Classic Ulcerative Pyoderma Gangrenosum Is A T Cell Mediated Disease Targeting Follicular Adnexal Structures: A Hypothesis Based On Molecular And Clinicopathologic Studies

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INTRODUCTION

Classic pyoderma gangrenosum (PG) is an ulcerative neutrophilic dermatosis that is the most common skin disease associated with inflammatory bowel disease (IBD). Diagnosis of PG is extremely challenging and treatment options are limited. Although it is most commonly thought to be a neutrophilic dermatosis, PG pathophysiology is actually poorly understood. The dominant hypothesis is that altered innate immunity leads to systemic autoinflammation (Ahronowitz et al., 2012).

An alternative view is that T cells are involved in PG pathophysiology (Antiga et al.; Doria et al., 2012), yet there are no current theories on autoimmune targets. Herein we attempt to gain insight into the pathophysiology of PG by characterizing the cellular and molecular events prior to ulcer formation and after ulcer healing.

MATERIALS & METHODS

Ten patients with history of classic ulcerative PG were included. All ten patients were asked about patterns of ulcer formation (Figure 1). Four PG patients with well-controlled disease underwent punch biopsies of PG scars and adjacent normal skin ("normal skin") for immunohistochemical analyses. Hypertrophic scars from healthy patients were used as an additional control ("control scar"). Scars from patients with biopsy-proven discoid lupus were also obtained to additionally control for post-inflammatory changes ("discoid scar").

Diagnosis of PG was verified by more than one board-certified dermatologist based on clinical history, physical examination findings, and biopsy findings.

Immunohistochemistry: Skin biopsies were paraffin-embedded, and 5 µm sections were stained. Biopsies were stained with H&E and Masson's trichrome. Giemsa stain was used to assess prevalence of mast cells. Immunohistochemical stains were performed with antibodies directed against markers listed in Figure 2. Differences in histological score are shown in Figure 3.

Gene expression analysis and qRT-PCR array: Differentially expressed genes of immunologic significance were identified in early pre-ulcerative PG papules versus normal skin by quantitative real-time PCR (qRT-PCR) array. Tissue samples were stabilized by RNAlater addition. Total RNA was extracted using RNeasy plus mini kit and the quantity and integrity of RNA was determined by fluorometry and 2200 TapeStation, respectively. Total RNA was reverse transcribed to cDNA using iScript (Bio-Rad) and qRT-PCR was performed using customized PrimePCR plates from Bio-Rad with GAPDH, TBP1 and HPRT1 as reference genes following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines. Using a custom qRT-PCR array with validated primer sets, SsoAdvanced Universal SYBR Green Supermix, and the CFX96 Touch Real-Time PCR Detection System, differential gene expression analysis and corresponding statistical analysis was performed.

Statistical Analysis: Differences in histological scores were assessed with one-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests. SigmaStat 4.0 was used to perform analyses. Bio-Rad CFX Manager was used to perform differential cytokine gene expression analysis and corresponding statistical analysis (Bio-Rad Laboratories, Hercules, CA).

RESULTS

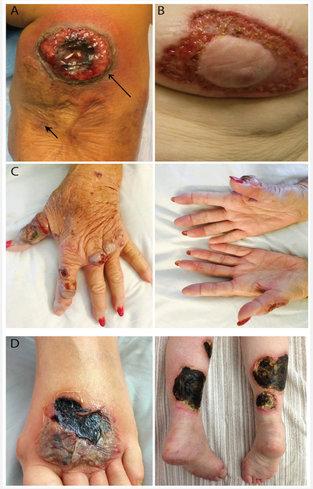

Figure 1. Patterns of ulcer formation in pyoderma gangrenosum. (A) PG scars do not re-ulcerate. Biopsy of a PG scar (short arrow) healed without incident, while biopsy of adjacent normal skin resulted in new ulcer (long arrow). (B) PG ulcers avoid the nipple-areolar complex, an area known to be devoid of pilosebaceous units. (C) Hand PG appears to affect only the dorsal surface of the hands. (D PG appears to spare the soles of the feet.

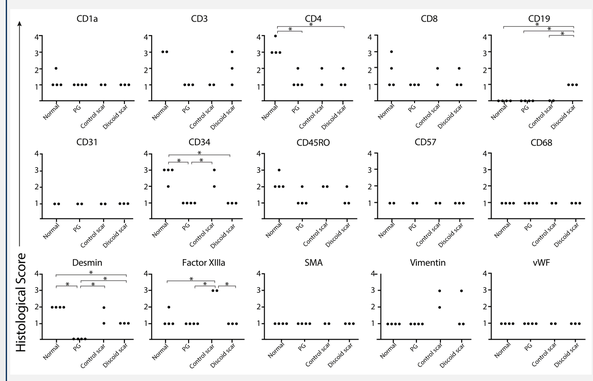

Figure 3. Pyoderma gangrenosum, scoring diagram. A blinded dermatopathologist scored each slide [1-10 positive cells/high powered field(HPF)=1, 11-20 positive cells/HPF=2, 21-30 positive cells/HPF=3 and >30 positive cells/HPF=4]. There is significantly less CD4+ ($p = 0.003$) and CD34+-expressing cells ($p \leq 0.001$) and undetectable desmin ($p \leq 0.001$) in a healed PG scar compared to the adjacent normal skin. Differences in histological scores between normal skin vs. PG scar vs. control scar vs. discoid scar were assessed with one-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests. Bonferroni error correction for multiple comparisons was calculated by dividing an alpha of 0.05 by the number of comparisons (n=6). SigmaStat 4.0 was used to perform statistical analyses on the histological scores.

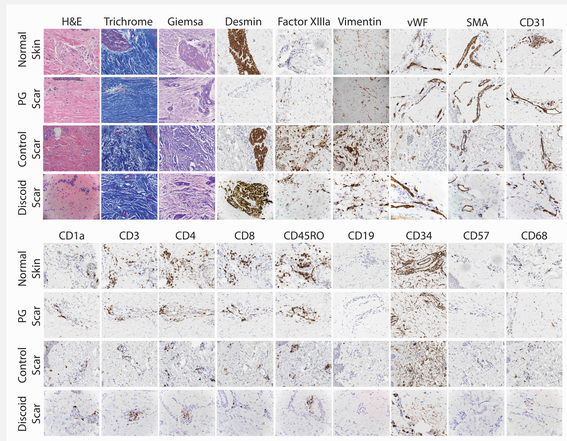

Figure 2. Detailed histological analysis of PG scars (n=4), adjacent normal skin, control scars, and discoid scars. Under 20x magnification, there was no significant difference between PG scars, normal adjacent skin, and discoid scars in the prevalence of mast cells (Giemsa), fibroblasts (vimentin), myofibroblasts (SMA), endothelial cells (CD31, vWF), macrophages (CD68), T cells (CD3), cytotoxic T cells (CD8), memory T cells (CD45RO), and NK cells (CD57). Adnexal structures are absent in PG scars (desmin) but present in normal adjacent skin, control scars, and discoid scars.

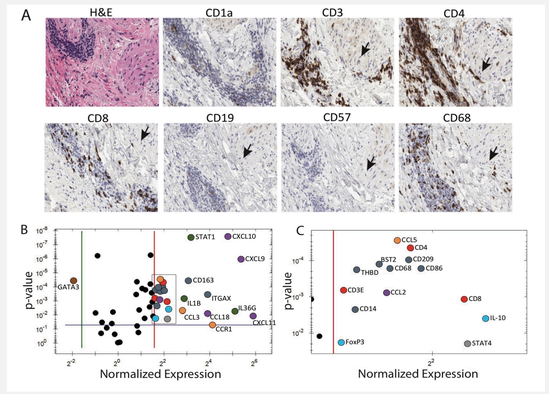

Figure 4. Pyoderma gangrenosum immunohistochemistry and gene expression analysis. (A) There is dense perivascular CD3+, CD4+ and to a lesser extent CD8+ infiltration. The close proximity to a hair follicle is evident by the arrector pili muscle (arrow). (B) Gene expression comparing cytokines in an early PG papule versus healthy skin. Among the 80 genes studied, the PG papule showed significant ($p \leq 0.05$) 26 upregulation and 1 downregulation genes compared to healthy skin. Genes that are significantly upregulated in PG are subgrouped based on its functions: antigen presenting cells- dark blue, T cells- red, IL-1 family (including IL-36G)- green , T cell chemoattractants- purple, regulatory T cells- light blue, neutrophil recruitment- orange, and Th1- grey. The top 4 up-regulated gene(s) include CXCL9, CXCL10, CXCL11 and IL36G. IL-8, and IL-17, were also strongly expressed in PG papules, but were undetectable in healthy normal skin, and therefore the ratio cannot be depicted in this diagram. IFNG was also detected in PG papules but absent in normal skin. (C) Expanded view of boxed area of Figure B. Bio-Rad CFX Manager was used to perform differential cytokine gene expression analysis and corresponding statistical analysis.

CONCLUSIONS

All PG patients reported that healed sites of previous ulceration are refractory to re-ulceration. Simultaneous biopsies of healed and uninvolved skin triggered ulceration only in the latter. Healed PG scars and body areas devoid of follicular adnexal structures are resistant to development of PG ulcers.

On immunohistochemistry, healed PG scars showed complete loss of pilosebaceous units, which were present in normal skin, and to a lesser extent in control scars, and discoid scars.

Early PG papules showed perivascular and peripilosebacceous T cell infiltrates, rather than neutrophils. These early inflammatory events were dominated by increased gene expression of *CXCL9, CXCL10, CXCL11, IL-8, IL-17, IFNG,* and *IL-36G*; and transcription factors consistent with Th1 phenotype.

PG results from aberrant cytokine expression and autoreactive T cells directed against follicular adnexal structures.

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