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Characterization of the Divergent Wound-Healing Responses Occurring in the Pathergy Reaction and Normal Healthy Volunteers[1]

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Behçet's disease (BD) is a multisystem inflammatory disorder of unknown etiology characterized by recurrent oral and genital ulcerations and uveitis, with varying other manifestations associated with vascular inflammation. A unifying feature of BD inflammation is the skin pathergy reaction (SPR), a nonspecific tissue hyperreactivity to minor trauma involving epithelial disruption. This study compared skin responses to needle prick in BD patients and normal healthy volunteers. Two study groups, each consisting of 10 BD patients with SPR[+] and 6 controls, were evaluated using either immunohistochemistry or quantitative real-time PCR to measure inflammatory cell and cytokine levels in biopsy specimens obtained serially from independent sites at 0, 8, and 48 h after needle prick. We found similar cellular infiltration patterns in response to needle prick in BD patients and controls between 0 and 8 h. Further development of this immune response was limited in skin of normal control subjects, with stable or decreased inflammatory mediators observed at 48 h. In contrast, in BD-derived skin specimens, increased influxes of mature dendritic cells, monocytes, and lymphocytes, including T regulatory cells, were noted by 48 h. Similarly, increases in cytokines (IFN-γ, IL-12 p40, IL-15), chemokines (MIP3-α, IP-10, Mig, and iTac), and adhesion molecules (ICAM-1, VCAM-1) were noted at 48 h in the skin of BD patients with SPR[+] but not in the skin of normal controls. These results suggest that, in contrast to the self-limited inflammation associated with epithelial disruption of normal skin, BD patients experience marked cellular influxes into the injury site, leading to an exaggerated lymphoid Th1-type response. *The Journal of Immunology,* **2006, 177: 6415–6421.**

Behçet's disease (BD)[4] is a complex multisystem inflammatory disorder of unknown etiology. It typically manifests as recurrent oral and genital ulcerations and uveitis, variably accompanied by symptoms affecting skin, the large vessels, gastrointestinal system, and CNS or other organs (1). The precise mechanisms of tissue destruction in BD have not been fully elucidated. Nevertheless, a growing number of reports have implicated T lymphocyte-mediated immune responses in the disease process (2–6).

A unifying feature of the inflammation observed in BD is the nonspecific hyperreactivity of tissue to minor trauma, termed the skin pathergy reaction (SPR). Although not always a reproducible indicator of disease activity and with a varied prevalence rate among different ethnicities, SPR remains the most diagnostically relevant lesion in BD patients (7). In clinical practice, SPR is induced by needle prick, with positive reactions manifesting as a papule or pustule developing by 48 h. Histologically, the SPR is characterized predominantly by macrophages and CD4^{+} T cells infiltrating the dermis, accompanied by strong HLA-DR staining of epidermal keratinocytes (8).

The histopathology of spontaneous papulopustular lesions in BD has been shown to be reproduced in the SPR (9). Thus, we hypothesized that study of immune processes triggered at the site of nonspecific trauma in BD patients may provide insights into the dysregulated immunity underlying other affected organ systems in these patients. Therefore, we compared the evolving inflammatory response to needle prick of the skin in BD patients with the response in normal healthy control subjects. We observed that by 48 h after needle prick, SPR[+] infiltrates were enriched for monocytes/dendritic cells (DCs) and T lymphocytes, including T regulatory cells (Tregs). This cellular response was associated with increased mRNA levels of IFN-γ and related signaling components and a corresponding increased expression of chemokines associated with Th1-type T cells and DCs.

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[4] Abbreviations used in this paper: BD, Behçet's disease; SPR, skin pathergy reaction; DC, dendritic cell; Tregs, T regulatory cell.

Materials and Methods

Patients, skin testing, and tissue samples

A total of 46 untreated BD patients (12 females, 34 males) fulfilling the International Study Group Criteria for BD (10) was enrolled into this study, along with 12 normal volunteers (4 females, 8 males). In each individual, an identical series of three skin wounds on each arm was made by oblique insertion of a 20-gauge needle into the dermis of skin (the needle insertion technique is similar to intradermal injection of PPD for tuberculosis skin testing). One skin wound site was then biopsied immediately, with a second wound site biopsied at 8 h. Biopsies were performed at 48 h on any of the remaining wound sites that developed a papular or pustular reaction characteristic of pathergy. The size of an induced pathergy reaction is small

(~3 mm), producing material sufficient only for either immunohistochemistry or RNA extraction from individual biopsy sites in a single patient. Hence, patients with BD were sequentially enrolled until 10 patients with positive SPRs at 48 h were accumulated, forming a group for analysis of cellular changes (study group 1). Patient enrollment was then continued until another group of 10 BD patients with positive SPRs at 48 h were accumulated for gene expression studies (study group 2). BD patients that failed to develop an SPR at 48 h, in general, did not have skin biopsies at this final time point. Normal controls for each of these two study groups consisted of normal volunteers receiving skin wounds and then undergoing sequential biopsies at each of the three time points. All skin biopsy samples were obtained with a 3-mm circular dermal punch after injection of 1% lidocaine solution into the hypodermis. Biopsy samples were either embedded in Tissue-Tek OCT Compound (Sakura Finetek) and frozen for immunohistochemistry processing (study group 1) or snap frozen directly in liquid nitrogen for mRNA extraction and RT-PCR analysis (study group 2). This protocol was approved by the Institutional Review Boards of Cerrahpasa Medical Faculty and The Rockefeller University.

The demographics and medical characteristics of enrolled BD patients were as follows. The mean age of BD patients was 30 years (range from 16 to 50) and the average disease duration was 4.5 years (range from 1 to 14). The mean age of the normal healthy controls was 34 years (range from 23 to 46). Of 46 patients, all had oral ulcerations, 31 had genital ulcers, 19 had erythema nodosum, 39 had papulopustular lesions, 13 had arthritis, 12 had uveitis, 6 had deep venous thrombosis, and 20 had a positive SPR. Consistent with previous published reports, there were no demographic or clinical differences discernible between BD patients with a positive or negative SPR in our study (11).

Immunohistochemistry processing and evaluation

OCT cryostat sections (6 μm) were air dried in a desiccator for 2 h, followed by acetone fixing for 2 min. After a brief PBS wash, sections were blocked for 30 min with 10% normal goat serum. Sections were subsequently incubated with primary mAbs or an isotype-matched control mAb and included mouse anti-human mAbs to CD1a, CD4 (Novocastra), CD25 (Serotec), CD11c, CD83, VCAM-1, ICAM-1, VLA-4 (BD Pharmingen), CD8, CD14, elastase (DAKO), and DC lysosome-associated membrane protein (Immunotech). For immunofluorescence imaging, Cy-3 coupled secondary mAbs were used (Jackson ImmunoResearch Laboratories). Cell nuclei were counterstained with Hoechst blue (Molecular Probes). Specimens processed for immunoperoxidase staining underwent an additional blocking step with 1% H_2O_2 in PBS for 15 min. Sequential incubations were performed with biotinylated goat anti-mouse IgG secondary mAb and HRP-conjugated avidin-biotin complexes (Vectastain ABC; Vector Laboratories).

An Olympus epifluorescence microscope, equipped with a CCD camera (Hamamatsu), was used for examination of the fluorescence stained sections. Six different digital images containing the most densely infiltrated areas were taken from each fluorescence or immunoperoxidase stained section using the ×20 objective. Quantification of histology and immunohistology was performed on digitally captured images using NIH Image 1.67 (⟨http://rsb.info.nih.gov/nih-image/⟩). Results are expressed as the mean number of positive stained cells for each section.

Real-time quantitative PCR

Total cellular RNA was isolated from the snap frozen tissue samples using a saw tooth rotor stator tissue homogenizer (PowerGen 700; Fisher Scientific) and the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Following treatment with 27 U of RNase-free DNase I (Qiagen), the RNA was eluted from the column with diethyl-pyrocarbonate-treated sterile water. The RNA concentration was determined by optical absorbance at 260 nm. RNA (50 ng) was then reverse transcribed and amplified by rTth DNA polymerase in a single tube assay using the TaqMan EZ RT-PCR kit (PerkinElmer Applied Biosystems) with gene-specific sense and antisense primers and a detection probe labeled on the 5′ end with the reporter dye 6-FAM. Primers and probes were designed using Primer Express software (PerkinElmer Applied Biosystems) and synthesized by PerkinElmer and have been previously described (12, 13). Primer pairs were chosen that crossed intron/exon borders whenever possible to avoid amplification of contaminating genomic DNA. Using an ABI Prism 7700 sequence detection system (PerkinElmer Applied Biosystems), duplicate samples were reversed transcribed and amplified under the following consecutive steps: 2 min at 50°C, 30 min at 60°C for reverse transcription, 5 min at 95°C for deactivation of uracil N-glycosylase (AmpErase; Applied Biosystems), followed by 40 amplification cycles of 15 s at 95°C, and 1 min at 60°C. Sequence-specific amplification was detected as an increased fluorescent signal of 6-FAM exceeding the threshold limit during

the amplification cycle. Quantification of gene-specific message levels was determined by comparing fluorescence intensity from unknown RNA samples to the fluorescence intensity of a standard curve generated from control mRNA levels. Amplification of the gene for human acidic ribosomal protein was performed on all samples tested to control for interspecimen variations in RNA amounts. The result for each gene was normalized to the quantity of human acidic ribosomal protein detected in the sample. Levels of gene-specific messages were graphed as normalized message units as determined from the standard curve. A no-template control was included in each amplification reaction to control for contaminating templates. For valid sample analysis the fluorescent intensity in the no-template control was required to be zero.

Statistical analysis

Data from this prospectively designed study are presented graphically as means plus SEM unless otherwise indicated. Nonparametric comparisons of differences between normal controls and BD patients were performed using SPSS software (version 11.0; SPSS) using the Wilcoxon signed-rank test and Mann-Whitney U test for paired and independent data, respectively. Significance is indicated at a value of $p \leq 0.05$.

Results

In this study we created a series of identical skin wounds in BD patients and in normal controls so that we could perform biopsies on individual lesions at different time points to characterize inflammatory cellular and genomic responses to injury. In BD patients, our analysis was focused mainly on patients who developed a positive SPR at 48 h. A limited number of specimens available from BD patients with a negative SPR were processed for mRNA analysis only, with results statistically similar to normal healthy controls with one exception as noted below.

Infiltrating leukocyte populations within the needle prick skin sites

Baseline skin biopsy specimens from all subjects at $t = 0$ h contained primarily T lymphocytes (Fig. 1). Few cells stained for the monocyte marker CD14 (Fig. 1C), whereas no cells expressed the
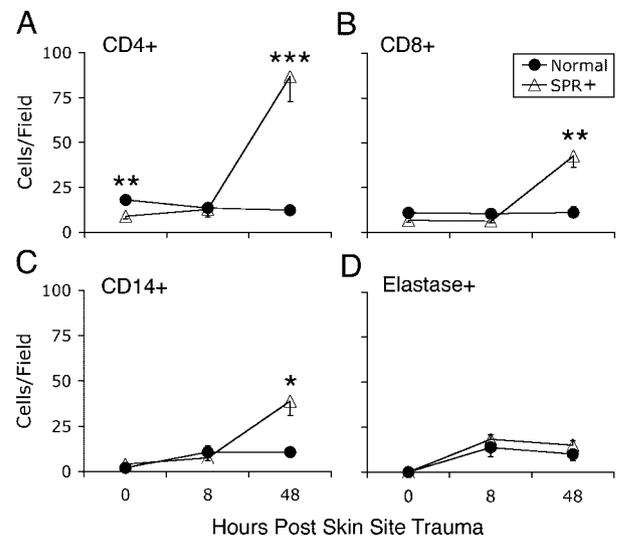
FIGURE 1. The effect of needle-induced trauma on skin-infiltrating leukocyte subsets. Serial skin biopsy specimens were from six normal healthy controls and 10 SPR$^+$ BD patients at 0, 8, and 48 h after needle prick. The frozen sections were stained for T lymphocytes expressing CD4 (A) or CD8 (B), for monocytes expressing CD14 (C), and for neutrophils expressing elastase (D) followed by Cy3-conjugated secondary Ab. Sections were digitally imaged by epifluorescence microscopy and counted using NIH Image software. Results are expressed as the mean number ± SEM of positively stained cells from the six most densely infiltrated areas analyzed at magnification ×200. *, $p = 0.01$; **, $p \leq 0.005$; ***, $p = 0.0001$ vs normal controls.
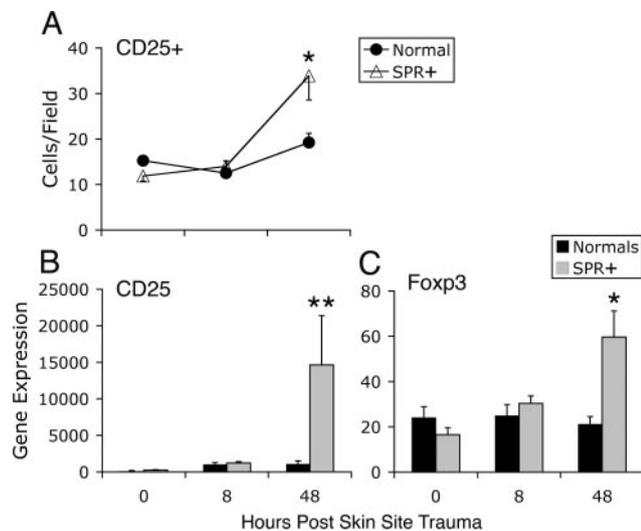
neutrophil marker elastase (Fig. 1D). There were significantly lower numbers of CD4+ staining cells in BD patient tissue sections compared with sections from normal controls, resulting in a CD4+ to CD8+ ratio of 1.3 vs 1.7, respectively (Fig. 1, A and B). By 8 h after needle prick of skin in both normal healthy subjects and BD patients, lymphocyte counts remained unchanged, whereas a large influx of neutrophils and monocytes was noted. Staining for lymphocytes, monocytes, and neutrophils remained unchanged between 8 and 48 h in skin of normal control subjects. Although neutrophil numbers in BD patient specimens also remained unchanged from 8 to 48 h, significant infiltrations of additional monocytes and lymphocytes were detected compared with that found in specimens of normal healthy controls.

Treg response to epithelial disruption

To further characterize the infiltrating lymphocytes, we stained sections for the IL-2Rα subunit (CD25) that is present on CD4+ Tregs (Fig. 2A). A significant increase in CD25+ staining cells was noted at 48 h in tissue sections from BD patients compared with sections from normal volunteers at 48 h or at earlier time points in either group. In skin biopsy specimens processed for mRNA analysis, significant increases at 48 h were also noted in CD25 mRNA expression in the SPR+ tissue sites from BD patients compared with sites from normal control specimens (Fig. 2B). Because CD25 is up-regulated on activated T lymphocytes, we measured mRNA expression of the more specific Treg marker Foxp3 (Fig. 2C). In skin specimens from normal volunteers, Foxp3 expression at later time points did not vary substantially from baseline levels. In contrast, progressively increased Foxp3 mRNA expression occurred in serial biopsy specimens from SPR+ subjects, suggesting augmented number of Tregs were infiltrating the inflamed SPR+ sites.

Activated DC infiltration of SPR+ sites at 48 h

We evaluated the infiltrating myeloid populations in our skin specimens for the presence of DCs. CD1a staining for Langerhans cells

was restricted to the epidermis and did not show any quantitative differences between groups at any time point (data not shown). CD11c+ cells in the dermis include both DCs and monocytes and were few in number at baseline in both groups (Fig. 3, A and D). By 8 h, increased dermal CD11c staining was detected in all subjects and was largely located in the perivascular regions of the dermis (Fig. 3, B and E). At 48 h, further increases in CD11c staining were observed only in the SPR+ biopsy sections from BD patients, while remaining relatively unchanged in skin sections from normal controls (Fig. 3, C and F, and graphed in G).

Chemokines play a key role in regulating the influx of inflammatory cells to skin in response to stimuli. The chemokine CCL2, also known as MCP-1, is produced by macrophages, DCs, endothelial cells, and fibroblasts in response to inflammatory stimuli and is an important chemoattractant for lymphocytes, monocytes, and DCs (14–16). Detectable skin expression of MCP-1 mRNA in response to needle prick was observed by 8 h in specimens from both groups with significantly higher MCP-1 mRNA expression noted in the BD patient-derived specimens compared with specimens from normal controls (Fig. 3H). At 48 h, MCP-1 mRNA levels had decreased to near baseline levels in normal control sites,
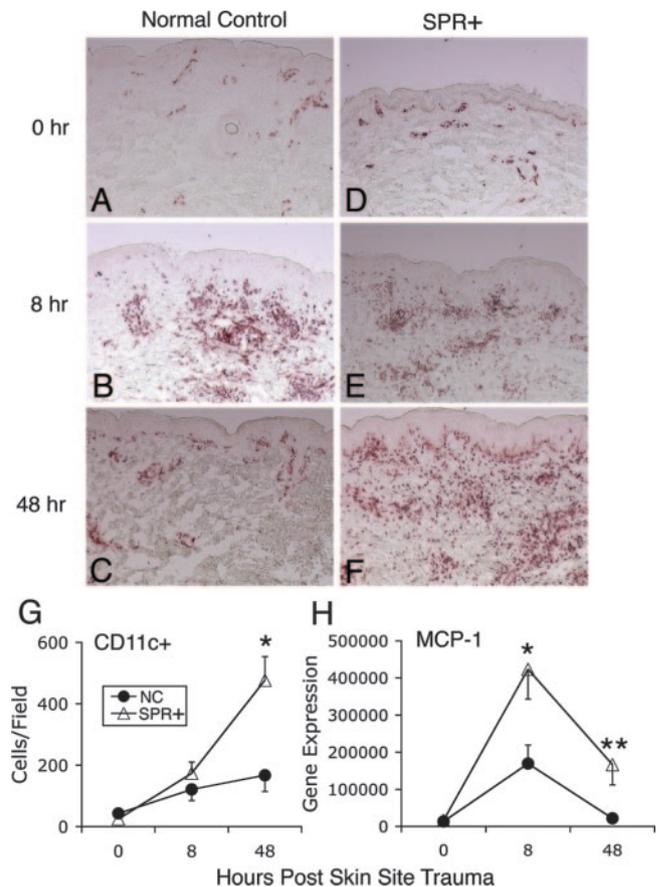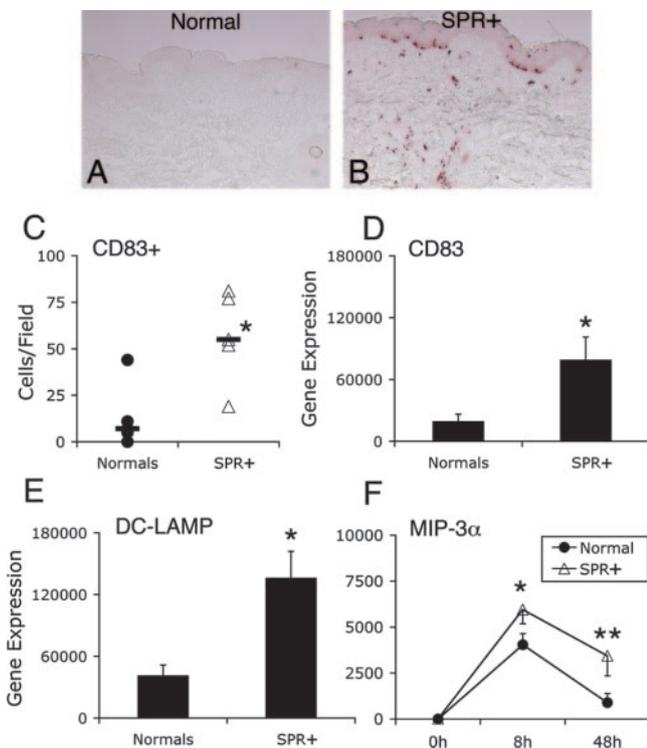

FIGURE 3. The effect of needle-induced trauma on skin-infiltrating CD11c+ mononuclear cells and dermal MCP-1 expression. CD11c+ staining by the Vectastain ABC immunoperoxidase method in representative, serially obtained skin biopsy specimens (t = 0, 8, and 48 h) from a normal healthy control (NC) (A–C) and from a SPR+ BD patient (D–F) is shown. Magnification, ×100. G, Quantification of CD11c+ staining at magnification ×100 is shown graphically for six normal healthy controls (NC) and eight SPR+ BD patients at 0, 8, and 48 h. H, Skin biopsy specimens from six normal controls and 10 SPR+ BD patients were processed for mRNA expression of MCP-1 using real-time quantitative RT-PCR, normalized to a housekeeping gene, and shown for t = 0, 8, and 48 h. Results are the mean ± SEM shown. *, p ≤ 0.04; **, p = 0.004.

FIGURE 2. The effect of needle skin trauma on dermal expression of CD25 and Foxp3. A, Tissue sections obtained serially from six normal healthy controls and 10 SPR+ BD patients at 0, 8, and 48 h were stained for the leukocyte activation marker CD25. Positive staining cells from each biopsy specimen were scored in six different ×200 fields and averaged. Skin biopsy specimens from a second group were processed for mRNA expression of CD25 (B) and Foxp3 (C) in normal healthy controls (■) and Behçet's patients (▨) using real-time quantitative RT-PCR, normalized to the housekeeping gene human acidic ribosomal protein. The average ± SEM is shown. *, p ≤ 0.03; **, p = 0.003 vs normal controls.

but remained elevated in the BD tissue specimens, corresponding to the CD14$^+$ (Fig. 1C) and CD11c$^+$ (Fig. 3, A–G) cellular infiltration patterns observed.

CD83 expression within the dermis is primarily due to activated DCs and was detected in stained sections by 48 h (Fig. 4, A–C), with significant increases seen in sections from SPR$^+$ sites over sections obtained from healthy controls. These large-appearing of CD83$^+$ cells were located primarily at the dermal-epidermal border (Fig. 4B) and thus suggestive of newly activated Langerhans cells migrating from the epidermis into the dermal layer. CD83 mRNA expression was also significantly increased in SPR$^+$ tissue specimens compared with biopsy specimens from normal controls at 48 h after needle prick-induced skin trauma (Fig. 4D). mRNA expression of another DC activation marker, DC lysosome-associated membrane protein, was similarly elevated at 48 h in SPR$^+$ biopsy specimens compared with specimens from normal control tissue (Fig. 4E).

Keratinocyte production of the chemokine CCL20 (MIP-3α) is up-regulated by inflammatory stimuli and is thought to play an important role in recruiting immature DCs and precursor DCs to the skin (17, 18). MIP-3α mRNA levels were detected in skin biopsy specimens by 8 h in both groups, with significantly higher expression found by 48 h in specimens from BD patients compared
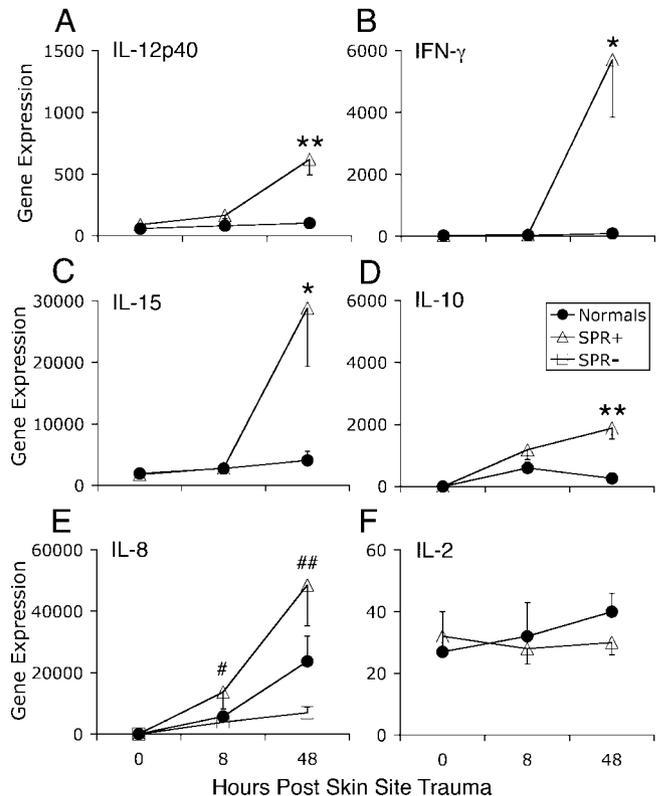
with levels found in specimens from normal control biopsies (Fig. 4F).

Up-regulation of Th1-associated cytokines in SPR$^+$ specimens at 48 h

We next used real-time RT-PCR analysis to evaluate cytokine expression in needle-pricked skin sites from BD patients and normal volunteers (Fig. 5). IL-12 is produced by activated DCs and promotes development of a Th1-type immune response as defined by activated T lymphocytes releasing IFN-γ (19). mRNA expressions of IL-12 p40 and IFN-γ were at low levels in baseline skin samples from normal controls and remained unchanged at 8 and 48 h post needle prick (Fig. 5, A and B). Although similar low levels of mRNA for IFN-γ and IL-12 p40 were also observed in BD skin specimens during the initial 8 h post needle prick, significantly increased mRNA expressions for both cytokines occurred by 48 h in SPR$^+$ sites.

IL-15 is produced in the skin by monocytes, macrophages, and keratinocytes and plays an important role in functional maturation of APCs and induction of a Th1-type response (20). IL-15 mRNA expression was limited during the initial 8 h of inflammatory response in all skin samples (Fig. 5C). At 48 h, IL-15 mRNA increased significantly in SPR$^+$ skin specimens while remaining low in normal control samples.

The Th2 cytokine IL-10 is produced by a wide range of cell types and is counterregulatory to Th1 immune responses. IL-10 mRNA was first detected at 8 h in the needle-pricked sites with

FIGURE 4. The effect of needle-induced trauma on skin-infiltrating DCs. CD83$^+$ staining by immunoperoxidase method in representative skin biopsy samples obtained 48 h after skin pin prick in a normal healthy control (A) and an SPR$^+$ BD patient (B). Magnification, ×100. C, Quantification of CD83$^+$ staining in 48 h tissue sections is shown graphically for five normal controls and six SPR$^+$ BD patients. Individual results and median values are shown. In skin biopsy samples obtained in parallel from six normal controls and 10 SPR$^+$ BD patients at 48 h, mRNA expression of CD83 (D) and DC lysosome-associated membrane protein (DC-LAMP) (E) was measured using real-time quantitative RT-PCR, normalized to a housekeeping gene. F, mRNA levels of MIP-3α are shown from serial skin biopsy samples obtained at 0, 8, and 48 h from six normal controls and 10 SPR$^+$ BD patients. Results are the mean ± SEM as shown. *, $p < 0.05$; **, $p = 0.007$ vs normal controls).

FIGURE 5. The effect of needle-induced trauma on skin mRNA expression of cytokines. mRNA expression of cytokines IL-12 p40 (A), IFN-γ (B), IL-15 (C), IL-10 (D), IL-8 (E), and IL-2 (F) from serial skin biopsy specimens obtained from six normal healthy volunteers, 10 patients with a positive SPR (SPR$^+$), and six patients with a negative SPR (SPR−) (IL-8 only) at baseline (0 h), 8, and 48 h after needle prick-induced skin trauma. Results are expressed as the mean ± SEM. *, $p \leq 0.02$; **, $p \leq 0.003$ vs normal controls; #, $p = 0.04$; ##, $p = 0.001$ vs SPR−.

significant up-regulation noted at 48 h in SPR$^+$ sites compared with normal control tissues (Fig. 5D). In addition, all skin samples showed mRNA up-regulation by 8 h for the potent neutrophil chemoattractant IL-8 (Fig. 5E), corresponding to observed elastase-positive neutrophil accumulation within skin infiltrates (Fig. 1D). Although additional expression of IL-8 mRNA was noted by 48 h, no significant differences were observed within or between normal controls and SPR$^+$ subjects. Interestingly, we found significantly lower IL-8 mRNA levels at 8 and 48 h in specimens from BD patients with negative SPR compared with patients with positive SPR (Fig. 5E). mRNA levels of the Th1 cytokine IL-2 (Fig. 5F) and the Th2 cytokine IL-4 (data not shown) were detected at low levels without significant differences within or between groups.
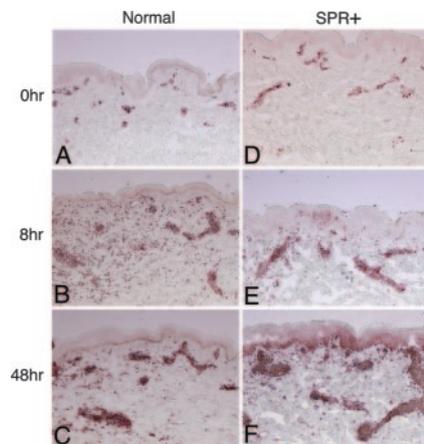
Because of high IFN-γ mRNA levels detected in SPR$^+$ skin specimens, we investigated downstream immune responses associated with IFN-γ, including IFN-γ-inducible protein (IP-10, CXCL10), monokine induced by IFN-γ (MiG, CXCL9), and IFN-γ-inducible chemoattractant (iTAC, CXCL11). These chemokines exert their action via CXCR3, which is highly expressed on activated T cells. We found that mRNA expressions of chemokines (IP-10, MiG, and iTAC) were low during the initial 8 h after skin trauma in both groups, with only SPR$^+$ sites at 48 h showing significant up-regulation of chemokine expression (Fig. 6). In addition, we also measured IFN-γ-induced transcription factors, including IFN regulatory factor-1 and STAT1 (Fig. 6). Significantly increased levels were noted of both transcription factors at 48 h in SPR$^+$ skin sites compared with levels found in normal control skin sites.

Adhesion molecule expression in the skin sites in response to needle prick

Vascular expression of adhesion molecules occurs in response to cytokine-mediated inflammation and is an important determinant of leukocyte migration into surrounding tissues. We examined the expression patterns of ICAM-1 and VCAM-1 in the developing SPR inflammation. Low levels of perivascular ICAM-1 staining at baseline in both groups became more diffuse by 8 h after skin trauma (Fig. 7, A and D, compared with B and E, respectively). At 48 h, ICAM-1 staining appeared to be resolving in the normal control tissues (Fig. 7C), whereas in the SPR$^+$ sites, ICAM-1 staining increased to include the keratinocytes of the lower epidermal layer (Fig. 7F). In contrast, staining for VCAM-1 and its ligand VLA-4 was observed only in sections from SPR$^+$ BD patients at 48 h (data not shown) and was not found in normal control specimens. Significantly increased VLA-4 mRNA expression was also noted at 48 h in eight SPR$^+$ tissue specimens compared with

FIGURE 7. Effect of needle skin trauma on dermal ICAM-1 expression. Representative serial skin biopsy specimens obtained from normal healthy controls (A–C) and in BD patients with positive SPR (SPR$^+$) (D–F) at 0, 8, and 48 h after needle-induced skin trauma. Sections are stained for the adhesion molecule ICAM-1 followed by visualization using the immunoperoxidase method and digitally recorded at magnification ×100.

specimens from six normal healthy volunteers (gene expression: normal controls 5,370 vs SPP+ 24,329; $p = 0.003$).

Discussion

The skin is an immunologic organ that is the first barrier to the outside environment. Any disruption of the skin's integrity results in a cascade of events to clear invading pathogens, repair damage, and restore homeostasis. We have previously shown clinically that BD patients have a normal healing rate after skin punch biopsy despite increased erythematous reactions (21). In this study comparing normal skin inflammatory responses to needle-induced trauma with the hyperreactive pathergy response occurring in BD patients, we found similar cellular infiltration patterns in normal volunteers and BD patients during the initial 8 h after needle prick. However, by 48 h post needle prick, infiltrating cell populations in our two patient groups differed significantly. Specimens from normal control biopsy sites showed minimal changes in infiltrating leukocytes numbers between 8 and 48 h, whereas specimens from SPR$^+$ sites of BD patients contained marked increases in lymphocytes, monocytes, and activated DCs and associated Th1-associated cytokines and chemokines during this period.

Our findings demonstrating an exaggerated Th1-type immune response in BD patients' SPR$^+$ sites are consistent with other published reports. In a study comparing cytokine and chemokine mRNA levels in BD lesional skin and pathergy sites with nontraumatized skin from normal volunteers, high mRNA levels of IL-8, IFN-γ, IL-12, IL-10, and MCP-1 were detected in BD specimens (3). In a separate study, high levels of IFN-γ, IL-12, and IL-18 were detected by immunohistochemistry techniques in skin lesions of BD patients with active disease, along with increased levels of Txk, a Th1-cell specific transcription factor (22). Our report differs from these past immunological studies of the pathergy reaction by its serial, comparative study of needle-prick skin trauma in both normal healthy controls and BD patients, thus providing the opportunity to explore in a step-wise manner how immune responses in BD patients diverge from the norm.

Studies in normal healthy volunteers or individuals undergoing routine surgery have shown that aseptic epithelial barrier disruption induces a limited inflammatory response, whereas mechanical injury without epithelial damage induces inflammatory cytokines without an accompanying cellular infiltration (23). Only persisting

FIGURE 6. IFN-γ-inducible chemokines and transcription factors in skin sites 48 h after needle-induced skin trauma. mRNA expression of chemokines (IP-10, MiG, and iTAC) and transcription factors (IFN regulatory factor-1 and STAT1) in skin biopsy specimens obtained at 48 h after needle prick from six normal volunteers and from SPR$^+$ sites on 10 BD patients. Results are expressed as the average ± SEM. All SPR values are significantly elevated compared with normal control values at $p \leq 0.003$ except iTAC at $p = 0.02$.

infection caused pronounced inflammatory cellular infiltrates in those otherwise healthy subjects. The delayed progressive inflammation in our SPR$^+$ BD patients 48 h after aseptic disruption of the epithelial barrier is in marked contrast to the responses observed in our normal controls and suggests either the presence in BD skin sites of a defective regulatory response or the continued presence of danger signals preventing normal early resolution of the inflammation.

Our finding that BD patients have reduced overall baseline numbers of CD4$^+$ T cells could contribute to dysregulation of their cutaneous immunity. Exaggerated inflammatory responses are associated with absence of CD4$^+$CD25$^+$ Tregs in animal models (24). However, we found that CD25$^+$ staining and mRNA expression for both CD25 and Foxp3 were increased significantly at the 48-h SPR$^+$ sites. Thus, Tregs are trafficking into the SPR$^+$ sites in BD patients, although our results do not reveal the functional level of these responding Tregs.

A counterregulatory Th2 response becomes evident in our specimens by 8 h post skin trauma, with detectable IL-10 levels noted in both groups; findings also shown by others in normal volunteers as early as 6 h (25). IL-10 has been described as an anti-inflammatory cytokine that counteracts Th1 immunity and decreases associated chemokine and endothelial adhesion molecule expression. Yet despite further increased IL-10 gene expression in 48-h SPR$^+$ sites, inflammatory indicators progressed in these specimens while remaining constant or decreasing in normal control skin, suggesting the possibility of either resistance of the responding inflammatory cells in the BD dermis to IL-10 modulatory effects or of persisting danger signals overriding IL-10 counterregulatory activity.

In both BD patients and normal controls, an early cellular event in immune responses to skin trauma was the similar infiltration by 8 h of elastase-positive neutrophils and myeloid cells bearing CD14 and CD11c, together with the simultaneous up-regulated expression of their potent chemoattractants, IL-8, MCP-1, and MIP-3α. This pattern of cell phenotype and chemokine expression in response to barrier disruption has been well defined in wound-healing studies. It shows that IL-8 and MCP-1 provide the first chemotactic gradient for the innate effector cell recruitment into traumatized skin sites, whereas MIP-3α is a potent chemokine for immature DC and Langerhans cell precursors (17, 18, 26). In our study, MCP-1 stands out among the early immunologic responses measured at 8 h for being significantly increased in BD-derived biopsy specimens compared with normal healthy skin specimens, with an increasing trend seen with MIP-3α. These findings are consistent with the subsequent increased CD11c$^+$ myeloid cell infiltration at 48 h in the SPR$^+$ sites, occurring with the appearance of mature CD83$^+$ dermal DCs.

The presence of activated dermal DCs within the SPR$^+$ site at 48 h also coincides with marked increases in lymphocyte numbers and IL-12 p40 levels. This finding may indicate the participation of antigenic material in the subsequent triggering of Th1-type responses in the SPR$^+$ sites as suggested by a report of TCR clonal expansion in dermal lymphocyte infiltrates from BD lesional tissues (27). In addition, it has also been shown that the SPR$^+$ incidence decreases in BD patients when performed on surgically cleaned skin (28). This finding suggests that a persisting danger signal within the damaged dermis, such as occurring from an innate immune failure to clear foreign Ag, leads to a compensatory cellular immune response similar to that expected in the normal skin response to active infection.

Alternatively, an underlying, primed immunity triggered by tissue injury may be responsible for causing a hyperreactive inflammatory response by DCs and lymphocytes. BD patients have been reported to have increased blood levels of inflammatory mediators, including IFN-γ, TNF-α, IL-2, IL-6, IL-12, IL-17, IL-18, MCP-1, vascular endothelial growth factor, and NO with IL-12, vascular endothelial growth factor, and soluble IL-2R levels related to their disease activity (5, 6, 29–31). However, other inflammatory disorders have high blood levels of inflammatory mediators without associated pathery. Perhaps instead, immune priming upon a constitutively abnormal leukocyte population is necessary for the pathergy response, as suggested by the appearance of SPR$^+$ in non-BD patients undergoing immune activating treatment with IFN-α for chronic myelogenous leukemia (32). Interestingly, other disease entities for which rare pathergy has also been described are lymphoproliferative disorders with underlying genetic abnormalities and include hairy cell leukemia and non-Hodgkin's lymphoma (33, 34).

In summary, this study is the first to directly compare the immunological responses over time to needle prick in normal, healthy skin with the hyperreactive responses occurring in BD patients. We show that, in contrast to the limited inflammation in normal controls to aseptically induced epithelial barrier damage, an exaggerated inflammatory response develops in the skin of BD patients between 8 and 48 h that is characterized by dermal infiltration of activated DCs and the presence of a Th1-type immunological cascade. Our findings of similar initial immune responses in BD patients and healthy normal controls to minor trauma, point potentially to either an inadequate innate immune response that provokes a compensating adaptive response, or insufficient regulatory mechanisms acting upon the adaptive response. Differences in the immunological response patterns demonstrated between our two study groups provide potential areas for further investigation in the pathogenic mechanisms underlying BD.

Acknowledgments

Disclosures

References

1. Suzuki Kurokawa, M., and N. Suzuki. 2004. Behçet's disease. *Clin. Exp. Med.* 4: 10–20.
2. Ben Ahmed, M., H. Houman, I. Ben Ghorbel, A. Braham-Sfaxi, M. Miled, K. Dellagi, and H. Louzir. 2003. Cytokine expression within mucocutaneous lesions of Behçet's disease: involvement of proinflammatory and Th1 cytokines. *Adv. Exp. Med. Biol.* 528: 343–346.
3. Ben Ahmed, M., H. Houman, M. Miled, K. Dellagi, and H. Louzir. 2004. Involvement of chemokines and Th1 cytokines in the pathogenesis of mucocutaneous lesions of Behçet's disease. *Arthritis Rheum.* 50: 2291–2295.
4. Frassanito, M. A., R. Dammacco, P. Cafforio, and F. Dammacco. 1999. Th1 polarization of the immune response in Behçet's disease: a putative pathogenetic role of interleukin-12. *Arthritis Rheum.* 42: 1967–1974.
5. Hamzaoui, K., A. Hamzaoui, F. Guemira, M. Bessioud, M. Hamza, and K. Ayed. 2002. Cytokine profile in Behçet's disease patients: relationship with disease activity. *Scand. J. Rheumatol.* 31: 205–210.
6. Turan, B., H. Gallati, H. Erdi, A. Gurler, B. A. Michel, and P. M. Villiger. 1997. Systemic levels of the T cell regulatory cytokines IL-10 and IL-12 in Behçet's disease: soluble TNFR-75 as a biological marker of disease activity. *J. Rheumatol.* 24: 128–132.
7. Ozarmagan, G., T. Saylan, G. Azizlerli, C. Ovul, and V. L. Aksungur. 1991. Re-evaluation of the pathergy test in Behçet's disease. *Acta Derm. Venereol.* 71: 75–76.
8. Gül, A., S. Esin, N. Dilsen, M. Konice, H. Wigzell, and P. Biberfeld. 1995. Immunohistology of skin pathergy reaction in Behçet's disease. *Br. J. Dermatol.* 132: 901–906.
9. Nazzaro, P. 1966. Cutaneous manifestations of Behçet's disease. In *International Symposium on Behçet's Disease, Rome*. M. Monacelli and P. Nazzaro, eds. Karger, New York, pp. 15–41.
10. International Study Group for Behçet's Disease. 1990. Criteria for diagnosis of Behçet's disease. *Lancet* 335: 1078–1080.
11. Krause, I., Y. Molad, M. Mitrani, and A. Weinberger. 2000. Pathergy reaction in Behçet's disease: lack of correlation with mucocutaneous manifestations and systemic disease expression. *Clin. Exp. Rheumatol.* 18: 71–74.
12. Chamian, F., M. A. Lowes, S. L. Lin, E. Lee, T. Kikuchi, P. Gilleaudeau, M. Sullivan-Whalen, I. Cardinale, A. Khatcherian, I. Novitskaya, et al. 2005.

Alefacept reduces infiltrating T cells, activated dendritic cells, and inflammatory genes in *Psoriasis vulgaris*. *Proc. Natl. Acad. Sci. USA* 102: 2075–2080.

13. Gottlieb, A. B., F. Chamian, S. Masud, I. Cardinale, M. V. Abello, M. A. Lowes, F. Chen, M. Magliocco, and J. G. Krueger. 2005. TNF inhibition rapidly downregulates multiple proinflammatory pathways in psoriasis plaques. *J. Immunol.* 175: 2721–2729.

14. Daly, C., and B. J. Rollins. 2003. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* 10: 247–257.

15. Nakamura, K., I. R. Williams, and T. S. Kupper. 1995. Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J. Invest. Dermatol.* 105: 635–643.

16. Xu, L. L., M. K. Warren, W. L. Rose, W. Gong, and J. M. Wang. 1996. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. *J. Leukocyte Biol.* 60: 365–371.

17. Dieu-Nosjean, M.-C., C. Massacrier, B. Homey, B. Vanbervliet, J.-J. Pin, A. Vicari, S. Lebecque, C. Dezutter-Dambuyant, D. Schmitt, A. Zlotnik, and C. Caux. 2000. Macrophage inflammatory protein 3α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* 192: 705–718.

18. Schutyser, E., S. Struyf, and J. Van Damme. 2003. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* 14: 409–426.

19. Puccetti, P., M. L. Belladonna, and U. Grohmann. 2002. Effects of IL-12 and IL-23 on antigen-presenting cells at the interface between innate and adaptive immunity. *Crit. Rev. Immunol.* 22: 373–390.

20. Alpdogan, O., and M. R. van den Brink. 2005. IL-7 and IL-15: therapeutic cytokines for immunodeficiency. *Trends Immunol.* 26: 56–64.

21. Mat, M. C., M. Nazarbaghi, Y. Tüzün, V. Hamuryudan, N. Hizli, S. Yurdakul, Y. Özyazgan, and H. Yazici. 1998. Wound healing in Behçet's syndrome. *Int. J. Dermatol.* 37: 120–123.

22. Nagafuchi, H., M. Takeno, H. Yoshikawa, M. S. Kurokawa, K. Nara, E. Takada, C. Masuda, M. Mizoguchi, and N. Suzuki. 2005. Excessive expression of Txk, a member of the Tec family of tyrosine kinases, contributes to excessive Th1 cytokine production by T lymphocytes in patients with Behçet's disease. *Clin. Exp. Immunol.* 139: 363–370.

23. Van Der Laan, N., L. F. de Leij, and H. J. ten Duis. 2001. Immunohistopathological appearance of three different types of injury in human skin. *Inflamm. Res.* 50: 350–356.

24. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.

25. Nickoloff, B. J., and Y. Naidu. 1994. Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. *J. Am. Acad. Dermatol.* 30: 535–346.

26. Engelhardt, E., A. Toksoy, M. Goebeler, S. Debus, E. B. Brocker, and R. Gillitzer. 1998. Chemokines IL-8, GROα, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am. J. Pathol.* 153: 1849–1860.

27. Esin, S., A. Gul, V. Hodara, M. Jeddi-Tehrani, N. Dilsen, M. Konice, R. Andersson, and H. Wigzell. 1997. Peripheral blood T cell expansions in patients with Behçet's disease. *Clin. Exp. Immunol.* 107: 520–527.

28. Fresko, I., H. Yazici, M. Bayramicli, S. Yurdakul, and C. Mat. 1993. Effect of surgical cleaning of the skin on the pathergy phenomenon in Behçet's syndrome. *Ann. Rheum. Dis.* 52: 619–620.

29. Akdeniz, N., M. Esrefoglu, M. S. Keles, A. Karakuzu, and M. Atasoy. 2004. Serum interleukin-2, interleukin-6, tumour necrosis factor-α and nitric oxide levels in patients with Behçet's disease. *Ann. Acad. Med. Singapore.* 33: 596–599.

30. Alpsoy, E., C. Cayirli, H. Er, and E. Yilmaz. 1998. The levels of plasma interleukin-2 and soluble interleukin-2R in Behçet's disease: a marker of disease activity. *J. Dermatol.* 25: 513–516.

31. Çekmen, M., C. Evereklioglu, H. Er, H. S. Inalöz, S. Doganay, Y. Türköz, and I. H. Özerol. 2003. Vascular endothelial growth factor levels are increased and associated with disease activity in patients with Behçet's syndrome. *Int. J. Dermatol.* 42: 870–875.

32. Budak-Alpdoğan, T., Demircay, Ö. Alpdoğan, H. Direskeneli, T. Ergun, A. Öztürk, A. Günay, S. Yavuz, N. Üskent, and T. Akoğlu. 1998. Skin hyperreactivity of Behçet's patients (pathergy reaction) is also positive in interferon alpha-treated chronic myeloid leukaemia patients, indicating similarly altered neutrophil functions in both disorders. *Br. J. Rheumatol.* 37: 1148–1151.

33. Burney, I. A., T. Moatter, T. Siddiqui, and N. Yaqoob. 2003. Pathergy in non-Hodgkin lymphoma. *J. R. Soc. Med.* 96: 127–129.

34. Kaplan, R. P., G. Newman, and D. Saperia. 1987. Pyoderma gangrenosum and hairy cell leukemia. *J. Dermatol. Surg. Oncol.* 13: 1029–1031.