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The population of T cells and new cytokines signature in psoriasis patients

Natalia Moreno-Castellanos, MSc, PhD

Correspondence: Prof Natalia Moreno-Castellanos, Professor, Centro de Investigaciones en Enfermedades Tropicales (CINTROP), Department of Basic Sciences, School of Medicine, Faculty of Health, University Industrial de Santander, Cra. 32 #29-31, Administrative Building 1, Floor 1, Office 205, Bucaramanga, 680001 Colombia. nrmorcas@uis.edu.co

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INTRODUCTION

Psoriasis is a disease characterised by skin inflammation, mediated by cellular components of the activated immune system and their products such as inflammatory cytokines. The most common clinical variant is known as "Psoriasis vulgaris", defined by formations of erythematous plaques, as the result of hyperproliferation of the epidermis, along to premature maturation of keratinocytes (parakeratosis) and thickening of the skin (acanthosis). Psoriatic disease constitutes an autoimmune disease, characterised by chronic inflammation in the absence of known infectious agents. This disease comprises different cell types like Th1, Th17, and regulatory T cells, essential components that contribute to the pathogenesis disease.[1,2]

Specifically, Th1 cells have been considered for a long time as the main factor responsible for pathogenesis. However, some advances have detected and considered Th17 cells in the dermis of patients with psoriasis as relevant factors in the disease pathogenesis. Th17 cells differentiate from native CD4+ T cells by their response to IL-1 and IL-6.[1,3]

On the other hand, Th17 maintenance mainly depends on IL-23, a cytokine produced by keratinocytes, Langerhans cells, dendritic cells, and macrophages. Likewise, Th17 cells produce IL-17, TNF-α, IL-6, IL-21, and IL-22 cytokines, which are regulated during chronic inflammatory disorders, and induce acanthosis and inflammatory dermatoses.[4] The functional importance of Th17 has attracted significant attention from researchers, as well as finding new signatures of cytokines associated with these cells in psoriatic disease. Among the cytokines that have not been described to date in individuals with psoriasis include IL9, IL26, and IL31. IL-9 intervenes actively on the responses of T cells by inducing the proliferation and differentiation of these cells. This interleukin has effects on B lymphocytes and hematopoietic stem cells and mediates immunity against parasitic infections. Also, IL-9 can be produced by Treg, Th2 and Th17; in this way, IL-9 needs IL-2 to its generation and IFN-γ for its inhibition.[5-7]

Otherwise, IL-26 is produced by monocytes, Th17 and memory T cells. This cytokine acts mainly on keratinocytes; it has a high affinity for heparin which suggests high traction for glycosaminoglycans. Besides, IL-26 relates to autoimmunity and tissue repair processes during inflammation, which indicates that IL-26 may have a role in the development of psoriasis pathophysiology.[2]

IL-31, an important cytokine in the inflammatory process, is expressed in monocytes and activated lymphocytes, and mainly produced by Th2 cells. Interestingly, IL-31 negatively influences the differentiation and function of Th1 and Th17, and its action on Th2 causes these T cells to produce more IL-4, IL-5, and IL-13. This cytokine stimulates the secretion of other proinflammatory cytokines, chemokines, indicating that IL-31 could function as a proinflammatory cytokine that participates in the polymorphonuclear cells, monocytes, and T lymphocytes recruitment to an in vivo inflammatory site in psoriasis.[8,9]

Thus, in addition to the accurate identification of the different types of Th17 cells (CD4+ and/or CD8+) present in the affected tissues by psoriasis, this study has been conducted aiming to detect new cytokines (IL9, IL26 and IL31) that could participate on psoriasis pathogenesis. The identification of new cytokine signatures has been shown as a useful molecular tool for the knowledge of the pathophysiological processes developed by the immune system in the generation and maintenance of autoimmune pathology.

METHODS

This study involved ten patients diagnosed with psoriasis, aged 32 and 48 years (five women and five men). Data collected included skin biopsy (6 mm diameter), and peripheral blood sample. The inclusion criteria assessed: i) Participants without systemic treatment for at least four weeks, or at least two weeks before getting the sample; ii) body surface area affected >10%; iii) Participants without predisposing conditions to infections, immune deficiency, or

suffering from any other disease. Control samples were obtained from five healthy individuals, through abdominoplasty and peripheral blood biopsies. The participants did not report any personal or family history related to inflammatory skin diseases. Samples were obtained following protocols approved by the Institutional Review Board of Reina Sofia University Hospital (Spain); the Declaration of Helsinki Principles was followed, and patients and healthy control individuals gave written, informed consent.

Human peripheral blood mononuclear cells (PBMC) were isolated by using density gradient centrifugation with Ficoll-. Paque (Sigma) according to previously described methods.[1]

T-cell proliferation was assessed using carboxyfluorescein diacetate N-succinimidyl ester labelling and stimulated with CD3 (Sigma). Briefly, once the cells were marked with CFSE, on day 0 both, T cells from peripheral blood and biopsies were resuspended in RPMI (Gibco) at concentrations of 1x106 cells / well and cultured with anti-CD3 (Sigma). In all cases, controls of T cells labelled with CFSE not stimulated with OKT3 (Sigma) were included.

Direct immunofluorescence assays were performed to characterise lymphocyte populations phenotype. Cells were collected at a rate of 1x105 cells/test. The samples were resuspended into Facs Flow solution (Sigma), followed by centrifugation. Once the supernatant was decanted, the cells were labelled with the corresponding monoclonal antibodies following the manufacturer recommendation. In all cases, an isotype control was included.

RNA isolation and purification were performed as described earlier.[10] Transcript levels for IFNγ, TGFβ, IL9, IL17A, IL-22, IL26 and IL31 were quantified by real-time PCR (7300 Real-Time PCR System). Primers and probes (Supplementary Table 1) were designed using the software Primer 3.0 software (Applied Biosystems) and purchased from Genosys (Sigma). All results were normalised for the expression of 18 S rRNA (Applied Biosystems),

and relative quantification was calculated using the ΔΔCt formula.[10] Relative mRNA expression was expressed as fold expression over the calibrator sample.

Data are expressed as the mean ± SEM. Statistical differences between mean values were analysed using two-way ANOVA or one-way ANOVA followed by Tukey's posthoc test if an interaction was detected. A P value < 0.05 was considered statistically significant. The statistical analyses were performed using the SPSS/Windows version 15.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

Initially, CD4+CD8+ lymphocytes (Fig. 1A and 1B, respectively) were isolated from skin biopsies and to a lesser extent cell, with a significant difference of CD4 + cells (P <0.01), and CD8 + cells (P <0.05) from 10 patients diagnosed with psoriasis compared to 5 healthy donors.

To carry out the characterisation of the different populations of expanded T cells of patients with psoriasis. As shown in Fig. 2A, the cells from individuals with psoriasis (CD3+CD4+) expressed 100% TCRαβ, indicating the antigenic recognition of the TCRαβ receptor, restricted by the major histocompatibility complex (MHC). Also, this cell showed 100% expression of the HLA-DR receptor demonstrating the presence of HLA type II surface receptor on T cells, which realise the functions of recognition and presentation of antigenic peptides to other cells, as well as an indication of CD3+CD4+ lymphocytes activation. Furthermore, the CD3+CD4+ T lymphocytes present in psoriatic skin, showed an effector/memory phenotype, expressing 100% of CD45-RO +, and the absence of CD45-RA, which suggest an antigenic contact of these lymphocytes, plus a co-stimulation process observed with CD45- RA-, CD28+ CD27-. An antigenic recognition and binding can explain this to the T lymphocyte receptor-TCR. Besides, the expression of CD28+ receptor in 97% of the lymphocytes, we demonstrated evidence of cell proliferation and activation signalling.

Also, it is clear, that, cells with expression of CD56 ≤ 0.5%, were not Natural killer (NK) cells. These T lymphocytes expressed CCR5 and CCR6 surface receptors, but they did not express CCR2 receptors.

On the other hand, Fig. 2B shows several surface markers observed in expanded CD3 + CD8 + cells from the biopsies of patients with psoriasis. Specifically, the CD3 + CD8 + population cell showed the following characterization: 100% TCRαβ, HLA-DR, CD45-RO +. This cell group was determined by CD28 + CD27-, types cells CD3 + CD8 + CD56-, and the expression of chemokines receptors, CCR5 and CCR6, on their surface. This result indicates the recognition of CD3 + CD8 + cells by the MHC, the presence of the HLA-II receptor, and therefore its activation, proving a memory phenotype involved inactivation and/or proliferation processes, and were not NK.

To evaluate cell proliferation in response to CD3 (previous labelling with CFSE), T cells from the biopsies of patients with psoriasis, as well as PBLs from healthy donors were stimulated with OKT3 stuck to a plaque. This assay was monitored for six days.

After each cell division, the fluorescence goes down. At 24 hours from the staining and the subsequent stimulus, 100 % of fluorescence is obtained. As there is a cell division after another, the CFSE is transferred to the daughter cells, and therefore the curve is displaced in the histogram and progressively decreases. Thus, each division reduces the fluorescence of CFSE by half, generating peaks in a logarithmic scale, where each bar indicates a new cell division. The first peak observed at 24 hours is used as control of CFSE. As shown in Supplementary Fig. 1, we can detect the proliferative dynamic observed. An illustrative example for each group studied, and the histograms obtained at 24 hours, 3, 4 and 6 days of culture are shown.

The results found showed a response from the studied T cells to an anti-CD3 stimulus in each patient diagnosed with psoriasis and healthy donors. Also, the cells exhibited optimal growth conditions for the following assays carried out during the experimental design.

The gene expression of T cells was performed by qualitative PCR, from five healthy donors, and biopsies from ten patients diagnosed with psoriasis (stimulated with OKT3 for 16 hours before the extraction of RNA to obtain activated T cells). We quantify the gene expression for the cytokines IFN-γ, TGF-β, IL-17, IL-26, IL-9, IL-22, and IL-31 (Fig. 3). The constitutive gene HPRT1 was used as an internal control. We found a high expression of IFN-γ, IL-17, IL-22, increased expression of cytokines not previously studied in patients with psoriasis such as IL9, IL26 and IL31 respect to healthy donors (Fig. 3). Additionally, TGF-β tends to increase in psoriatic patients, but there are no significant changes.

DISCUSSION

In the present work, we focus on T cells characterisation, and the identification and molecular definition of the so-called cytokine signatures in psoriasis patients (supplementary Fig. 2). Our results confirm the presence of CD3+/CD4+ cells, and CD3+/CD8+ cells in a psoriatic plaque, previously described,[1] suggesting an important role of the lymphocytes on keratinocytes control growth, as well as their contribution to initiate, stabilise, or inhibit the expression of psoriasis in the skin.[1] This was confirmed by the data obtained from psoriasis affects, in which CD3+/CD4+ cells, and in less proportion CD3+/CD8+ cells were presented in the lesions. The phenotypic characteristics show that in some cases, T cells can express chemokine receptors on their surface (CCR6), as a selective marker for Th17 cells, which produces IL-17.[1,3]

In our trials, Th17 cells exhibited a characteristic CD4+CD8-TCRαβ+, or CD4-CD8+ TCRαβ+ phenotype. Several studies have demonstrated the expression of all CD161 in human Th17.[11] Also, it has been determined Th17 cells as CCR6+CXCR3- predominantly, and a

significant percentage of these cells are positive for IL-17A, and IFNγ (a characteristic of Th1 cells). The cell percentage is known as"Th17/Th1" cells.[11] Th17/Th1 cells were found in the study, confirming previous results by Annunziato et al[4] regarding the characteristic phenotype of the cells.

Among the cytokines found with a broad relevance in the study conducted for patients diagnosed with psoriasis, and non-previously reported for this autoimmune pathology are the cytokines IL-26 and IL-31, high expressed in psoriatic patients respect to the control. These cytokines are expressed in keratinocytes and epithelial cells.[2,9] Thus, their presence and high relevance in psoriasis are due to the IL-26 and IL-31 were frequently found in this pathology.[9] Their expression may be involved in inflammatory processes, and cell activation can be supported based on different studies carrying out the association between the two cytokines and inflammation mechanisms in atopic skin,[2,8,9] and their induction of chemokines such as CCL17, CCL22, and CCL1,[8,9,12] related to the inflammatory process.[8,9,13]

For this pathology, the TGF-β gene was high expressed without significant change, which may indicate inhibition of keratinocytes growth. This can be related to the founds described by Han et al., in which he discusses the central role of TGF-β in the hyperproliferation of keratinocytes on the epidermis of patients with psoriasis and correlating it closely with the severity of psoriatic disease,[6] explaining the presence of cytokine TFG-β in some patients. Even though the expression of TGF-β is highly variable in patients with psoriasis, its finding plays an essential role in the process of autoimmune disease.

Moreover, T helper 17 cells (Th17) can produce cytokines such as IL-17, IL-22, and IFNγ in psoriasis, which were highly expressed in all psoriatic patients of the study. Although the Th17 cells population was low for the study, this type of cells play an important role in psoriatic disease due its ability to produce IL-17, a cytokine present in the epidermis in response to the autoantigenic potential of the HLA I molecule found in the keratinocytes, which are

involved in the pathology.[14] The co-expression of IL-17 and IFNγ was observed for the Th17 cells in this study and might be explained by the ability of Th17 lymphocytes from psoriatic plaques to kill target cells through TCR/CD3, demonstrating the presence of these two cytokines in psoriatic biopsies.[1] Additionally, many authors have described the Th17 cells as critical mediators of epidermal growth, cell differentiation, and local inflammation, main characteristics in psoriasis, giving to CD8+/IL17+, or CD4+/CD17+ cells high importance concerning its functional activity.[12]

Finally, we founded IL-9 high expression in psoriasis biopsies; This cytokine may actively work on the T cells response by inducing their proliferation and differentiation. IL-9 is mainly produced by Th17 and Th9 lymphocytes.[5-7] The role of IL-9 in psoriasis is oriented towards the hyperproliferation of keratinocytes and Th17 lymphocytes in the psoriatic lesion, due to its proliferative and differentiation mechanisms.[5,7]

To this, patients diagnosed with psoriasis exhibit frequently the occurrence of cytokines IL-31 and IL26 expression in the mRNA transcripts, which may be probably the cause of cell activation and the triggering of a pro-inflammatory response.[2,15] Although Th17 lymphocytes are not so frequent, they produce other types of cytokines such as IL-17, INFγ, and IL-9, which act directly on the inflammatory process and can be associated with the pathogenesis of psoriatic disease, as well. Though most cytokines are related to the increase of inflammatory processes, it has been found a type of cytokine TGFβ that do not change, which is oriented towards the inhibition process. Thus, the cytokines described in the present study, and less studied in previous researches, have shown an important function in psoriatic disease and the development of autoimmune disease.

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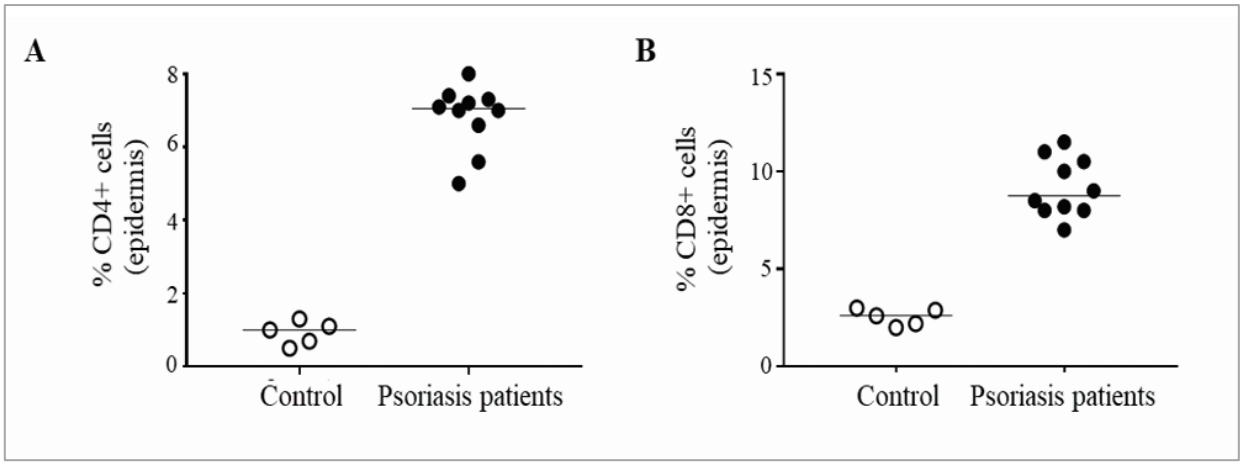


Fig. 1 T cells Percentage. T cells percentage obtained from epidermal samples of (●) 10 patients with psoriasis and (o) 5 control patients. (a)% of CD4 + epidermal cells with a mean ± SEM of 1.0 ± 0.14 for healthy patients (controls), and 7.1 ± 0.28 for patients with psoriasis, respectively, $p < 0.001$. (b)% of CD8 + epidermal cells with a mean ± SEM of 2.5 ± 0.2 for healthy patients (controls), and 9.1 ± 0.5 for patients with psoriasis, respectively, $p < 0.001$.

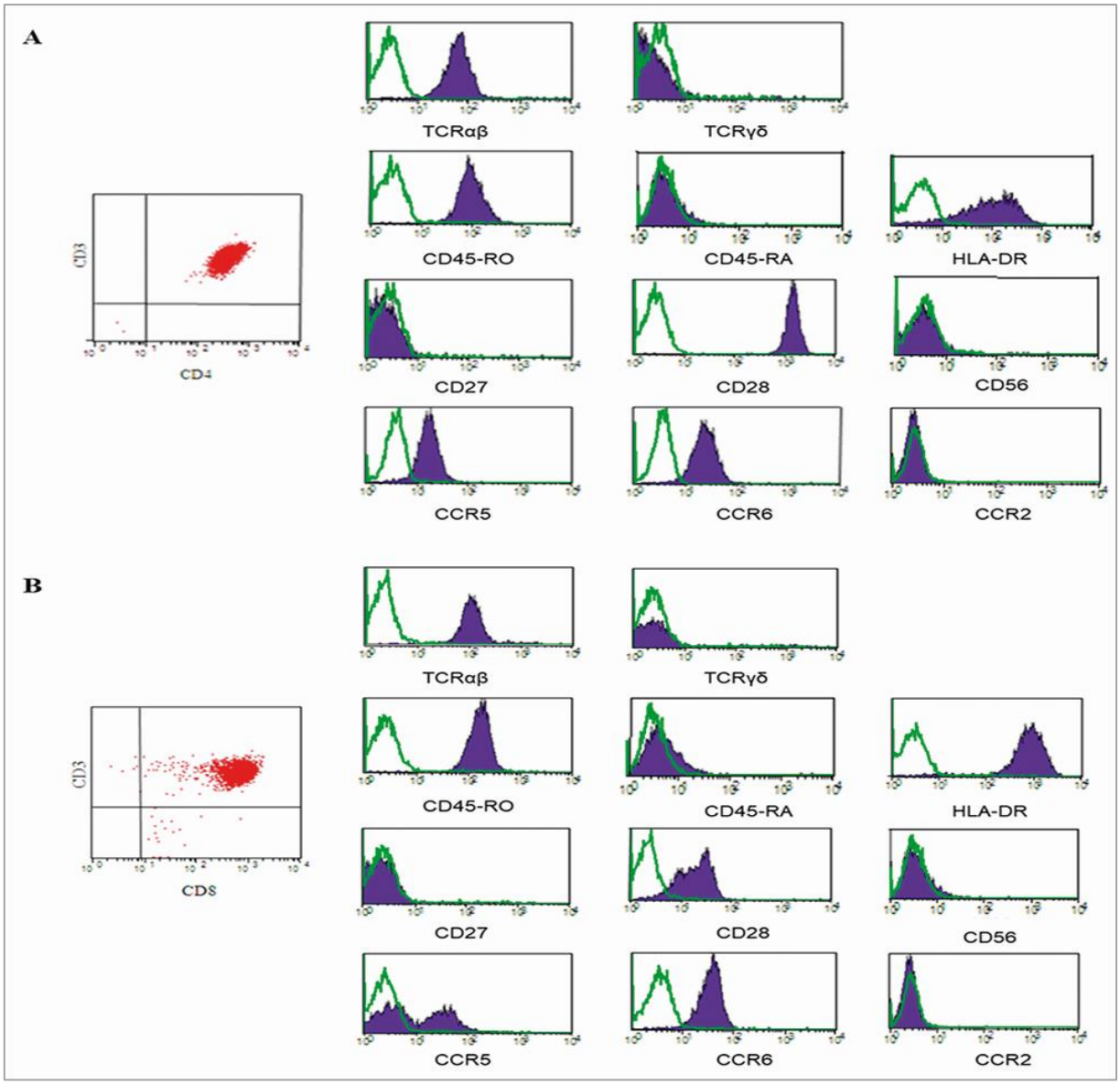


Fig. 2 Phenotypic characterization of CD3 + T cells. Representative image of the surface markers expressed by lymphocytes T (a) CD3 + CD4 + (b) CD3 + CD8 +, widened from a non-healthy patient's biopsy (diagnosed with psoriasis). Within the surface markers include: TCPαβ, TCRγδ, CD45-RO, CD45-RA, HLA-DR, CD27, CD28, CD56, CCR5, CCR6 and CCR2.

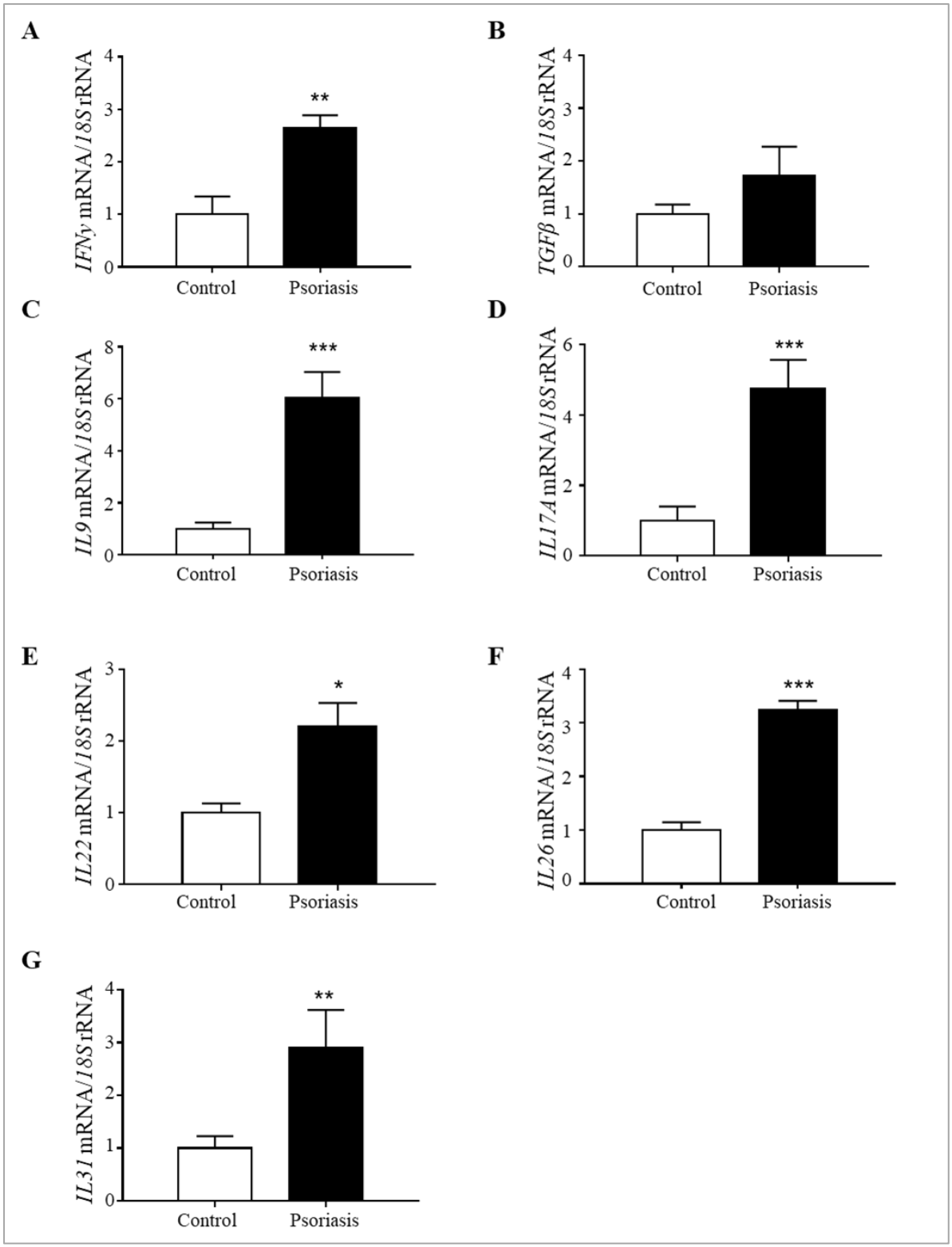


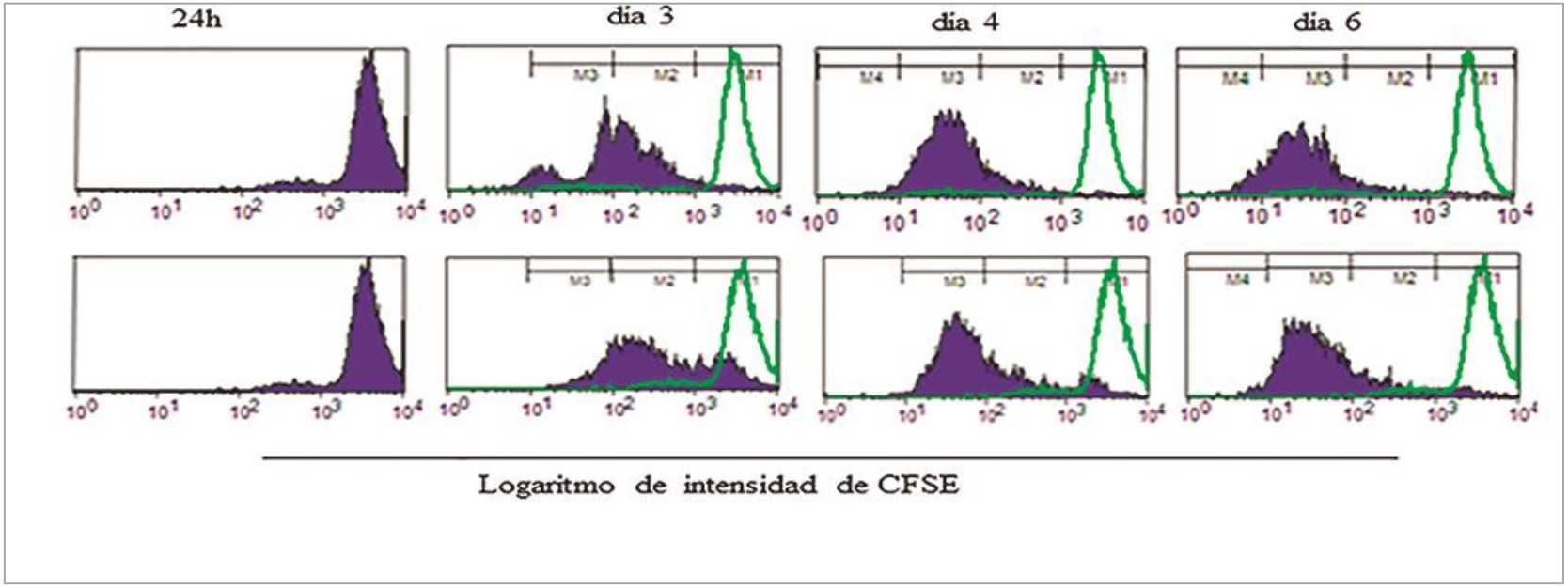
Fig. 3 Cytokine signature. Bar graphs show the mRNA biopsies expression of IFNY (a), IGF-β (b), IL9 (c), IL17A (d), IL22 (e), IL26 (f) and IL31 (g) obtained from control subjects and psoriasis patients. Gene expression in control participants was assumed to be 1. Values are the mean ± SEM. Differences between groups were analyzed by one-way ANOVA followed by Scheffe's test. *P<0.05; **P<0.01, ***P <0.001 vs. control subjects.

APPENDIX

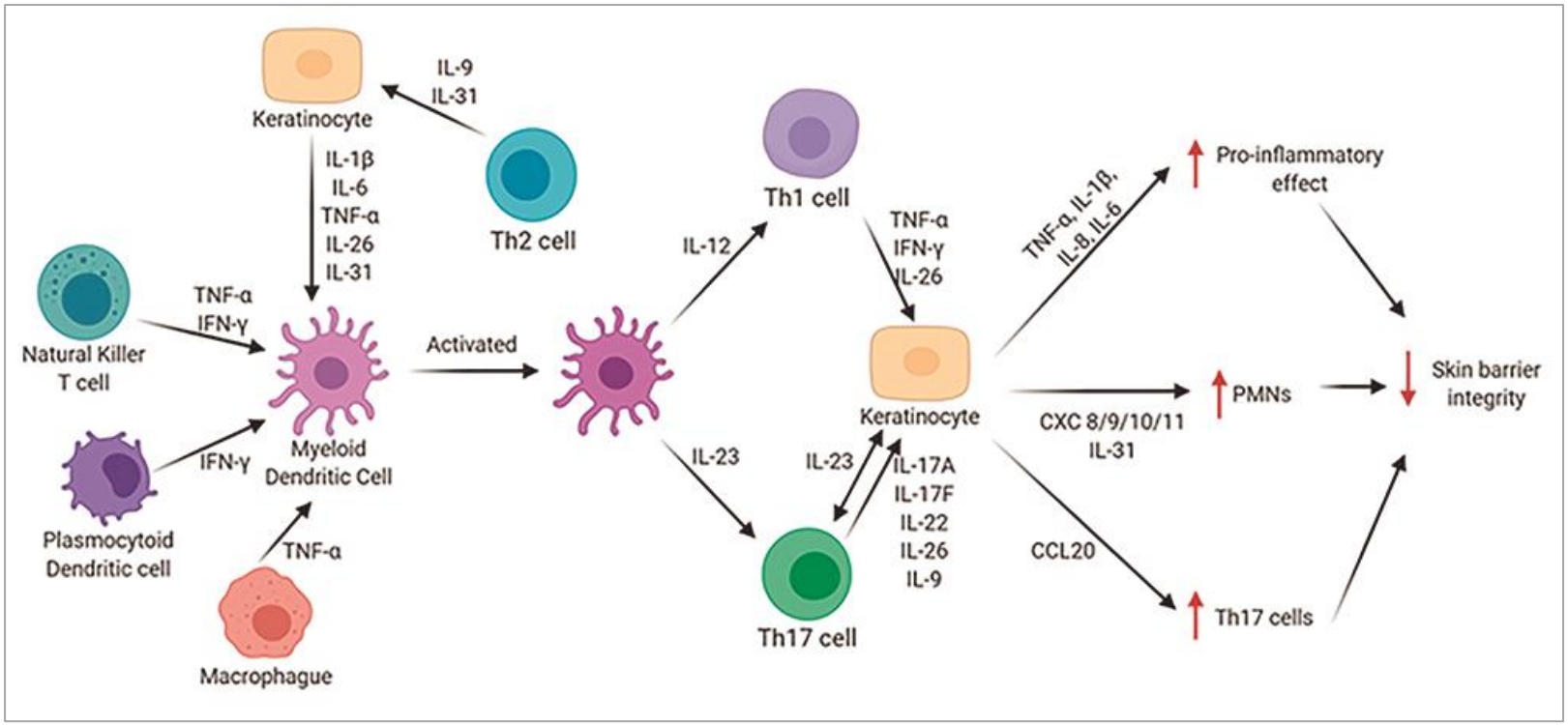
Supplementary Table 1. Sequences of primers and TaqMan® probes.

Gene (GenBank accesion no.)	Oligonucleotide sequence (5'-3')
IFNγ (NM_000619.2)	
Forward	TGACCAGAGCATCCAAAAGA
Reverse	CTCTTCGACCTCGAAACAGC
Probe	FAM- CCAACGCAAAGCAATACATG-TAMRA
TGFβ (NM_000660.6)	
Forward	GGGACTATCCACCTGCAAGA
Reverse	CCTCCTTGGCGTAGTAGTCG
Probe	FAM- TATCGACATGGAGCTGGTGA -TAMRA
IL9 (NM_000590.1)	
Forward	CCTCTGACAACTGCACCAGA
Reverse	TTGCCTCTCATCCCTCTCAT
Probe	FAM- CCACTGATTTTCAGTCGGGT-TAMRA
IL17A (NM_002190.3)	
Forward	ACCAATCCCAAAAGGTCCTC
Reverse	GGGGACAGAGTTCATGTGGT
Probe	FAM-GCAATGAGGACCCTGAGAGA-TAMRA
IL22 (NM_020525.5)	
Forward	CTCCTTCTCTTGGCCCTCTT
Reverse	GTTCAGCACCTGCTTCATCA
Probe	FAM-CTTCATGCTGGCTAAGGAGG-TAMRA
IL26 (NM_018402.1)	
Forward	GGAACATTGTCCCAAGCTGT
Reverse	TGCAGTTGACCAAAAACGTC
Probe	FAM-ATGGCTCAAAGCAACGATTC-TAMRA
IL31 (NM_001014336.1)	
Forward	CGACGTCTGTGCTCTTTCTG
Reverse	CAGCGTGTAATTCTGGGACA
Probe	FAM-CCCTCTCGAAGATGCTTTTG-TAMRA

IFNγ, interferon gamma; *TGFβ*, transforming growth factor beta; *IL9*, interleukin 9; *IL17A*, interleukin 17A; *IL22*, interleukin 22; *IL26*, interleukin 26; *IL31*, interleukin 31.



Supplementary Fig. 1. Proliferation of CD3 + lymphocytes. Proliferation assay in T cells from (above) psoriasis biopsy, (below) PBLs from healthy donors. At 24 hours, day 3, day 4, and day 6 after stimulation with CD3.



Supplementary Fig. 2. Cells and key mediators involved in the pathogenesis of psoriasis. Immune cells produce cytokines like (tumour necrosis factor α [TNF-α], interferon-α [IFN-α], interferon-γ [IFN-γ], IL-1β, IL-6, IL-26 and IL-31) that activate myeloid dendritic cells. Activated dendritic cells present antigens and secrete mediators such as interleukin-12 and interleukin-23, leading to the differentiation of T helper cells (Th17 and Th1). T cells, in turn, secrete mediators (e.g., IL-17A, IL-17F, IL-22, IL-26 and IL-9) that activate keratinocytes and induce the production of proinflammatory cytokines (TNF-α, IL-1β, IL-8, IL-6), chemokines (CXCL8/9,/10/11 and IL-31 that increase polymorphonuclear cells and CCL20 that increase Th17 cells. Those tree changes induce the reduction of the skin barrier integrity and the psoriasis plaque.