Charaterization of the Divergent Wound-Healing Responses Occurring in the Pathergy Reaction and Normal Healthy Volunteers

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Behçet’s disease (BD) 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.

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...
<p>(~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 Cerrapahsa Medical Faculty and The Rockefeller University.</p>
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 chemotactic signal 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,
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 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 48 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 ≤ 0.02; **, p ≤ 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) and transcription factors (IFN regulatory factor-1 and STAT1) in skin biopsy specimens obtained at 48 h after needle trauma in both groups, with only SPR⁻ sites 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 specimens from six normal healthy volunteers (gene expression: normal controls 5,370 vs SPR⁺ 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 Tsk, a Th1-cell specific transcription factor (22).

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 = 0.003 except iTAC at p = 0.02.

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.
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 MIP3-α 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 pathergy. 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.

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Disclosures
The authors have no financial conflict of interest.

References


