

Lymphocyte subsets in peripheral blood of patients with moderate-to-severe versus mild plaque psoriasis

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Received: 1 March 2007 / Revised: 25 November 2007 / Accepted: 26 November 2007 / Published online: 19 December 2007
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Abstract In several studies peripheral blood T-cells have been quantified, yet few data are available on lymphocyte subsets in moderate-to-severe psoriasis (in terms of extent and activity of lesions) versus mild psoriasis. The objective is to compare lymphocyte subsets in peripheral blood of patients with moderate-to-severe disease (PASI-score ≥ 12) to patients with mild disease (PASI-score < 12) and to healthy subjects. By means of flow cytometry method, lymphocytes in peripheral blood of 27 patients with psoriasis and 10 healthy controls were characterized. The absolute number of total lymphocytes was markedly decreased in patients with moderate-to-severe psoriasis as compared to patients with mild disease and normal subjects. Cellcounts of all analysed subsets were found to be increased in more severe psoriasis, except for CD8+CD45RO+ cells. The under-representation of CD8+CD45RO+ cells is compatible with the dynamics of acquired immunity, which requires a time lag after the relapse of the lesions to differentiate from CD45RA+ naive cells.

Keywords Psoriasis · Lymphocytes · Peripheral blood · Severity of disease · T cells

Introduction

Psoriasis is a common inflammatory skin disease characterized by hyperproliferation of keratinocytes. It is a well

established fact that T-cells play an important role in the pathogenesis of psoriasis [6, 9]. Indeed, treatments such as anti-CD4 [5, 15, 19], anti-CD11 [5, 13] and anti-CD25 [13], targeting at specific T-cells, have shown to be effective in psoriasis. Several T-cell subsets seem to play a primary role. Major classes are CD4+, CD8+, CD45RO+ and CD45RA+ T-cells. Recently, NK-T cells have been suggested to play an additional role in the regulation of immunity, through the release of cytokines [3, 14, 16]. NK-T cells are cells bearing both T cell receptors as well as natural killer-cell specific receptors such as CD161. It has been shown that circulating NK-T cells are significantly reduced in some autoimmune diseases [8, 17].

The large body of research on the role of T-cells in psoriasis has been focused on lesional T-cells. This has resulted in the hypothesis that there is a final common pathway responsible for the development of chronic plaque psoriasis, which may involve specific antigen recognition by T-cells that results in stimulation of keratinocyte proliferation. In psoriasis, CD4+ cells seem to be important mainly in the early phase of plaque development. These cells are found predominantly in the upper dermis, whereas CD8+ cells have proven to be relevant during chronic phases and are found predominantly in the epidermis [21], although others have found a more early involvement of CD8+ T-cells in the development of psoriatic plaques [22]. A large number of activated T-cells have been shown in clinically involved skin of psoriatic patients, but also uninvolved skin contains a significant number of T-cells. In healthy skin, however, T-cells can hardly be found. It has been suggested that circulating T-cells are activated and subsequently recruited from the circulation during the development of psoriatic plaques [2, 10].

Several studies have investigated peripheral blood T-cells of psoriatic patients [1–3, 7, 10, 12, 14, 20, 21].

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These studies indicated that the total amount of T-cells in patients is comparable or slightly increased as compared to that found in normal subjects. No relevant differences have been shown with respect to total T-cell counts and T-cell subsets including CD4+, CD8+, CD45RO+ and CD45RA+ cell counts. Only few studies comprise a comparison between mild and more severe forms of psoriasis, using clinical severity indicators such as the Psoriasis Area and Severity Index (PASI-score) [4, 18].

The present study compares specific circulating lymphocyte subsets in patients with mild psoriasis, patients with moderate-to-severe psoriasis and normal subjects.

Materials and methods

Patients and controls

Fifteen patients with moderate-to-severe psoriasis vulgaris (12 male and three female aged 19–66, mean age 46.2 years) and 12 patients with mild psoriasis (seven male and five female, aged 34–72, mean age 52.8 years) from our outpatient department participated in this study. Mean PASI-scores in both groups were 20.97 ± 2.55 (mean \pm SEM) and 6.11 ± 1.27 , respectively. Patients were classified into one of both groups based on their PASI-score. We considered all patients with a PASI-score <12 as having a “mild”, and all patients with a PASI >12 as having a “moderate-to-severe” psoriasis [4, 18].

Patients were free of any systemic therapy for at least 4 weeks and did not use any topical therapy in the last 2 weeks. Peripheral blood was obtained from all subjects with their written informed consent.

Control samples were collected from 10 healthy volunteers without any history or signs of skin disease (four male and six female aged 24–49, mean age 33.8 years).

Preparation of PBMC's

For each patient, the exact amount of blood withdrawn was determined by measuring the height of the column of blood in the tube, which was subsequently converted to the corresponding volume in microliter.

PBMC's were isolated from heparinized blood by density centrifugation on polyester gel (Becton Dickinson Vacutainer™ CPT™, Franklin Lakes NJ, USA). After filtering through a 70 μ m cellstrainer, cells were washed twice.

For flow cytometry, single-cell suspensions (concentration 5×10^5 cells ml^{-1}) were stained in 1% fetal calf serum in phosphate-buffered saline (PBS) at concentrations recommended by the manufacturer. Additionally, 450 μ l propidium iodide (PI) in PBS was added to each sample in order to exclude non-viable cells from analysis.

The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD4, FITC-conjugated anti-CD8, phycoerythrin (PE)-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-CD94 and PE-conjugated anti-CD161 (all from Immunotech, Marseille, France), as well as FITC-conjugated anti-CD45RA (Becton Dickinson, San Jose, CA, USA) and FITC-conjugated anti-CD45RO (DAKO, Glostrup, Denmark).

Flowcytometric analysis

Cells were analyzed with an EPICS Elite flow cytometer (Coulter, Luton, UK), using the forward scatter as a discriminator. Lymphocytes were identified by gating on CD45 and side and forward scatter properties. All samples were processed within 18 h of phlebotomy.

The following (combinations of) PE and FITC-conjugated reagents were used to determine the expression of each antigen or antigen combination on lymphocytes derived from peripheral blood: CD4+ (marker for T-helper cells), CD8+ (marker for cytotoxic T cells), CD45RA+ (marker for naïve T-cells), CD45RO+ (marker for memory T-cells), CD94+ (marker for NK-cells, T cells), CD161+ (marker for NK-cells, T-cells with memory phenotype), CD4+ CD45RA+, CD4+ CD45RO+, CD8+ CD45RA+, CD8+ CD45RO+, CD8+ CD94+ and CD8+CD161+.

Determination of absolute numbers of lymphocytes

Enumeration of positive cells was performed by adding Flow Count Beads (Beckman Coulter, Fullerton, CA, USA) to the cell suspension of PBMCs. An automatic stop after a defined amount of beads was programmed on the flow cytometer. Absolute counts of lymphocyte subsets per blood sample were calculated by determining the ratio of the beads to the cell population and then multiplying this ratio by the number of beads in the tube. By dividing this count by the amount of microliters in the tube, a relative absolute lymphocyte count in cells μl^{-1} blood withdrawn was obtained. Analysis was performed with Verity software.

Ratios for CD4+/CD8+ cells, CD45RO+/CD45RA+ cells, CD4+CD45RO+/CD4+CD45RA+ cells and CD8+CD45RO+/CD8+CD45RA+ cells were calculated afterwards, with data derived from this analysis.

Statistical analysis

Data-entry and analysis was performed using Statistica 6.0 software. Means and standard deviations were calculated for each parameter and were tested with one-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

Results

In total, 27 patients with psoriasis and 10 normal subjects without signs or symptoms of skin disease were included in the present study. Table 1 summarizes the demographic details and psoriasis related characteristics of the patients with moderate-to-severe psoriasis, patients with mild psoriasis and normal subjects. Four out of 12 patients with mild psoriasis had shown a minimal increase in the extent of lesions; in the others the skin abnormalities were stable. With respect to the 15 patients with moderate-to-severe-psoriasis, 12 had an increase of the extent of lesions during the previous 4 weeks, whereas the other three had been stable.

Total cell counts

The total amount of gated cells (per microliter blood withdrawn) in normal subjects was $2,220 \pm 301$ cells μl^{-1} (mean \pm SEM). The cell-count in mild psoriasis was $1,923 \pm 230$ cells μl^{-1} . In contrast, patients with moderate-to-severe psoriasis had cell counts of 498 ± 95 cells μl^{-1} . No statistically significant difference was observed between normal subjects and patients with mild psoriasis. However, a highly significant difference ($p < 0.0001$) was observed between mild and severe psoriasis. The CD4+/CD8+ ratios in all three groups were in the same range, meaning no shift occurred in the distribution of these subgroups of peripheral blood lymphocytes.

The total number of lymphocytes, and the CD4+ and CD8+ counts of the patients with moderate-to-severe

disease were also routinely measured at the GLP certified Central Haematology Laboratory in our hospital, in which the whole blood lysing method is the standard. Comparison of both methods reveals an acceptable resemblance in results, with lower counts of maximum 10%.

Lymphocyte subsets

Lymphocyte subsets as a percentage of total gated cells are summarized in Fig. 1. CD4+, CD8+, CD45RO+ and CD45RA+ cells are increased in patients with severe psoriasis as compared to patients with mild psoriasis and normal subjects ($p < 0.0001$ in all cases).

No significant differences were observed between patients with mild psoriasis and normal subjects.

Double labeling of lymphocytes revealed that the percentages of CD4+CD45RO+, CD4+CD45RA+ and CD8+CD45RA+ cells were significantly increased in moderate-to-severe versus mild psoriasis ($p < 0.001$ in all cases), whereas the percentage of CD8+CD45RO+ cells was not increased significantly (Fig. 2). Cell ratios for CD4+/CD8+ cells, CD45RO+/CD45RA+ cells, CD4CD45RO+/CD4CD45RA+ cells and CD8CD45RO+/CD8CD45RA+ cells are summarized in Fig. 3.

Lymphocytes expressing NK cell receptors

CD94+ and CD161+ cells, expressed as a percentage of total cells were increased in patients with moderate-to-severe psoriasis, as compared to patients with mild psoriasis

Table 1 Demographic and psoriasis related characteristics of patient and subjects participating in the study (mean \pm SEM)

		Moderate-to-severe psoriasis	Mild psoriasis	Normal subjects
Demographics	Age (years)	46.2 \pm 3.45	52.8 \pm 3.49	33.8 \pm 3.1
	Male:female ratio	12:3	7:5	4:6
Psoriasis related characteristics	PASI-score	20.97 \pm 2.55	6.11 \pm 1.27	NA
	Duration (years)	16.7 \pm 2.7	22.2 \pm 2.5	NA

Fig. 1 CD4, CD8, CD45RO and CD45RA subpopulations, expressed as a percentage of total lymphocyte counts. Comparison between normal subjects, patients with mild- and patients with moderate-to-severe psoriasis. (*: $p < 0.0001$)

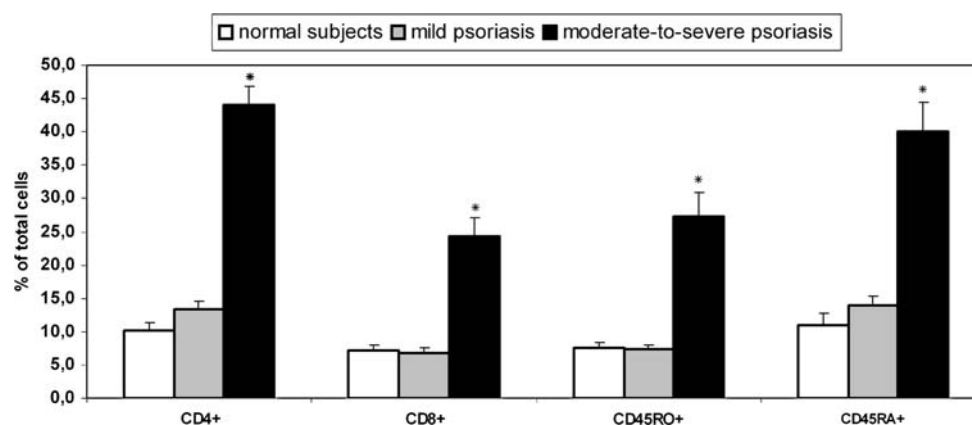


Fig. 2 Expression of CD4+CD45RO+, CD4CD45RA+, CD8+CD45RO+ and CD8CD45RA+ cells, as percentage of the total amount of lymphocytes, in three subgroups: moderate-to-severe psoriasis, mild psoriasis and normal subjects (*: $p < 0.001$)

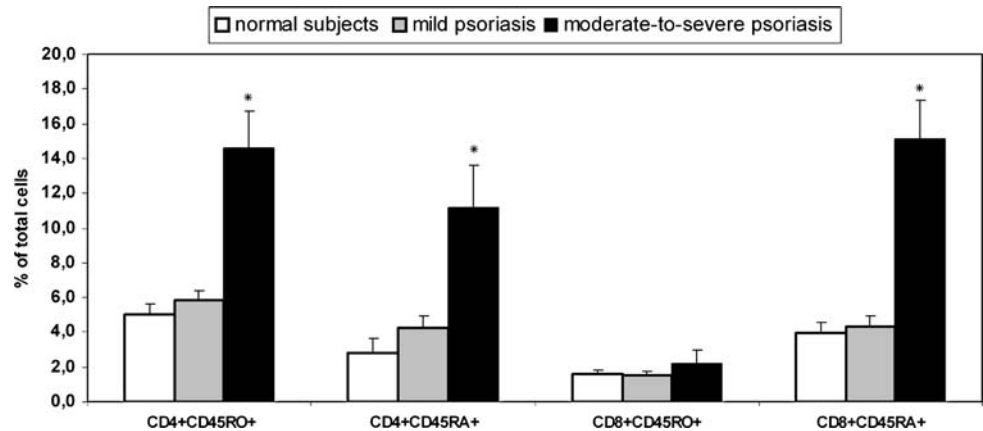
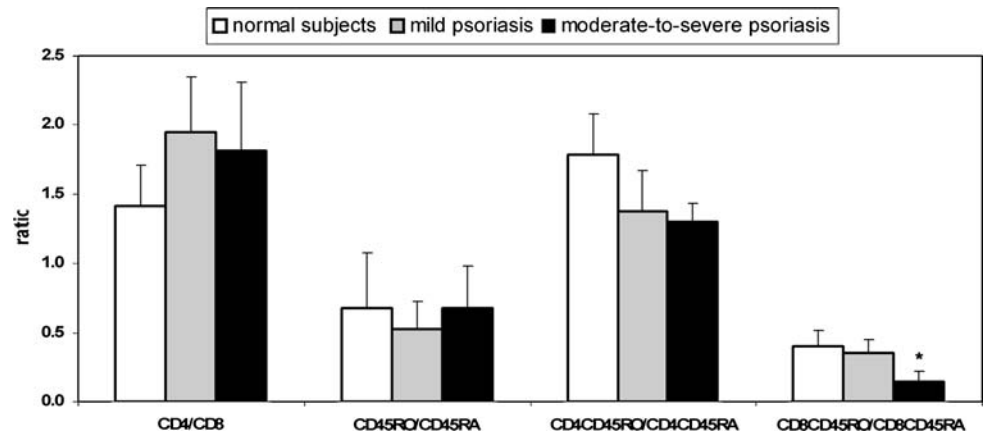


Fig. 3 Ratios of CD4/CD8, CD45RO+/CD45RA+, CD4CD45RO+/CD4CD45RA+ and CD8CD45RO+/CD8CD45RA+ in normal subjects, mild and moderate-to-severe psoriasis (*: $p < 0.05$)



and normal subjects ($p < 0.05$ and $p < 0.001$, respectively). Figure 4 summarizes these observations. Again no statistically significant differences were observed between normal subjects and patients with a mild form of psoriasis.

With respect to double stained cells, we observed an increase in the expression of both CD8+CD94+ cells as well as CD8+CD161+ cells (markers for NK T subpopulations) in patients with a more extensive form of psoriasis, expressed as a percentage of the total amount of cells, as compared to patients with mild psoriasis and normal subjects

($p < 0.008$ and $p < 0.009$, respectively, results shown in Fig. 5).

Correlation between PASI-score and lymphocyte subsets and cells expressing NK receptors

Linear regression analysis revealed no significant correlation between the individual PASI-scores and the quantifications of lymphocyte subsets or cells with NK-cell receptors. Correlation analysis between disease activity and peripheral

Fig. 4 CD94 and CD161 subpopulations, expressed as a percentage of total lymphocyte counts. Comparison between normal subjects, patients with mild- and patients with moderate-to-severe psoriasis (*: $p < 0.05$ and $p < 0.001$ respectively)

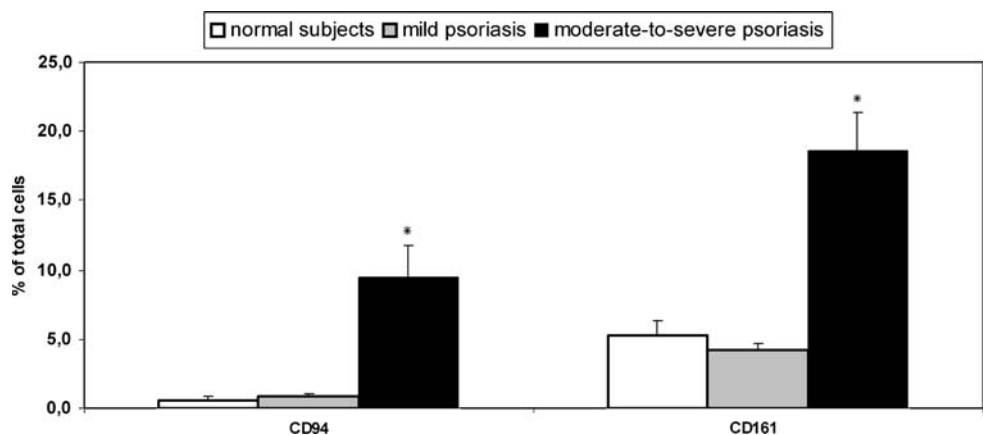
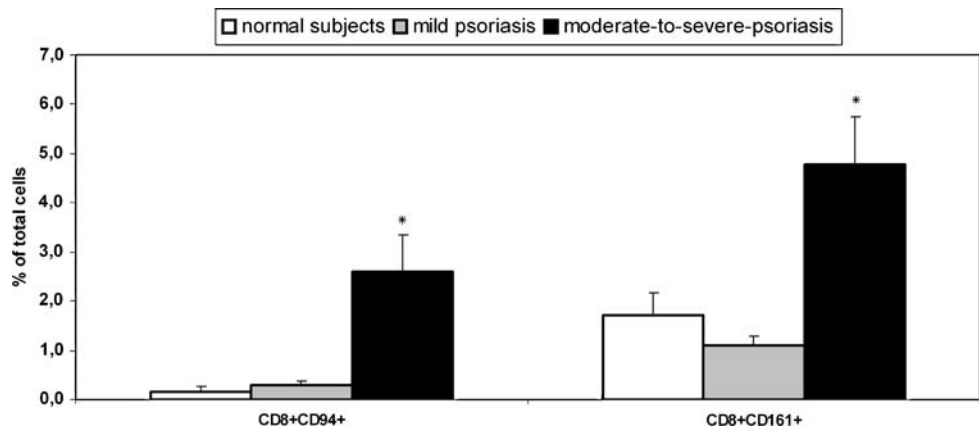


Fig. 5 Cellcounts of CD8+CD94+ and CD8+CD161+ cells as percentage of total lymphocytes (*: $p < 0.009$)



lymphocytes did not reveal a difference between patients with spreading lesions and those with stable plaque psoriasis.

Discussion

The results of this study show marked differences in absolute cellcounts between the three patient groups. Our main goal was to investigate whether there is a difference in the occurrence of biomarkers on circulating lymphocytes in mild psoriasis versus more severe forms of the disease. One of the criteria to qualify for a treatment with major systemic therapies such as a biological is often a minimum PASI-score of 12 [4, 18]. We were interested whether this cut-off point also implies a differentiation between mild versus moderate/severe psoriasis in terms of T-cell and NKT-cell subsets. The results of the present study have shown us that there are indeed marked differences between mild psoriasis and the more severe forms of disease defined by cut-off point PASI 12.

A decrease of total lymphocyte counts, as percentage of PBMC, and an increase in the percentage of CD4+CD45RO+ cells in patients with moderate-to-severe psoriasis has been reported before [10]. These patients (32 subjects) had an average PASI of 25.7 (10–48), and all had had an exacerbation following infection. Patients had a mixed morphology, from guttata psoriasis to chronic plaque psoriasis. In the same study, however, a decrease in the amount of CD4+ cells was described, which is in contrast with the findings in our study. Other authors have reported no significant difference in total T-cells as percentage of PBMC counts between patients with psoriasis and normal subjects [3]. These patients (14 subjects) had an average PASI of 23.5 ± 10.6 . However, no information was given whether these patients had relapsing psoriasis. In the present study, the patients with severe psoriasis had an extension of lesions during the wash out period (average increase in PASI: 4.9). Therefore, the severity in our patients not only comprises the extent of lesions but also activity of lesions with progression and therefore, approaches the

characteristics of the patients reported by Lecewicz-Torún et al. [10], who also reported a decrease of T-cells in peripheral blood in psoriasis (expressed as percentage of total PBMC). The decrease of lymphocyte counts, expressed as a percentage, in peripheral blood in patients with moderate-to-severe psoriasis is the reverse of the substantial accumulation of lymphocytes in psoriatic skin. The most likely explanation for this paradox is an increased influx of cells into the skin of patients with severe disease. Further studies, in which simultaneous measurements of lesional and circulating T cells will be performed, need to be carried out to support this hypothesis.

A striking observation is the increased percentage of both CD4+ cells and CD8+ cells in patients with moderate-to-severe psoriasis as compared to mild psoriasis and normal subjects, without a change in CD4/CD8 ratio. So far, reports on the CD4+/CD8+ ratio in psoriasis have been at variance [10, 11].

With respect to the double labeled lymphocytes, both the percentage of CD4+CD45RO+ cells as well as the percentage of CD4+CD45RA+ cells is increased in patients with moderate-to-severe psoriasis, in contrast to the absolute decrease of total lymphocytes. The relative count of both subsets is increased, whereas their ratio shows a tendency to decrease ($p = 0.0509$). Thus, with increasing severity of disease, the increase in CD4+CD45RA+ cells seems to exceed the increase in CD4+CD45RO+ cells. An explanation for this phenomenon is the preferential migration of CD4+CD45RO+ cells into lesional skin in extensive disease, resulting in a relative decrease of this subset of CD4+ lymphocytes in peripheral blood. This hypothesis supports earlier studies where CD4+CD45RO+ cells have been shown to be the major players in psoriatic plaques. Indeed, CD4+ cells appear in a very early phase of the psoriatic process. In a previous study, CD4+ cells have been detected in the distant uninvolved skin of patients with active psoriasis [22]. The percentage of CD8+CD45RO+ cells in patients with moderate-to-severe psoriasis approached the percentages in patients with mild psoriasis and normal subjects, in

contrast with the CD8+CD45RA+ cells, which did show a significant increase in peripheral blood. The impressive under-representation of CD8+CD45RO+ lymphocytes as compared to CD8+CD45RA+ lymphocytes in moderate to severe psoriasis suggests a preferential influx of CD8+CD45RO+ cells into the skin. Both CD8+ cells, as well as CD45RO+ cells are abundantly present in psoriatic lesional skin. Previously, we have shown that in the outer margin of the spreading psoriatic plaque the number of CD8+ cells and CD45RO+ cells were increased [22]. Therefore, the relative under-representation in peripheral blood of CD45RO+ cells, in particular the CD8+ CD45RO+ cells, most likely reflect the influx of these cells into the skin in patients with moderate-to-severe psoriasis in an active phase of disease. However, further studies are needed to support this hypothesis. Lymphocyte counts, preferably T-cells, in skin as well as in the peripheral blood should be monitored during a longer period of time, e.g., during exacerbation and during disease-free intervals. The CD8+CD94+ cells and CD8+CD161+ cells show a marked increase in patients with moderate-to-severe psoriasis as compared to mild psoriasis and normal subjects. The increases of these lymphocytes expressing NK receptors in peripheral blood are accompanied with increases of these cells in the psoriatic lesion.

The results of the present study suggest that in peripheral blood of patients with moderate-to-severe psoriasis, in contrast to the vast presence of T-cells in skin lesions, the absolute lymphocyte counts are profoundly decreased, without any change of CD4/CD8 ratio. The relative under-representation of CD45RO+ cells, as compared to CD45RA+ cells, in peripheral blood most likely results from a massive influx of memory effector T-cells into the psoriatic lesions in an active phase of disease. Both in blood and skin of patients with moderate-to-severe psoriasis the percentages of CD94+ and CD161+ cells are increased. These results are compatible with the functions of these cells. CD45RO+ cells differentiate from CD45RA+ cells and represent acquired immunity, whereas NK-T cells are able to act without prior sensitization, representing innate immunity [3]. In further research, more selective lymphocyte markers are required in order to investigate the role of peripheral blood T-cells in the pathogenesis of psoriasis, and correlation studies between peripheral blood T-cells and T-cells in skin are required in order to understand the dynamics of compartmentalization of T-cell subsets belonging to either the acquired or the innate immunity system in psoriasis.

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