

A Single Intradermal Injection of IFN- γ Induces an Inflammatory State in Both Non-Lesional Psoriatic and Healthy Skin

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Psoriasis is a chronic, debilitating, immune-mediated inflammatory skin disease. As IFN- γ is involved in many cellular processes, including activation of dendritic cells (DCs), antigen processing and presentation, cell adhesion and trafficking, and cytokine and chemokine production, IFN- γ -producing Th1 cells were proposed to be integral to the pathogenesis of psoriasis. Recently, IFN- γ was shown to enhance IL-23 and IL-1 production by DCs and subsequently induce Th17 cells, which are important contributors to the inflammatory cascade in psoriatic lesions. To determine whether IFN- γ indeed induces the pathways expressed in psoriatic lesions, a single intradermal injection of IFN- γ was administered to an area of clinically normal, non-lesional (NL) skin of psoriasis patients and biopsies were collected 24 hours later. Although there were no visible changes in the skin, IFN- γ induced many molecular and histological features characteristic of psoriatic lesions. IFN- γ increased a number of differentially expressed genes in the skin, including many chemokines concomitant with an influx of T cells and inflammatory DCs. Furthermore, inflammatory DC products tumor necrosis factor (TNF), inducible nitric oxide synthase, IL-23, and TNF-related apoptosis-inducing ligand were present in IFN- γ -treated skin. Thus, IFN- γ , which is significantly elevated in NL skin compared with healthy skin, appears to be a key pathogenic cytokine that can induce many features of the inflammatory cascade of psoriasis.

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INTRODUCTION

Psoriasis is an inflammatory skin disease affecting ~2% of the population of the United States, and is considered one of the most prevalent cell-mediated inflammatory diseases (Lebwohl, 2003). Psoriatic lesions are typically erythematous scaly plaques, and patients are classified based on the severity of their disease, with a gradient ranging from mild to moderate-to-severe based on the body surface area involved. Psoriatic lesions are characterized by increased infiltration of both activated T cells and dendritic cells (DCs) compared with normal or uninvolved (non-lesional, NL) psoriatic skin (Lowes *et al.*, 2007). Through the use of biological agents and

therapeutic treatments, many immune pathways involved in psoriasis are being elucidated (Nograla *et al.*, 2010).

Historically, psoriasis was considered to be a predominantly type-1, IFN- γ -mediated disease, based on the significant IFN- γ genomic signature in lesional skin (LS; Lew *et al.*, 2004; Suarez-Farinas *et al.*, 2010). Flow cytometric analysis revealed overexpression of IFN- γ in both circulating T cells and those isolated from LS (Austin *et al.*, 1999). In recent years, focus has shifted to the role of IL-17 and the contributions of Th17 cells to the pathogenesis of autoimmunity. Although IFN- γ inhibits the production of IL-17 by T cells *in vitro* (Harrington *et al.*, 2005; Park *et al.*, 2005), Th17 cells cultured with IL-12 are able to produce IFN- γ (Annunziato *et al.*, 2007). The impact of the coproduction of IL-17 and IFN- γ has recently been underscored in a murine model of diabetes, where Th17 cells become more pathogenic once they acquired the ability to produce IFN- γ (Bending *et al.*, 2009; Martin-Orozco *et al.*, 2009). In human autoimmune disease, Th1 and Th17 cells often coexist (Aggarwal *et al.*, 2003; Murphy *et al.*, 2003; Langrish *et al.*, 2005; Annunziato *et al.*, 2007). Our group has identified a population of T cells that coproduce IFN- γ and IL-17 in psoriatic lesions (Lowes *et al.*, 2008), induced by DCs isolated from the dermis of psoriatic lesions, but not by DCs from healthy skin (Zaba *et al.*, 2009a). This difference in

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Abbreviations: DC, dendritic cell; DEGs, differentially expressed genes; iNOS, inducible nitric oxide synthase; LS, lesional skin; NL, non-lesional skin; RT-PCR, real-time PCR; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

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immunostimulatory potential has been attributed, in part, to the presence of an additional population of CD11c⁺CD1c⁻ inflammatory DCs that are found in psoriatic, but not healthy skin (Zaba *et al.*, 2009a). *In situ*, inflammatory DCs express IL-23 and IL-12, which may potentially drive Th1/Th17 cell responses (Lowes *et al.*, 2005; Guttman-Yassky *et al.*, 2008). However, the initial stimulus for this cascade *in situ* is currently unknown. Recent studies have implicated IFN- γ as a possible contributor, as it can program myeloid DCs to produce IL-23 and IL-1, promoting IL-17 production by memory T cells (Kryczek *et al.*, 2008). Thus, the contributions of IFN- γ to the pathogenesis of psoriasis are now being re-evaluated.

Before the discovery that IFN- γ has a role in autoimmunity, IFN- γ was used as therapy for several diseases including rheumatoid arthritis, psoriatic arthritis, psoriasis, and lepromatous leprosy infections (Morhenn *et al.*, 1987; Cannon *et al.*, 1989; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Veys *et al.*, 1997). Regular cutaneous injections of IFN- γ had some efficacy in the treatment of these diseases; however, a subset of patients developed focal skin reactions at the site of injection (Morhenn *et al.*, 1987; Cannon *et al.*, 1989; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Veys *et al.*, 1997). Injection site reactions (often assessed several weeks after starting IFN- γ) were characterized by induration, erythema, epithelial thickening, increased expression of HLA-DR, and accumulation of T cells and monocytes in the dermis (Nathan *et al.*, 1986; Morhenn *et al.*, 1987; Barker *et al.*, 1989, 1990; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990). These studies suggested that prolonged exposure to IFN- γ might initiate changes in the skin that are consistent with a psoriatic phenotype, and thus IFN- γ may be involved in the initial development of psoriatic lesions.

To address the role of IFN- γ in the induction of the inflammatory cascade in psoriasis and to identify the early underlying changes that occur before the development of a visible lesion, we analyzed responses to a single intradermal injection of IFN- γ at an early 24-hour time point in psoriasis patients with mild disease (<10% body surface area) and healthy volunteers. In psoriasis patients, IFN- γ injections were administered to an area of NL skin, which allowed the early genomic and histological IFN- γ responses to be determined. Although there were no visible changes in the skin, IFN- γ induced a genomic signature and cellular infiltration pattern that was similar to lesional psoriatic skin. Many inflammatory cytokines and chemokines were upregulated concurrent with increases in multiple dermal cell populations, including CD3⁺ T cells and CD11c⁺ DCs. Notably, inflammatory DCs (CD11c⁺CD1c⁻) were increased in the skin after IFN- γ injection, in addition to enhanced expression of their inflammatory products—tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS), IL-23p19, and TNF-related apoptosis-inducing ligand (TRAIL). Furthermore, Ingenuity Pathway Analysis identified pathways involved in lymphoid tissue development that were enriched in the transcriptome of the skin after IFN- γ injection. IFN- γ induced the lymphoid-organizing chemokine, CCL19,

and associated organization of T cells and DC clusters, suggesting that IFN- γ may induce lymphoid tissue neogenesis before the development of visible lesions. Thus, this *in vivo* study suggests that IFN- γ can prime an inflammatory environment in the skin that has many features of psoriasis.

RESULTS

A single intradermal injection of IFN- γ was biologically active in the skin

To confirm that the injected IFN- γ was biologically active, we assessed HLA-DR, a protein known to be upregulated by IFN- γ after intradermal injection (Nathan *et al.*, 1986, 1990). There were increased HLA-DR⁺ cells in both normal and NL skin after injection of IFN- γ , but not in the placebo control, indicating that the IFN- γ was functional (Supplementary Figure S1 online). Clinically, a single injection of IFN- γ did not induce the visible development of a psoriasis-like lesion at the site of injection, as had been seen after a month of repeated IFN- γ injections (Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990). Histologically, epidermal thickness was not altered by IFN- γ injection in either group (Supplementary Figure S2a online), and expression of keratin 16, a measure of altered keratinocyte differentiation, was not induced (Supplementary Figure S2b and c online). However, there were marked genomic and cellular changes induced by IFN- γ in both the groups.

IFN- γ -induced genomic changes in the skin consistent with a psoriatic phenotype

Microarray analysis was performed to compare the IFN- γ response in healthy and psoriatic skin using fold change >2 and false discovery rate <0.01. The 'IFN- γ response' was defined as the differentially expressed genes (DEGs) in IFN- γ -treated skin compared with placebo for psoriasis patients and healthy individuals. Using this definition, 706 unique DEGs (964 probe sets) were upregulated and 547 DEGs (708 probe sets) were downregulated in healthy IFN- γ -treated skin. There were 775 upregulated DEGs (1055 probe sets) and 719 downregulated DEGs (900 probe sets) in IFN- γ -treated psoriatic skin (Supplementary Table S1 online). Although the IFN- γ response (fold change) of many genes was higher in psoriasis than in healthy skin, these differences were not statistically significant after correcting for multiple hypotheses with the Benjamini-Hochberg procedure. In addition, there was very strong correlation in the response to IFN- γ in both the groups ($r=0.96$; Supplementary Figure S3 online), further supporting that there was no difference in IFN- γ -induced gene expression between healthy controls and psoriasis patients. Therefore, hereafter, we present data mostly from psoriatic NL skin.

IFN- γ induced the expression of numerous genes in the skin that are known to contribute to inflammation and that have been implicated in the pathogenesis of psoriasis. A selected list of inflammatory DEGs that are upregulated in psoriasis (Suarez-Farinas *et al.*, 2010), as well as after IFN- γ injection, is presented in Table 1. This list included cytokines and chemokines, as well as other inflammatory products. Expression of select known IFN- γ -induced genes (CXCL9,

Table 1. Microarray fold change values of select inflammatory genes upregulated in skin after IFN- γ injection compared with placebo

Symbol	Description	IFN- γ response (fold change) ¹	
		Psoriasis	Healthy
CXCL11	Chemokine (C-X-C motif) ligand 11	161.89	57.09
CXCL9	Chemokine (C-X-C motif) ligand 9	128.78	29.87
CCL8	Chemokine (C-C motif) ligand 8	126.85	41.36
CXCL10	Chemokine (C-X-C motif) ligand 10	71.38	25.86
CCR1	Chemokine (C-C motif) receptor 1	23.10	7.78
CX3CL1	Chemokine (C-X3-C motif) ligand 1	19.81	12.18
CCL2	Chemokine (C-C motif) ligand 2	12.00	7.09
CCR2	Chemokine (C-C motif) receptor 2	9.54	4.23
CCL5	Chemokine (C-C motif) ligand 5	8.16	5.47
CCL13	Chemokine (C-C motif) ligand 13	5.58	3.66
CXCR4	Chemokine (C-X-C motif) receptor 4	5.27	1.40
CCL7	Chemokine (C-C motif) ligand 7	4.09	2.00
CCR5	Chemokine (C-C motif) receptor 5	3.45	2.37
CCL4	Chemokine (C-C motif) ligand 4	3.43	1.43
CCL19	Chemokine (C-C motif) ligand 19	2.55	3.78
IL12RB2	Interleukin 12 receptor, beta 2	37.54	14.41
STAT1	Signal transducer and activator of transcription 1	9.02	4.07
TNF	Tumor necrosis factor	7.09	3.09
LTb	Lymphotoxin beta (TNF superfamily, member 3)	4.24	4.15
IL6	Interleukin 6	4.13	1.66
IL7R	Interleukin 7 receptor	3.26	2.9
IL2RG	Interleukin 2 receptor, gamma	4.76	2.3
IL1B	Interleukin 1, beta	2.14	1.61
S100A9	S100 calcium-binding protein A9	11.96	10.92
TLR2	Toll-like receptor 2	9.89	5.49
TLR1	Toll-like receptor 1	8.50	4.85
TNFSF10	TNF superfamily, member 10 (TRAIL)	5.73	4.35
S100A8	S100 calcium-binding protein A8	4.80	5.02
S100A12	S100 calcium-binding protein A12	4.08	2.27
S100A7	S100 calcium-binding protein A7	3.47	5.23
ICAM1	Intracellular adhesion molecule 1	18.61	8.68
VCAM1	Vascular cell adhesion molecule 1	6.31	5.16

Abbreviations: FCH, fold change; FDR, false discovery rate; TRAIL, TNF-related apoptosis-inducing ligand.

¹Although the IFN- γ response of many genes was higher in psoriatic than in healthy skin, these differences were not statistically significant (FCH range=0.25-3.63, FDR=0.999 for all genes).

Color code: blue=chemokines, yellow=cytokines/transcription factors, orange=inflammatory molecules, and green=adhesion molecules.

CXCL10, CCL2, and CX3CL1) was verified by real-time PCR (RT-PCR; Figure 1). Additional DEGs were confirmed by RT-PCR, and the IFN- γ response measured by RT-PCR was strongly correlated with the microarray data (Supplementary Figure S4 online). Furthermore, using gene set enrichment analysis, four published psoriasis gene sets (Suarez-Farinas *et al.*, 2010) correlated with the IFN- γ response in psoriasis

patients (Supplementary Table S2 online), indicating that IFN- γ can induce many genes that are present in psoriasis. Ingenuity Pathway Analysis was also used to identify the statistically significant biological functions represented by these IFN- γ -induced DEGs (Supplementary Table S2 online). The top functions represented by the upregulated DEGs after IFN- γ injection were consistent with the pathways involved

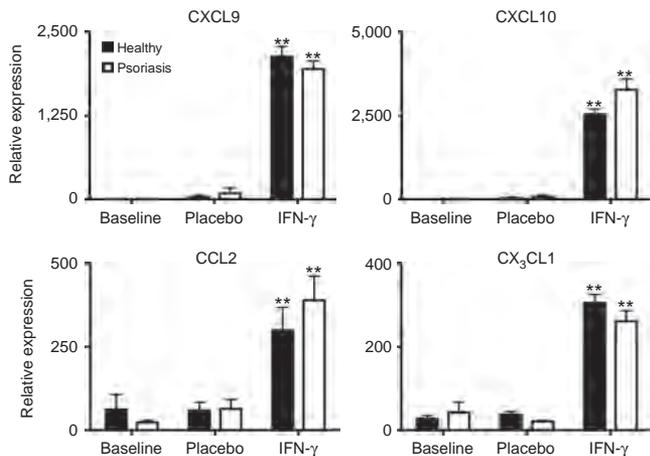


Figure 1. IFN- γ injection induces genomic expression of known IFN- γ -regulated chemokines. mRNA expression levels normalized to human acidic ribosomal protein (hARP) for the T-cell (CXCL9 and CXCL10) and dendritic cell (DC; CCL2 and CX₃CL1) chemokines in baseline, placebo, and IFN- γ -injected healthy (black bars, $n = 10$) and psoriatic skin (white bars, $n = 10$). Error bars represent the mean \pm SEM. IFN- γ -injected skin was compared with placebo. ** $P < 0.01$.

in psoriasis, including inflammatory response, inflammatory disease, cellular movement and development, immune cell trafficking, and cell-mediated immune responses. Thus, the IFN- γ response in skin reflects an inflammatory molecular phenotype.

IFN- γ -induced T cells and inflammatory myeloid DCs infiltration into the skin

Given the upregulation of many chemokines by IFN- γ , we evaluated the cellular infiltrates induced by IFN- γ in the skin. Previous studies have found an increased number of T cells in the skin 3 days after repeated IFN- γ injections (Nathan *et al.*, 1986, 1990). Here, we showed that even 24 hours post IFN- γ injection, there was a significantly increased number of CD3⁺ T cells in the skin (Supplementary Figure S5 online).

DCs and their inflammatory products have also been implicated in the pathogenesis of psoriasis. Thus, we sought to determine whether IFN- γ induced the infiltration of DCs into skin. We have previously classified CD11c⁺CD1c⁺ DCs in normal skin as 'resident' DCs. In psoriatic LS, an additional population of inflammatory DCs, which express CD11c, but not CD1c, are present (Zaba *et al.*, 2009a). IFN- γ induced greater numbers of CD11c⁺ DCs in the skin, whereas CD1c⁺ DCs remained constant throughout all the samples analyzed (Figure 2a and b). As there is no exclusive marker for inflammatory DCs, an approximation of their numbers can be calculated by subtracting the number of CD1c⁺ DCs from the number CD11c⁺ cells (Johnson-Huang *et al.*, 2010). Figure 2c shows significantly increased numbers of CD11c⁺CD1c⁻ inflammatory DCs in both normal and NL skin only after IFN- γ injection. To further analyze these cells, we performed two-color immunofluorescence with antibodies against CD11c and CD1c. Although the majority of CD11c⁺ cells in the placebo-treated skin co-expressed

CD1c (yellow cells), a large proportion of CD11c⁺ cells in IFN- γ -treated skin were CD1c negative (Figure 2d), confirming the presence of inflammatory DCs. We have previously generated a list of DEGs specific for psoriatic inflammatory CD1c⁻ DCs (Zaba *et al.*, 2010). Using gene set enrichment analysis, the inflammatory CD1c⁻ DC DEGs were highly enriched in the IFN- γ response genes (Supplementary Table S2 online; enrichment score = 0.38, normalized enrichment score = 1.8, $P < 0.0001$, for both the groups). Thus, IFN- γ can induce the accumulation of inflammatory DCs, both at the cellular and genomic levels, even in normal skin where they are typically absent.

IFN- γ -induced products of inflammatory myeloid DCs in the skin

Inflammatory DCs in psoriasis have been previously shown to produce inflammatory mediators, including TNF, iNOS, IL-12/23p40, and IL-23p19, and express TNFSF10/TRAIL—a putative new marker of inflammatory DCs (Lowe *et al.*, 2005; Zaba *et al.*, 2007, 2010; Guttman-Yassky *et al.*, 2008). To determine whether the DCs induced by IFN- γ could be functioning as inflammatory DCs, we assessed the presence of these inflammatory products. Gene expression of TNFSF10/TRAIL is elevated by IFN- γ (Table 1), and, similarly, immunofluorescent labeling identified many CD11c⁺ cells in the skin co-express TRAIL⁺ after IFN- γ injection (Figure 3a). In comparison with baseline and placebo controls, which had minimal TNF and iNOS staining, co-expression of these mediators with CD11c⁺ DCs was increased in IFN- γ -treated skin (Figure 3b and c). In addition, by using RT-PCR, we detected a significant increase in IL-23p19 messenger RNA (mRNA) expression in the skin after IFN- γ injection, and a trend toward increased IL-12p35 and IL-12/23p40 (Figure 3d). These data are in line with a recent study that described a role of IFN- γ in priming DCs *ex vivo* to produce IL-23 (Kryczek *et al.*, 2008). This *in vitro* priming stimulated IL-17 production by T cells, and thus we assessed expression of IL17A mRNA in the skin after IFN- γ injection by RT-PCR. Despite increased levels of IL-23 after IFN- γ injection, we did not find mRNA expression of IL17, or two IL-17-regulated genes *CCL20* and *DEFB4*, in the skin (data not shown). As biopsies were taken only 24 hours after the injection of IFN- γ , there may not have been sufficient time to induce a Th17 cell response in NL skin. Taken together, these findings further suggest that IFN- γ primes an inflammatory environment.

Molecules involved in ectopic lymphoid neogenesis were induced by IFN- γ

During chronic inflammation, ectopic lymphoid tissue develops in the peripheral non-lymphoid organs (Aloisi and Pujol-Borrell, 2006). Previously, on the basis of analysis of upregulated DEGs, the existence of these structures within psoriatic LS skin has been proposed (Lew *et al.*, 2004). In addition, organized clusters of T cells and DCs are often seen in LS skin (Lowe *et al.*, 2007). Ingenuity Pathway Analysis biological function pathways of lymphoid tissue structure and development, hematological system development and

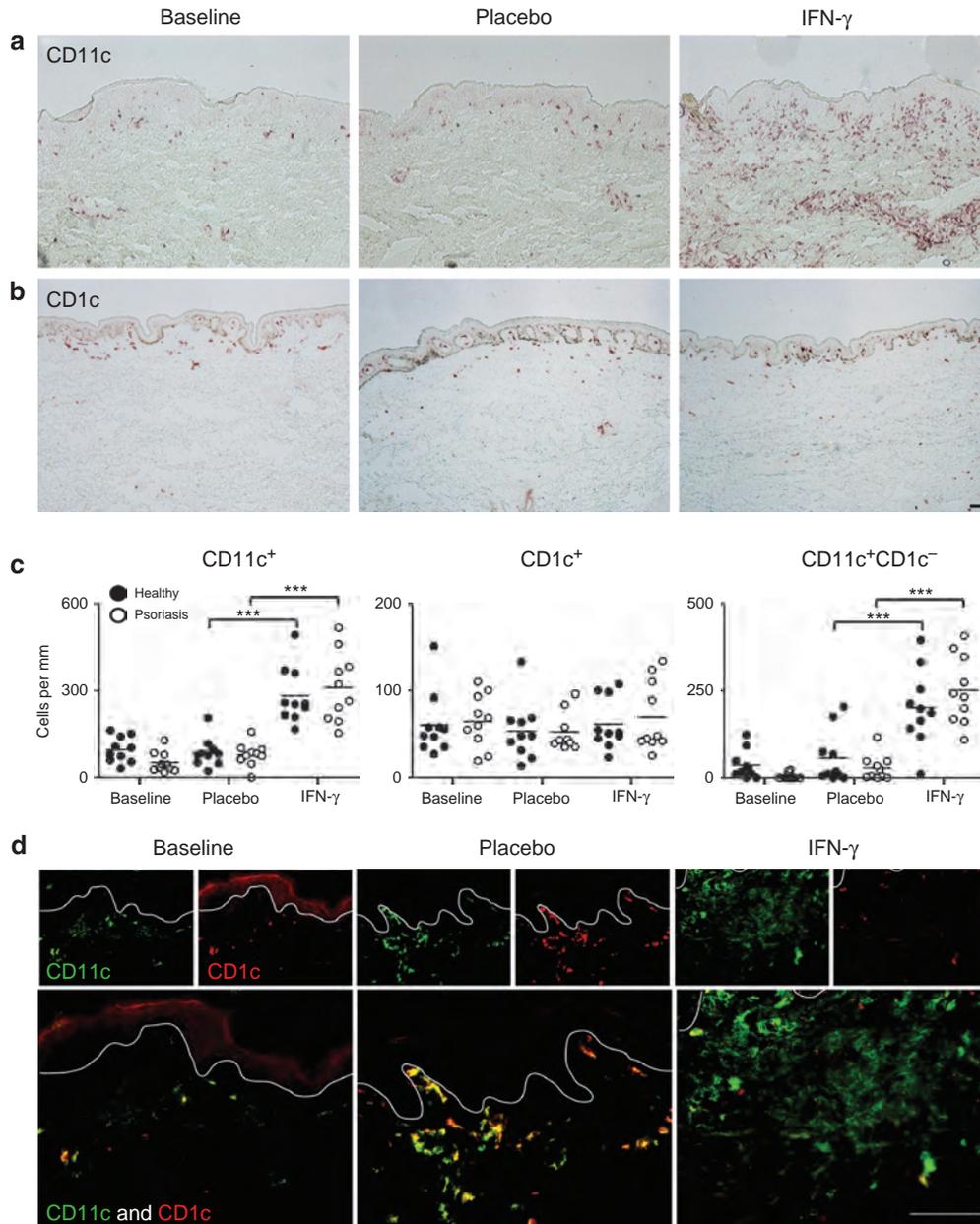
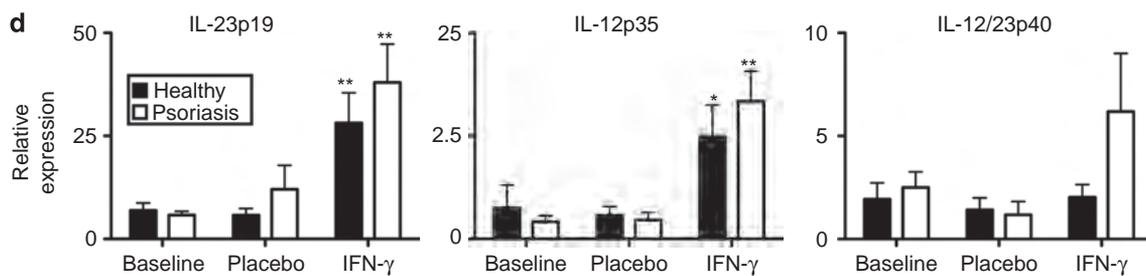
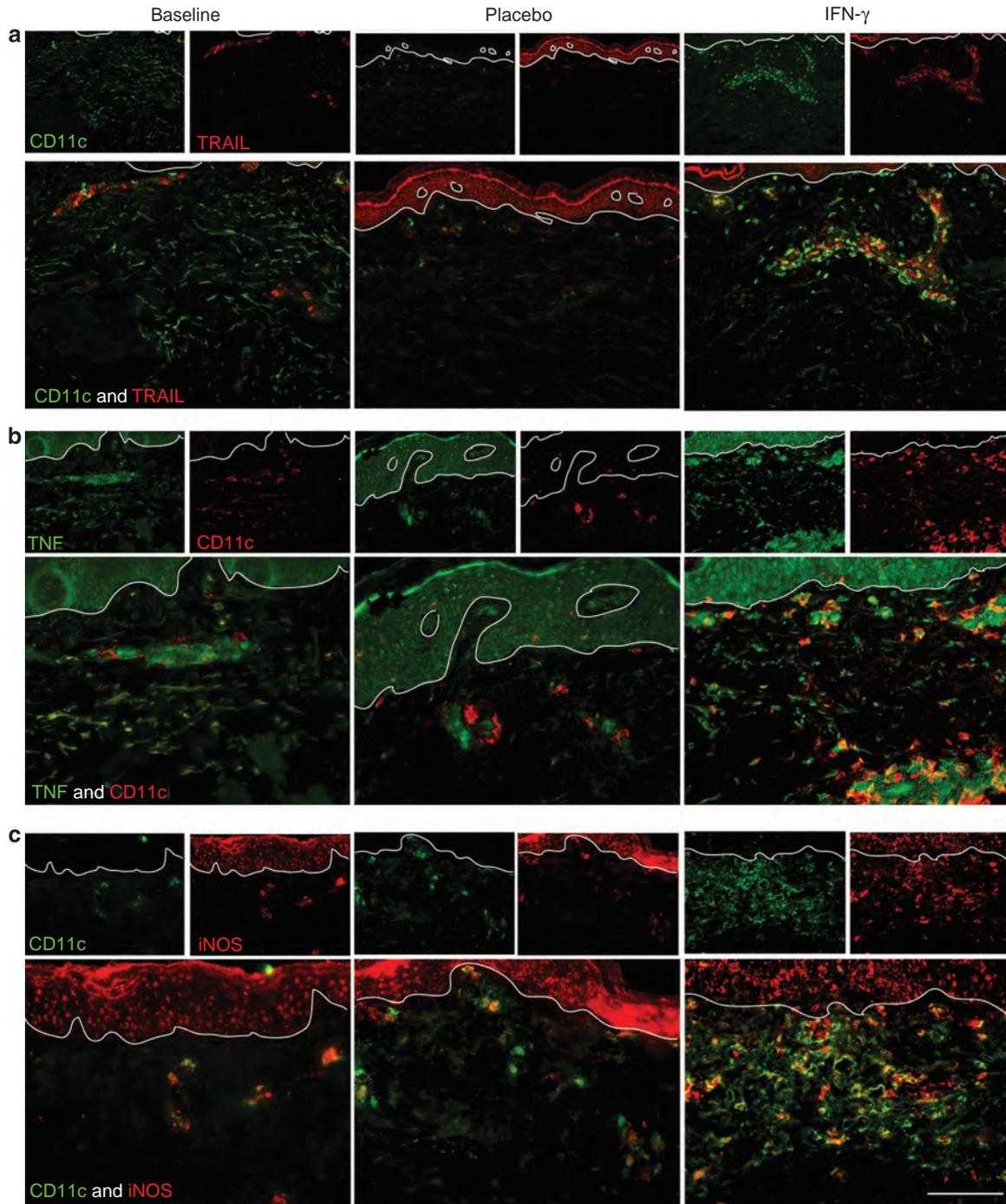


Figure 2. IFN- γ injection induces dermal inflammatory myeloid dendritic cells (DCs). Representative immunohistochemistry of (a) CD11c⁺ and (b) CD1c⁺ cells in psoriatic skin. (c) Quantification of CD11c⁺, CD1c⁺, and inflammatory DCs (CD11c⁺ minus CD1c⁺ cells) in healthy (black circles) and psoriatic skin (white circles). Each circle represents a different patient. IFN- γ -injected skin is compared with placebo. *** $P < 0.001$. (d) Two-color immunofluorescence of CD1c⁺ DCs (red) and CD11c⁺ DCs (green). The white line delineates the dermal-epidermal junction. Bar = 100 μ m.

function, tissue morphology, and hematopoiesis were significantly represented by the IFN- γ -induced genes (Supplementary Table S2 online). Genes involved in the ectopic lymphoid neogenesis were further investigated by RT-PCR and protein staining. Lymphotoxin- β , which is critical in the development of lymphoid structures (De Togni *et al.*, 1994; Banks *et al.*, 1995; Fütterer *et al.*, 1998), was elevated by IFN- γ (Figure 4a). Lymphoid tissue-organizing chemokines, CXCL12/SDF-1, CXCL13, and CCL21, were also assessed, but were unchanged by IFN- γ (Supplementary Figure S4 online). IFN- γ did, however, increase the expression of

CXCR4, the receptor for CXCL12/SDF-1, as well as regulator of G protein signaling 16, which is involved in the retention of CXCR4⁺ cells at sites of inflammation (Hsu *et al.*, 2008). CCL19, an important factor involved in the organization of T cells and DCs (Fukuyama *et al.*, 2006), was significantly increased by IFN- γ (Figure 4a). Consistent with CCL19 upregulation, CD11c⁺ DCs and CD3⁺ T cells in IFN- γ -injected skin were organized in clusters reminiscent of the T-cell zones of lymph nodes (Figure 4b). The development of lymphoid tissue is also associated with high endothelial venules marked by peripheral node addressin, and IFN- γ



enhanced the expression of peripheral node addressin in the skin (Figure 4c). Finally, at both the mRNA (Table 1) and protein (Figure 4d) levels, IFN- γ induced the expression of ICAM-1 and VCAM-1, adhesion molecules found in secondary and tertiary lymphoid organs. Taken together, these data suggest that IFN- γ may indeed induce some facets of ectopic lymphoid neogenesis in the skin.

IFN- γ as a biomarker of psoriasis disease potential and/or severity

The molecular and histological responses to IFN- γ injection were similar between healthy and psoriatic skin. This finding suggested that the difference between psoriatic patients and controls might be upstream of the “response” to IFN- γ , potentially in the induction of IFN- γ , with psoriatic patients having a lower threshold for IFN- γ production than the healthy controls. To address this question, IFN- γ mRNA expression was compared across NL and LS skin of patients with varying severities of psoriasis, from mild psoriasis (this study) to moderate-to-severe psoriasis, published previously (Johnson-Huang *et al.*, 2010). The lowest level of IFN- γ expression was found in healthy skin, with an increasing gradient of IFN- γ mRNA expression as disease severity worsened. IFN- γ expression was significantly increased in NL skin from moderate-to-severe psoriasis patients compared with mild NL skin, and was highest in LS psoriatic skin (Figure 5a). Moreover, an intradermal injection of IFN- γ into mild NL skin induced IFN- γ mRNA at a level between NL and LS skin of moderate-to-severe patients.

To evaluate whether the level of IFN- γ mRNA in the skin correlated with a downstream genomic effect, we analyzed two lists of DEGs from comparison of NL versus healthy skin by Ingenuity Pathway Analysis. The IFN signaling pathway was significantly represented ($P=0.03$) by DEGs in moderate-to-severe NL skin (Zaba *et al.*, 2009b), but not by DEGs in mild NL skin compared with healthy skin. For comparison, the IFN signaling pathway was also significantly represented by the DEGs in IFN- γ -treated NL skin ($P<10^{-9}$). The range of IFN- γ expression and activation of downstream signaling molecules, based on disease severity, suggests that IFN- γ may be a useful biomarker of psoriasis disease potential.

We also compared cell counts across the range of disease states and showed that CD3⁺ T cells and CD11c⁺CD1c⁻ inflammatory DCs followed a similar pattern, with an increase in cells as disease severity worsened (Figure 5b). The patterns of increasing cellularity and IFN- γ mRNA expression with psoriasis disease progression were associated with an enrichment of psoriasis genes in the IFN- γ response DEGs (by gene set enrichment analysis). Thus, we sought to

determine how the IFN- γ response genes compared with moderate-to-severe NL and LS psoriasis. Using unsupervised hierarchical clustering, we found that there were two main clusters of samples: (1) healthy skin samples clustered with NL skin and (2) IFN- γ -treated skin clustered with LS psoriatic skin (Supplementary Figure S6 online). Taken together, these data suggest that injection of IFN- γ may induce an inflammatory state with some features of LS psoriatic skin.

DISCUSSION

Factors that induce the progression of psoriatic NL skin into LS skin, and why some patients have more severe disease than others are still unclear. Here, we have analyzed the molecular and cellular changes that occur in the skin in response to IFN- γ , and found that a single intradermal injection of IFN- γ induced an inflammatory environment in both healthy and psoriatic skin. We showed upregulation of many chemokines and inflammatory cytokines, as well as the influx of inflammatory cells, most notably T cells and inflammatory DCs. In addition, IFN- γ induced features of lymphoid tissue, including organization of T-cell and DC clusters. At the genomic level, psoriasis gene sets (DEGs in LS skin compared with NL) (Suarez-Farinas *et al.*, 2010) were highly enriched in the IFN- γ response genes, indicating a significant degree of overlap of these genes. Thus, the inflammatory response induced by IFN- γ in the skin resembled some of the changes that occur in psoriatic lesions.

The observation that the response to IFN- γ was similar in both NL psoriatic skin and healthy skin indicates that the pathogenic etiology in psoriasis is not in the “response” to IFN- γ *per se*, but may be further upstream. Conceivably, the threshold to produce IFN- γ may be different between healthy controls and psoriasis patients. Consistently, elevated IFN- γ expression has been found in NL psoriatic skin compared with healthy skin (Uyemura *et al.*, 1993; Guttman-Yassky *et al.*, 2008; Kryczek *et al.*, 2008). Here, we also show that IFN- γ mRNA expression increased, even in NL skin, as psoriasis became more severe, suggesting that psoriasis patients have a greater propensity to produce IFN- γ than the healthy controls. The increase in IFN- γ expression in psoriasis patients has been linked to polymorphisms in the *IL4* and *IL13* genes, resulting in the hypofunction of these IFN- γ -inhibitory cytokines (Elder, 2009).

Which cells contribute to the elevated expression of IFN- γ in NL compared with healthy skin is not known. Slightly increased numbers of activated CD4⁺ and CD8⁺ T cells have been found in NL skin (Baker *et al.*, 1984; Placek *et al.*, 1988), which may account for augmented IFN- γ . Here, we also show that as psoriasis progresses, the numbers of CD3⁺

Figure 3. IFN- γ injection induces expression of known products of inflammatory myeloid dendritic cells (DCs). Two-color immunofluorescence of CD11c⁺ DCs with inflammatory products: (a) tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), (b) TNF, and (c) inducible nitric oxide synthase (iNOS) in baseline, placebo, and IFN- γ -injected psoriatic skin. The white line delineates the dermal-epidermal junction. Bar = 100 μ m. (d) mRNA expression levels normalized to human acidic ribosomal protein (hARP) for additional DC cytokines in healthy (black bars, $n=10$) and psoriatic skin (white bars, $n=10$). Error bars represent the mean \pm SEM. IFN- γ -injected skin is compared with placebo. * $P<0.05$, ** $P<0.01$.

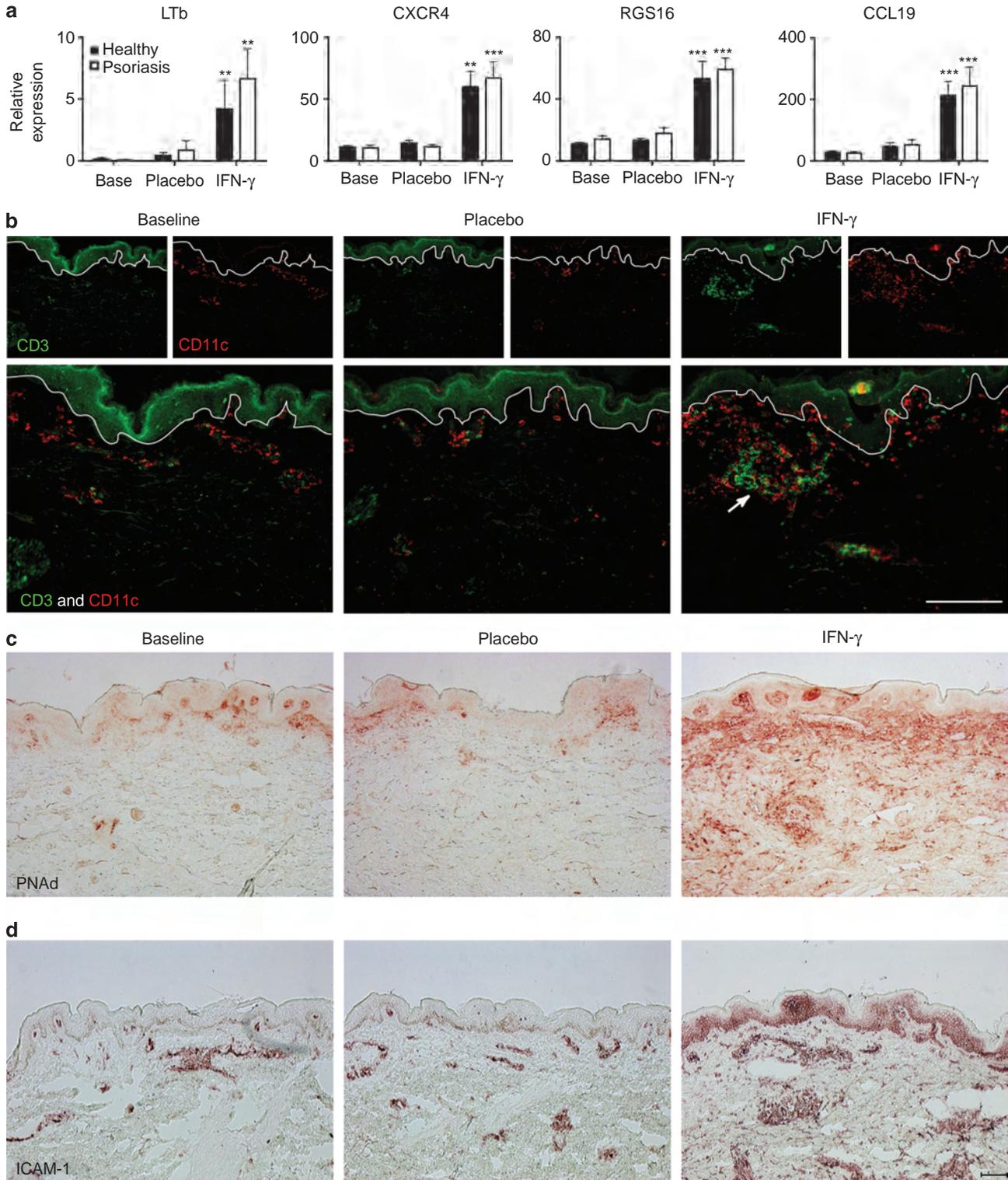


Figure 4. IFN- γ injection induces lymphoid-like structures and expression of known lymphoid genes and proteins. (a) mRNA expression levels normalized to human acidic ribosomal protein (hARP) for the lymphoid genes in healthy (black bars, $n=10$) and psoriatic skin (white bars, $n=10$). Error bars represent the mean \pm SEM. IFN- γ -injected skin is compared with placebo. ** $P<0.01$, *** $P<0.001$. (b) Two-color immunofluorescence of CD11c⁺ dendritic cells (DCs; red) and CD3⁺ T cells (green) in psoriatic skin. White arrows denote DC/T-cell clusters. The white line delineates the dermal-epidermal junction. Representative immunohistochemistry of (c) peripheral node addressin (PNAAd) and (d) ICAM-1 in psoriatic skin. Bar = 100 μ m.

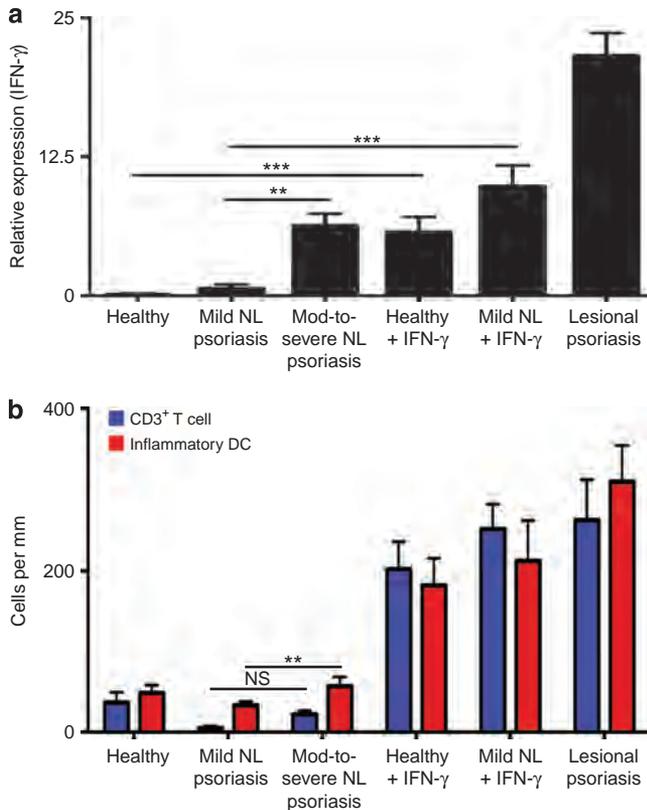


Figure 5. The gradient of IFN- γ mRNA expression parallels T-cell and dendritic cell (DC) infiltration as psoriasis becomes more severe. (a) IFN- γ mRNA expression in healthy ($n=10$), non-lesional (NL) skin from mild ($n=10$, body surface area (BSA) <10%) versus moderate-to-severe ($n=9$, BSA >10%) psoriasis patients, IFN- γ -injected healthy ($n=10$) or mild NL psoriatic ($n=10$) skin, and psoriatic lesional (LS; $n=9$) skin. Error bars represent the mean \pm SEM. ** $P<0.01$. *** $P<0.001$. (b) Quantification of CD3⁺ T cell (blue bars) and CD11c⁺CD1⁻ inflammatory DC (red bars) counts per mm of skin in patients in a. Mod, moderate; NS, nonsignificant.

T cells in NL skin increase, with the highest numbers being in LS skin (Figure 5b), consistent with previous reports (Bos *et al.*, 1983; Krueger, 2002). Additional cell types may also be contributing to the elevated IFN- γ . A significant proportion of mast cells in NL skin showed positive IFN- γ staining (Ackermann *et al.*, 1999). Neutrophils have also been implicated as a source of IFN- γ (Ethuin *et al.*, 2004), although they are rare in NL skin. In addition, keratinocytes in NL skin express CD1d, which can stimulate CD161⁺ NK-T cells to produce large amounts of IFN- γ (Bonish *et al.*, 2000). In another genomic study comparing NL skin to healthy skin, Gudjonsson *et al.* (2009) showed that innate defense genes are elevated in NL skin, raising the possibility that psoriatic NL skin may be “pre-activated”. Despite these changes, not all NL skin evolves into LS skin, indicating that a secondary trigger, perhaps coupled with the presence of factors like IFN- γ , may induce lesion formation in psoriatic skin, but not in healthy individuals who lack IFN- γ .

Previous studies using a skin organ culture system where NL psoriatic skin was cultured *in vitro* with IFN- γ showed that the epidermal changes induced by IFN- γ were partially

mediated by IL-1 β (Wei *et al.*, 1999). In this study, although there was abundant DC infiltration and cytokine production (including IL-1 β and IL-23), with concomitant T-cell infiltration after IFN- γ injection, these changes were insufficient to induce full plaque development, possibly because there was no Th17 cell cytokine production. The fact that the epidermis was not altered by this regime of IFN- γ is consistent with the lack of IL-17. The dose of IFN- γ may be insufficient for the induction of T-cell responses or the early 24 hours time point may not have allowed enough time to induce T-cell activation and polarization, and if biopsies were taken at later time points, the T-cell phenotype may have been altered. Along these lines, previous reports found that epidermal changes and visible lesions resulted after a month of continuous IFN- γ injections (Nathan *et al.*, 1986; Morhenn *et al.*, 1987; Barker *et al.*, 1989, 1990; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990), although the induction of IL-17 and IL-22 was not assessed in these studies.

In conclusion, the present study strengthens the hypothesis that IFN- γ is a crucial mediator of skin inflammation. Although this study examines the effect of IFN- γ only in healthy and psoriasis patients, it may provide a framework for evaluating other diseases where IFN- γ is considered to have a major pathogenic role (Dardalhon *et al.*, 2008).

MATERIALS AND METHODS

Study design and skin biopsies

We conducted a research study under a Rockefeller University Institutional Review Board-approved protocol in which healthy volunteers and patients with mild psoriasis ($n=10$ per group) received single intradermal injections of IFN- γ (Actimmune, 1×10^6 IU) and saline (placebo) in areas of the skin that were clinically normal (NL skin). Saline was used as a control, as the inactive components of the Actimmune solution were not available. After 24 hours, biopsies of both the sites were taken; biopsies at baseline were also taken before injections. Each biopsy was cut into two: half was stored in optimum cutting temperature for cryosections and the other half was snap-frozen in liquid nitrogen for RNA extractions. Written informed consent was obtained and the study was performed in adherence with the Declaration of Helsinki Principles. NL and LS tissue biopsies ($n=9$ pairs) from moderate-to-severe psoriasis patients were obtained under a Rockefeller University Institutional Review Board-approved protocol (NCT ID: 00220025), which has been previously published (Johnson-Huang *et al.*, 2010).

Immunostaining

Skin sections were stained for immunohistochemistry and immunofluorescence as previously described (Fuentes-Duculan *et al.*, 2010). All the antibodies used for staining are listed in Supplementary Table S3 online.

mRNA extraction and RT-PCR

RNA extraction and RT-PCR using Taqman gene expression assays (Supplementary Table S4 online) were performed as previously described (Chamian *et al.*, 2005). Custom primers for IFN- γ and IL-12/23p40 were generated as previously described (Chamian *et al.*, 2005). Data normalized to *hARP* housekeeping gene were quantified

by software provided with Applied Biosystems (Carlsbad, CA) PRISM 7700 (Sequence Detection Systems, version 1.7). Normalized PCR data were log₂ transformed before statistical analysis.

Microarray hybridization

Human Genome U133 2.0. arrays (Affymetrix, Santa Clara, CA) were used. See Supplemental Materials and Methods for details.

Statistical analysis

For comparison of RT-PCR data and cell counts from IFN- γ -injected skin versus respective placebo-treated skin, the Wilcoxon matched pairs test was used. Mann-Whitney *U* test was used to compare unpaired moderate-to-severe NL and LS with IFN- γ - or placebo-treated skin. A *P*-value <0.05 was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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