RESEARCH ARTICLE

Proinflammatory cytokine responses in patients with psoriasis

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ABSTRACT. ***Background:*** **Psoriasis is one of the most common, immune-mediated, chronic inflammatory skin diseases. Proinflammatory cytokines play an important pathogenetic role at a local level.** ***Objective:*** **To assess whether the proinflammatory cytokines IL-1β, IL-6, IL-17, IL-22 and TNF-α are released systemically during psoriasis.** ***Methods:*** **Peripheral blood mononuclear cells (PBMCs) were isolated from 30 patients with psoriasis and 30 healthy volunteers. Cytokine production was assessed in supernatants using an enzyme immunoassay after stimulation of PBMCs with microbial stimuli. In addition, flow cytometry was used to determine the subsets of monocytes involved and the intracellular TNF-α production in monocytes.** ***Results:*** **IL-17 levels were significantly higher in the supernatants of PBMCs from psoriatic patients after stimulation with phytohemagglutinin. TNF-α production was also significantly higher in cells from psoriatic patients after stimulation with all stimuli, as compared with health volunteers. Similar changes were not found for the other cytokines. A statistically significant difference was observed between patients and controls for inflammatory $CD14^+/CD16^+$ monocytes ($p<0.0001$) and patrolling CD14-/$CD16^+$ monocytes.** ***Conclusion:*** **Hyper-production of TNF-α is documented in psoriasis. These results support the concept that there is a systemic, proinflammatory component in psoriasis.**

Key words: psoriasis, IL-1β, IL-6, IL-17, IL-22, TNF-α, monocytes

Psoriasis is a common, persistent, and relapsing skin disorder, affecting approximately 2% of the population [1]. The pathogenesis of psoriasis remains unknown, but an overwhelming inflammatory reaction in the skin is believed to play an important role. Triggering factors such as emotional stress, trauma, infectious agents, and drugs activate a complex immunological cascade leading to skin inflammation and accelerated epidermal and vascular growth [2, 3].

The cytokine pattern observed in the psoriatic plaque shows an increased expression of IL-1β, L-6, IL17, IL-22 and TNF-α [4-8]. IL-1 is also called a primary cytokine, since it can independently initiate a number of mechanisms capable of triggering inflammation [7]. IL-6 acts as an autocrine mitogen in psoriatic epidermis, and, in synergy with IL-1 and TNF-α, contributes to cellular hyperproliferation through its action on the epidermal growth factor receptor (EGF) [9]. IL-17 is a proinflammatory cytokine, responsible for expanding and maintaining the Th17 pathway. This pathway has been the subject of many studies because of its relevance in the development and management of psoriasis [10]. IL-22 is a member of the IL-10 cytokine family, produced by several different cellular sources including Th17 cells, natural killers cells (NK), and Th22 cells [11]. It works synergistically with IL-17 to enhance the expression of anti-microbial peptides that are increased in psoriasis [12]. IL-22 mediates the epidermal acanthosis and abnormal differentiation of keratinocytes that are the main pathological findings in psoriasis [13]. Acting through transmembrane receptors, TNF-α is a pleiotropic cytokine produced by many different cell types, especially cells of monocytic lineage [14, 15]. Its central role in psoriasis has come to light through observations of the efficacy of anti-TNF-α biological therapies in psoriasis and psoriatic arthritis [16-22]. TNF-α levels were found to be elevated in psoriatic skin lesions [8, 23, 24]. There is increasing evidence that psoriasis also has an important systemic component. This is supported by the presence of inflammatory-mediated co-morbidities in psoriatic patients, and by the favorable impact of anti-TNF blocking agents on the course of the disease [25-27]. These observations prompted several questions: is TNF-α the main proinflammatory cytokine implicated in the pathogenesis of psoriasis; is its overproduction mediated only at a local level in the skin, or can it also be overproduced by circulating monocytes?

In order to provide answers to these questions, we assessed the production of proinflammatory cytokines by

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circulating monocytes from psoriatic patients. In addition, we investigated the subpopulations of monocytes involved in TNF-α production.

PATIENTS AND METHODS

Patients

The study protocol was approved by the Ethics Committee of the "ATTIKON" University Hospital (06/07-07-08). Written informed consent was provided by all enrolled patients and healthy volunteers.

Patients with chronic plaque psoriasis who had not received any systemic treatment during the previous six months were included in the study. The diagnosis of psoriasis was made on clinical and histological grounds. Patients with erythrodermic, pustular and psoriatic arthritis were excluded from the study. The severity of psoriasis was assessed according to the psoriasis area and severity index (PASI-score) for each patient [28]. Moreover, patients were evaluated using the Dermatology Life Quality Index (DLQI) [29]. The control group included healthy, age- and sex-matched volunteers with no personal or family history of psoriasis.

Laboratory procedures

Cell stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients and healthy volunteers after gradient centrifugation over Ficoll (Biochrom, Berlin, Germany). After three consecutive washings in ice-cold PBS, pH 7.2 (Biochrome), PBMCs were counted on a Neubauer plate with trypan blue exclusion of dead cells. They were then diluted in RPMI 1640 enriched with 2 mM of L-glutamine and 10 mM of pyruvate (Biochrom), 500 mg/mL of streptomycin and 100 U/mL of penicillin, and suspended in duplicate in 96-well plates at a density of 5×10^5 cells/well. PBMCs were incubated for 24 hours and five days at 37°C in 5% CO_2 with the following stimuli: 10 ng/mL of lipopolysaccharide of *Escherichia coli* O55:B5 (LPS, Sigma, St. Louis, USA), a purified TLR-4 agonist; 5 μg/mL Pam_3Cys (EMC microcollections, Tübingen , Germany), a purified TLR-1 and TLR-2 agonist; 5 μg/mL of phytohemagglutinin (PHA, EMC microcollections, Tübingen, Germany), which is a lymphocyte mitogen; and 5×10^5 colony-forming units/mL of each of the following heat-killed isolates; *Candida albicans*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* (MRSA). In the case of incubation lasting five days, the growth medium was supplemented with fetal bovine serum (FBS, Biochrom) at a final concentration of 10%. The PBMCs from one patient and one healthy control were run in parallel each day of the experiment. After incubation, the plates were centrifuged and the supernatants were collected and stored at -70°C until assayed.

Using an enzyme immunoassay (R&D, Minneapolis, USA), concentrations of TNF-α, IL-1β and IL-6 were measured in cell culture supernatants after 24 hours of incubation; concentrations of IL-17 and IL-22 were measured after 5 days of incubation. The lower limits of detection were 20 pg/mL for TNF-α, 20 pg/mL for IL-1β, 20 pg/mL for IL-6, 10 pg/mL for IL-17 and 10 pg/mL for IL-22.

Subpopulation of monocytes

Whole blood was incubated for 15 minutes, in the dark, with the following fluorocolor-conjugated monoclonal antibodies: anti-CD14 to fluorochrome fluorescein isothiocyanate (FITC, emission 525 nm, Immunotech, Marseille, France), anti-CD16 to fluorochrome phycoerythrin (PE, emission 525 nm, Immunotech) and anti-CD45 to fluorochrome phycoerythrin-Cyanin 5.1 (PC5, emission 680 nm, Immunotech). Red blood cells were lysed, and white blood cells were fixed using a lyse-fix solution (VunersaLyse Solution, Beckman Coulter containing 2% formaldehyde). Isotypic IgG controls were used for each patient. Cells were analyzed after running through Cytomics FC flow cytometry (Beckman Coulter Co, Miami, Florida) with gating for monocytes based on their characteristic SS scattering and CD45 expression. Absolute counts were determined using Fluorospheres (Flow Count, Immunotech).

Two mL of whole blood were placed into sterile, heparinized, 25 cm^3 cell culture flasks. Blood was incubated at 37°C in 5% CO_2 for eight hours with/without 10 ng/mL of LPS in the presence of 10 μg/mL Brefeldin A (AppliChem, Darmstadt Germany) [30]. The non-adherent blood cells were collected, while adherent monocytes were thoroughly washed with Hank's solution (Biochrom), harvested with a 0.25% trypsin–0.02% EDTA solution (Biochrom) and centrifuged. After reconstitution with PBS, adherent monocytes were fixed; following permeabilization cells (Intraprep, Immunotech), cytoplasmic staining with anti-TNF-α monoclonal antibody to the fluorochrome PE (immunotech) was performed. Specimens were analyzed through flow cytometry with gating on monocytes based on their characteristic SS scatter and CD45 expression. Isotypic IgG controls were used for each patient.

Stimulation of macrophages

In some patients and healthy controls, PBMCs were incubated for one hour at 37°C in 5% CO_2 with RPMI supplemented with 2mM glutamine. Non-adherent lymphocytes were subsequently discarded, while the adherent monocytes were thoroughly washed with Hank's solution, then harvested using a trypsin/0.02% EDTA solution, and measured in a Neubauer chamber with trypan blue exclusion of dead cells. Monocytes were incubated at a density of 1×10^5 cells/mL for five days with 10% FBS to induce macrophage differentiation. On the third day, the medium was refreshed [31]. After five days, the supernatant was removed and the cells were stimulated with/without 5×10^5 cfu/mL of heat-killed isolate *P. aeruginosa* and *S. aureus*. After 24 hours of incubation, supernatants were collected and stored at -70°C until assayed. Concentrations of TNF-α were measured in supernatants by an enzyme immunoassay.

Statistical analysis

All data were analyzed using the statistical package for social science (SPSS 17.0) for Windows. Results were expressed as means ± SE. Comparisons between groups were made by the Mann-Whitney U test. The level of statistical significance was determined at a p value of 0.05 after adjustment for multiple comparisons.

Table 1
Demographic characteristics of psoriatic patients and controls enrolled in the study.

	Patients	Controls
	Mean ± SD	Mean ± SD
Sex (Male/Female)	16/14	16/14
Age	43 ± 11.8	44.7 ± 2.3*
PASI score	11.62 ± 5.7	
DLQI score	12.53 ± 4.41	

*pNS compared with patients

RESULTS

Thirty patients with proriasis (sixteen males and fourteen females), and thirty age- and sex-matched healthy controls were enrolled in the study. Demographic characteristics, as well as values for PASI and DLQI scores are shown in *table 1*.

IL-17 concentrations in supernatants after stimulation of PBMCs with bacterial stimuli are presented in *figure 1*. High levels of IL-17 were only found after stimulation with PHA, a strong mitogenic factor for lymphocytes. In contrast, no statistically significant difference was documented for IL-1β, IL-6 and IL-22 between patients and healthy controls. (*figure 1*).

Although PBMCs from patients released significantly higher amounts of TNF-α in response to all stimuli, the clinical severity of the psoriasis did not correlate statistically with increased TNF-α production by monocytes.

In order to investigate which monocyte subpopulation is potentially responsible for the increased production of TNF-α in psoriatic patients, the absolute counts of monocyte subpopulations were determined in fifteen patients and fifteen matched controls (*figure 2A*). FACS analysis is shown in *figure 2B*. Although the absolute counts of $CD14^+/CD16$- cells did not differ between patients and controls, the absolute counts of $CD14^+/CD16^+$ monocytes and $CD14^-/CD16^+$ patrolling monocytes were found to be significantly higher in patients compared with controls.

These findings prompted us to hypothesize that the higher levels of TNF-α found in the supernatants of stimulated PBMCs from patients are produced by the two aforementioned monocyte subpopulations. With a view to exploring this hypothesis, TNF-α production stimulated by LPS was measured in whole blood using flow cytometry in five patients and matched controls. As shown in *figure 3*, patients showed increased TNF-α expression produced by patrolling monocytes as compared to healthy controls.

To define whether these monocytes continue to produce increased amounts of TNF-α after their differentiation into tissue macrophages, circulating monocytes were matured

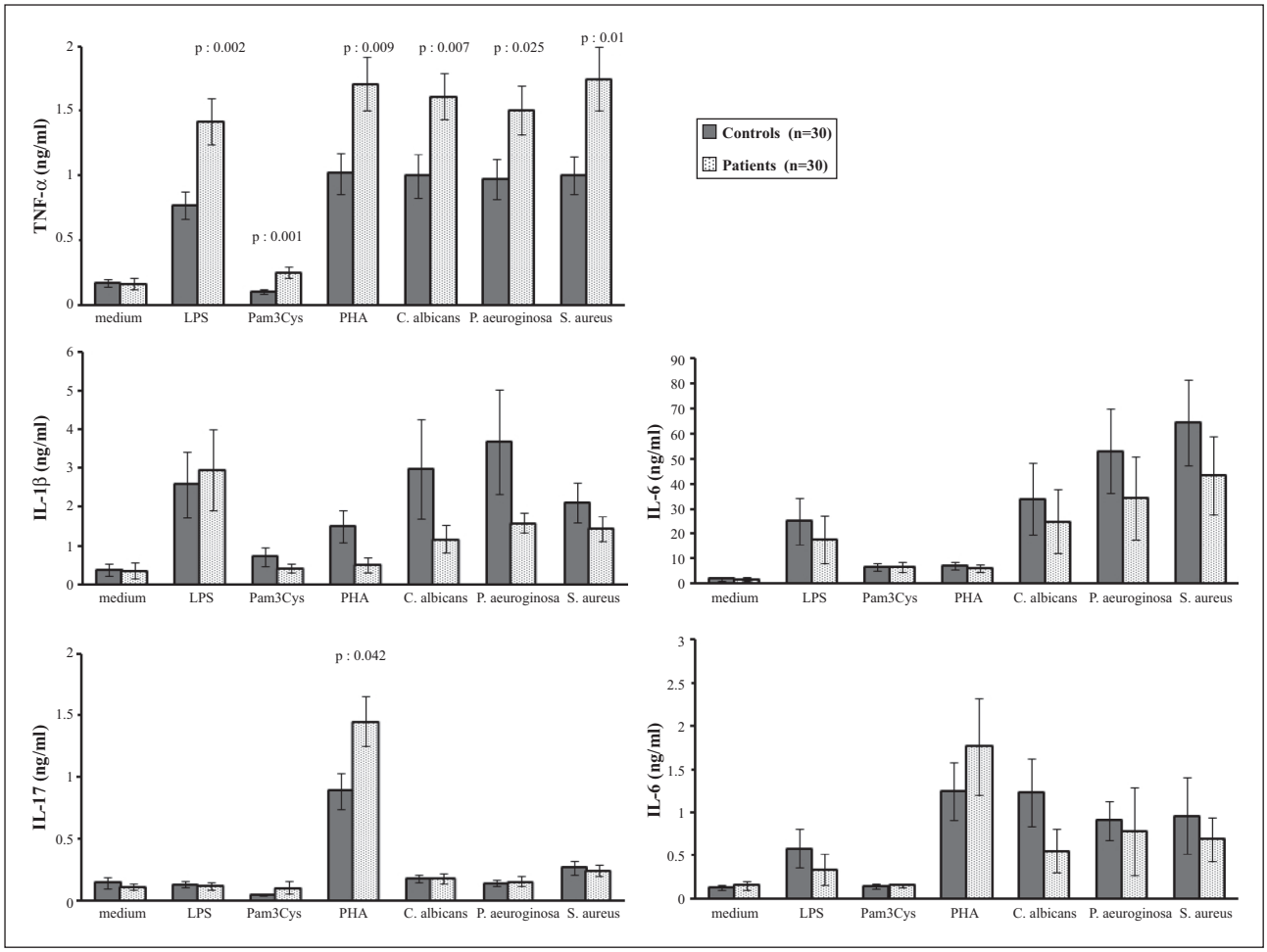

Figure 1
Cytokine production from circulating monocytes: PBMCs from 30 healthy controls (gray bars) and 30 psoriatic patients (shaded bars) were isolated and stimulated with purified bacterial endotoxin LPS, Pam3Cys, PHA and heat-killed isolates of *C. albicans*, *P. aeruginosa* and *S. aureus*. Production of the proinflammatory cytokines IL-1β, IL-6, L-17,IL-22 and TNF-α was measured in supernatants. P-values indicate statistically significant differences between patients and controls.

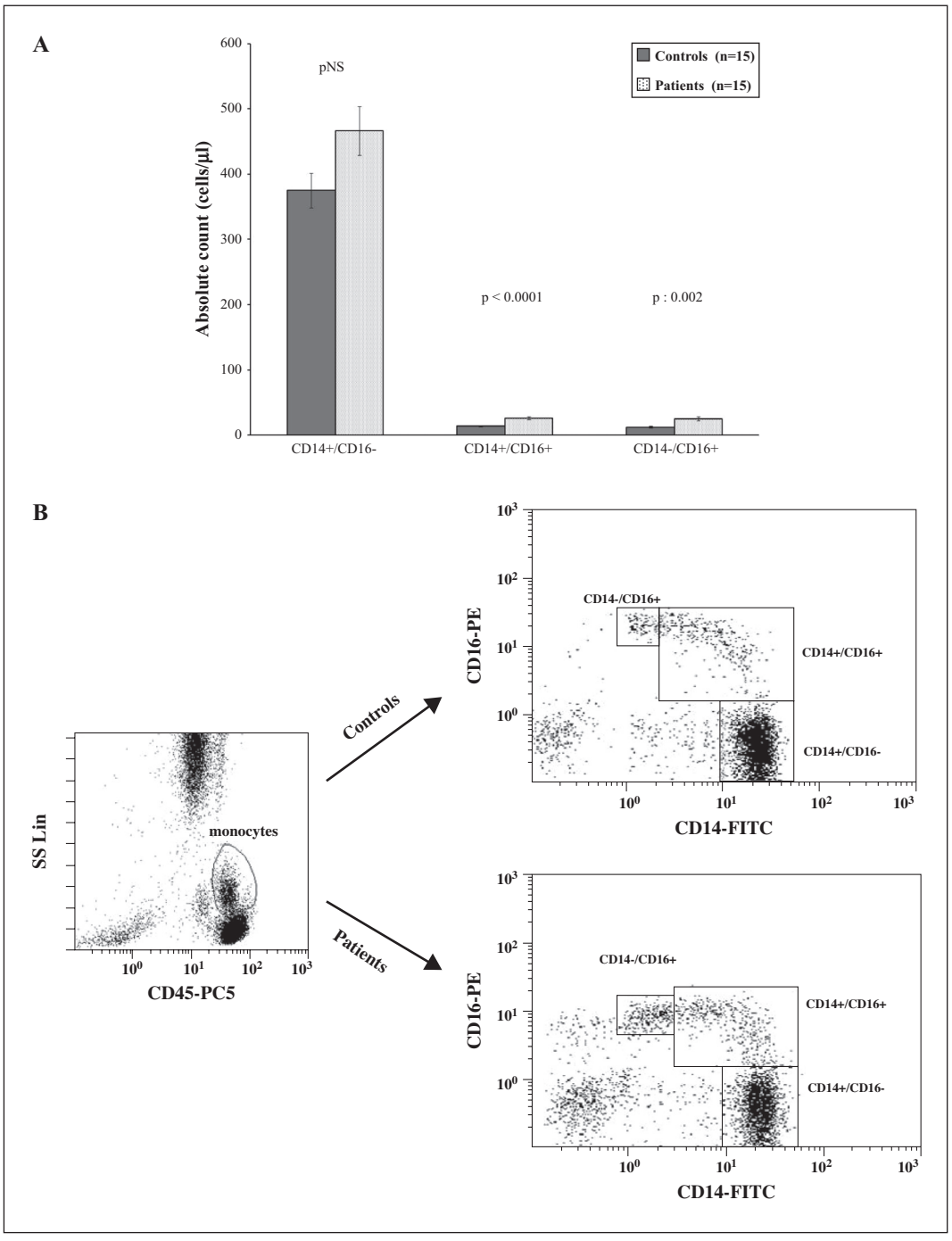

Figure 2

A) Subpopulations of monocytes in psoriatic patients and controls. Absolute counts of inflammatory and patrolling monocytes in 15 psoriatic patients and 15 healthy volunteers were performed using flow cytometry. A statistically significant difference was observed between patients and controls for inflammatory $CD14^+/CD16^+$ and patrolling $CD14^-/CD16^+$ monocytes. **B)** FACS analysis of inflammatory and control subpopulations of monocytes in psoriatic patients and controls.

ex-vivo to tissue macrophages; then they were stimulated for TNF-α production. As illustrated in *figure 4*, macrophages from patients produced greater concentrations of TNF-α following stimulation with whole bacteria, as compared to healthy controls.

DISCUSSION

In the present study, we found that circulating mononuclear cells from patients with psoriasis are capable of producing increased amounts of TNF-α as a response to bacterial stimuli compared with healthy controls. Absolute counts of inflammatory CD14+/CD16+ and patrolling $CD14^-/CD16^+$ monocytes were found to be increased in patients compared with controls, and patrolling monocytes were found to be responsible for the increased TNF-α levels. The increased production capacity persisted even after *ex-vivo* maturation of monocytes into tissue macrophages. No significant differences were documented between patients and controls for the production of IL-1β, IL-6 and IL-22. Production of IL-17 was higher in cells isolated from patients after PHA stimulation. These results demonstrate that psoriasis may have an important

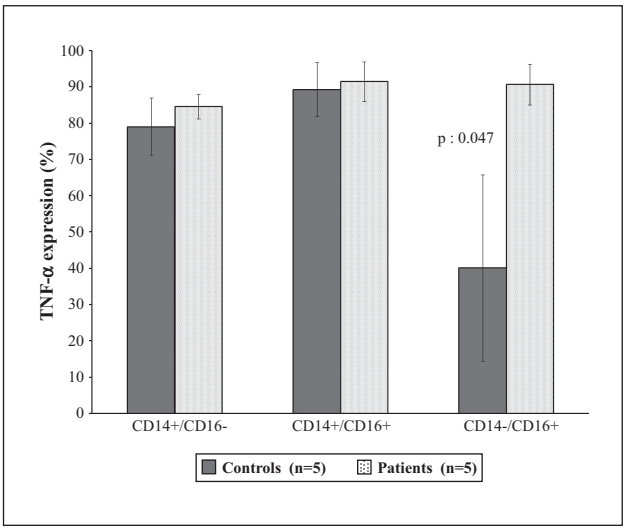

Figure 3
Intracellular determination of %TNF-α expression by inflammatory and patrolling monocytes in the presence and absence of LPS and Brefeldin A was performed using flow cytometry. No TNF-α production was observed in the absence of LPS (data not shown). Increased TNF-α expression by patrolling monocytes was observed in five psoriatic patients.

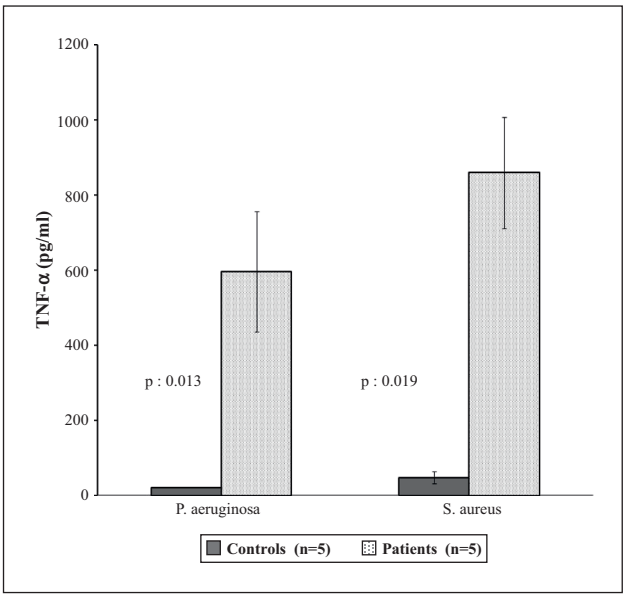

Figure 4
Peripheral blood monocytes from five psoriatic patients and their matched controls were incubated for five days in the presence of plasma to induce macrophage differentiation. TNF-α levels produced by macrophages after stimulation with *P. aeruginosa*, and *S. aureus* were determined. An increased production of τNF-α was noted in psoriatic patients after stimulation with *P. aeruginosa* and *S. aureus*.

systemic component, the pathogenesis of which is closely linked with increased TNF-α production by monocyte subpopulations.

Psoriasis is an immune-mediated disease, with a central immunopathogenic role for proinflammatory cytokines at the affected skin site [32]. Both keratinocytes and immune cells secrete various chemokines and cytokines leading to overactivation of the immune response [33]. Both the local expression patterns and the efficacy of anti-IL17 therapies suggest that Th17 cells play a crucial role in the pathogenesis of the disease [34, 35]. In the present study, we found that TNF-α in patients with psoriasis had a tendency to be monocyte- rather than Th1 cell-derived, suggesting that circulating T cells are not the only source of cytokine production. Since circulating monocytes migrate into tissues and differentiate into macrophages, it can be speculated that TNF-α overproduction by monocytes continues after differentiation into macrophages.

TNF-α is a potent inflammatory cytokine that is highly expressed in psoriatic skin and participates in all stages of psoriatic plaque development [8, 23, 24]. Its role in the pathogenesis of psoriasis is crucial and has been demonstrated by the efficacy of anti-TNF-α therapies [16-20]. Our study corroborates the systemic role of TNF-α in psoriasis. Increased levels of TNF-α produced by circulating monocytes were observed, and this overproduction appears to continue even when they differentiate into macrophages. In this context, psoriasis might be considered as a TNF-α-specific disease.

The serum cytokine profile of psoriatic patients has also been investigated [36-38]. Although concentrations of IL-1β and IL-6 were found to be increased in the serum of patients, current results do not indicate circulating monocytes as a site of overproduction of these proinflammatory cytokines. Some studies describe increased serum concentrations of IL-17 in psoriatic patients [36]. In the present study, IL-17 levels were significantly elevated in supernatants of the PBMCs from psoriatic patients when these were stimulated with PHA, a strong mitogenic factor for lymphocytes. In a study by Boniface *et al.* [39], IL-22 concentrations were described to be elevated in the serum of psoriatic patients and in culture supernatants of lesional psoriatic skin compared with healthy controls. In contrast, in PBMCs, IL-22 levels were not significantly different between patients and healthy volunteers. Our study using stimulated PBMCs corroborates these findings.

It is well known that blood monocytes and tissue macrophages are the main producers of TNF-a [40, 41]. In line with the findings of several published studies regarding serum TNF-α levels in psoriatic patients, we found elevated TNF-α levels in supernatants in the PBMCs, and increased absolute counts of patrolling $CD14^-/CD16^+$ monocytes in psoriatic patients as compared with healthy controls. In addition, there is evidence to suggest that these subpopulations of monocytes are responsible for the increased TNF-α production by monocytes. Our results provide further support to the concept that TNF-α is a major effector in the immunological response in psoriasis, and that other cells and pathways are involved in its pathogenesis.

Further studies are necessary to elucidate the complex immunological mechanisms underlying psoriasis. As our knowledge expands, new methods of assessing disease severity could develop and, more importantly, new and more effective therapeutic approaches might emerge.

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