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T Cell-Regulated Neutrophilic Inflammation in Autoinflammatory Diseases

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Previous studies of acute generalized exanthematous pustulosis, a peculiar drug hypersensitivity reaction, suggested that CXCL8-producing T cells regulate sterile, polymorphonuclear neutrophil-rich skin inflammations. In this study, we test the hypothesis of whether CXCL8-producing T cells are present in autoinflammatory diseases like psustular psoriasis and Behçet’s disease. Immunohistochemistry of normal skin revealed few CD4+ and CD8+ T cells, few CXCL8+ cells, and no neutrophilic infiltration, whereas in acute exacerbations of atopic dermatitis, numerous CD4+ T cells but few CD8+ T cells, neutrophils, or CXCL8+ cells were detected. In contrast, a pronounced infiltration of neutrophils and of predominantly CD4+ T cells was observed in skin biopsies from pustular psoriasis, Behçet’s disease, and acute generalized exanthematous pustulosis, with infiltrating T cells strongly positive for CXCL8 and the chemokine receptor CCR6. Skin-derived T cell clones from pustular skin reactions were positive for CCR6 but negative for CCR8 and secreted high amounts of CXCL8 and GM-CSF, together with IFN-γ and TNF-α after in vitro stimulation. Moreover, some skin-derived T cell clones from Behçet’s disease and from pustular psoriasis predominantly produced CXCL8 and GM-CSF, but failed to secrete IL-5 and IFN-γ. These cells might represent a particular subset as they differ from both Th1 as well as Th2 T cells and are associated with a unique, neutrophil-rich sterile inflammation. Our findings suggest that CXCL8/GM-CSF-producing T cells may orchestrate neutrophil-rich pathologies of chronic autoinflammatory diseases like pustular psoriasis and Behçet’s disease. The Journal of Immunology, 2005, 175: 7678–7686.

Infiltration of polymorphonuclear neutrophils (PMN) into the tissue is a classical hallmark of bacterial infection, but occurs also in some autoimmune diseases. The recruitment of PMN to sites of bacterial infection is fast and does presumably not involve the adaptive immune system. One main mechanism is the activation of the complement system leading to complement split-products that recruit and activate leukocytes. In addition, bacterial surfaces and products can activate phagocytes via pattern recognition receptors of the innate immune system, leading to enhanced phagocytosis and cytokine/chemokine secretion, and further promote leukocyte recruitment into sites of infection.

In contrast to autoimmune diseases, sterile neutrophilic inflammations lack autoreactive antibodies and are therefore also termed autoinflammatory diseases. It is not clear how PMN are recruited in these instances where no pyogenic bacteria are present and no damage of tissue cells due to bacterial products occurs. An interesting model disease for such PMN-rich sterile inflammations is a peculiar drug hypersensitivity reaction, called acute generalized exanthematous pustulosis (AGEP), where >100 pustules appear on the skin, associated with fever and leukocytosis (1–5). Interestingly, this sterile pustular skin reaction can be induced by epicutaneous skin tests using the drug causing the hypersensitivity reaction (6, 7). When investigating this disease, we previously detected drug-specific T cells secreting high amounts of CXCL8 (IL-8) and GM-CSF (6, 8, 9). A further characterization of these CXCL8-secreting T cells revealed that the majority also released IFN-γ and the proinflammatory cytokine TNF-α, whereas a few produced high levels of IL-4 and IL-5. Together with cytotoxic T cells, these CXCL8-secreting T cells precede PMN infiltration into the dermis and epidermis. Cytotoxic T cells are thought to be responsible for vesicle formation by direct perforin/granzyme B mediated killing of keratinocytes, as well as FasL-mediated cell lysis (10). In parallel with this cell infiltration, resident keratinocytes are induced to express chemokines, in particular CXCL8. The newly formed intraepidermal vesicles are initially filled by perforin/granzyme B-positive as well as CXCL8/GM-CSF-secreting T cells. Within hours, these vesicles are transformed into pustules that are filled with PMN. Thus, a combination of CXCL8-secreting keratinocytes and T cells seems to modulate this Ag-dependent recruitment of PMN (6, 8, 9).

As in AGEP, PMN infiltration, and formation of sterile pustules can be observed in certain autoinflammatory diseases like Behçet’s disease and pustular psoriasis (11–13), which are considered to be T cell-mediated diseases (14, 15). In these neutrophilic autoinflammatory reactions, CXCL8 seems to play an important role as patients with Behçet’s disease have elevated systemic levels of CXCL8 that correlate with disease activity (16). Likewise, several studies reported elevated expression of CXCL8 in psoriatic skin (17, 18).

In this study, we addressed the question of to what extent CXCL8-secreting T cells can be detected in skin lesions of Behçet’s disease and pustular psoriasis in comparison to skin from patients with acute
atopic dermatitis, AGEP, and healthy individuals. In healthy individuals, ~2% of circulating T cells also produced CXCL8 (6). Based on immunohistochemistry and cloning of skin-derived T cells, our data support the concept that in autoinflammatory diseases with PMN involvement, T cells themselves may orchestrate the neutrophil-rich inflammation by secreting high amounts of CXCL8. Some of the T cell clones (TCC) derived from inflamed skin produced predominantly CXCL8 and GM-CSF, but only low amounts of Th1- or Th2-typical cytokines, and may represent a functionally distinct and unique neutrophil-regulating T cell subset, which contributes to PMN-rich inflammations.

Materials and Methods

Patients’ characteristics

Patients with different autoinflammatory pustular diseases were included in this study. We analyzed three patients with Behçet’s disease (BD1, BD2, BD3) and four patients with pustular psoriasis (PP1, PP2, PP3, PP4). For comparison, three patients with acute AGEP (AG1, AG2, AG3), four donors of healthy human skin, and two patients with acute lesions of atopic dermatitis were included. Table I summarizes the clinical characteristics of the patients. The study was approved by the Ethical Committee of the University of Bern.

Staining of biopsy specimens

Punch-biopsy specimens (4 mm) were obtained from affected skin (pustular eruption) and were divided in two equal parts, one for T cell elution and one for immunohistochemistry. Biopsies used for immunohistochemistry were snap-frozen in tissue-embedding medium (TissueTek OCT Compound; Sakura Finetek) and stored at −80°C. The following Abs were used for single immunostainings: anti-CD4 (1-150, clone MT 310; DakoCytomation), anti-CD8 (1-100, clone DK25; DakoCytomation), anti-IL-8 (CXCL8, 1/100, clone C20791A; BD Biosciences/BD Pharmingen), anti-neutrophil elastase (1/300, clone NP57; DakoCytomation), anti-CCR6 (1/200, clone 53103.111; R&D Systems), and anti-CCL20 (1/20, clone C20791A; BD Biosciences/BD Pharmingen). For the culture of T cell lines (TCL) or TCC, the CM was enriched with 100 U/ml human IL-2 (Proleukin; Roche Pharma).

Evaluation of sections

Slides were analyzed on a Leica microscope DM500 by the same investigator. CD4, CD8, and CXCL8+ cells were identified by their characteristic morphology and by their membrane and/or cytoplasmatic staining. In each section, staining was assessed by analyzing 8–12 fields in the dermis at ×400 magnification. The eyepiece had a grid of 1 cm² and covered 0.0625 mm² of the biopsy. Mean values were determined to compare the cell density and CXCL8 expression among the different diseases.

Culture medium (CM)

CM consisted of RPMI 1640 (52400-25; Invitrogen Life Technologies) supplemented with 10% pooled heat-inactivated human AB serum (Swiss Red Cross), 2 mMol/l glutamine (K0202; Biochrom), 100 μg/ml streptomycin, and 100 IU/ml penicillin (4-01F00-H; Amimed). For the culture of TCL or TCC, the CM was enriched with 100 U/ml human IL-2 (1/200, clone ATU01; R&D Systems).

Isolation of skin-infiltrated T cells from pustular skin eruptions

The second half of the biopsy specimen was first washed with Ca²⁺/Mg²⁺-free HBSS. The fatty tissue was cut off, the remaining tissue mechanically disaggregated to very small pieces, and kept for 6–12 h at 4°C before being washed in Ca²⁺/Mg²⁺-free HBSS. Nonspecific binding was blocked by preincubation for 20 min with PBS containing rabbit serum and Fc receptor blockers (Beckmann Coulter), detecting CD3, CD4, CD8, and CXCL8+ mAbs (R&D Systems). CCR8 staining procedures were performed as described (21). Phenotype characterization and expression of surface CD3, anti-CD4, anti-CD8 (all from BD Pharmingen), and anti-CCR6 mAbs (1/200, clone E0460; DakoCytomation) and thereafter with ABComplex/AP (K0367; DakoCytomation). Finally, all sections were developed with a red staining. The incubation of the slides with anti-CD4 Ab was followed by the ABComplex/AP (K0367; DakoCytomation) and finally developed with fuchsin substrate-chromogen (K0597; DakoCytomation) and counterstained with Mayer’s hematoxylin. Omission of either primary Ab served as negative control, resulting in only one color staining.

Flow cytometry analysis

Aliquots containing 10⁵ cells were stained with fluorochrome-conjugated Abs in 50 μl of buffer (PBS/1% FCS/0.02% NaN₃) for 15–30 min at 4°C and analyzed on a Coulter EPICS XL-MCL, or a Cytomics FC 500 Flow Cytometer (both Beckmann Coulter). Monoclonality of generated TCC was shown by TCR β-chain staining using a panel of 24 mAbs against different β-chain genes (Beckmann Coulter), detecting ~75% of all β-chain families as described (21). Phenotype characterization and expression of surface markers of TCL and TCC was performed using fluorochrome-labeled anti-CD3, anti-CD4, anti-CD8 (all from BD Pharmingen), and anti-CCR6 mAbs (R&D Systems). CCR8 staining procedures were performed as described elsewhere (22).

Table I. Characteristics of patients with Behçet’s disease (BD1-BD3), pustular psoriasis (PP1-PP4), and AGEP (AG1-AG2) a,b

<table>
<thead>
<tr>
<th>Patients</th>
<th>Disease</th>
<th>Age (years)/Sex (M/F)</th>
<th>Drug Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1</td>
<td>Pustular psoriasis</td>
<td>59/F/unknown</td>
<td>Tacrolimus, flutetasone, + salicylic acid (topical) naproxen, metoprolol, + ibersartan (oral)</td>
</tr>
<tr>
<td>PP2</td>
<td>Pustular psoriasis</td>
<td>69/F/53 years</td>
<td>Betamethasone (topical), UV</td>
</tr>
<tr>
<td>PP3</td>
<td>Pustular psoriasis</td>
<td>46/F/4 years (recurrent)</td>
<td>Prednisolone (discontinued 5 days before biopsy)</td>
</tr>
<tr>
<td>PP4</td>
<td>Pustular psoriasis</td>
<td>36/M/3 wk</td>
<td>Infliximab (last injection 1 mo ago)</td>
</tr>
<tr>
<td>BD1</td>
<td>Behçet’s disease</td>
<td>44/M/4 years</td>
<td>Prednisone</td>
</tr>
<tr>
<td>BD2</td>
<td>Behçet’s disease</td>
<td>31/M/5 years</td>
<td>Acetylsalicly acid</td>
</tr>
<tr>
<td>BD3</td>
<td>Behçet’s disease</td>
<td>44/M/8 years</td>
<td>Leflunomide, colchicine</td>
</tr>
<tr>
<td>AG1</td>
<td>AGEP</td>
<td>38/F/acute</td>
<td>Hypersensitivity to nickel (screw for orthopedic surgery)</td>
</tr>
<tr>
<td>AG2</td>
<td>AGEP</td>
<td>43/M/acute</td>
<td>Hypersensitivity to prednisone and hydrocortisone</td>
</tr>
<tr>
<td>AG3 b</td>
<td>AGEP</td>
<td>30/F/cured</td>
<td>Hypersensitivity to amoxicillin</td>
</tr>
</tbody>
</table>

a PP, pustular psoriasis; BD, Behçet’s disease, AG, AGEP.

b Previously characterized as patient AP (8), cells obtained from positive patch test reaction.
Cytokine and chemokine detection in cell-culture supernatants

A total of $5 \times 10^6$ T cells were stimulated in 200 μl of CM for 48 h in flat-bottom 96-well plates coated with anti-CD3 (Okt3, 1 μg/ml) and soluble anti-CD28 (1 μg/ml) mAb as costimulatory factor in the presence of low amounts of IL-2 (40 U/ml). Supernatant of unstimulated T cells was taken as control and proliferation was measured by thymidine incorporation overnight as described (8). The following cytokine and chemokines ELISA sets were used: IL-5 and CXCL8 (BD Pharmingen); IFN-γ, TNF-α, IL-4, IL-5, and CXCL8 (Diaclone); GM-CSF (R&D Systems). Duplicate samples were diluted (1/10, 1/100, and 1/1000 as indicated) and measured according to standard protocols of the corresponding ELISA set. The detection limit of the assays performed was 1 pg/ml for IL-4, 6 pg/ml for IFN-γ, 8 pg/ml for IL-5, 13 pg/ml for TNF-α, and 16 pg/ml for CXCL8 and GM-CSF.

**Statistical analysis**

Statistical analyses were performed using the Kruskal-Wallis nonparametric ANOVA test. Values of $p < 0.05$ were considered significant.

**Results**

**Characterization of T cells, neutrophils, and CXCL8$^+$ cells in healthy human skin, acute atopic dermatitis, pustular psoriasis, and Behçet’s disease**

Immunostainings of T cells, neutrophils, and CXCL8 in healthy human skin, acute atopic dermatitis, and pustular autoimmune diseases are shown in Fig. 1. In normal human skin, few CD4$^-$ and some CD8$^+$ T cells could be detected, which were mainly located within the dermis (Fig. 1, A and B), whereas in acute exacerbation of atopic dermatitis, more CD4$^-$ and CD8$^+$ T cells were found (Fig. 1, E and F). Analysis of pustular psoriasis (Fig. 1, I and J), Behçet’s disease (Fig. 1, M and N), and acute AGEP (Fig. 1, Q and R) revealed an augmented presence of CD4$^-$ and CD8$^+$ T cells compared with normal skin. The majority of T cells were located within the dermis.

The number of dermis-infiltrating CD4$^+$ T cells in atopic dermatitis patients revealed a 1.6-fold increase compared with normal skin, while patients PP3, BD1, BD3, and AG2 had a 1.1- to 2.6-fold increase (Fig. 2A). The number of CD8$^+$ T cells in the dermis of diseased skin was increased 1.7- to 2.6-fold compared with normal skin (Fig. 2B).

PMN, as revealed by staining for neutrophil elastase, were absent in normal skin (Fig. 1C), and rare in atopic dermatitis (Fig. 1G), while a high number of neutrophils was found in pustular psoriasis (Fig. 1K), Behçet’s disease (Fig. 1O), and AGEP (Fig. 1S), where PMN were the dominant cell type within the subcorneal pustules. Furthermore, PMN prominently infiltrated the epidermis underlying the pustules and the papillary dermis.

We have previously demonstrated that AGEP patients show an enhanced CXCL8 production by keratinocytes and also by the dermal cell infiltrate (8). In this study, we compared CXCL8 production in patients with autoinflammatory diseases, AGEP, acute atopic dermatitis and normal skin. CXCL8$^+$ cells were barely detectable in normal skin (Fig. 1D) and in atopic dermatitis (Fig. 1H). However, a strong CXCL8 production was detected in the keratinocytes and also in the cell infiltrates of the autoinflammatory diseases. In pustular psoriasis and in AGEP, the CXCL8$^+$ cells

**FIGURE 1.** Immunohistochemical staining of CD4, CD8, neutrophil elastase, and CXCL8 in normal skin and inflamed tissue. In normal skin (A and B) and in atopic dermatitis (E and F), a low number of CD4$^-$ and CD8$^+$ cells was observed compared with pustular psoriasis (I and J, patient PP3), Behçet’s disease (M and N, patient BD1), and AGEP (O and R, patient AG2). The majority of the T cells were located in the dermis, and most of them were CD4$^-$. Neutrophil elastase was absent in normal skin (C) and barely detectable in atopic dermatitis (D), whereas a high number of PMN could be detected in all autoinflammatory diseases (K, O, and S) situated characteristically within the subcorneal pustules where the PMN are the dominant cell type. CXCL8 was barely detectable in normal skin (D) and in atopic dermatitis (H). In pustular psoriasis (L) and in AGEP (T), CXCL8$^+$ cells were distributed in the entire epidermis and in dermal infiltrates. In Behçet’s disease (P), CXCL8$^+$ cells were augmented and mainly located in the basal and suprabasal layers of the epidermis and to a lesser degree in the dermis. Original magnifications, $\times 100$. Downloaded from http://www.jimmunol.org/ by guest on April 19, 2017
were characteristically distributed in the entire epidermis and in the dermal infiltrate (Fig. 1, L and T). In Behçet’s disease, an augmented presence of CXCL8 \(^{+}\) cells was mainly located in the basal and the suprabasal layers of the epidermis, and to a lesser degree in the dermis (Fig. 1 P).

Dermal CXCL8 \(^{+}\) cells were counted to compare the chemokine production by skin homing cells (in healthy skin) or skin-infiltrating cells (in inflamed skin). Most of the cells in the dermis of normal skin were negative for CXCL8 probably because they were not activated. Also, the dermis of patients with atopic dermatitis contained only a few CXCL8 \(^{+}\) cells (increase in atopic dermatitis: 1.2-fold). However, in patients PP3, BD1, BD3, and AG2, the number of CXCL8 \(^{+}\) cells was significantly elevated compared with normal skin, namely 37-, 27-, 31-, and 42-fold, respectively (Fig. 2 C). When all positive cells in the epidermis and dermis were counted the increase was even higher (58- to 81-fold; data not shown). This difference of CXCL8 staining between normal/atopic skin and skin with neutrophilic inflammation is rather dramatic, considering the 2-fold only increase of CD4 \(^{+}\) T cells in autoinflammatory diseases. We attribute this difference to the activation of skin homing and immigrating T cells in patients with autoinflammatory diseases, which produce CXCL8, whereas T cells present in normal skin hardly produce any CXCL8.

To confirm that indeed the skin-infiltrating CD4 \(^{+}\) cells produced CXCL8, we performed double staining with anti-CD4 and anti-CXCL8 Abs. As shown in Fig. 3 (patient PS3 with pustular psoriasis), some CD4 \(^{+}\) and CXCL8 \(^{+}\) double-positive cells could be detected, together with single CD4 \(^{+}\) T cells or single CXCL8 \(^{+}\) cells.

In summary the immunohistochemical analysis of pustular psoriasis, Behçet’s disease, and AGEP demonstrated many skin-infil-
trating T cells (CD4⁺ > CD8⁺) as well as a high amount of the neutrophil-attracting chemokine CXCL8 that is not only produced by keratinocytes but also by CD4⁺ T cells.

**CCR6 and CCL20 (MIP-3α) expression in normal human skin and in autoinflammatory diseases**

Skin homing T cells and T cells infiltrating the skin under inflammatory conditions can be discriminated by expression of different chemokine receptors. It has previously been demonstrated that T cells infiltrating lesional skin in psoriasis and AGEP express high levels of the chemokine receptor CCR6, whereas in atopic dermatitis lower levels were found (6, 23–27). In agreement with this concept, we found that CCR6 was expressed only scarcely in the epidermis or dermis of normal skin (Fig. 4A) and acute atopic dermatitis (Fig. 4C). In contrast, in pustular psoriasis (Fig. 4E), Behçet’s disease (Fig. 4G), and AGEP (Fig. 4I), many CCR6⁺ cells were detectable, which were located predominantly close to the basal layers in the epidermis and around vessels in the dermis. In addition, we analyzed the expression of CCL20, an inflammatory chemokine, which is the ligand for CCR6. In normal skin, only a few CCL20⁺ cells were detectable (Fig. 4B), whereas CCL20 was augmented in atopic dermatitis (Fig. 4D). Substantially higher immunoreactivity for CCL20 was seen in all three autoinflammatory diseases, where the entire epidermis and the dermal infiltrates were stained (Fig. 4, F, H, and J).

In summary, these data indicate that neutrophilic inflammations of the skin correlate with strong local CCL20 production and immigration of CCR6⁺ T cells.

**Characterization of skin-derived T cells**

TCC from skin biopsy specimens of patient PP1, BD1, AG2, and AG3 were generated by direct cloning after isolation, and from patients PP2, PP3, PP4, and AG1 by cloning of TCL generated from the affected skin.

It has previously been demonstrated that T cells homing to healthy human skin are characterized by CCR8 expression (22). By investigating T cells eluted from sterile skin papules or eruptions, we detected CCR8 expression only on 2 of 11 and on 1 of 12 analyzed TCC from pustular psoriasis and Behçet’s disease, respectively (Fig. 5A). In contrast, we detected the expression of CCR6 on most TCC eluted from the diseased skin, whereas it was hardly detectable on TCC from normal skin (Fig. 5B, Ref. 22, and data not shown).

Fifty-five of 59 skin-derived TCC from patients with Behçet’s disease and pustular psoriasis were CD4⁺ and all expressed the αβ TCR. There was some preferential use of a certain TCR-Vβ chain in the TCC directly cloned from the skin of patient PP1 with pustular psoriasis: 3 of 10 TCC expressed the TCR-Vβ2 (data not shown), CD4⁺ TCC were selected for further investigation. Where cloning was performed from a TCL, only one clone of those expressing the same TCR-Vβ chain was further analyzed to avoid a potential overrepresentation of TCC derived from the same progenitor T cell.

**Cytokine and chemokine production of TCC**

Skin-derived TCC from healthy individuals and patients PP1, PP2, PP4, BD1, AG2, and AG3 were stimulated with anti-CD3/anti-CD28 Abs for 48 h and cytotoxicity (IL-4, IL-5, IFN-γ, TNF-α, GM-CSF) and chemokine (CXCL8) secretion was determined in the supernatant (Fig. 6 and Table II). In total, 13 TCC were analyzed from patients with pustular psoriasis, 13 from Behçet’s disease, 10 from AGEP, and 15 from normal skin. In each disease, TCC with different cytokine secretion patterns and several CXCL8 secreting clones were found. We classified them into Th1- (high IFN-γ), Th2- (high IL-5), and Th0-like (intermediate amounts of IFN-γ and IL-5) cells depending on their cytokine secretion (Table II). High amounts of IL-4 (>1 ng/ml) were secreted by half of the Th2 clones, but never in the absence of IL-5. TNF-α was produced in high amounts (>1 ng/ml) by all Th1 and by most of the Th2 cells. The cytokine secretion pattern of all these T cells is summarized in Table II and data are shown in Fig. 6.

In pustular psoriasis, 2 TCC had a Th1-, 2 TCC a Th2-, and 2 TCC a Th0-like secretion pattern. Six of 13 clones produced CXCL8 (difference between stimulated and unstimulated >0.2 ng/ml), of which 1 Th1 and 1 Th2 clone secreted even >1 ng/ml. GM-CSF was also found in the supernatant of every clone (0.5–27.1 ng/ml), and 11 of 13 clones released >1 ng/ml GM-CSF. We detected 4 of 13 clones which did not belong to the Th1 or Th2 phenotype, because they secreted small amounts of IFN-γ and IL-5, but produced CXCL8 (>0.2 ng/ml) together with GM-CSF (>0.2 ng/ml). Only 1 of these predominantly CXCL8-producing cells secreted >1 ng/ml TNF-α. Three of 13 TCC produced only low amounts of all tested cytokines.

In Behçet’s disease, 5 TCC had a Th1, 1 TCC a Th2, and 5 TCC a Th0 profile. CXCL8 secretion was higher and more frequently detectable (12 of 13 clones: >0.2 ng/ml) than in psoriasis, and 4 Th1 and 3 Th0 clones released even >1 ng/ml CXCL8. GM-CSF production was similar to psoriasis and all of the TCC analyzed secreted high amounts (1.1–12.9 ng/ml). In this disease 2 of 13 TCC produced predominantly CXCL8, GM-CSF, and TNF-α (all >1.0 ng/ml) but no or very low amounts of Th1 and Th2 typical cytokines.

Six of 13 TCC from patient BD1 released substantial amounts of CXCL8 even without any previous in vitro stimulation (0.2–2.1 ng/ml, clones BD1 S10, S33, S34, S35, S66, and S154; data not shown).

The cytokine secretion of 10 skin-derived TCC from 2 patients with AGEP is shown in Fig. 6C (8). These clones secreted different
cytokines in variable amounts: four TCC with a Th1-, 1 TCC with a Th2-, and 4 TCC with a Th0-like secretion pattern could be detected, but no TCC that produced only CXCL8 and GM-CSF. In fact, the CXCL8 production of these skin derived TCC was lower (0-1.2 ng/ml) than from drug-specific TCC obtained from the peripheral blood (8).

Upon stimulation with anti-CD3/anti-CD28 Abs, TCC derived from normal skin produced high amounts of different cytokines and the majority tended to produce a Th0-like cytokine secretion pattern without a clear bias for one or the other phenotype. In contrast to immunohistochemistry, where no CXCL8 staining of the resting T cells was detected, substantial amounts of CXCL8 (10 of 15 >0.2 ng/ml, thereof 4 of 15 >1 ng/ml) and GM-CSF (1.4-12.8 ng/ml) were secreted after in vitro stimulation by several of these clones derived from healthy skin. Chemokine production was in a similar range as the production by TCC derived from patient’s skin, but each CXCL8 producer secreted also IFN-γ or IL-5 in considerable amounts.

Discussion

Many autoimmune diseases are considered to be T cell-regulated diseases, as some have a well-documented HLA association, and therapy targeted to T cells is often efficient. Some of these autoimmune diseases have been further classified as Th1- or Th2-mediated diseases, with Th1-like diseases featuring a high production of IFN-γ, of complement-fixing Ab isotypes, and/or of activation of monocytes (28-33). Other autoimmune reactions are classified as Th2-like diseases, with high IL-4, IL-13, and IL-5 production by T cells, or high IgE and IgG4 synthesis and mast cell and eosinophil activation (34-36). However, this classification fails to explain the involvement of PMN in sterile inflammatory reactions as seen in many autoimmune/autoinflammatory diseases. Indeed, the

FIGURE 6. Characterization and cytokine/chemokine secretion of skin-derived CD4+ TCRab+ TCC from patients with pustular psoriasis, Behçet’s disease, AGE, and from normal skin. TCC of patients PP1, PP2, and PP4 with pustular psoriasis (A), of patient BD1 with Behçet’s disease (B), patients AG2 and AG3 with AGE (C), and from two different donors of normal skin NS1, NS2 (D) were stimulated with anti-CD3/anti-CD28 Abs, and the cytokine secretion patterns were determined. The differences between stimulated and unstimulated values are given. The mean cytokine/chemokine secretion of the unstimulated cells was always <0.2 ng/ml, except for CXCL8 in TCC from patient BD1 (unstimulated levels of CXCL8 0-2.1 ng/ml, mean: 0.5 ng/ml). Cytokine (IL-4, IL-5, IFN-γ, TNF-α, GM-CSF) and chemokine (CXCL8) levels are shown as dot plots, where each symbol represents one TCC. TCC are grouped into different cytokine secretion patterns: Th1-like (gray open symbols), Th2-like (gray filled symbols), Th0-like (black open symbols), predominantly CXCL8/GM-CSF-producing T cells (black filled symbols), and “low producers” (black-gray symbols).
view that PMN may act as T cell dependent effector cells is not widely accepted, probably due to the fact that PMN can clearly act without T cell regulation and that they appear often at the site of inflammation even before a specific immune response has developed. However, this feature of PMN does not rule out that T cells may still influence PMN recruitment and use them as effector cells in certain T cell-regulated inflammations (12).

The data presented here extend our previous work on CXCL8- and GM-CSF-secreting T cells, which we found in AGEP (6, 8–10) and could be related to an Ag-driven development of a neutrophilic inflammation. In this study, we describe the occurrence of CXCL8/GM-CSF-producing T cells—rare in normal skin, but much more abundant in cutaneous manifestations of Behçet’s disease and pustular psoriasis. Although these diseases are chronic, the manifestations in the skin with pustule formation reflect an acute process that is similar to AGEP. By investigating these chronic relapsing neutrophilic diseases, we detected T cells which predominantly produce high amounts of CXCL8 and GM-CSF and thus might represent a unique T cell subset.

Schaerli et al. (22) have recently shown that the T cell population present in normal skin is characterized by the expression of CCR8. These CCR8+ T cells respond to the ligand CCL-1 (I-309) and are clearly distinct from the CCR6+ CCR8+ T cells immigrating during an inflammatory reaction. These CCR8+ T cells may represent skin homing T cells, which are found in healthy skin and were reported to produce a preferential Th1-like cytokine secretion pattern after ex vivo stimulation (22), suggesting that skin-derived, CCR8+ T cells might be involved in the initiation of type 1 responses via secreting TNF-α and IFN-γ (29, 30, 32, 37). Our data on normal skin-derived TCC confirm a high TNF-α production after in vitro stimulation with anti-CD3/anti-CD28 Abs. Some TCC showed high IFN-γ production and about half of them showed a simultaneous IFN-γ and IL-5 production, suggesting more of a Th0-like secretion pattern for healthy skin (Table II). Moreover, in some TCC a rather high CXCL8 production could be detected after in vitro stimulation. Interestingly, we consistently observed a weak CXCL8 staining of a few T cells in normal skin in immunohistochemical experiments, which was similar to the CXCL8 production by basal keratinocytes. Thus, some skin-derived CCR8+, CCR6− T cells might not only be involved in the initiation of type 1 responses but may also start PMN recruitment.

The immunohistochemistry of patients with sterile, pustular skin eruptions in the context of a systemic autoinflammatory disease revealed a substantially denser, lymphocyte rich cell infiltrate (mainly CD4+ and some CD8+) than in normal skin. The majority of T cells detected were immigrating, inflammatory T cells, as they expressed CCR6, the receptor for CCL20 (MIP-3α). This chemokine was indeed strongly expressed in vivo in all three PMN-rich inflammations, namely Behçet’s disease, pustular psoriasis, and AGEP, but only to a low degree in atopic dermatitis, and almost

### Table II. Cytokine secretion patterns of skin-derived TCC

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of TCC Tested (n)</th>
<th>Th1-Like Cytokine Secretion Pattern</th>
<th>Th2-Like Cytokine Secretion Pattern</th>
<th>Th0-Like Cytokine Secretion Pattern</th>
<th>Predominant CXCL8 and GM-CSF Secretion</th>
<th>Low Producers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IFN-γ &gt; 8 ng/ml</td>
<td>IL-5 &gt; 1 ng/ml</td>
<td>IFN-γ &gt; 1 ng/ml and IL-5 &gt; 0.2 ng/ml together</td>
<td>Non-Th1, Th2, Th0 but CXCL8 &gt; 0.2 ng/ml and GM-CSF &gt; 0.2 ng/ml</td>
<td>Non-Th1, Th2, Th0, and no CXCL8 production</td>
</tr>
<tr>
<td>Pustular psoriasis</td>
<td>13</td>
<td>2 (1/0/1)</td>
<td>2 (1/0/1)</td>
<td>4 (0/3/1)</td>
<td>3 (3/0/0)</td>
<td></td>
</tr>
<tr>
<td>Behçet’s disease</td>
<td>13</td>
<td>5 (1/0/4)</td>
<td>1 (0/1/0)</td>
<td>2 (0/0/2)</td>
<td>0 (0/2/0)</td>
<td></td>
</tr>
<tr>
<td>AGEP</td>
<td>10</td>
<td>4 (2/0/2)</td>
<td>1 (1/0/0)</td>
<td>1 (1/0/0)</td>
<td>1 (1/0/0)</td>
<td></td>
</tr>
<tr>
<td>Normal skin</td>
<td>15</td>
<td>6 (0/0/3)</td>
<td>7 (2/4/1)</td>
<td>0 (2/4/1)</td>
<td>2 (2/0/0)</td>
<td></td>
</tr>
</tbody>
</table>

* TCC were grouped into Th1-like (IFN-γ > 8 ng/ml), Th2-like (IL-5 > 1 ng/ml), and Th0-like cells (IFN-γ > 1 ng/ml together with IL-5 > 0.2 ng/ml). Predominantly CXCL8/GM-CSF-producing T cells do not belong to Th1 (<8 ng/ml IFN-γ), nor to Th2 (<1 ng/ml IL-5), nor to Th0 (no simultaneous IFN-γ and IL-5 production). They are characterized by medium to high CXCL8 (>0.2 ng/ml) production together with GM-CSF (>0.2 ng/ml). “Low producers” secreted low amounts of all cytokines/chemokines. CXCL8-producing TCC are shown in each group (~, 0.2-1 ng/ml; ++, >1 ng/ml). Details are shown in Fig. 6.

![FIGURE 7. Composition of T cells contributing to CXCL8/GM-CSF production and neutrophilic inflammation in the skin. In healthy individuals, CCR8+ skin homing T cells have the ability to produce CXCL8, after stimulation only (illustrated by dashed line). In acute neutrophilic inflammations, CXCL8 production occurs mainly by skin-infiltrating CCR6+ T cells, which also produce moderate amounts of TNF-α, IFN-γ, and IL-4/IL-5 (Th0) or are already biased to produce a more restricted set of cytokines (Th1, occasionally Th2). In chronic relapsing neutrophilic inflammations, CXCL8/GM-CSF derive from various CCR6+ T cells, some of which produce mainly CXCL8/GM-CSF, but not other cytokines (ThCXCL8). The strength of the arrows reflects the relative contribution of the various subsets.](http://www.jimmunol.org/)
absent in normal skin. Pustules, if detectable, were filled with PMN, which were also present dispersed throughout the tissue. CXCL8 was detected in skin biopsies of PMN-rich inflammations where it was expressed in keratinocytes, but also abundantly in skin-infiltrating T cells of patients with Behçet’s disease, pustular psoriasis, and AGEP. This supplements previous reports that describe the enhanced production of CXCL8 by keratinocytes (12, 18) and neutrophils (17) in pustular psoriasis, and elevated CXCL8 serum levels in Behçet’s disease (16, 38–40). Double staining confirmed the presence of CD4+ T cells that produce the chemokine CXCL8 in inflamed tissue. Of note, this is a special feature of PMN-rich inflammations and not a general feature of inflammatory skin reactions, as CXCL8 production was low in the inflammatory tissue of patients with acute atopic dermatitis, which represents more a Th2-type, eosinophilic inflammation largely devoid of PMN.

T cells derived from skin lesions were cloned and expanded in vitro by PHA stimulation and characterized for their cytokine production after a culture period of 6–8 wk. Still, these TCC had the phenotype of their origin, as the TCC from the inflammatory skin reaction expressed CCR6. After stimulation, the TCC derived from pustular skin lesions produced different cytokines: a few TCC had a Th2 phenotype (IL-4/IL-5 high), while the majority were Th1 T cells (IFN-γ high), which may explain why Behçet’s disease and psoriasis are normally classified as Th1-like diseases (29, 30, 32).

The majority of Behçet’s disease-derived and a substantial part of psoriasis-derived TCC produced high amounts of CXCL8 and GM-CSF, which may promote the recruitment and survival of PMN in the tissue (6, 41–43). This finding supports the observation of Mochizuki et al. (44) that TCC established from pustular lesions of patients with Behçet’s disease have an enhanced CXCL8 mRNA expression. Interestingly, most of the Behçet’s disease-derived TCC secreted substantial levels of CXCL8 already without stimulation, which was not observed in TCC from patients with pustular psoriasis, and thus might be peculiar for Behçet’s disease. Moreover, the CXCL8 production of Behçet’s disease-derived TCC was actually higher than that of TCC derived from the skin of patients with AGEP or psoriasis. The high levels of CXCL8 and GM-CSF secreted by stimulated TCC might explain and at least contribute to the PMN-rich skin inflammation. In addition, most of these TCC produced also TNF-α and IFN-γ (=Th1-like cells with CXCL8 production), which are two cytokines known to enhance CXCL8 production by local tissue cells like keratinocytes (45), and might thus also contribute to the PMN recruitment.

The analysis of the chronic autoimmune, PMN-rich inflammations revealed some TCC that might represent a new, peculiar T cell subset, clearly distinct from Th1 and Th2: they produced almost exclusively CXCL8 together with GM-CSF (high in five of six) or TNF-α (high in three of six), but low amounts of Th1 or Th2 typical cytokines. These T cells are also different from the low cytokine producers (Table II) and are found only in chronic PMN-rich inflammatory reactions like Behçet’s disease and pustular psoriasis, but were not detectable in normal skin or in AGEP, which normally lasts only a few days. In analogy to the differentiation of T cells into Th1 or Th2 cells (46), several factors such as the local cytokine environment, the type of APCs, the type of Ags, the nature/strength of the TCR-mediated signals and possibly also repetitive stimulations during the chronic course of the disease may play a role in the development of such predominantly CXCL8/GM-CSF-producing T cells. However, our data also indicate that such T cells do not appear to be essential for inducing pustule formation, as pustules appeared as well if the immigrating T cells secreted CXCL8 and GM-CSF together with other cytokines (CXCL8 producing Th1/Th0 cells; see Fig. 7).

In conclusion, our data suggest that CXCL8 production by T cells may play an important role in sterile neutrophilic inflammations, where CCR6+ T cells are attracted by the high local production of CCL20. These immigrating T cells produce cytokines like IFN-γ and TNF-α as well as CXCL8 and GM-CSF and are found in acute and chronic diseases, while only in chronic relapsing diseases like pustular psoriasis and Behçet’s disease some T cells secrete predominantly CXCL8 but less of Th1 or Th2 cytokines, and may thus represent a unique T cell subset. The combined action of these T cells, together with tissue-derived cytokines/chemokines, could be responsible for the recruitment and survival of PMN in the affected tissue (Fig. 7). How T cells are stimulated to produce high amounts of CXCL8 in these diseases remains still enigmatic. The ensuing immune response results in an immunopathology which is clearly distinct from a Th1- or Th2-mediated reaction, and distinguishes sterile neutrophilic inflammatory disorders. In view of the great importance of neutrophilic reactions in clinical medicine, it is necessary to better investigate these PMN orchestrating T cells, as well as which drugs may modify this peculiar inflammatory response.

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