The Majority of Epidermal T Cells in Psoriasis Vulgaris Lesions can Produce Type 1 Cytokines, Interferon-γ, Interleukin-2, and Tumor Necrosis Factor-α, Defining TC1 (Cytotoxic T Lymphocyte) and TH1 Effector Populations:[1] a Type 1 Differentiation Bias is also Measured in Circulating Blood T Cells in Psoriatic Patients

Lisa M. Austin, Maki Ozawa, Toyoko Kikuchi, Ian B. Walters, and James G. Krueger

Laboratory for Investigative Dermatology, The Rockefeller University. New York, New York, U.S.A.

Psoriasis vulgaris is a skin disease potentially mediated by pro-inflammatory cytokines produced by type 1 lesional T cells. The capability of individual T cells to produce these cytokines in lesional skin is not known. In this study we measured the ability of lesional and peripheral blood T cells to produce intracellular interferon-γ, tumor necrosis factor-α, interleukin-2, interleukin-4, and interleukin-10 proteins as detected by flow cytometric analysis. Cytokine synthesis was induced by activation with ionomycin/phorbol myristate acetate (in the presence of Brefeldin A, which inhibits the exocytosis of these cytokines). After stimulation, we found relatively high percentages of epidermal CD8 and CD4 T cells capable of producing interferon-γ, tumor necrosis factor-α, and interleukin-2, whereas few T cells, < 11%, expressed interleukin-4 or interleukin-10. Hence both $CD8^+$ and $CD4^+$ T cells are capable of type 1 effector functions (TC1 and TH1, respectively). This activation scheme was repeated on peripheral blood T cells from psoriatic patients *versus* healthy controls, where we also found a type 1 bias. In order to evaluate quantitatively the type 1 cytokine bias, we compared the frequency of type 2 interleukin-4 producing *versus* type 1 interferon-γ producing T cells in our assay and found a shift towards type 1 producing cells. This shift reveals a type 1 differentiation bias in both lesional areas and in the peripheral blood, which may indicate an imbalance within the T cell population, which is contributing to the chronic or sustained immunologic activation of T cells found in this disease. ***Key words: interleukin-4/interleukin-10/intracellular/ratio. J Invest Dermatol 113:752–759, 1999***

Psoriasis vulgaris is a human inflammatory cell-mediated autoimmune disorder (Uyemura *et al*, 1993; Gottlieb *et al*, 1994, 1995; Bata-Csorgo *et al*, 1995a). Lesional skin T cells are hypothesized to contribute pro-inflammatory/type 1 (T1) cytokines to initiate and/or maintain the cell-mediated keratinocyte hyperplasia in inflammatory lesions. Reverse transcription–polymerase chain reaction cytokine profiles of psoriatic skin reveal the potential production of many pro-inflammatory cytokines such as interleukin (IL)-1 α , IL-1 β, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, TNF-β, transforming growth factor-α and -β, and granulocyte/macrophage colony-stimulating factor (Grossman *et al*, 1989; Baker *et al*, 1991; Gomi *et al*, 1991; Uyemura *et al*, 1993; Vollmer *et al*, 1994; Olaniran *et al*, 1996) with a few reports of IL-10 and IL-5 (Olaniran *et al*, 1996) in psoriatic lesions. As the majority of studies show little IL-4, IL-5, or IL-10 message in lesional skin (Uyemura *et al*, 1993; Schlaak *et al*, 1994; Asadullah *et al*, 1998), lesional T cells may not be able to produce these regulatory cytokines. Thus indicating that specific type 2 (T2) cytokine producing cells are potentially absent from lesional skin. Although we realize that exceptions are found for IL-2, TNF-α, and IL-10, for ease of explanation in this study, we will refer to T1 cells as those that produce IFN-γ, TNF-α, and IL-2 and T2 cells as those that produce IL-4 and IL-10 for reasons which shall become apparent in this study.

In addition to studies showing the presence of T1 cytokine message in lesional skin, investigators have looked for protein expression, receptor expression, and signs of biologic activity of the primary potential immunoregulatory cytokines IFN-γ and TNF-α. Whereas IFN-γ and its receptor has been shown to be relatively scarce in the epidermis by immunohistochemistry (Livden *et al*, 1989; Nickoloff and Mitra, 1992) other cytokines such as TNF-α (Nickoloff *et al*, 1991) and the TNFR1 (Nickoloff and Mitra, 1992; Kristensen *et al*, 1993) are clearly present in the lesional epidermis. Biologic activity for both of these cytokines

Manuscript received December 18, 1998; revised July 15, 1999; accepted for publication July 22, 1999.

Reprint requests to: Dr. Lisa M. Austin, Laboratory for Investigative Dermatology, Box 178, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, U.S.A. Email: austinl@rockvax.rockefeller.edu

Abbreviations: CTL, cytotoxic T lymphocyte; T1, type 1; T2, type 2.

[1]Austin LM, Ozawa M, Kikuchi T, Krueger JG: Intracellular TNF-α, IFN-γ, and IL-2 identify TC1 and TH1 effector populations in psoriasis vulgaris plaque lymphocytes. Single-cell analysis by flow cytometry. *J Invest Dermatol* 110:649, 1998 (abstr.)

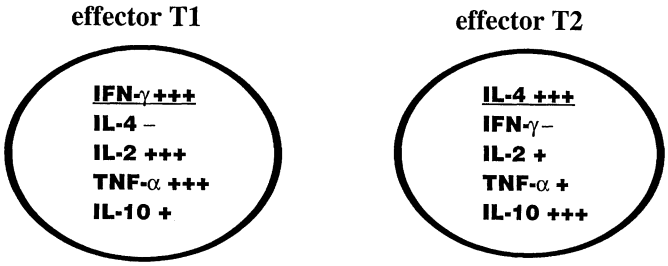

Figure 1. Cytokine expression related to differentiation status for the two primary effector types of lymphocytes. This diagram shows the rationale for cytokine comparisons shown in this study (Del Prete, 1998).

in the epidermis is suggested by the expression of ICAM-1, HLA-DR, IL-8, and IP-10 on and in lesional keratinocytes (Gottlieb *et al*, 1988; Griffiths *et al*, 1989; Nickoloff *et al*, 1989; Barker *et al*, 1990; Paukkonen *et al*, 1992) as IFN-γ and TNF-α treatment of keratinocytes *in vitro* can upregulate the expression of these molecules (Barker *et al*, 1990; Groves *et al*, 1995).

IFN-γ and TNF-α coexpression is found in differentiated T1 effector cytotoxic T lymphocyte (CTL) (Res *et al*, 1994) and differentiated CD4 T cells (Mosmann *et al*, 1986). Therefore, we outlined an identification scheme for T1 effectors (**Fig 1**) based on studies in mouse models and *in vitro* culture experiments with human cells as published by other investigators (Prussin, 1997; Del Prete, 1998). Although we did not expect to find many T2 cells, we did look for them as some message for IL-4 and IL-10 had been reported in lesions. Immature or "naïve" (T0) coexpress IFN-γ and IL-4 whereas T1 *versus* T2 effector designations were simply based on exclusionary IFN-γ or IL-4 expression, respectively.

Therefore, the goals of this study were: (i) to determine the overall capability of lesional epidermal T cells and blood T cells from psoriasis vulgaris patients to produce five cytokines: IFN-γ, IL-4, TNF-α, IL-2, and IL-10, in relation to control blood T cells, and (ii) to determine the capability of lesional epidermal CD8 and CD4 T cell subsets to produce IFN-γ, TNF-α, and IL-2. As no specific antigen has been clearly implicated in psoriasis, and as different superantigens stimulate cytokine production in specific subsets which may or may not be directly involved with psoriatic lesions, we chose a global *in vitro* stimulation procedure with ionomycin (the Ca^{2+} ionophore A23187) in conjunction with the phorbol ester 12-O-tetradecanoyl-phorbol 13-acetate (PMA). This type of stimulation procedure, in the presence of inhibitors of cytokine exocytosis, Brefeldin A or monesin, is currently being used in several laboratories to determine the capability of freshly isolated human T cells to produce intracellular cytokines. Ionomycin/PMA stimulates the production of our five cytokines whereas Brefeldin A will inhibit their exocytosis from the cell thus allowing us to measure the capability of each cell to produce these cytokines (Picker *et al*, 1995; Jason and Larned, 1997; Suni *et al*, 1998). The binding of anti-cytokine fluorochrome conjugated antibodies to permeabilized cells was analyzed by flow cytometry to reveal an overall potential for cytokine production and to provide an insight into the differentiation status and the effector function of T cells in psoriatic patients.

MATERIALS AND METHODS

Patients Eighteen adult patients with moderate-to-severe psoriasis vulgaris (14 male, four female, ages 31–69, median age 40) were enrolled in this study which was approved by the Rockefeller University Hospital Institutional Review Board. Enrollment excluded patients with guttate, erythrodermic, or pustular psoriasis. With one exception, no arthritic involvement was reported. There were two additional criteria for admission to this study. First, they were untreated for a period of at least 1 mo prior to their biopsy, and second > 10% of their body surface was covered with lesions. Control blood T cells were obtained from 10 people without a personal history of skin disease (four male, six female, ages 21–65, median age 35) plus one control blood sample from an unknown person without visible or hematologic evidence of disease, purchased from the New York Blood Center.

Biopsies At the time of blood collection, split-thickness skin biopsies were taken from lesional areas of skin and processed for flow cytometry as described (Austin *et al*, 1998). Briefly, the biopsies were rinsed twice in phosphate-buffered saline containing Gentamicin (PBS-G) at 100 μg per ml (Gibco-BRL, Gaithersburg, MD) and held at 4°C for 1 h before processing. The biopsies were rinsed again and placed in 0.5% Dispase (Sigma, St Louis, MO) for 12–15 h at 4°C, then rinsed in PBS-G and trypsinized for 10–20 min at 37°C in trypsin/ethylenediamine tetra-acetic acid 0.25 mg per ml (Clonetics, Walkersville, MD). The trypsin was neutralized in RPMI containing 5% normal human serum (C-Six Diagnostics, Mequon, WI), HEPES buffer (1 mM) (Gibco-BRL), and gentamicin (0.5 μg per ml). After disassociating the tissue, large pieces were pulled away and the cells were pelleted at approximately 500× *g*. Small, lymphocyte-sized live (Trypan blue-negative) epidermal cells were loaded on to 96-well plates at 5×10^4 to 1×10^5 cells or with blood (PBMCs or ER^+ fractions) at $1–2 \times 10^5$ cells in 100 ml of 5% normal human serum and incubated approximately 5–8 h at 37°C. Typically, 25,000–50,000 epidermal cells were analyzed with 1–8% of the cells live $CD3^+$ T cells. Although CD3 is unaffected by trypsinization, the overnight incubation is necessary for recovery of both CD4 and CD8 expression. We tested this procedure on control blood lymphocytes and found that T cells regain the original percentage of CD4 and CD8 T cells.

Cytokine staining The skin cells and peripheral T cells were stained for cytokine production (Picker *et al*, 1995) and as described in the Becton Dickinson technical bulletin (Becton Dickinson, Mansfield, MA) on the FastImmune Cytokine System, based on Brefeldin A. Brefeldin A optimally inhibits cytokine exocytosis of IL-2, IFN-γ, and TNF-α. Representative levels of IL-4 and IL-10 can also be detected with this method [PharMingen technical bulletin (PharMingen, San Diego, CA): I/C Cytokine Staining Protocol 5.19.1997]. This procedure was modified for smaller cell numbers by activating and staining cells in 96-well plates, cell numbers as described above. The cells were activated with 1 μg per ml ionomycin (Calbiochem-Novabiochem Corporation, San Diego, CA) and 25 ng PMA per ml; unactivated cells were given an equal amount of stock solution vehicle without the activation chemicals. Ten micrograms Brefeldin A per ml (Sigma) was added to both activated and unactivated samples. Our preliminary studies on lymphocyte cell lines showed that Brefeldin A alone did not induce cytokine production. After 5–7 h of activation, phenotypic surface staining was accomplished by adding the antibodies directly to the wells for 10–15 min, pelleting the cells by centrifuging at approximately 500× *g* for 3–5 min, followed by one rinse with FACs wash (PBS, Ca- and Mg-deficient, with 0.1% fetal bovine serum and 0.01% sodium azide). The cells were fixed for 10 min at room temperature in 200 μl of 1 × FACs $Lyze^{BD}$, rinsed one time with 250 μl of FACs wash. The cells were incubated in 200 μl of 1 × FACs $PERM^{BD}$ (mixed well) for 10 min at room temperature, rinsed once with 250 μl of FACs wash, resuspended in 100 μl of FACs wash, then cytokine antibodies or isotype control antibodies were added for 30 min at room temperature. Following incubation in cytokine antibodies, the cells were rinsed with 150 μl of FACs wash for the first rinse followed by one rinse with 250 μl of FACs wash. Cells were resuspended in 1% formaldehyde in FACs wash and analyzed within 12 h. The samples were analyzed with three or four color staining using a FACSCalibur Flow Cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). The percentage of cytokine staining was determined by subtracting control staining from test staining. In general, control staining levels were established by setting quadrant markers with > 98% negative staining. When more than one sample from an individual was stained for the same cytokine, sample values were averaged for that individual.

Antibodies The antibodies used in this analysis were IL-2 fluorescein isothiocyanate (FITC), IFN-γ FITC or phycoerythrin (PE), TNF-α PE, IL-4 PE with isotype controls IgG_1 FITC for the IL-2, IgG_{2a} FITC, or PE for the IFN-γ and platelet IgG_1 PE (regular IgG_1 PE with unbound PE removed) for the IL-4 and TNF-α, Simultest IFN-γ FITC/IL-4 PE, Simultest Control IgG_{2a} FITC/platelet IgG_1 PE (all from Becton Dickinson). TNF-α with anti-trinitrophenol (TNP) mouse IgG_1 isotype control from PharMingen was used in the first three of 14 experiments as TNF-α was not yet available from Becton Dickinson. IL-10 and isotype controls were from Caltag Labs (Burlingame, CA) except for two experiments using PharMingen antibodies. The cells were stained either for 3 or 4 fluorochrome analysis. CD3 PerCP is used for three-color analysis whereas CD3 APC was used for four-color analysis. The following antibodies were used for phenotypic analysis: IgG_1 FITC/PE/PerCP/APC, CD3 PerCP/APC, CD4 PE/PerCP, CD8 PE/PerCP, and CD69 FITC (Becton-Dickinson).

Data analysis Four types of comparisons were made. The first was a peripheral blood lymphocyte analysis based on two groups: control, nonpsoriatic healthy volunteers (n = 11) and patients with moderate-to-severe untreated psoriasis vulgaris (n = 14). These groups were compared with a Mann–Whitney U test. The second comparison of the T2/T1 ratios (IL-4/IFN-γ) were done between the two groups of blood lymphocytes (n = 8 each) and then psoriatic blood lymphocytes were compared with epidermal lymphocytes (n = 6 each) with the Student's t test. The third comparison of psoriatic blood lymphocytes (n = 14) to lesional epidermal lymphocytes (n = 8–11) were compared with the Mann–Whitney U test. The fourth matched comparisons were done with the Student's paired t test for CD8 *versus* CD4 comparisons. $CD8^+CD3^+$ *versus* $CD4^+CD3^+$ information is based on direct $CD8^+CD3^+$ gating with direct $CD4^+CD3^+$ gating (control blood) or $CD8^-CD3^+$ gating, from the epidermis. Significance was considered at $p \leq 0.05$ at the one-tail level.

RESULTS

Few peripheral blood T cells produce pro-inflammatory cytokines unless stimulated with ionomycin/PMA The single cell CD3 population was defined by combined gating based upon gated APC $CD3^+$ or PerCP $CD3^+$ T cells and single viable cells defined by forward scatter for cell size (FSC) *versus* side scatter for cell granularity (SSC) identification. Cytokine synthesis within individual T cells was determined by co-staining with FITC- and/or PE-conjugated anti-cytokine antibodies. **Figure 2**(***A***) shows isotype controls and **Fig 2**(***B***) the relative lack of T1 cytokine production in unstimulated T cells treated with Brefeldin A. In fact, few blood or lesional T cells produced detectable levels of IFN-γ, TNF-α, or IL-2, whereas some IL-4 and IL-10 positive cells were detected (**Table I**). As shown in **Fig 2**(***C, D***), *in vitro* stimulation with ionomycin/PMA induced IFN-γ, TNF-α, or IL-2 in a sizeable fraction of blood T cells from both controls and psoriatic patients. The majority of T cells that produced IFN-γ coexpressed high levels of TNF-α (**Fig 2*C, D,*** *arrows*), whereas many cells that produced IL-2 also produced high levels of TNF-α (not shown). The majority of cells producing IL-2 and IFN-γ were in separate populations with low percentages of double-positive cells (not shown).

Psoriatic blood T cells contain a larger population of IFN-γ and TNF-α producing cells than controls, after stimulation with ionomycin/PMA T cell populations capable of producing intracellular cytokines induced by ionomycin/PMA were evaluated in blood T cells from 14 patients with untreated psoriasis vulgaris and 11 controls. On average, these T cells showed similar expression of combined intracellular and extracellular CD69 staining (**Fig 3**). T cells from psoriatic patients contained, on average, a larger population of cells producing each cytokine with the exception of IL-4 (**Fig 3**). The inflammatory/effector cytokines IFN-γ (23% *vs* 12%, p = 0.03) and TNF-α (50% *vs* 36%, p = 0.048) were found in significantly higher frequencies within the *psoriatic* T cell populations. Although IL-2 expression was usually higher for the patient T cells, these approached statistical significance (p = 0.09). The percentages of $IL\text{-}4^+$ T cells were similar between psoriatic and control blood, although the percentages of $IL\text{-}10^+$ T cells appeared slightly higher, there was no significant difference between the values.

IL-4 and IFN-γ ratios indicate a shift towards T1 differentiation in psoriatic blood T cells One way to compare the balance of T1 *versus* T2 T cell differentiation is to calculate the ratio between IL-4 and IFN-γ producing cells (IL-4/IFN-γ ratio) (Barna *et al*, 1994). As discrete populations of T cells synthesize only IL-4 or IFN-γ (**Fig 4*A***), we calculated these ratios for peripheral blood T cells (**Fig 4*B***). The mean of ratios in psoriatics was 0.15, significantly lower than the mean of 0.45 calculated for control T cells (p = 0.007), indicating a definite shift of circulating T cells towards T1 cytokine production.

Analysis of cytokine production by T cells isolated from psoriatic lesions, after stimulation with ionomycin/PMA With the stimulation and analysis methods used for peripheral blood, total epidermal cell suspensions from psoriatic lesions were analyzed for cytokine expression. T cells were identified as described above for blood samples by combined gating on both viable cells (SSC *versus* FSC) and $CD3^+$ cells (CD3 *versus* SSC) as shown in **Fig 5**(***A***). Isotype control levels for these $CD3^+$ lymphocytes were established for each anti-cytokine antibody (not shown) and with unactivated cells as demonstrated for IFN-γ and TNF-α (**Fig 5*B***), IL-2 (**Fig 5*C***), IL-4 (**Fig 5*D***), and IL-10 (**Fig 5*E***). Please note that only $CD3^+$ T cells are plotted in **Fig 5**(***B–E***). The overall percentages of cytokine production were determined for psoriatic blood $CD3^+$ T cells and intraepidermal $CD3^+$ T cells isolated from untreated psoriatic lesions (n = 11) (**Fig 6*A***). The major cytokines produced

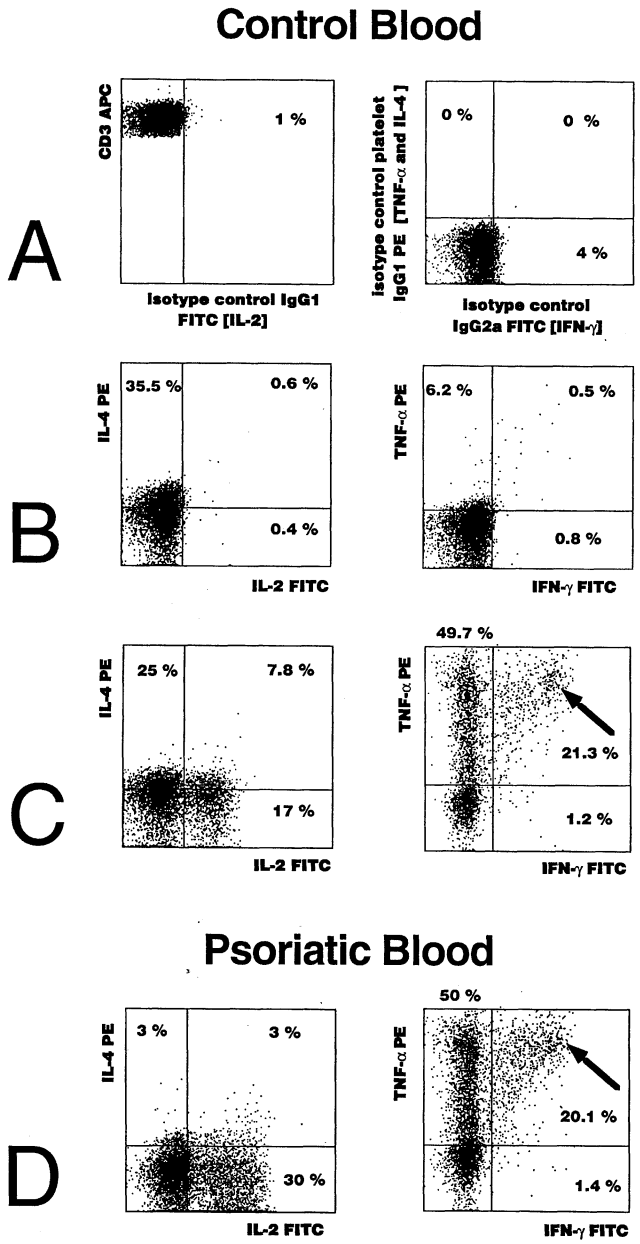

Figure 2. *In vitro* ionomycin/PMA stimulation of T cells from both control and psoriatic blood T cells primarily induces IFN-γ, TNF-α, and IL-2. (*A*) Isotype control staining of stimulated control blood T cells. (*B*) Intracellular cytokine staining (in the presence of Brefeldin A), of freshly isolated normal control blood T cells (*C*) as compared with stimulated control blood T cells stained with the same anti-cytokine antibodies and (*D*) intracellular cytokine staining of stimulated psoriatic patient blood T cells. Isotype controls shown are typical for unactivated and activated T cells. The *arrow* points to the majority of $IFN\text{-}\gamma^+$ T cells which are in the $TNF\text{-}\alpha^{hi}$ region for both psoriatic and control blood.

Table I. Little T1 intracellular cytokine expression is detected in unactivated cells incubated in Brefeldin A and appropriate concentrations of vehicle but without ionomycin and PMA[a]

Cytokine	Normal control blood	Psoriatic patient blood	Patient epidermis
IL-2	0.7% ± 0.5 (5)	0.3% ± 0.3 (14)	0.7% ± 0.5 (5)
IFN-γ	0.9% ± 0.7 (5)	0.4% ± 0.2 (14)	0.9% ± 0.7 (5)
TNF-α	1.5% ± 1.3 (5)	4.2% ± 4.0 (14)	1.5% ± 1.3 (5)
IL-4	2.3% ± 2.0 (3)	2.1% ± 1.0 (10)	5.1% ± 2.0 (3)
IL-10	18.9% ± 9.3 (4)	15.0% ± 5.5 (12)	15.0% ± 9.3 (4)

[a]Mean ± SEM (n).

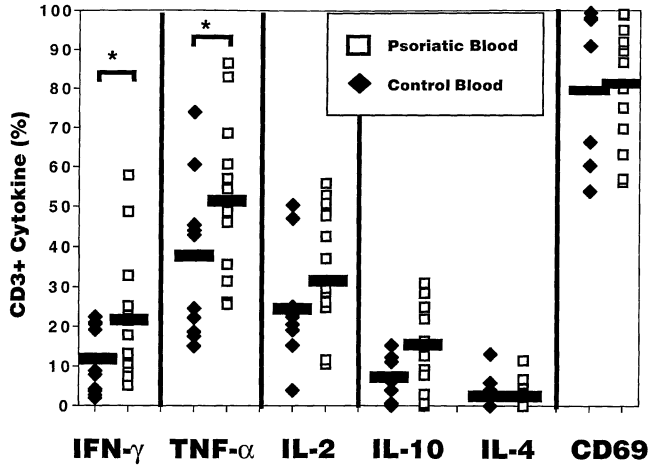

Figure 3. Psoriatic patients' peripheral blood T cells contain larger populations of T1 cytokine producing cells. Cytokine expression in activated peripheral blood cells between control (n = 8–11) and psoriatic patients (n = 14). The psoriatic patients T cells have larger populations producing IFN-γ (p = 0.03) and TNF-α (p = 0.048). The percentage of $CD69^+$ T cells demonstrates the similar number of permeabilized T cells expressing CD69 after stimulation with ionomycin/PMA in the presence of Brefeldin A.

by intraepidermal T cells were TNF-α, IL-2, and IFN-γ with some T cells producing IL-4 and very few cells producing IL-10. Population means for the number of cells producing IFN-γ and TNF-α were marginally higher for intraepidermal cells compared with blood cells in the same group of patients whereas total numbers of $CD69^+$ T cells were similar between these groups. IL-4/IFN-γ ratios also demonstrate a T1 shift in the epidermal T cells at 0.18, which is comparable with the ratio of 0.13 of matched patient blood values, n = 6 (**Fig 6*B***). Overall, intraepidermal T cells show an even greater bias towards T1 cytokine production than in psoriatic blood with the slightly larger IFN-γ and TNF-α populations and fewer IL-10 producing cells (13% *vs* 4%).

Comparison of potential for cytokine production between $CD4^+$ and $CD8^+$ T cell subsets in psoriatic lesions Using four-color cytometric analysis, with APC CD3 to identify T cells with PerCP CD4 or PerCP CD8 to subset these $CD3^+$ cells, we studied the capability for ionomycin/PMA to induce cytokines within these subsets from psoriatic skin lesions (n = 3), psoriatic blood (n = 8), and control blood (n = 11, not shown). The paired t test for CD8/CD4 comparisons was used for information from the same individual (**Fig 7**). We found that in both control blood (16% *vs* 11%, p = 0.001, not shown) and psoriatic blood (11% *vs* 5%, p = 0.01), more $CD8^+$ T cells than $CD4^+$ T cells expressed IFN-γ. This bias was previously reported by others for control blood (North *et al*, 1996; Jason and Larned, 1997). We also found that a much larger population of lesional intraepidermal $CD8^+$ T cells produced IFN-γ than CD4 (53% *vs* 23%, p = 0.03). Comparatively, we found the population of intraepidermal CD8

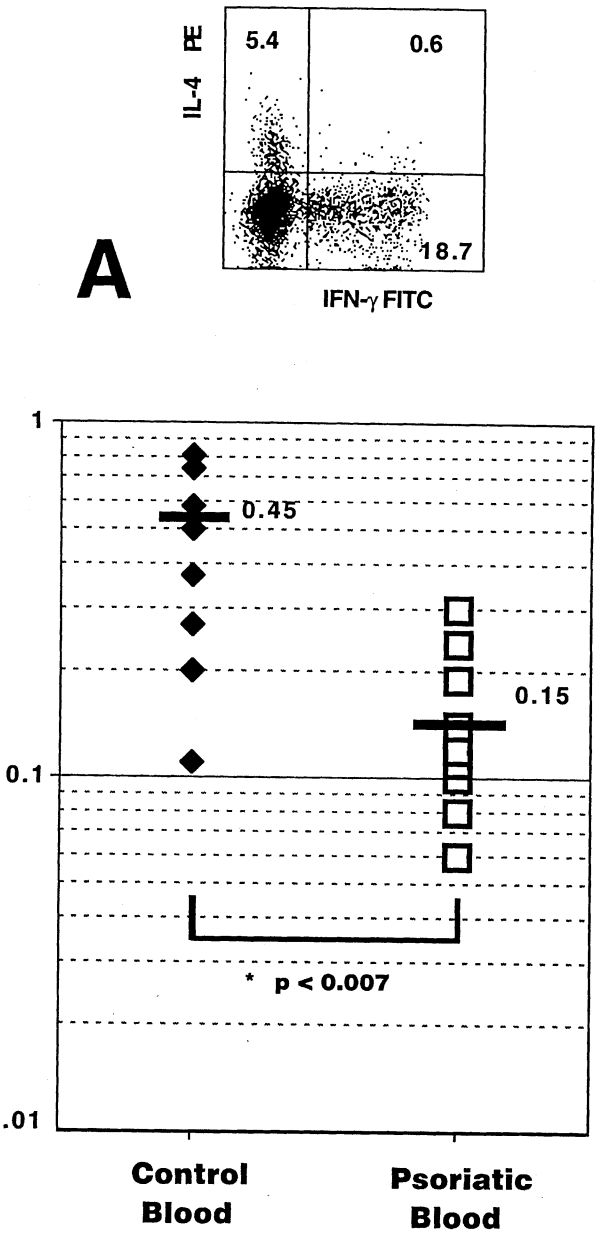

Figure 4. Patients' T cells demonstrate a T1 differentiation shift with lower IL-4/IFN-γ ratios. (*A*) IFN-γ *versus* IL-4 populations in peripheral blood T cells of control volunteers and patients, a patient sample is shown and (*B*) the psoriatic blood IL-4/IFN-γ ratio (n = 14) is lower than the ratio found in normal blood (n = 8) (p = 0.007).

producing IFN-γ was much greater than in psoriatic blood or control blood at 53% *vs* 11% *vs* 16% (respectively). Psoriatic blood CD8 and CD4 contained similarly sized $TNF\text{-}\alpha^+$ populations (35% *vs* 37%) in contrast to control blood in which more CD8 make TNF-α than CD4 (34% *vs* 19%, p = 0.001). High percentages of both $CD8^+$ and $CD4^+$ T cells from the epidermis produced TNF-α (73% *vs* 59%, marginal significance at p = 0.07). Again, the overall percentage of these TNF-α producing cells was higher in lesional epidermis than in blood, 73% *vs* 35% for CD8, and 59% *vs* 37% for CD4. As expected, more $CD4^+$ T cells in psoriatic blood produced IL-2 than $CD8^+$ T cells (23% *vs* 15%, p = 0.04), which we also found in control blood (23% *vs* 16%, p = 0.02) (Prussin and Metcalfe, 1995; Jason and Larned, 1997). It was surprising that more $CD8^+$ T cells in psoriatic lesions produced IL-2 compared with intraepidermal $CD4^+$ T cells (57% *vs* 45%, marginal significance at p = 0.06) as ionomycin/PMA stimulation

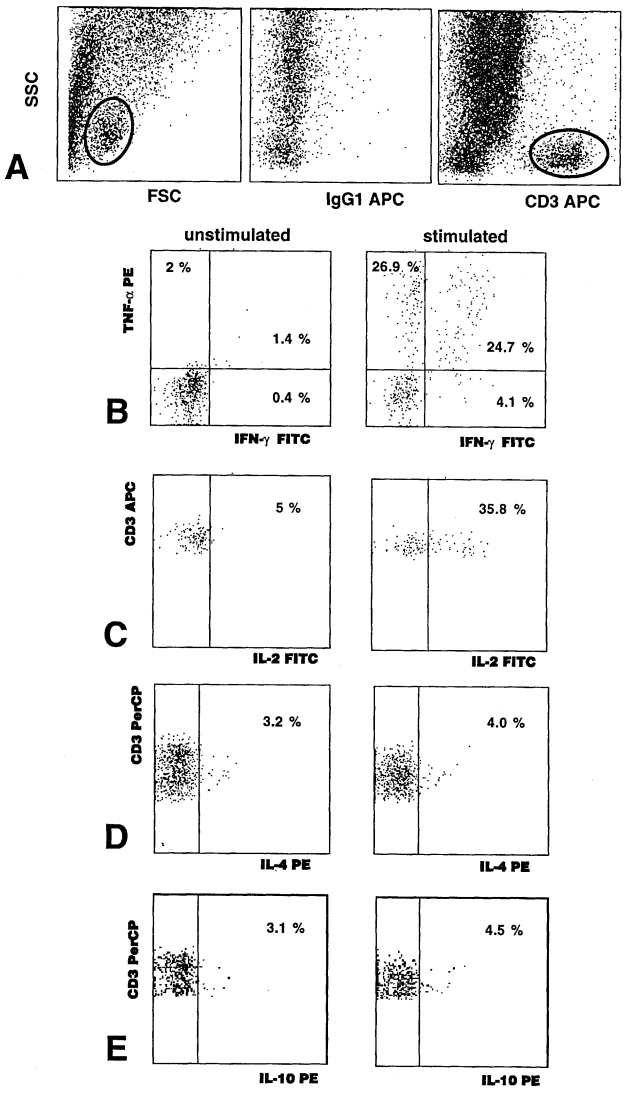

Figure 5. Lesional epidermal CD3$^+$ T cells express T1 cytokines when stimulated *in vitro*. (*A*) representative dot plots of epidermal cells which show FSC (size) *versus* SSC (granularity) information in addition to CD3$^+$ staining compared with its isotype control. For the following dot plots of CD3$^+$ T cells, the first column shows unactivated epidermal T cells whereas the second column shows the *in vitro* activated epidermal T cells (*B*) IFN-γ and TNF-α, (*C*) IL-2, (*D*) IL-4, and (*E*) IL-10.

of control blood T cells induces statistically significant greater numbers of CD4 T cells producing intracellular IL-2.

DISCUSSION

Autoimmune disorders and inflammatory reactions are currently segregated into cell-mediated T1 or T2 categories (Mosmann and Sad, 1996). These designations are primarily based on IFN-γ$^+$ *versus* IL-4$^+$ amounts, respectively, as measured directly from tissue or from tissue-derived clones for organ-specific disorders such as rheumatoid arthritis (RA) (T1), multiple sclerosis (T1), and Grave's disease (T2). T2 designations can be confusing as there are two ways in which T2 cells function. First, T2 cells are shown to function *in vitro* to influence antibody responses. Secondly, they are implicated but not proven to downregulate T1 responses (D'Elios and Del Prete, 1998). Inflammatory skin diseases have also been assigned effector type designations which are clearly aligned with the detection of intracellular cytokine staining patterns. T1 cytokine production is found in cell-mediated inflammation as shown with delayed-type hypersensitivity responses and in tuberculoid leprosy, whereas T2 cytokines are found in atopic dermatitis and lepromatous leprosy. Within individual T cell subsets, both CD4 (H) and CTL (C) contain T1 and T2 subsets designated TH1 and TH2 (Mosmann *et al*, 1986), TC1 and TC2 (Salgame *et al*, 1991), respectively.

Because psoriasis vulgaris lesions have shown a strong T1 bias based on cytokine message, researchers sought to show that lesional T cells produce these cytokines. And indeed, indirect evidence for T1 cytokine producing cells in lesional skin was obtained from T cell lines and clonal cell lines grown nonspecifically from psoriatic skin biopsies. These lines were primarily CD4$^+$ with some CD8$^+$ lines, many secreting IFN-γ and TNF-α (Michel *et al*, 1987; Barna *et al*, 1994; Prinz *et al*, 1994; Vollmer *et al*, 1994; Bata-Csorgo *et al*, 1995b; Jones *et al*, 1996) as measured either by reverse transcription–polymerase chain reaction of the cells, or by enzyme-linked immunosorbent assay from culture medium. To avoid bias of results from clonal selection (Kelso and Groves, 1997), however, it is more accurate to analyze T cells in or from psoriatic lesions before clonal expansion. Recently, ≈16% of T cells isolated directly from psoriatic lesional epidermis were found to be capable of producing IFN-γ after ionomycin/PMA stimulation (Szabo *et al*, 1998).

With the *in vitro* activation and intracellular cytokine staining reported here, we found numerous lesional epidermal and blood T cells with the capability to express T1 cytokines IFN-γ, TNF-α, and IL-2 whereas few cells express the T2 associated cytokines IL-4 or IL-10. This type of cytokine profile parallels the relative kinds of cytokine expression by bulk mRNA measures as published by these other investigators. Furthermore, we found it very interesting that a T1 differentiation bias could be detected in peripheral blood T cells in these psoriatic patients when compared with healthy controls. This finding is analogous to the recent report

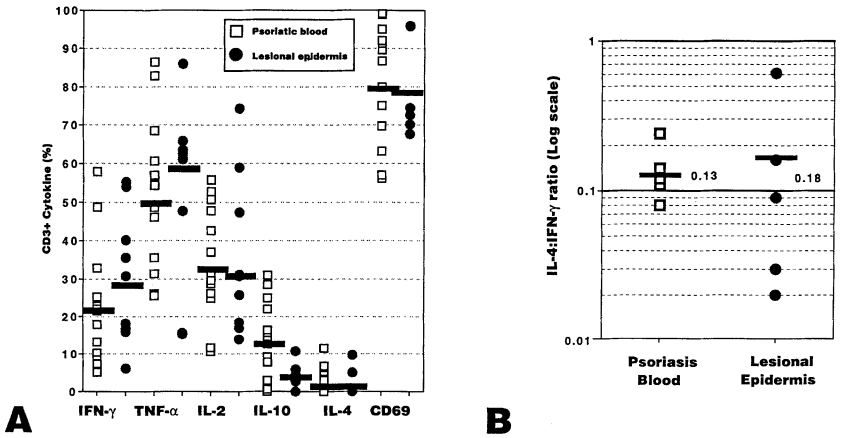

Figure 6. The size of populations expressing each cytokine is similar between the epidermis and blood. (*A*) The average size of the epidermal lesional population producing each cytokine was statistically similar (with marginal significances in some cases) to the peripheral blood population (*B*) IL-4:IFN-γ ratios were determined and also found to be similar for blood and epidermal T cells for each patient (n = 6 for patient matched samples). Although the majority of lesional epidermal T cells express CD69 on variable numbers of cells prior to ionomycin/PMA stimulation, these values represent the total CD69 expression of permeabilized T cells showing similar percentages between blood and skin T cells after ionomycin/PMA treatment in the presence of Brefeldin A.

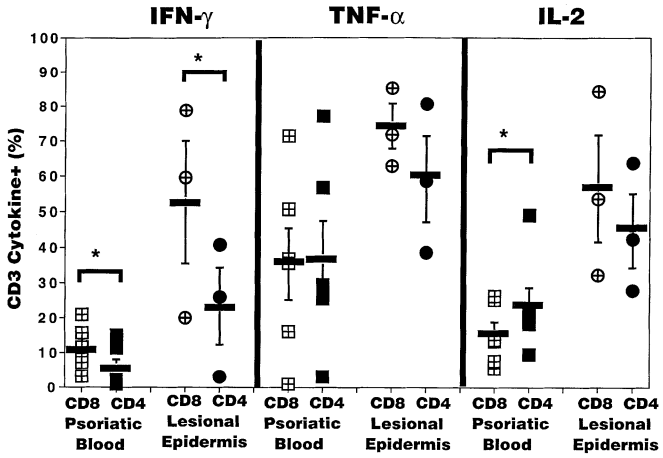

Figure 7. Both $CD8^+$ and $CD4^+$ peripheral blood cells and epidermal cells from psoriatic patients can produce IFN-γ, TNF-α, and IL-2 after stimulation with ionomycin/PMA. (*A*) IFN-γ expression is consistently higher in the $CD8^+$ population when compared with the $CD4^+$ T cells in the epidermis ($p = 0.03$), with a similar finding in psoriatic blood ($p = 0.01$) and normal blood ($p = 0.001$, $n = 11$, not shown). (*B*) TNF-α expressing populations, whereas marginally significant ($p = 0.07$) between the subsets, are greater in the epidermal CD8 population than in the patients' peripheral blood ($p = 0.05$). And, whereas there is still no difference between the subsets of TNF-α expressing cells in the psoriatic blood, in normal blood significantly more $CD8^+$ $CD3^+$ cells were expressing TNF-α than $CD4^+$ $CD3^+$ cells ($p = 0.001$, $n = 11$, not shown). (*C*) Lesional $CD8^+$ $CD3^+$ populations were marginally larger than $CD4^+$ $CD3^+$ cells ($p = 0.06$) and were larger than IL-2^+ $CD8^+$ $CD3^+$ populations in patient's peripheral blood populations ($p = 0.04$) whereas in both sources of peripheral blood cells, psoriatic and normal, more $CD4^+$ T cells express IL-2 than CD8 T cells ($p = 0.04$ and $p = 0.02$, respectively). Cells were gated for $CD8^+CD3^+$ whereas the $CD4^+CD3^+$ information is based on both direct $CD4^+CD3^+$ gating (normal blood and two each of the psoriatic epidermal and psoriatic blood samples) and indirect $CD8^-CD3^+$ gating, depending on sample availability (normal volunteers $n = 11$, psoriatic blood $n = 6$, psoriatic epidermis $n = 3$).

of a T2 bias found in peripheral blood T cells of patients with atopic dermatitis using a similar type of ionomycin/PMA stimulation with flow cytometric analysis (Sato *et al*, 1998). This T2 bias was found in both CD4 and CD8 blood T cells, although atopic dermatitis is mainly considered a CD4-mediated skin disorder. Although ionomycin/PMA bypasses antigen-specific activation pathways, and some cells which may not have been actively producing cytokines may become capable of doing so, the specificity for the cytokine type is maintained. As such, this method is useful in defining cytokine producing potential (as indicating the overall extent of differentiation within the population) rather than the ongoing production of cytokines by the individual cells in the inflamed tissue or in blood. Such capability would be better measured by intracellular staining of T cells after antigen-specific stimulation (Picker *et al*, 1995; Suni *et al*, 1998) as we found that the majority of lesional and blood T cells did not produce measurable amounts of intracellular pro-inflammatory cytokines without *in vitro* stimulation.

We were able to quantitate a T1 cytokine differentiation bias in blood and lesional T cells with the use of IL-4/IFN-γ ratios. The IL-4/IFN-γ ratios of the control blood T cells were significantly different than those from psoriatic blood T cells and emphasized the shift towards bulk T cell T1 cytokine production. And, although, overall, fewer circulating blood T cells than epidermal T cells expressed cytokines after ionomycin/PMA treatment, the expression pattern within the populations was very similar to that of T cells from the epidermis. Many investigators have used IFN-γ *versus* IL-4 comparisons and ratios to measure the extent of differentiation within T cell populations in disease conditions, between T cell lines from different types of diseases and from normal individuals (Sunder-Plassmann *et al*, 1996; Guo *et al*, 1997; Roura-Mir *et al*, 1997; Schoendorf *et al*, 1997) or between clonal lines induced to differentiate along either a T1 or T2 pathway (Sunder-Plassmann *et al*, 1996). Similar measures of T1 differentiation shifts were detected with intracellular staining, after *in vitro* stimulation procedures, in T cells of other diseases such as RA (Dolhain *et al*, 1996) Lyme arthritis (Gross *et al*, 1998), and Behçet's disease (Sugi-Ikai *et al*, 1998). In contrast, T2 shifts are observed as expected in atopic dermatoses (Wierenga *et al*, 1990; Ferry *et al*, 1997; Nakazawa *et al*, 1997, 1998). In a comparison with published information on RA synovial fluid cells, psoriatic epidermal T cells contained larger numbers of differentiated T cells with a stronger T1 bias than T cells in RA synovial fluid. Unlike RA synovial fluid, we found few lesional epidermal cells expressing IL-4 or IL-10, and virtually no T0 cells that coexpress IL-4 and IFN-γ (Morita *et al*, 1998). Our results also suggest that T1 *versus* T2 T cell frequencies can be altered when growing lymphocytes from psoriatic lesions (Barna *et al*, 1994).

As expected, based on our earlier study where we showed that a major portion of epidermal T cells were activated ($CD25^+$ GMP-17^+) CTL (Austin *et al*, 1998) the majority of CD8 T cells can produce IFN-γ and TNF-α as do many resident CD4 T cells. Therefore, any T1 cytokine-mediated effector functions in lesional epidermis would be contributed to by both CTL and CD4 T cells. As there usually are more CTL (>60%) in lesional epidermis than CD4 (<40%), a phenomenon similar to synovial fluid in RA (Morita *et al*, 1998), the CTL contribution towards the cytokine contribution could be significant. Some of the epidermal T cells in our assay did not produce measurable amounts of cytokine. Many of these cells were $CD4^+$ T cells. There are several explanations for this including that these cells may not produce cytokine at levels high enough for cytometric detection due to their stage in the cell cycle, i.e., their effector contribution towards the lesional pathophysiology is finished in these established lesions. Alternatively they may represent nonfunctioning suppressor-like cells (Croft *et al*, 1994). The epidermal CD8 enrichment is unlike the dermis which has the more typical CD4 (>60%)/CD8 (<40%) ratio as found in blood, and thus epidermal effector cells may provide a unique contribution to the psoriatic phenotype.

There is continuing controversy over the potential function of the CD4 and CD8 lesional T cells in psoriasis. For example, it has been suggested that CD4 T cells are pathogenic effectors whereas CD8 T cells function as suppressors (Bos and Rie, 1999). The predominance of T1 cytokine producing capability for both the CD4 and CD8 subsets of epidermal T cells, suggests a role for both T cell subsets as T1 effectors.

It is now evident that enhanced CTL effector function is induced by CD4 T cells which can be mediated through the dendritic cells (Bhardwaj *et al*, 1994). Thus preactivated CD4 T cells provide signals necessary for the "preconditioning" of the dendritic cells, which in turn enhance CTL effector functions (Bennett *et al*, 1998; Ridge *et al*, 1998; Schoenberger *et al*, 1998). Therefore, the CD4 T cell's primary role in plaque formation may be to boost CTL cytokine effector function, as implied by the results in this study. In addition, the potentially low numbers of psoriatic lesional T cells capable of expressing IL-10 after ionomycin/PMA treatment could allow the persistence of the psoriatic lesions (Abdullah *et al*, 1989) as mediated through antigen-presenting cells (Nestle *et al*, 1994; Mitra *et al*, 1995). Differentiation towards T1 T cells is influenced by the presence of bioactive IL-12 (Croft *et al*, 1994) of which the bioactive form was recently found in the lesions of psoriatic plaques (Yawalkar *et al*, 1998).

This study defines the cytokine potential of intralesional $CD8^+$ T cells as pro-inflammatory with their ability to produce IFN-γ and TNF-α. A similar CTL effector cell has been described (Guidotti *et al*, 1996), which had the ability to produce effector cytokines without inflicting large-scale cellular death. This may be the type of effector we find in psoriatic lesions. The exact nature of the CTL contribution to the psoriatic phenotype, however, is unknown. A cytolytic granule associated protein associated with CTL activation, GMP-17, is found *in vivo* in psoriatic lesional

skin (Austin *et al*, 1998). We do not yet, however, know if these activated CTL are actually producing and secreting pro-inflammatory cytokines within the lesion. Of the three T1 cytokines we assayed, IFN-γ and IL-2 are the two cytokines which could directly contribute to the induction of and worsening of the plaques. Subcutaneous injections of IFN-γ have been shown to induce lesions in nonlesional areas of psoriatic patient's skin and worsen pre-existing psoriatic skin plaques (Fierlbeck *et al*, 1990). If IL-2 is expressed in the lesion, it may function to support and maintain lesional T cell activation via the numerous $CD25^+$ T cells found within the lesions (Krueger *et al*, 1995) as functional studies indicate in a mouse model for diabetes (von Herrath *et al*, 1995). Therefore, as the majority of intraepidermal lymphocytes in many psoriatic patients are phenotypically acivated effector CTL ($GMP\text{-}17^+CD8^+CD3^+$) (Austin *et al*, 1998) and intraepidermal CTL have the ability to produce effector cytokines (IFN-γ and TNF-α), these CTL are the primary candidate effector cells for cytokine mediation in this disease.

This research was supported in part by a Postdoctoral Fellowship from the Irvington Institute for Immunological Research; by a General Clinical Research Center Grant (M01-RR00102) from the National Center for Research Resources at the National Institutes of Health; by NIH grants CA54215 and AI39214, by grants or gifts from the Carl J. Herzog Foundation, the American Skin Association, the Carson Family Charitable Trust and Seragen, Inc., Dr James Murphy, and Jean Stein. The authors thank Dr Todd R. Coven, Pat Gilleaudeau, RN, BSN, and Dr Lauren Burack for assistance with this study. A special thank you for Dr Ann Ho, RU Laboratory on the Biology of Addictive Diseases, for assistance with the statistical analysis.

REFERENCES

Abdullah NA, Torres BA, Basu M, Johnson HM: Differential effects epidermal growth factor, transforming growth factor-alpha, and vaccinia virus growth factor in the positive regulation interferon-gamma production. *J Immunol* 143:113–117, 1989

Asadullah K, Sterry W, Stephanek K, *et al*: IL-10 is a key cytokine in psoriasis. *J Clin Invest* 101:783–794, 1998

Austin LM, Coven TR, Bhardwaj N, Steinman R, Krueger JG: Intraepidermal lymphocytes in psoriatic lesions are activated GMP-17 (TIA-1) +CD8+CD3+ CTLs as determined by phenotypic analysis. *J Cutan Pathol* 25 (2):79–88, 1998

Baker JN, Jones ML, Mitra RS, *et al*: Modulation keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. *Am J Pathol* 139:869–876, 1991

Barker JNWN, Sarma V, Mitra RS, Dixit VM, Nickoloff BJ: Marked synergism between tumor necrosis factor-α and interferon-γ in regulation keratinocyte-derived adhesion molecules and chemotactic factors. *J Clin Invest* 85:605–608, 1990

Barna M, Snijdewint FGM, van der Heijden FL, Bos JD, Kapsenberg ML: Characterization lesional psoriatic skin T lymphocyte clones. *Acta Dermatol Venereol (Stockh) Suppl.* 186:9–11, 1994

Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD: Intralesional T-lymphocyte activation as a mediator psoriatic epidermal hyperplasia. *J Invest Dermatol* 105:89S–94S, 1995a

Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD: Kinetics and regulation human keratinocyte stem cell growth in short-term primary ex vivo culture. *J Clin Invest* 95:317–327, 1995b

Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR: Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393:478–480, 1998

Bhardwaj N, Bender A, Gonzalez N, Bui LK, Garrett MC, Steinman RM: Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J Clin Invest* 94:797–807, 1994

Bos JD, Rie MAD: The pathogenesis of psoriasis: Immunology and speculations. *Immnol Today* 20 (1):40–46, 1999

Croft M, Carter L, Swain SL, Dutton RW: Generation polarized antigen-specific CD8 effector populations: Reciprocal action interleukin (IL) −4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J Exp Med* 180:1715–1728, 1994

D'Elios M, Del Prete G: Th1/Th2 balance in human disease. *Transplant Proc* 30: 2373–2377, 1998

Del Prete G: The concept type-1 and type-2 helper T cells and their cytokines in humans. *Int Rev Immunol* 16 (3–4):427–455, 1998

Dolhain RJEM, van der Heiden AN, ter Haar NT, Breedveld FC, Miltenburg AMM: Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints patients with rheumatoid arthritis. *Arthritis Rheum* 39:1961–1969, 1996

Ferry B, Antrobus P, Huzicka I, Farrell A, Lane A, Chapel H: Intracellular cytokine expression in whole blood preparations from normals and patients with atopic dermatitis. *Clin Exp Immunol* 110:410–417, 1997

Fierlbeck G, Rassner G, Muller C: Psoriasis induced at the injection site recombinant interferon gamma. Results immunohistologic Invests. *Arch Dermatol* 126: 351–355, 1990

Gomi T, Shiohara T, Munakata T, Imanishi K, Nagashima M: Interleukin 1alpha, Tumor necrosis factor alpha and interferon gamma in psoriasis. *Arch Dermatol* 127:827–830, 1991

Gottlieb AB, Lifshitz B, Fu SM, Staiano-Coico L, Wang CY, Carter DM: Expression of HLA-DR molecules by keratinocytes and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. *J Exp Med* 164:1013–1028, 1986

Gottlieb AB, Luster AD, Posnett DN, Carter DM: Detection of a gamma interferon-induced protein (IP-10) in psoriatic plaques. *J Invest Dermatol* 90:565, 1988

Gottlieb S, Johnson R, Gilleaudeau P, Woodworth T, Nylen P, Gottlieb AB, Krueger JG: A specific lymphocyte toxin (DAB389IL-2) reverses epidermal keratinocyte activation in psoriasis: improvement correlates with elimination intraepidermal CD8+ lymphocytes. *J Invest Dermatol* 102:531, 1994

Gottlieb SL, Gilleaudeau P, Johnson R, Estes L, Woodworth TG, Gottlieb AB, Krueger JG: Response psoriasis to a lymphocyte-selective toxin (DAB_{389}IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nature Med* 1:442–447, 1995

Griffiths CEM, Voorhees JJ, Nickoloff BJ: Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: Modulation by recombinant gamma interferon and tumor necrosis factor. *J Am Acad Dermatol* 20:617–629, 1989

Gross DM, Steere AC, Huber BT: T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis. *J Immunol* 160:1022–1028, 1998

Grossman RM, Krueger J, Yourish D, *et al*: Interleukin-6 is expressed in high levels in psoriatic skin and stimulates proliferation cultured human keratinocytes. *Proc Natl Acad Sci USA* 86:6367–6371, 1989

Groves RW, Allen MH, Ross EL, Barker JNWN, MacDonald DM: Tumour necrosis factor alpha is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression. *Br J Dermatol* 132:345–352, 1995

Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV: Intracellular inactivation the hepatitis B virus by cytotoxic T lymphocytes. *Immunity* 4:25–36, 1996

Guo J, Rapoport B, McLachlan SM: Cytokine profiles in vivo activated thyroid-infiltrating T cells cloned in the presence or absence interleukin 4. *Autoimmunity* 26:103–110, 1997

von Herrath MG, Allison J, Miller JFAP, Oldstone MBA: Focal expression interleukin-2 does not break unresponsiveness to 'self' (viral) antigen expressed in β cells but enhances development autoimmune disease (diabetes) after initiation an anti-self immune response. *J Clin Invest* 95:477–485, 1995

Jason J, Larned J: Single-cell cytokine profiles in normal humans: comparison flow cytometric reagents and stimulation protocols. *J Immunol Methods* 207:13–22, 1997

Jones CD, Guckian M, El-Ghorr AA, Gibbs NK, Norval M: Effects phototherapy on the production cytokines by peripheral blood mononuclear cells and on systemic antibody responses in patients with psoriasis. *Photodermatol Photoimmunol Photomed* 12:204–210, 1996

Kelso A, Groves P: A single peripheral CD8+ T cell can give rise to progeny expressing type 1 and/or type 2 cytokine genes and can retain its multipotentiality through many cell divisions. *Proc Natl Acad Sci USA* 94:8070–8075, 1997

Kristensen M, Chu CQ, Eedy DJ, Feldmann M, Brennan FM, Breathnach SM: Localization tumour necrosis factor-alpha (TNF-α) and its receptors in normal and psoriatic skin: epidermal cells express the 55-kD but not the 75-kD TNF receptor. *Clin Exp Immunol* 94:354–362, 1993

Krueger JG, Wolfe JT, Nabeya RT, *et al*: Successful ultraviolet B treatment psoriasis is accompanied by a reversal keratinocyte pathology and by selective depletion intraepidermal T cells. *J Exp Med* 182:2057–2068, 1995

Livden JK, Nilsen R, Bjerke JR, Matre R: In situ localization interferons in psoriatic lesions. *Arch Dermatol Res* 281:392–397, 1989

Michel L, Mencia-Huerta JM, Benveniste J, Dubertret L: Biologic properties LTB4 and paf-acether in vivo in human skin. *J Invest Dermatol* 88 (6):675–681, 1987

Mitra RS, Judge TA, Nestle FO, Turka LA, Nickoloff BJ: Psoriatic skin-derived dendritic cell function is inhibited by exogenous IL-10. Differential modulation B7-1 (CD80) and B7-2 (CD86) expression. *J Immunol* 154(6):2668–2677, 1995

Morita Y, Yamamura M, Kawashima M, *et al*: Flow cytometric single-cell analysis cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 41:1669–1676, 1998

Mosmann TR, Sad S: The expanding universe T-cell subsets: Th1, Th2 and more. *Immunol Today* 17:138–146, 1996

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL: Two types murine helper T cell clone. 1. Definition according to profiles lymphokine activities and secreted proteins. *J Immunol* 136:2348–2357, 1986

Nakagawa S, Aiba S, Tagami H: Decreased frequency interferon-gamma producing $CD4^+$ cells in the peripheral blood patients with atopic dermatitis. *Exp Dermatol* 7:112–118, 1998

Nakazawa M, Sugi N, Kawaguchi H, Ishii N, Nakajima H, Minami M: Predominance type 2 cytokine-producing $CD4^+$ and $CD8^+$ cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 99:673–682, 1997

Nestle FO, Turka LA, Nickoloff BJ: Characterization dermal dendritic cells in psoriasis. Autostimulation T lymphocytes and induction Th1 type cytokines. *J Clin Invest* 94:202–209, 1994

Nickoloff BJ, Mitra RS: Intraepidermal psoriatic cytokine network involves gamma interferon, transforming growth factor-alpha, and their cell surface receptors: Dysregulation rather than deficiency. *J Invest Dermatol* 99:882–883, 1992

Nickoloff BJ, Mitra RS, Elder JT, Fisher GJ, Voorhees JJ: Decreased growth inhibition by recombinant gamma interferon is associated with increased transforming growth factor-alpha production in keratinocytes cultured from psoriatic lesions. *Br J Dermatol* 121:161–174, 1989

Nickoloff BJ, Karabin GD, Barker JN, *et al*: Cellular localization interleukin-8 and its inducer, tumor necrosis factor-alpha in psoriasis. *Am J Pathol* 138:129–140, 1991

North ME, Ivory K, Funauchi M, Webster ADB, Lane AC, Farrant J: Intracellular cytokine production by human $CD4^+$ and $CD8^+$ T cells from normal and immunodeficient donors using directly conjugated anti-cytokine antibodies and three-colour flow cytometry. *Clin Exp Immunol* 105:517–522, 1996

Olaniran AK, Baker BS, Paige DG, Garioch JJ, Powles AV, Fry L: Cytokine expression in psoriatic skin lesions during PUVA therapy. *Arch Dermatol Res* 288:421–425, 1996

Paukkonen K, Naukkarinen A, Horsmanheimo M: The development of manifest psoriatic lesions in linked with the invasion of $CD8^+$ T cells and $CD11c^+$ macrophages into the epidermis. *Arch Dermatol Res* 284:375–379, 1992

Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR, Maino VC: Direct demonstration cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86:1408–1419, 1995

Prinz JC, Gross B, Vollmer S, Trommler P, Strobel I, Meurer M, Plewig G: T cell clones from psoriasis skin lesions can promote keratinocyte proliferation in vitro via secreted products. *Eur J Immunol* 24:593–598, 1994

Prussin C: Cytokine flow cytometry: Understanding cytokine biology at the single-cell level. *J Clin Immunol* 17:195–204, 1997

Prussin C, Metcalfe DD: Detection intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods* 188:117–128, 1995

Res PC, Struijk L, Leow A, Daha MR, van den Elsen PC, Breedveld FC: Inflamed joints patients with rheumatoid arthritis contain T cells that display in vitro proliferation to antigens present in autologous synovial fluid. Functional analysis on the basis synovial-fluid-reactive T-cell clones and lines. *Hum Immunol* 40:291–298, 1994

Ridge JP, Di Rosa F, Matzinger P: A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. *Nature* 393:474–477, 1998

Roura-Mir C, Catalfamo M, Sospedra M, Alcalde L, Pujol-Borrell R, Jaraquemada D: Single-cell analysis intrathyroidal lymphocytes shows differential cytokine expression in Hashimoto's and Graves' disease. *Eur J Immunol* 27:3290–3302, 1997

Salgame P, Abrams JS, Clayberger C, Goldstein H, Convit J, Modlin RL, Bloom BR: Differing lymphokine profiles functional subsets human CD4 and CD8 T cell clones. *Science* 254:279–282, 1991

Sato A, Tsuji K, Yamamura M, *et al*: Increased type 2 cytokine expression by both CD4+ CD45RO+ T cells and CD8+ CD45RO+ T cells in blood circulation is associated with high serum IgE but not with atopic dermatitis. *J Invest Dermatol* 111(6):1079–1084, 1998

Schlaak JF, Buslau M, Jochum W, Hermann E, Girndt M, Gallati H, Meyer zum Buenschenfelde K-H: T cells involved in psoriasis vulgaris belong to the Th1 subset. *J Invest Dermatol* 102:145–149, 1994

Schoenberger SP, Toes REM, van der Voort EIH, Offringa R, Melief CJM: T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* 393:480–483, 1998

Schoendorf T, Engel H, Lindemann C, von Kolhagen H, Ruecker AA, Mallmann P: Cellular characteristics peripheral blood lymphocytes and tumour-infiltrating lymphocytes in patients with gynaecological tumours. *Cancer Immunol Immunother* 44:88–96, 1997

Sugi-Ikai N, Nakazawa M, Nakamura S, Ohno S, Minami M: Increased frequencies interleukin-2- and interferon-γ-producing T cells in patients with active Behçet's disease. *Invest Ophthalmol Vis Sci* 39:996–1004, 1998

Sunder-Plassmann R, Breiteneder H, Zimmermann K, Strunk D, Majdic O, Knapp W, Holter W: Single human T cells stimulated in the absence feeder cells transcribe interleukin-2 and undergo long-term clonal growth in response to defined monoclonal antibodies and cytokine stimulation. *Blood* 87:5179–5184, 1996

Suni MA, Picker LJ, Maino VC: Detection antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods* 212:89–98, 1998

Szabo SK, Hammerberg C, Yoshida Y, Bata-Csorgo Z, Cooper KD: Identification and quantitation of interferon-γ producing T cells in psoriatic lesions: Localization to both $CD4^+$ and $CD8^+$ subsets. *J Invest Dermatol* 111:1072–1078, 1998

Uyemura K, Yamamura M, Fivenson DF, Modlin RL, Nickoloff BJ: The cytokine network in lesional and lesion-free psoriatic skin is characterized by a T-helper type 1 cell-mediated response. *J Invest Dermatol* 101:701–705, 1993

Vollmer S, Menssen A, Trommler P, Schendel D, Prinz JC: T lymphocytes derived from skin lesion patients with psoriasis vulgaris express a novel cytokine pattern that is distinct from T helper type 1 and T helper type 2 cells. *Eur J Immunol* 24:2377–2382, 1994

Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Hansen HM, Kapsenberg ML: Evidence for compartmentalization functional subsets $CD4^+$ T lymphocytes in atopic patients. *J Immunol* 144:4651–4656, 1990

Yawalkar N, Karlen S, Hunger R, Brand CU, Braathen LR: Expression of interleukin-12 is increased in psoriatic skin. *J Invest Dermatol* 111:1053–1057, 1998