3, and a cumulative score between 0 and 19 was recorded for each biopsy. The observer was blinded (J.H.E).

Statistical analysis. Values are expressed as the mean ± 2 SD. To compare the treatment group with controls, we used the Mann–Whitney *U*-test. To evaluate the differences between before treatment, during and after treatment, the normality of each type of measurement was evaluated using a KS test based on the residuals from a simple linear model using patient and time as factors. In no case was normality close to being rejected ($P > 0.4$ in all cases). Hence, one-way repeated-measures ANOVA was used. However, to evaluate the differences between the two treatment groups, two-way repeated-measures ANOVA was used. Three patients who received combined treatment were not evaluated at week 8 because they had started another psoriasis treatment due to exacerbations: two of

those patients at week 4 (Fig. 1A; BL3 and BL6) and one patient at week 7 (Fig. 1A; BL1). For these patients, PASI evaluation was made at the time point their study participation was terminated, and they were not included in the analysis at week 8. All measurements were taken using SIGMASTAT 3.1 (Systat Software, San Jose, CA, USA). A P-value ≤ 0.05 was considered statistically significant.

Results

Clinical evaluation

In order to evaluate whether clinical improvement of psoriasis following bathing in geothermal seawater combined with NB-UVB and NB-UVB alone is preceded by changes in systemic inflammatory markers, the clinical efficacy of each treatment regimen was evaluated first. As shown in Fig. 1C, both treatment regimens demonstrated significant clinical improvements. Furthermore, the data suggested that patients receiving combined treatment demonstrated better clinical response, measured by the PASI score, than patients treated only with NB-UVB. This was seen both after one week (% improvement: combined treatment 37.3 ± 10.3 versus NB-UVB treatment 18.3 ± 8.9, $P < 0.05$) and after three weeks (67.3 ± 11.9 versus 22.0 ± 12.0, $P < 0.0001$). However, this was not the main aim of the study, and larger cohort and another control group would be needed to fully address this interesting observation.

Interestingly, bathing in the Blue Lagoon immediately following skin punch biopsy resulted in no infections and only minor skin irritation resolving in few days. In addition, the above clinical findings were confirmed by the histological Trozak's score where patients in both treatment groups showed a significant histological improvement at week 3 (Trozak's score: BL treatment = 10.3 ± 5.5 versus NB-UVB treatment = 8.0 ± 4.6; Fig. 2).

Immunological evaluation

Skin homing and positive correlation with PASI score

To understand the role of adhesion molecules, chemokines and their receptors in cutaneous lymphocyte homing in patients with psoriasis, we evaluated intercellular adhesion molecule 1 (ICAM-1), E-selectin (CD62E), CD11c, two chemokine receptors (CCR4 and CCR10) and $\alpha E\beta 7$ integrin (CD103) on peripheral blood mononuclear cells before, during and after each treatment regimen. In the active stage of the disease (W0) and compared with healthy control, patients with psoriasis had higher percentage of circulating CLA+ T cells expressing CD103 (median 5.7 versus 1.5%; $P < 0.05$), CCR10 (median 5, 1 versus 1.7%; $P < 0.05$) and co-expressing CD103/CCR4 (median 11.4 versus 0.8%; $P < 0.05$) and CCR4/CCR10 (median 3.7 versus 1.2%; $P < 0.05$) (Fig. 3A). In addition, a positive correlation between PASI and circulating CD103+ T cells ($r = 0.6036$; $P < 0.05$) and CLA+ T cells expressing

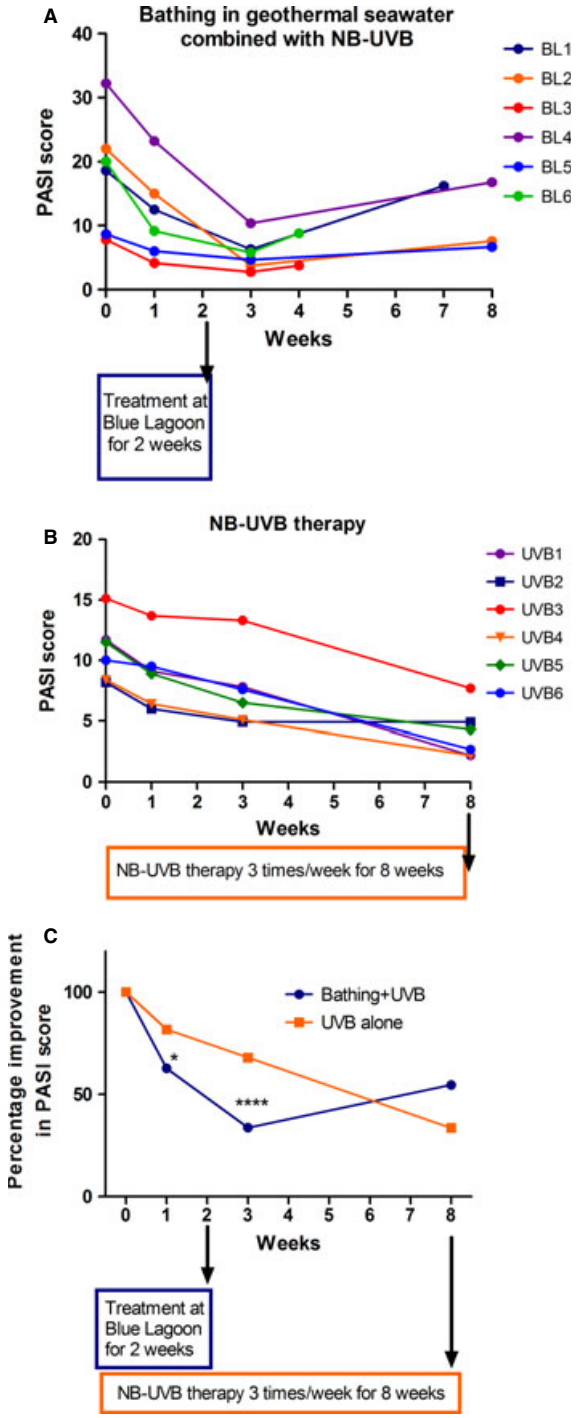

Figure 1 XY plots showing Psoriasis Area and Severity Index (PASI) score in psoriasis patients treated with bathing in geothermal seawater combined with NB-UVB therapy (A) and NB-UVB therapy alone (B), as well as the median percentage improvement in PASI score with each treatment (C). All patients were examined before treatment (0), and at 1 (W1), 3 (W3) and 8 (W8) weeks of treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

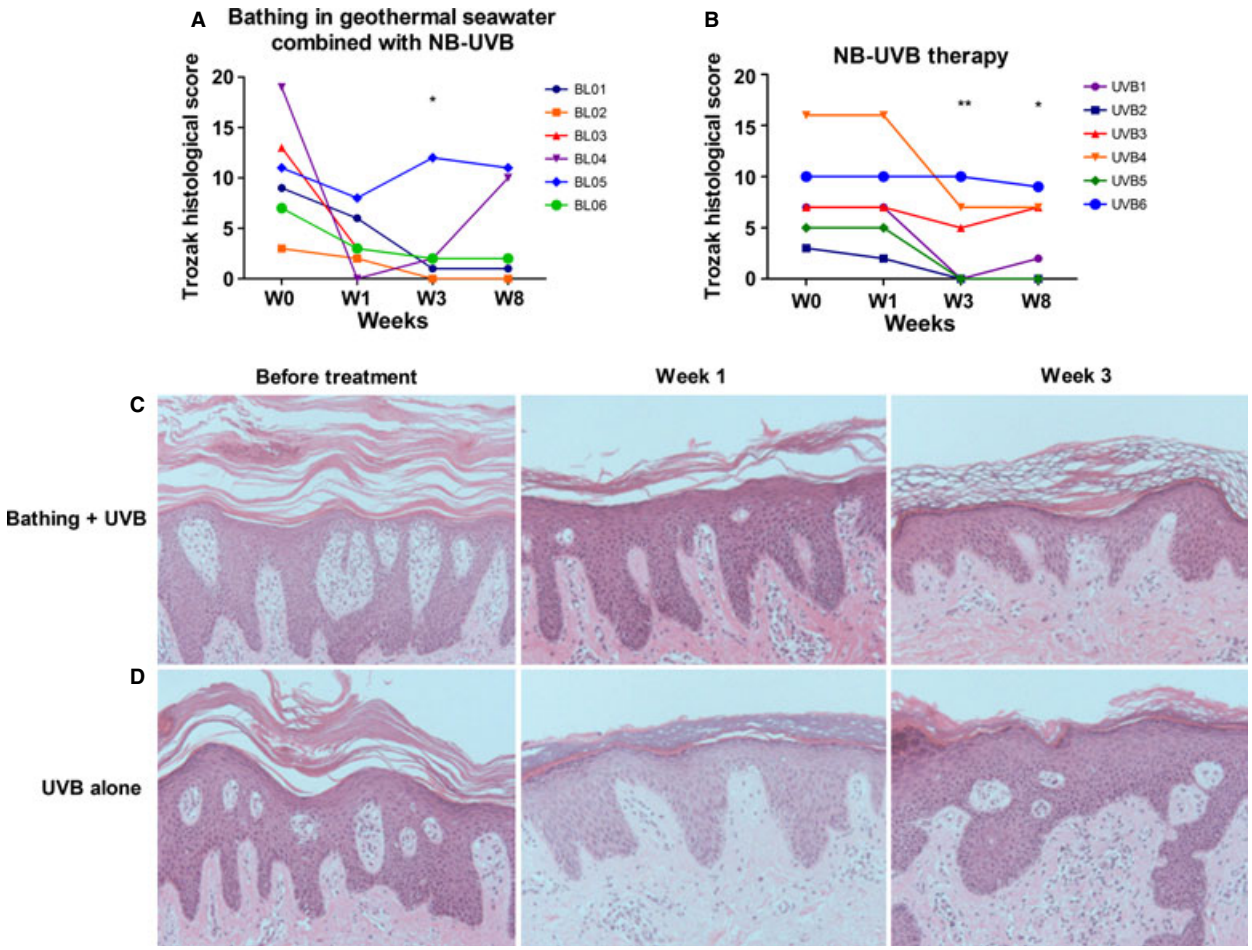

Figure 2 Histological assessment using Trozak´s grading system before, after 1, 3 and 8 weeks of treatment. Psoriasis patients who received bathing in geothermal water combined with UVB therapy (A) and NB-UVB therapy alone (B). Representative photographs from one patients who received bathing + UVB (C) and NB-UVB therapy alone (D). *$P < 0.05$, **$P < 0.01$.

CCR10 ($r = 0.7360$; $P < 0.01$) was similarly observed. No therapeutic changes were found regarding the expression of ICAM-1, CD62E, CD11c and other activation markers, such as CD25 and HLA-DR (data not shown).

In addition, patients receiving combined treatment had a significant reduction in CLA+ T cells expressing CCR4 or CD103 (68–74% reduction at W3, $P < 0.001$), while patients treated with NB-UVB alone did not (Fig. 3A). Furthermore, this reduction in CLA+CCR4+ T cells was predominantly confined to those who also expressed the CD103 integrin. Thus, no CLA+ T cells that co-expressed CD103 and CCR4 were detected in the circulation after 3 weeks (W3) in patients receiving combined treatment ($P < 0.05$; Fig. 3A). Both treatment groups achieved a significant reduction in CLA+ T cells that expressed CCR10 (71% reduction versus 44% reduction at W3; $P < 0.001$ versus $P < 0.05$; Fig. 3A). A marked reduction was also observed of circulating CLA+ T cells that co-expressed CCR4 and CCR10 in the combined treatment group (3.5% before treatment and 0.7% at W3; 80% reduction; $P < 0.01$; Fig. 3A).

Thus, the increased proportion of skin-homing T cells expressing CD103 and the chemokine receptors CCR4 and CCR10 was significantly reduced following clinical and histological improvements of psoriasis.

Effector T cell phenotype and its clinical correlation in psoriasis

To investigate the expression profile of circulating Th1/Tc1 and Th17/Tc17 cells in patients with psoriasis and its clinical correlation, their phenotypes were investigated amongst both CD4+/CD45RO+ and CD8+/CD45RO+ T cells. As expected in the active stage of the disease, patients with psoriasis had higher percentage of circulating CD4+ T cells expressing IFN-γ, TNF-α, IL-22 and IL-17 as compared with healthy controls (median 5.93 versus 2.06%, 9.08 versus 0.73%, 3.19 versus 0.33% and 4.78 versus 0.42%, respectively, $P < 0.05$ for all four subsets;

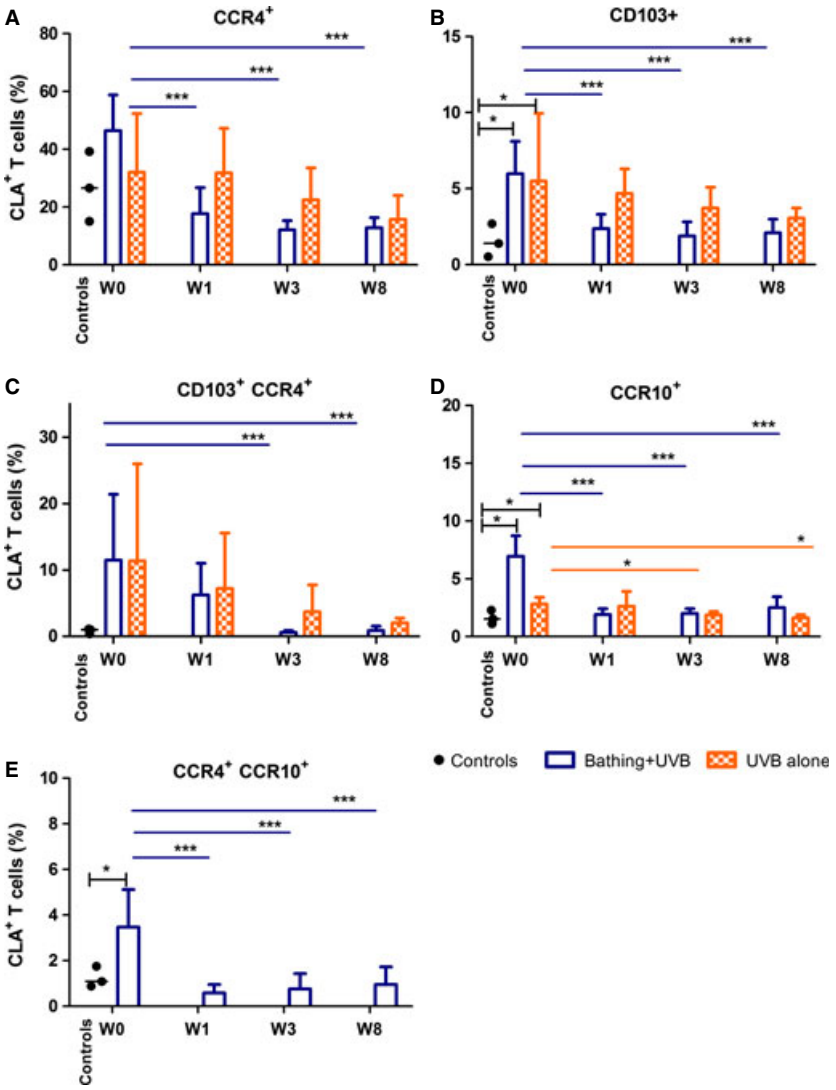

Figure 3 Circulating CLA+ T cells expressing CD103, CCR10 and CCR4/CCR10 are increased in psoriasis. The percentage of CLA+ T cells expressing CCR4 (A), CD103 (B), CCR10 (D) and co-expressing CCR4/CD103 (C) and CCR4/CCR10 among unstimulated cells from 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bating in geothermal seawater combined NB-UVB treatment (blue bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3 (W3) and 8 (W8) weeks after treatment. Data expressed as mean ± SD, except controls expressed as scatter dot with median. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 4A). Furthermore, this was also observed for the CD8+ phenotype expressing IFN-γ, IL-22 and IL-17 (median 6.93 versus 2.37%, 2.39 versus 0.81% and 2.22 versus 0.89%, respectively, $P < 0.05$ for all three subsets; Fig. 5A).

When evaluating the clinical efficacy with its corresponding immunological profile, patients receiving combined treatment showed a marked reduction (81%) in circulating Th17 (IL-23R+CD4+ T cells) after only one week of treatment (Fig. 4A). This was also reflected by a 53% reduction in the amount of IL-23R expressed (MFI) by these cells ($P < 0.05$, data not shown) and their IL-17/IL-22 cytokine secretion. In contrast, such immunological Th17 inflammatory response improvement was only detected after 8 weeks of NB-UVB treatment (4a). Furthermore, both of the treatment protocols resulted in a significant reduction in Tc17 T cells (producing IL-17 and IL-22; Fig. 5A). Finally, a similar reduction was also noted for the Th1 and Tc1 phenotype (IFN-γ and TNF-α production, Figs. 4A and 5A, $P < 0.05$).

Discussion

The role of skin-homing, Th1 and Th17 immune response in the immunopathology of psoriasis is demonstrated in this study. In addition, the importance of Tc1 and Tc17 immune response is also suggested. Finally, NB-UVB therapy induced excellent clinical improvement preceded by a reduction in these above systemic inflammatory markers, strongly suggesting that immune modulation

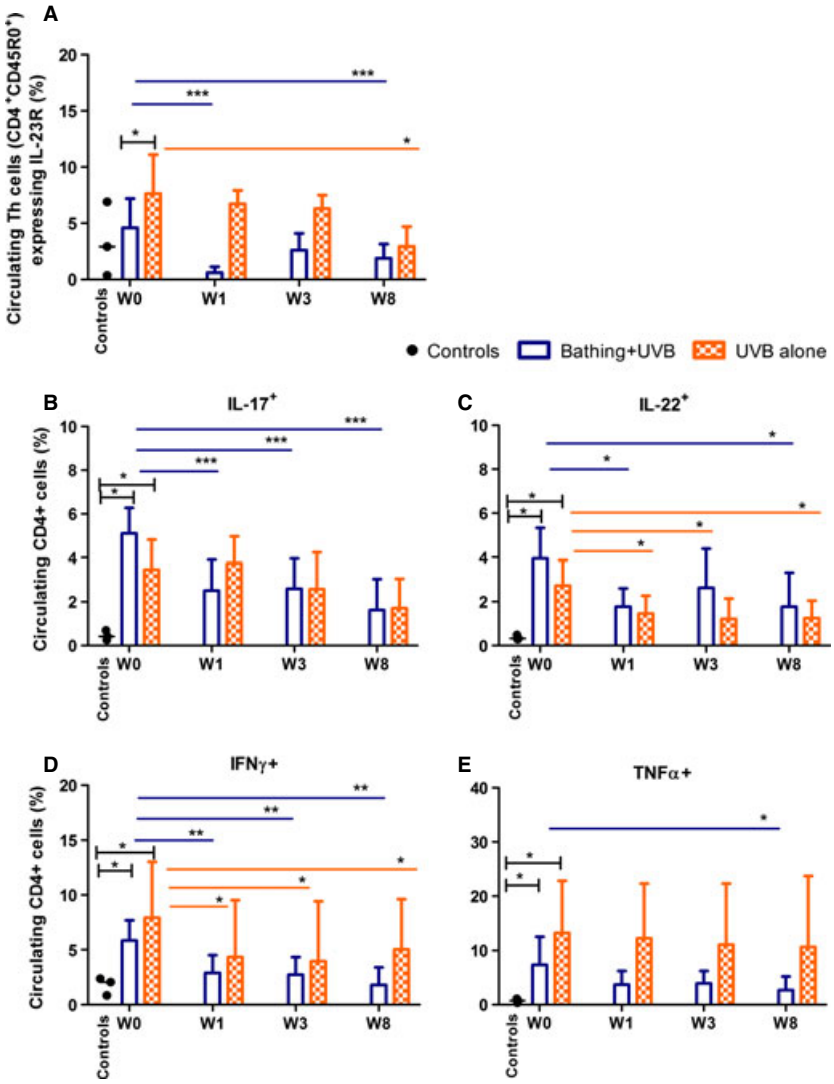

Figure 4 Decreases of the percentage of circulating Th17 and Th1 over time in 12 psoriasis patients, either receiving bathing in geothermal seawater combined with NB-UVB treatment or NB-UVB treatment alone. (A) The percentage of circulating Th17 cells (CD4+CD45R0+IL-23R$^+$), (B) Il-17, (C) Il-22, (D) IFNγ and (E) TNFα producing CD4+ T cells in 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bathing in geothermal seawater combined NB-UVB treatment (blue bars) and 6 receiving NB-UVB therapy alone (orange bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3(W3) and 8 (W8) weeks after treatment. Data expressed as mean $\pm$ SD, expect controls expressed as scatter dot plot with median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mediated the observed clinical effect. Furthermore, an improvement by histological assessment is clearly demonstrated substantially validating the observed clinical improvements by using 'Trozak's score' as a measure of treatment efficacy.

There is evidence suggesting that bathing in the geothermal seawater without NB-UVB treatment has a beneficial clinical effect [1, 2]. It has also been noted that the scaling of psoriasis lesions disappears quickly, and the lesions get thinner with less erythema, indicating that bathing in this geothermal seawater has a direct anti-inflammatory effect on psoriatic lesions [2]. Another study demonstrated the beneficial effects of bathing in geothermal seawater where NB-UVB treatment after bathing gave an additional clinical effect compared with NB-UVB treatment alone [5], thus supporting our observation that bathing in the geothermal seawater might provide some additional clinical effect that was further reflected by the reduction in potential pathogenic T cells in the peripheral blood.

Psychological stress has been reported to influence psoriasis severity [17]. Inpatient treatment at the BL clinic in a relaxed environment might reduce stress and thereby indirectly improve the psoriasis lesions in addition to the UVB-induced effects. Immunological studies show that psychological stress increases the numbers of various immunological cells in the peripheral blood of patients with psoriasis, including HLA-DR$^+$ T cells, and decreases

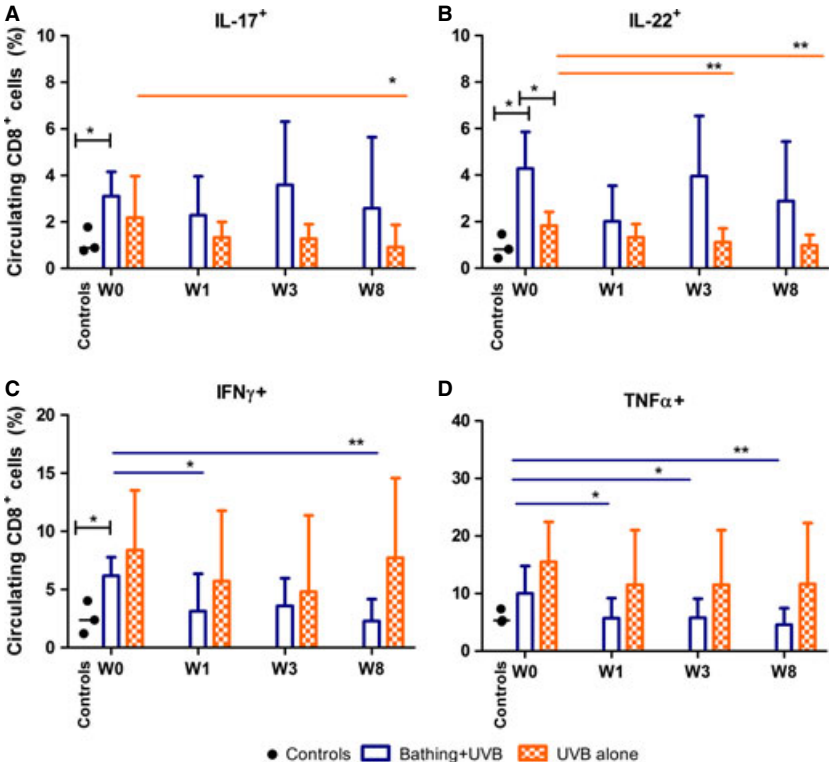

Figure 5 Decreases of the percentage of circulating Tc17 and Tc17 over time in 12 psoriasis patients, either receiving bathing in geothermal seawater combined with NB-UVB treatment or NB-UVB treatment alone. The percentage of circulating IL-17 (A), IL-22 (B), IFNγ (C) and TNFα (D) producing CD8+ T cells in 3 healthy individuals (controls), 12 psoriasis patients; 6 treated with bathing in geothermal seawater combined NBUVB treatment (blue bars) and 6 receiving NB-UVB therapy alone (orange bars). All patients were studied before commencing treatment (W0), during treatment (W1), and at 3 (W3) and 8 (W8) weeks after treatment. Data expressed as mean ± SD, except controls expressed as scatter dot plot with median. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the numbers of CD25$^+$ T cells [18]. However, in our study, the numbers of T cells expressing HLA-DR$^+$ and CD25$^+$ did not change significantly in the peripheral blood with both treatments, indicating that stress did not influence the outcome of our study.

The therapeutic properties of combined treatment with salt water baths and natural UV radiation (climatotherapy) and bathing in thermal water (spa therapy) have been known since ancient times [19, 20]. Today, it is being practised in many countries in the form of combination treatment of salt or thermal water baths and artificial UV radiation (balneotherapy) [21, 22]. Some studies indicate that the main therapeutic effect of climatotherapy at the Dead Sea can be attributed primarily to the sunshine and secondarily to the hypersaline seawater [21, 23]. Other studies show that balneotherapy with Dead Sea salt solution soaks in combination with NB-UVB therapy is superior to NB-UVB therapy alone [24, 25], which could be attributed to increased photosensitivity of the skin to UV radiation [26, 27]. We do not think that explains the results in our study for two reasons. As mentioned above, there are studies showing that bathing in the geothermal seawater without NB-UVB treatment has a beneficial clinical effect [1, 2]. In addition, the cumulative dose of NB-UVB therapy in this current study was only 10 treatment sessions for patients bathing in geothermal seawater combined with NB-UVB therapy compared with 24 sessions for patients treated with NB-UVB therapy alone.

However, the agents responsible for these beneficial effects of bathing in saline or thermal water have not been fully elucidated but most likely involve chemical [26, 28, 29], thermal [30], mechanical [2] and immunomodulatory effects [28, 31]. Furthermore, studies have shown that bathing in salt solutions has been associated with increased photosensitivity of the skin to UV radiation [26, 27]. Even though balneotherapy and spa therapy are widely used, the immune modulatory mechanisms are only partly understood. Few studies have shown immunomodulatory effects on epidermal Langerhans cells, inhibition of Th1 differentiation and cytokine production from keratinocytes [28, 31]. One recent study from Korea [32] showed that thermal spring water suppressed the expression of pro-inflammatory cytokines in human keratinocytes 'in vitro' as well as the differentiation of mouse CD4$^+$ T cells into Th1, Th2 and Th17 cells.

CCR4 has been found to be abundantly expressed on circulating T cells with a skin-homing CLA^+ phenotype [33] in normal subjects as well as in patients with psoriasis [34], which is consistent with our results. In contrast, CCR10 and CD103 are weakly expressed in the peripheral blood of normal subjects and nearly undetected in normal skin [35, 36]. In addition, CCR10 is expressed by a minority (approximately 30%) of circulating CLA^+ T cells [37]. However, both CCR10 and CD103 have been found in the inflamed psoriatic lesions [35, 36]. Their involvement in the immunopathogenesis of psoriasis is further suggested by our findings demonstrating the increased proportion of circulating skin-homing CLA^+ T cells co-expressing the tissue retention integrin CD103 and/or the chemokine receptors CCR4 and CCR10. More importantly, they had a positive correlation with the clinical improvements observed in the study, thus implicating the role of directing $CCR4^+/CCR10^+$ and $CD103^+$ subset of skin-homing T cells (CLA^+) into psoriasis plaques during the active stage of the disease. CLA+, CD103+ T cells, various adhesion molecules as well as activation markers did not change significantly during or after both treatment protocols. These findings demonstrate that the changes observed in our study are not only explained by the significant overall decrease in the inflammatory state of the patients following the treatment protocols. Today, it is known that CCR6 is a common chemokine receptor on Th17 T cells [38], but it is not included in our study. It is unfortunate, but at the time that our study was conducted, the role of CCR6 as a Th17 marker was being debated and unclear.

The immunopathogenesis of psoriasis has been connected to both Th1 and Th17 effector cells, and our observation that IL-17, IL-22 and IFNγ levels in the blood of patients with psoriasis returned to baseline with effective therapy supports this notion [9–11, 39]. Furthermore, the increased proportion of IL-17-/IL-22-producing $CD8^+$ T cells in the peripheral blood compared to healthy controls suggests their involvement in the immunopathogenesis of psoriasis, which has also been implicated by others [40]. In addition, the involvement of Tc17 cells in the immunopathogenesis was also evident by the positive correlation with individual clinical improvement measures. Similar to our findings, the therapeutic effectiveness of NB-UVB therapy has been associated with the corresponding Th1/Th17 pathway in psoriasis. In addition, in that study the role of innate immunity in psoriasis was suggested [41]. This has particularly been evaluated by the role of various Toll-like receptors in psoriasis. Thus, the expression of TLR2 has been found to be overexpressed in keratinocytes in psoriatic lesions [42], a finding also observed in our study with an increased expression of TLR2 on circulating monocytes ($CD14^+$) and dendritic cells ($CD11c^+$) in the peripheral blood of patients with psoriasis (data not shown). This study reflects the complexity behind the immunopathogenesis of psoriasis. It also reflects the following major confounding immunological elements. First, it confirms the importance of IFN-γ-, TNF-α-, IL-17- and IL-22-driven inflammatory response. Secondly, it suggests that these inflammatory cytokines are originating from both $CD4^+$ and $CD8^+$ T cells. Finally, this suggests that the inflammatory response is most likely predominantly driven by skin-homing tissue retaining T cells expressing the chemokine receptors CCR4 and CCR10.

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Conflict of interest

This study was conducted in collaboration with Blue Lagoon Ltd. and Landspitali University Hospital of Iceland.

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