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# Differential effects of phototherapy, adalimumab and betamethasone/calcipotriol on effector and regulatory T cells in psoriasis

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**Short title:** Regulatory T cells as therapeutic target in psoriasis

## ABBREVIATIONS

| | |
|---|---|
| DCs | Dendritic cells |
| DDCs | Dermal dendritic cells |
| NB-UVB | Narrow band ultraviolet B |
| PASI | Psoriasis area and severity index |
| SD | Standard deviation |
| Teff | Effector T cells |
| Treg | Regulatory T cells |




## Summary

***Background*** Psoriasis is a chronic T cell-mediated skin disease with marked social and economic burdens. Current treatments are unsatisfactory, with unpredictable remission times and incompletely understood modes of action. Recent advances in our understanding of the pathogenesis of psoriasis identify the imbalance between $CD4^+$ T effector cells, particularly the T helper (Th) 17 subset, and regulatory T cells (Treg) as key to the development of psoriatic lesions, and therefore a novel therapeutic target.

***Objectives*** To quantify in patients the effects of three commonly used psoriasis treatment modalities on the Th1, Th2, Th17 and Treg subsets, and test whether any change correlates with clinical response.

*Methods* Using flow cytometry to enumerate Th1, Th2, Th17 and Treg subsets in blood and skin of psoriatic patients before and after receiving any of the following treatments; narrow band UVB (NB-UVB), adalimumab and topical betamethasone/calcipotriol combination (Dovobet®)

***Results*** All patients responded clinically to treatments. NB-UVB significantly increased the numbers of circulating and skin Treg, while, by contrast, adalimumab reduced Th17 cells in these compartments, and Dovobet had dual effects by both increasing Treg and reducing Th17 cells.

***Conclusions*** The differential effects reported here for the above-mentioned treatment modalities could be exploited to optimize or design therapeutic strategies to better overcome the inflammatory drivers and restore the Th17/Treg balance in psoriasis.

**Key words:** Psoriasis; Th17/Treg subsets; Dovobet®; NB-UVB; adalimumab

## INTRODUCTION

Psoriasis is a chronic relapsing skin disease that affects 2-4% of the population in western countries.[1] Although not contagious, psoriasis greatly impacts on the patients' quality of life, and has been linked with social stigmatisation, discomfort, and psychological distress.[2] Despite the availability of many treatments, responses remain unsatisfactory, and the frequent relapses, together with absence of long-term control, make psoriasis a challenging condition. This therapeutic inadequacy reflects the need for a more complete understanding of the cellular and molecular determinants of psoriasis. An important advance has been recognition that the disease is mediated by T cells, and that different $CD4^+$ T helper (Th) cell or T regulatory (Treg) subsets can drive or control the pathogenic responses. For many years, psoriasis was classified as a Th1 mediated skin disease.[3] However, the recent description of abundant Th17 cells in psoriasis lesions,[4] and the evidence of impaired Treg function,[5] have led to a new model in which the disease is caused by an imbalance between inflammatory effector T (Teff) cells, represented by Th17 and to a lesser extent Th1 subsets, and Treg cells.[5] Although skewing a Th17 bias back towards Treg is emerging as a rational

therapeutic goal in psoriasis, it is not known how effectively conventional and more recent treatments correct this balance, and whether any such restoration correlates with clinical improvement. Non-selective treatments such as cyclosporine,[6] inhibit both Teff and Treg, leading to rebound of psoriasis on stopping therapy. Narrow band UVB (NB-UVB) is a popular and effective tool; yet the molecular mechanisms underlying its efficacy are incompletely understood.[7] More recently, anti TNF-α reagents such as adalimumab have emerged as valuable biologic agents with significant anti-inflammatory effects, although, with introduction to regular clinic use, they are associated with side effects, loss of efficacy and paradoxical cases of psoriasis.[8] Dovobet®, containing betamethasone and the synthetic vitamin D derivative calcipotriol, is widely used in clinical practice owing to its complementary anti-proliferative and anti-inflammatory mechanisms.[9] Vitamin D3 analogues were also found to have beneficial effects in murine models of autoimmune, allergic, and inflammatory diseases.[10] Overall, we suggest that better understanding of the mechanisms of success of these treatments can lead to innovation of new, more targeted, therapeutic strategies with fewer side effects. In this study we quantified the effect of NB-UVB, adalimumab and Dovobet® on circulating and skin T cells, with particular interest in the critical balance between Th17 and Treg cells, and correlated these changes to clinical response measured by psoriasis area and severity index (PASI) score. In particular, we wanted to test whether the most effective treatments would skew this balance in favour of Treg, which are predicted to be important for immune tolerance and prevention of autoimmunity.

## PATIENTS AND METHODS

### Patients

This study was approved by the North of Scotland Research Ethics Committee and MHRA according to the Declaration of Helsinki protocols. All participants signed written informed consent. Thirty four patients (23 M and 11 F; mean age 50 ± 14.5) were recruited from Aberdeen Royal Infirmary, Scotland, UK with moderate to severe psoriasis untreated with topical therapy for 2 weeks or with

systemic therapy for 4 weeks. Blood samples, and 6 mm punch biopsies were taken from lesional skin at baseline and 6 weeks after treatment with each of: NB-UVB (*n* = 15), adalimumab (*n* = 11) and Dovobet® (*n* = 8). Non-lesional 6 mm punches were taken from one group of patients (*n* = 8) at baseline. The number of subjects studied was limited by the restricted inclusion and exclusion criteria as we were looking for treatment naïve patient to avoid bias in results. Lesional biopsies were taken from the centre of plaques and non lesional samples were taken from the adjacent psoriasis free skin. Post treatment samples were collected from cleared psoriasis skin in same site of pre-treatment samples. We chose 6 weeks for post treatment samples as data from previous similar experiments showed significant immune response to treatment as early as 4-6 weeks. Psoriasis patients for whom the decision to treat with NB-UVB was made, were exposed to known amounts of NB-UVB three times weekly according to a standard escalating protocol based on minimal erythema dose (MED). MED is defined as the dose that caused just perceptible erythema 24 hours after irradiation. Standard dose ranges between 0.55 and 3.13 J/cm2. Whole-body UV-B was given in a Waldmann 7001 cabinet (Waldmann GmbH, Germany), incorporating twenty-four 100-W Philips TL-01 fluorescent lamps (311-313 nm). We recruited additional NB-UVB candidates to give 6 mm punch skin biopsies at baseline and 2 weeks after treatment. Adalimumab is a preloaded pen device which automatically injects the drug by subcutaneous (SC) infusion. It was done by the patient at home after initial illustration and training by doctor or the biologic nurse. Dose: 80 mg subcutaneously at week 0, 40 mg at week one, and then every other week thereafter. Betamethasone/calcipotriol (Dovobet®) is prescribed as a twice daily dose for six weeks to the affected areas. The selection of patient to treatment arm was not part of the study and was solely dictated by the clinical need. Control samples were provided by psoriasis-free patients who attended the dermatology clinic for excision of naevi.

**Samples**

Skin samples were physically disaggregated *ex vivo* for 1 minute using 50 µM medicons in the Medimachine (BD Biosciences, USA), allowing for collection of viable lymphocytes[11] and the cell suspension was then used for culture or flowcytometry. PBMC were obtained by density gradient centrifugation (Ficoll-Paque; Amersham Biosciences, USA).

**Flow cytometric analyses**

Cell suspensions were analysed using BD LSRII research flowcytometry, after staining with combinations of the following antibodies and their isotype controls: Anti-CD4-APC-CY7 (BD Pharmingen, USA), anti CD25-Alexa Fluor 700 (BD Pharmingen, USA), anti CCR4-PE-CY7 (BD Pharmingen, USA), anti CCR6-Alexa Fluor 647 (BioLegend), anti CD127-Alexa Fluor 647 (BD Pharmingen, USA), anti FoxP3-Alexa Fluor 488 (BD Pharmingen, USA), anti GATA-3-Alexa Fluor 647 (BD Pharmingen, USA), and anti Tbet-PE (Santa Cruz). For intracellular staining, cells were fixed and permeabilised using the cytofix/Cytoperm kit (BD Biosciences, USA) as per manufacturer's protocol. Data were analysed using FlowJo® version 7.6 (Tree Star Inc., USA). Lymphocyte population was selected then identification of pure $CD4^+$ proportion of T cells was gated on isotype control. The cell populations were calculated as percentages of $CD4^+$ T cells to the whole lymphocytic count and presented as mean $\pm$ SD. Identification of different T cell subsets and gating is shown in Figure S6, S7, S8 and S9.

### Statistical analysis

Statistical analyses were carried out using Prism® graphpad 5 for windows; version 5.02, 2008. Two-tailed Paired T test was used to compare proportions of cells in lesional versus non-lesional skin, and blood of patients. Mann-Whitney U test was used to compare the proportions of cells between blood and skin of patients and healthy controls. The changes in proportions of T cell subsets with treatment were correlated to changes in PASI score using Spearman correlation. The level of significance was taken as $P \leq 0.05$.

## RESULTS

### Clinical response

To study the clinical and immunological effects of three different therapies, 34 patients with moderate to severe psoriasis were recruited and received 6-week treatment regimens of NB-UVB (*n=15*), adalimumab (*n=11*), or Dovobet® (*n=8*). All patients responded clinically to treatment with mean (±SD) PASI for each treatment group decreased from 5.7 ± 4.3 to 1.5 ± 1.2, 16.03 ± 6.1 to 5.6 ± 5, and 5.03 ± 5 to 1.7 ± 8, respectively.

### The skin and blood of psoriasis patients (*n = 8*) contain significantly higher proportions of Th17 cells and lower proportions of Treg compared to healthy controls (*n = 5*)

The proportions of $CD4^+$ Th cells with Th1, Th2, Th17 and Treg phenotypes were compared in lesional and non-lesional skin, and in blood, of psoriasis patients at enrolment to the study. In line with reports describing the phenotypes of these subsets, and our own previous studies[4], we used the characteristic combinations of markers $CD4^+Tbet^+$, $CD4^+GATA\text{-}3^+$, $CD4^+CCR4^+CCR6^+IL\text{-}23R^+$ and $CD4^+CD25^+FoxP3^+CD127^{lo}$ respectively to identify Th1, Th2, Th17 and Treg cells. The choice of Th17 markers was based on literature indicating significant expression of the characteristic combination of

CCR4$^+$ and CCR6$^+$ on Th17 cells[12-14], and CCR6 is critically required for IL-23 induced psoriasis lesions[15]. IL-23 helps in the development of Th17 cells[16,17] and the IL-23R is only expressed on activated T cells[18]. RoRγT is the transcription factor for Th17 cells[19], and is also expressed by dermal γδ T cells[15]. We did not rely on IL- 17A expression to identify Th17 cell population, because IL-17A can be produced by other cells, including γδ T cells, CD8$^+$ memory T cells, eosinophils, neutrophils, monocytes and mast cells, contributing to the common pole of IL-17 in psoriasis. [20-23] Due to this complex origin and phenotyping of Th17 cells, we chose to combine the characteristic chemokines (CCR4$^+$ CCR6$^+$), with IL-23R for purification and identification of Th17 cells population. Examples of representative flow cytometric data from the blood and skin of patients and healthy controls are illustrated in Figures S1 and S2, and the results are summarized in Figure 1. The most striking findings were that patients' lesional skin and blood contained both significantly higher proportions of Th17 cells compared to respective samples from healthy controls ($P$ = 0.0008, 0.004 respectively), and lower proportions of Treg ($P$ = 0.0004, 0.001 respectively). Treg proportions were also significantly lower in patients' non-lesional skin versus healthy donors' skin ($P$ = 0.007). The highest proportions of Th17 cells were found in lesional skin, which were also significantly higher than in patients' non-lesional skin ($P$ = 0.0008). Although Th1 cell numbers were generally higher in blood and skin samples from patients versus healthy controls, these increases were not significant, and there were also no differences in the proportions of Th2 cells in samples between the groups. Taken together, these findings support the view that an imbalance between Th17 and Treg cells contributes to psoriasis pathogenesis.

**NB-UVB therapy significantly increased the percentages of circulating and skin regulatory T cells (Treg)**

The most marked effect of NB-UVB on the CD4$^+$ subsets of patients was shown on Treg. Examples of flow cytometric analyses are illustrated in Figure S3 a-c. A summary of NBUVB effects on various T cell subsets are shown in Figure 2. In the 15 tested samples, mean circulating and skin Treg percentages were significantly increased from 2.96 $\pm$ 0.6 to 8.92 $\pm$ 1.28 ($P$ = 0.0001), and from 1.47 $\pm$

0.8 to 13.6 ± 2.53 ($P$ = 0.0003), respectively after treatment. Th1 cells were also significantly reduced from 4.3 ± 0.9 to 1.2 ± 0.6 ($P$ = 0.007) in the blood, and from 6.01 ± 0.9 to 1.5 ± 0.5 ($P$ = 0.02) in the skin, while Th2 cells were significantly increased from 0.6 ± 0.4 to 3.05 ± 2.1 in the blood ($P$ = 0.04), and from 2.7 ± 0.5 to 4.7 ± 2.7 in the skin ($P$ = 0.01). A reduction in Th17 cells was not statistically significant either in the blood or the skin. Strikingly, the change in skin Treg (Δ Treg), but not in the other subsets, was positively correlated to clinical improvement assessed by change in PASI score (Δ PASI) (Figure S3 d).

**Adalimumab therapy significantly reduced Th17 proportions without significant effect on Treg**

Patients treated with adalimumab for 6 weeks exhibited a different pattern of shifts in the proportions of $CD4^+$ T cell subsets from those seen after NB-UVB treatment, with the main effects seen on Th17 cells. Examples of flow cytometric analyses are illustrated in Figure S4 a-c and a summary is displayed in Figure 3. The greatest effect of adalimumab was on the percentages of Th17 cells, which were significantly decreased from 7.1 ± 0.4 to 2.7 ± 0.1 in the blood ($P$ = 0.05), and from 21.7 ± 11.6 to 9.5 ± 3.8 in the skin ($P$ = 0.005). The proportions of Th1 and Th2 cells showed small non-significant increases, in both the blood and the skin, and there was also no statistically significant effect of adalimumab on Treg, with opposing trends for small decreases in blood but small increases in skin. The fall in skin Th17 numbers, but none of the other changes, was related to clinical improvement, with Δ skin Th17 significantly correlated to Δ PASI. (Figure S3 d).

**Treatment with Dovobet ointment significantly affects Th1, Th2, Th17 and Treg**

Dovobet® is a combination of betamethasone dipropionate and active vitamin D (calcipotriol), and has a well-established therapeutic role in psoriasis. We showed highly significant, reciprocal systemic effects of Dovobet on Th17 and Treg after treatment for 6 weeks. Examples of flow cytometric

analyses are illustrated in Figure S5 a-c, and summarized in Figure 4. Dovobet® significantly increased the percentages of Treg in the circulating (from 3.3 ± 0.6 to 7.3 ± 0.9, *P* = 0.005) and in the skin (from 1.9 ± 1.4 to 8.7 ± 2.3, *P* = 0.01), and decreased those of Th17 cells (from 4.3 ± 1.1 to 3.4 ± 0.4, *P* = 0.003 and from 8.7 ± 0.9 to 5.3 ± 1.8, *P* = 0.01 respectively). The percentages of Th1 cells were also significantly decreased in the skin (*P* = 0.01), while Th2 cells were increased in the blood (*P* = 0.01). The fall in circulating Th17 numbers, but none of the other changes, was related to clinical improvement, since ΔTh17 blood correlated significantly to Δ PASI (Figure S5 d).

## DISCUSSION

The present study of immunological changes associated with three effective treatments for psoriasis has, for the first time, revealed differential effects on $CD4^+$ T cell subsets and identified changes that correlate with clinical improvement. Patterns of change in the proportions of Teff and Treg subsets in the circulation and skin, including those that correlated with clinical improvement, depended on treatment type. The design of the study, comparing patients before and after treatment, allowed significant results to be obtained whilst applying stringent inclusion and exclusion criteria, and with patients allocated to different treatment arms on the basis of clinical decisions. The relatively short treatment interval focused on initial response rates, which are higher than those expected in longer studies that include relapses. Taken together, the results build on the understanding of psoriasis pathogenesis as an imbalance between $CD4^+$ subsets by demonstrating that a range of current treatments targets the imbalance in different ways, and point to the possibility of improved therapies in future.

NB-UVB enhanced the number of Treg, reduced Th1 and had no significant effect on Th17, while by contrast, adalimumab lowered Th17 cells without noticeable effects on either Th1 or Th17. The topical Dovobet® ointment, however, was remarkable for its reciprocal effect in reducing effector (Th1 and Th17) subsets whilst increasing the proportion of Treg. Moreover, the increase in UV-induced Treg, and the Th17 falls caused by adalimumab and Dovobet®, were correlated to improvements in PASI score. These results collectively emphasize the importance to therapeutic efficacy of restoring a Th17/Treg imbalance in psoriasis and indicate that successful treatment can target either one or both sides of the imabalance. Previous reports showed that psoriasis lesional Treg are dysfunctional[5] or numerically deficient[24]. Our data showed significant differences in Treg numbers between patients and healthy controls, supporting previous findings[25,26], and indicating that Treg are important to maintain skin immune homeostasis in healthy skin[27]. The demonstration of Th17 enriched in psoriasis lesions compared to normal skin and healthy donor skin strongly supports the model of a Th17/Treg immune disturbance in psoriasis and reflects the frequent occurrence of skin homing $CD4^+$T lymphocytes ($CCR4^+CCR6^+$) in psoriasis plaques, providing more evidence that activation and recruitment of Th17 cells is pivotal for psoriasis pathogenesis[4]. Our findings suggest that lesional Treg are numerous but either are dysfunctional or overwhelmed by chronic inflammatory microenvironments due to high numbers of Th17 cells and their cytokines. This in agreement with data shown by Keijsers *et al.*[28] and Rodriguez *et al.*[29], who reported increased percentages and absolute numbers of lesional FoxP3-expressing T cells, as well as, production of IL-17 and IFN-Y from these cells when compared to non-lesional skin, supporting the concept of T cell plasticity[30]. The reciprocal increase in Treg and fall in Th17 numbers in response to Dovobet® may be an example of a treatment driving subset plasticity in a beneficial direction. The striking increase in $CD4^+CD25^+Foxp3^+CD127^+_{lo}$ Treg percentages correlated to clinical response to NB-UVB supports the notion that enhanced Treg are key to improve psoriasis through restoration of immune tolerance, and the results also raise the possibility that a fall of Th1/2 ratio contributes to beneficial effects of NB-UVB. Our use of $CD127_{lo}$ and $Foxp3^+$ in addition to $CD25^+$ to better define the Treg

population confirmed earlier suggestions that restoration of dysfunctional $CD4^+CD25^+$T cells could be achieved via induction of $CD127l_o$ $CD4^+CD25^+$T cells[31]. Although not statistically significant, NB-UVB may have reduced Th17 cells, possibly secondary to Treg stimulation. Notwithstanding such a secondary effect NB-UVB emerges as a possible therapeutic tool to enhance Treg without compromising the immune defending role of Th17 cells. The effect of NB-UVB on resident dermal dendritic cells (DDCs) to boost Treg has been the focus of a relatively recent study[32]. Furthermore, previous work in our laboratory highlighted a strongly correlated Treg response to serum vitamin D level following two weeks of NB-UVB therapy[33], but other mediators such as NO may also be responsible for the induction by phototherapy of a $CD4^+CD25^+FoxP3^-$ Treg population from $CD4^+CD25^-$ $FoxP3^-$ cells[34]. We showed for the first time in psoriasis that 6 weeks of treatment with adalimumab significantly reduced the percentages of lesional and peripheral blood Th17 cells with a strong correlation to clinical response ($r^2$= -0.8, $P$ = 0.03). Other studies have revealed that TNF-α antagonists suppressed Th17[35] and Th22 signaling[36], while others reported enhancement of Th1/Th17 cell activation in peripheral blood and inhibition of T cell responses in the skin[37]. Although it has also been reported that anti TNF-α reagents can increase $CD4^+CD25^+FoxP3^+$ cells in responding patients[25,26], we did not see such effect. This may be because we used combined markers for Treg identification or due to the use of a different anti TNF-α in the other studies. There has been a growing interest in the role of vitamin D3 and analogues in the prevention of immune mediated diseases. Some studies suggested that vitamin D deficiency is associated with autoimmune conditions[38], and active vitamin D analogues may prevent such disease by induction of Treg cells[33]. Strikingly, Dovobet® in our study showed significant systemic effects on Teff and Treg. Moreover, the fall in circulating Th17 cells was significantly correlated to clinical response ($r^2$ =-0.7, $P$ = 0.04). This systemic effect of Dovobet® may be mediated by the response to vitamin D by plasmacytoid DCs[39]. Previous studies showed that vitamin D arrests the maturation of DCs and increases the level of regulatory cytokines, IL-10 and TGF-β in murine models of allergic asthma[40]. Thus, it is plausible that Dovobet® improves psoriasis primarily by inducing Treg, and that the effect on Th17 cells is

secondary to the release of suppressive cytokines such as TGF-β and IL-10. However, the direct suppressive effects of betamethasone on Teff responses are also likely. In conclusion, this study identified immunological mechanisms of action of three commonly used psoriasis treatments. The results not only support the model of psoriasis as an immune-mediated disease, in which there is a shift in the balance between Treg cells that maintain self-tolerance towards a predominance of pathogenic Th17 cells, but the work also has implications for optimizing existing, or developing new, treatment regimens. This study reveals that enhancing Treg proportions without necessarily compromising Th17 function is an effective treatment approach. The demonstration that existing treatments for psoriasis can exert selective effects on Treg and Teff subsets will also encourage a search for new strategies that restore tolerance without compromising protection against infectious disease.

## CONFLICT OF INTEREST

The authors state no conflicts of interest.


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[#] Equal contribution

## FIGURES

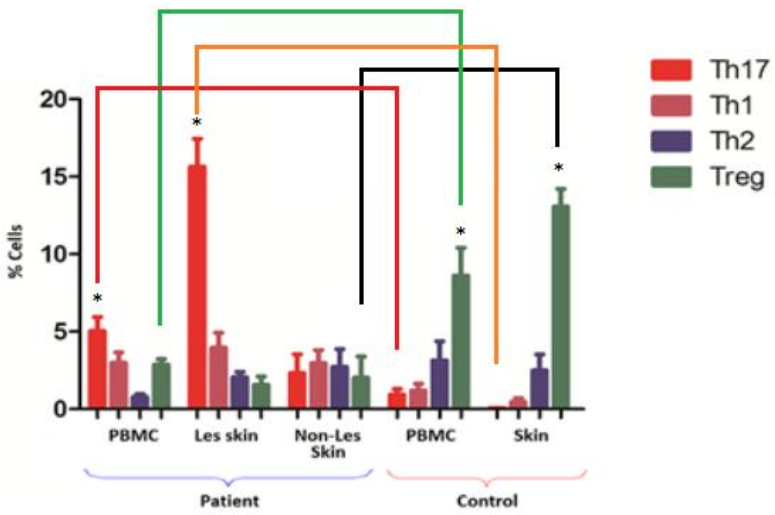


**Figure 1:** Proportions of $CD4^+$cell subsets from lesional, non-lesional skin, and blood of psoriasis patients at baseline ($n$ = 8), and from skin and blood of healthy controls ($n$ = 5). Flow cytometric analyses classified $CD4^+$ T cells as Th1 ($CD4^+Tbet^+$), Th2 ($CD4^+GATA\text{-}3^+$), $CD4^+CCR4^+CCR6^+IL\text{-}23R^+$ (Th17) and $CD4^+CD25^+FoxP3^+CD127^{lo}$ (Treg). Note the significant (*) higher proportions of Treg in the skin and blood of healthy donors compared to patients ($P$ = 0.0004, 0.001) respectively. The proportions of Th17 cells were significantly higher in the blood ($P$ = 0.004) and lesional skin ($P$ = 0.0008) of patients when compared with the respective samples in the control. Also skin Treg in healthy donors were significantly higher when compared to non-lesional Treg of patients ($P$ = 0.007).

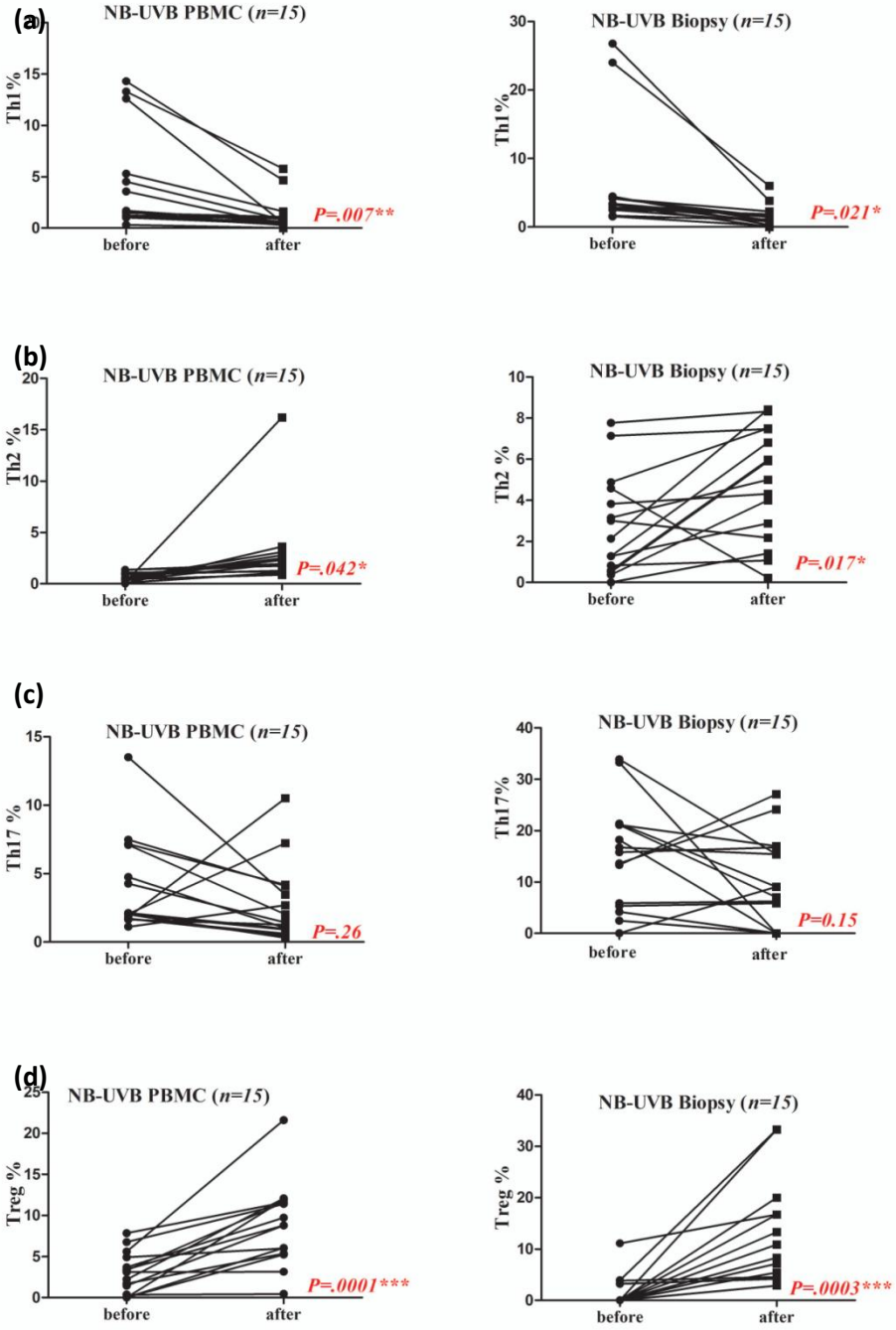


**Figure 2:** The effects of NB-UVB treatment of patients with psoriasis on T cell subsets; Th1, Th2, Th17 and Treg. Summary of flow cytometric data showing in proportions of T cell subsets circulating, and in lesional biopsies, from psoriasis patients before and after treatment. Significant changes are indicated. (a) The proportions of Th1 ($CD4^+$T-$bet^+$) cells, (b) The proportions of Th2 (CD4+GATA-3+) cells, (c) The proportions of Th17 ($CD4^+CCR4^+CCR6^+$IL-$23R^+$) cells, (d) The proportions of Treg ($CD4^+CD25^+FoxP3^+CD127^{lo}$) cells.

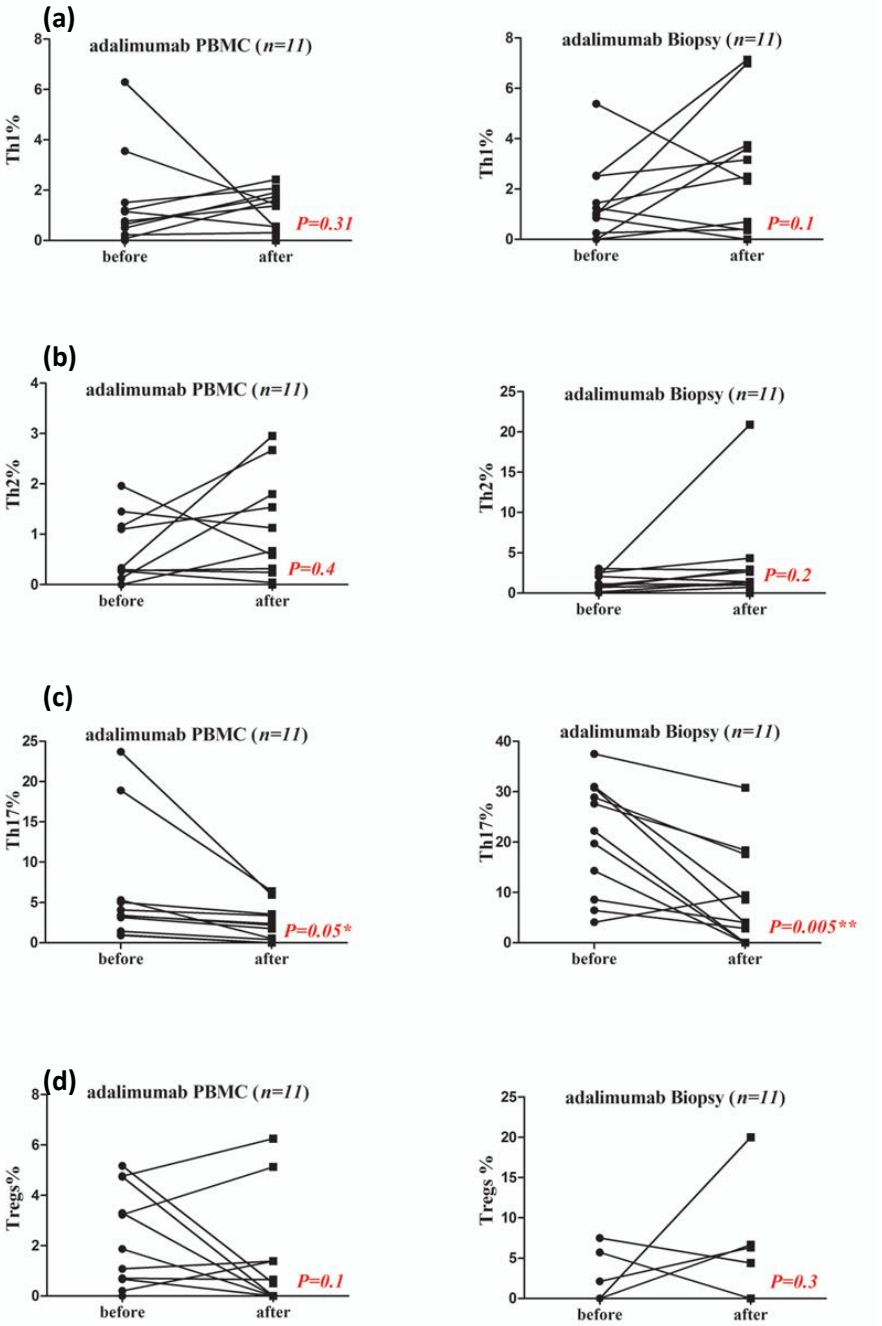


**Figure 3:** The effects of adalimumab treatment of patients with psoriasis on T cell subsets; Th1, Th2, Th17 and Treg. Summary of flow cytometric data showing in proportions of T cell subsets circulating, and in lesional biopsies, from psoriasis patients before and after treatment. Significant changes are indicated. (a) The proportions of Th1 ($CD4^+Tbet^+$) cells, (b) The proportions of Th2 (CD4+GATA-3+) cells, (c) The proportions of Th17 ($CD4^+CCR4^+CCR6^+IL\text{-}23R^+$) cells, (d) The proportions of Treg ($CD4^+CD25^+FoxP3^+CD127^{lo}$) cells.

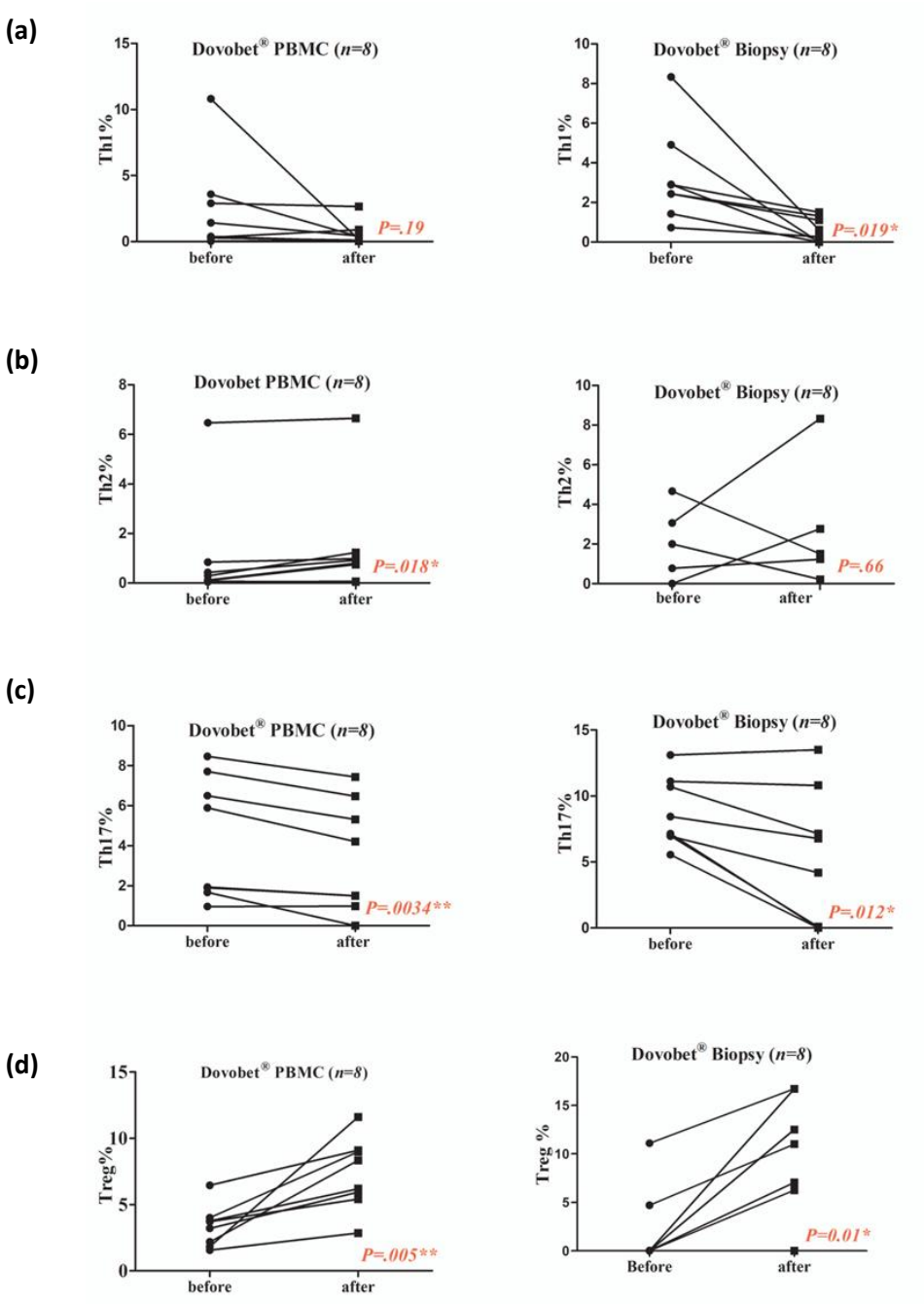


**Figure 4:** The effects of Dovobet® treatment of patients with psoriasis on T cell subsets; Th1, Th2, Th17 and Treg. Summary of flow cytometric data showing in proportions of T cell subsets circulating, and in lesional biopsies, from psoriasis patients before and after treatment. Significant changes are indicated. (a) The proportions of Th1 ($CD4^+Tbet^+$) cells, (b) The proportions of Th2 (CD4+GATA-3+) cells, (c) The proportions of Th17 ($CD4^+CCR4^+CCR6^+IL\text{-}23R^+$) cells, (d) The proportions of Treg ($CD4^+CD25^+FoxP3^+CD127^{lo}$) cells.