A single intradermal injection of IFN-γ induces an inflammatory state in both non-lesional psoriatic and healthy skin

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Abstract

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, important contributors to the inflammatory cascade in psoriasis lesions. To determine if IFN-γ indeed induces the pathways expressed in psoriasis lesions, a single intradermal injection of IFN-γ was administered to an area of clinically normal, non-lesional 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 psoriasis 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 TNF, iNOS, IL-23, and TRAIL were present in IFN-γ-treated skin. Thus, IFN-γ, which is significantly elevated in non-lesional skin compared to healthy skin, appears to be a key pathogenic cytokine that can induce many features of the inflammatory cascade of psoriasis.

Introduction

Psoriasis is an inflammatory skin disease affecting approximately 2% of the population of the United States, and is considered one of the most prevalent cell-mediated inflammatory diseases (Lebwohl, 2003). Psoriasis lesions are typically erythematous scaly plaques, and patients are classified based on the severity of their disease, with a gradient ranging from

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Conflict of Interest

The authors have no conflicts.

mild to moderate-to-severe based on body surface area (BSA) involved. Psoriatic lesions are characterized by increased infiltration of both activated T cells and dendritic cells (DCs) compared to 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 (Nograles *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 (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 lesional skin (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 co-production 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; Annunziato *et al.*, 2007; Langrish *et al.*, 2005; Murphy *et al.*, 2003). Our group has identified a population of T cells that co-produce IFN-γ and IL-17 in psoriasis lesions (Lowes *et al.*, 2008), induced by DCs isolated from the dermis of psoriasis lesions, but not by DCs from healthy skin (Zaba *et al.*, 2009a). This difference in 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 (Guttman-Yassky *et al.*, 2008; Lowes *et al.*, 2005). 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 reevaluated.

Before the discovery that IFN-γ plays a role in autoimmunity, IFN-γ was used a therapy for several diseases including rheumatoid arthritis, psoriatic arthritis, psoriasis and lepromatous leprosy infections (Cannon *et al.*, 1989; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Morhenn *et al.*, 1987; 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 (Cannon *et al.*, 1989; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Morhenn *et al.*, 1987; 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 (Barker *et al.*, 1989, 1990; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Morhenn *et al.*, 1987; Nathan *et al.*, 1990; Nathan *et al.*, 1986). 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 psoriasis 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% BSA) 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, TNF, iNOS, IL-23p19, and TRAIL. Furthermore, Ingenuity Pathway Analysis (IPA) 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 cell 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 IFNγ was biologically active in the skin

In order to confirm that the injected IFN-γ was biologically active, we assessed human leukocyte antigen-DR (HLA-DR), a protein known to be upregulated by IFN-γ after intradermal injection (Nathan *et al.*, 1990; Nathan *et al.*, 1986). There were increased HLA-DR$^+$ cells in both normal and NL skin after injection of IFN-γ, but not the placebo control, indicating the IFN-γ was functional (Fig S1). 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 (Fig S2a), and expression of keratin 16 (K16), a measure of altered keratinocyte differentiation, was not induced (Fig S2b–c). However, there were dramatic genomic and cellular changes induced by IFN-γ in both 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 (FCH) >2 and false discovery rate (FDR) <0.01. The "IFN-γ response" was defined as the differentially expressed genes (DEGs) in IFN-γ-treated skin compared to 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 (Table S1). Although the IFN-γ response (FCH) of many genes was higher in psoriasis than healthy skin, these differences were not statistically significant after correcting for multiple hypotheses with the Benjamini-Hochberg procedure. Additionally, there was very strong

correlation in the response to IFN-γ in both groups (r=0.96; Fig. S3), further supporting that there was no difference in IFN-γinduced 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 IFNγ-induced genes (CXCL9, CXCL10, CCL2, and CX_3CL1) was verified by real-time PCR (Fig. 1). Additional DEGs were confirmed by RT-PCR, and the IFN-γ response measured by RT-PCR was strongly correlated with the microarray data (Fig. S4). Furthermore, using gene set enrichment analysis (GSEA), four published psoriasis gene sets (Suarez-Farinas *et al.*, 2010) correlated with the IFN-γ response in psoriasis patients (Table S2), indicating that IFN-γ can induce many genes that are present in psoriasis. Ingenuity Pathway Analysis (IPA) was also used to identify the statistically significant biological functions represented by these IFN-γ-induced DEGs (Table S2). The top functions represented by the upregulated DEGs after IFN-γ injection were consistent with pathways involved in psoriasis, including *inflammatory response, inflammatory disease, cellular movement and development, immune cell trafficking, 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.*, 1990; Nathan *et al.*, 1986). Here, we showed that even after only 24 hours post-IFN-γ injection, there were significantly increased $CD3^+$ T cells in skin (Fig. S5).

Dendritic cells and their inflammatory products have also been implicated in the pathogenesis of psoriasis. Thus, we sought to determine if IFN-γ induced infiltration of DCs into skin. We have previously classified $CD11c^+CD1c^+$ DCs in normal skin as "resident" DCs. In psoriatic lesional skin, 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, while $CD1c^+$ DCs remained constant throughout all samples analyzed (Fig. 2a–b). As there is no exclusive marker of 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). Fig. 2c shows significantly increased numbers of $CD11c^+CD1c^-$ inflammatory DCs in both normal and non-lesional skin only after IFN-γ-injection. To further analyze these cells, we performed two-color immunofluorescence with antibodies against CD11c and CD1c. While 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 (Fig. 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 GSEA, the inflammatory

CD1c⁻ DC DEGs were highly enriched in the IFN-γ response genes (Table S2; ES=0.38, NES=1.8, p<0.0001, for both 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/TNF-related apoptosis-inducing ligand (TRAIL), a putative, new marker of inflammatory DCs (Guttman-Yassky *et al.*, 2008; Lowes *et al.*, 2005; Zaba *et al.*, 2007; Zaba *et al.*, 2010). In order to determine if 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 (Fig. 3a). In comparison to 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 (Fig. 3b–c). Additionally, using RT-PCR, we detected a significant increase in IL-23p19 mRNA expression in the skin after IFN-γ injection, and a trend towards increased IL-12p35 and IL-12/23p40 (Fig. 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 skin (data not shown). As biopsies were taken only 24 hours after 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 peripheral non-lymphoid organs (Aloisi and Pujol-Borrell, 2006). Previously, based on analysis of upregulated DEGs, the existence of these structures within psoriasis LS skin has been proposed (Lew *et al.*, 2004). Additionally, organized clusters of T cells and DCs are often seen in LS skin (Lowes *et al.*, 2007). IPA biological function pathways of *lymphoid tissue structure and development*, *hematological system development and function, tissue morphology,* and *hematopoiesis* were significantly represented by the IFN-γ-induced genes (Table S2). Genes involved in ectopic lymphoid neogenesis were further investigated by RT-PCR and protein staining. Lymphotoxin-β (LTb), which is critical in development of lymphoid structures (Banks *et al.*, 1995; De Togni *et al.*, 1994; Futterer *et al.*, 1998), was elevated by IFN-γ (Fig. 4a). Lymphoid tissue organizing chemokines, CXCL12/SDF-1, CXCL13, and CCL21 were also assessed, but were unchanged by IFN-γ (Fig. S4). IFN-γ did, however, increase expression of CXCR4, the receptor for CXCL12/SDF-1, as well as regulator of G protein signaling 16 (RGS16), which is involved in retention of CXCR4⁺ cells in sites of inflammation (Hsu *et al.*, 2008). CCL19, an important factor involved in organization of T cells and DCs (Fukuyama *et al.*, 2006), was significantly increased by IFN-γ (Fig. 4a).

Consistent with CCL19 upregulation, CD11c^{+} DCs and CD3^{+} T cells in IFN-γ injected skin were organized in clusters reminiscent of T cell zones of lymph nodes (Fig. 4b). The development of lymphoid tissue is also associated with high endothelial venules (HEVs) marked by peripheral node addressin (PNAd), and IFN-γ enhanced expression of PNAd in the skin (Fig. 4c). Finally, at both the mRNA (Table 1) and protein (Fig. 4d) levels, IFN-γ induced 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 psoriasis patients having a lower threshold for IFN-γ production than 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 to mild NL skin, and was highest in LS psoriatic skin (Fig. 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 if 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 IPA. 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 to 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 DC followed a similar pattern, with an increase in cells as disease severity worsened (Fig. 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 GSEA). Thus, we sought to determine how the IFN-γ response genes compared to 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 psoriasis skin (Fig. S6). 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. Additionally, 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 to 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 psoriasis 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 to healthy skin (Guttman-Yassky *et al.*, 2008; Kryczek *et al.*, 2008; Uyemura *et al.*, 1993). 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 healthy controls. The increase in IFN-γ in psoriasis patients has been linked to polymorphisms in the *IL4* and *IL13* genes resulting in hypo-function of these IFN-γ-inhibitory cytokines (Elder, 2009).

Which cells contribute to the elevated expression of IFN-γ in NL compared to 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^{+} T cells in NL skin increase, with the highest numbers being in LS skin (Fig. 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. Additionally, 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.*, showed that innate defense genes are elevated in NL skin, raising the possibility that psoriatic NL skin may be "pre-activated" (Gudjonsson *et al.*, 2009). 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 healthy individuals which 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-1β (Wei *et al.*, 1999). In this study, although there was abundant DC infiltration and cytokine production (including IL-1β 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 lack of IL-17. The dose of IFN-γ may be insufficient for the induction of T cell responses or the early 24 hour 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 (Barker *et al.*, 1989, 1990; Fierlbeck and Rassner, 1990; Fierlbeck *et al.*, 1990; Morhenn *et al.*, 1987; Nathan *et al.*, 1990; Nathan *et al.*, 1986), 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 play 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/group) received single intradermal injections of IFN-γ (Actimmune, 1×10^6 IU) and saline (placebo) in areas of 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 sites were taken; biopsies at baseline were also taken prior to injections. Each biopsy was cut in two: half was stored in OCT for cryosections, and half 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).

Immuno-staining

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

mRNA extraction and real-time PCR

RNA extraction and real-time PCR using Taqman gene expression assays (Table S4) 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 PRISM 7700 (Sequence Detection Systems, version 1.7). Normalized PCR data were $\log_2$ transformed before statistical analysis.

Microarray hybridization

Human Genome U133 2.0 arrays (Affymetrix Inc, 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, a Wilcoxon matched pairs test was used. A Mann-Whitney U test was used to compare unpaired moderate-to-severe NL and LS to IFN-γ or placebo-treated skin. A p-value less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

DC	Dendritic cell
BSA	body surface area
NL	non-lesional skin
LS	lesional skin
FCH	fold change
FDR	false discovery rate
RT-PCR	real-time polymerase chain reaction
IPA	Ingenuity Pathway Analysis
HLA-DR	human leukocyte antigen-DR
ICAM-1	intracellular adhesion molecule-1
K16	keratin 16
DEGs	differentially expressed genes
GSEA	gene set enrichment analysis
HEVs	high endothelial venules
PNAd	peripheral node addressin
LTb	lymphotoxin-β
RGS16	regulator of G protein signaling 16
TRAIL	TNF-related apoptosis-inducing ligand

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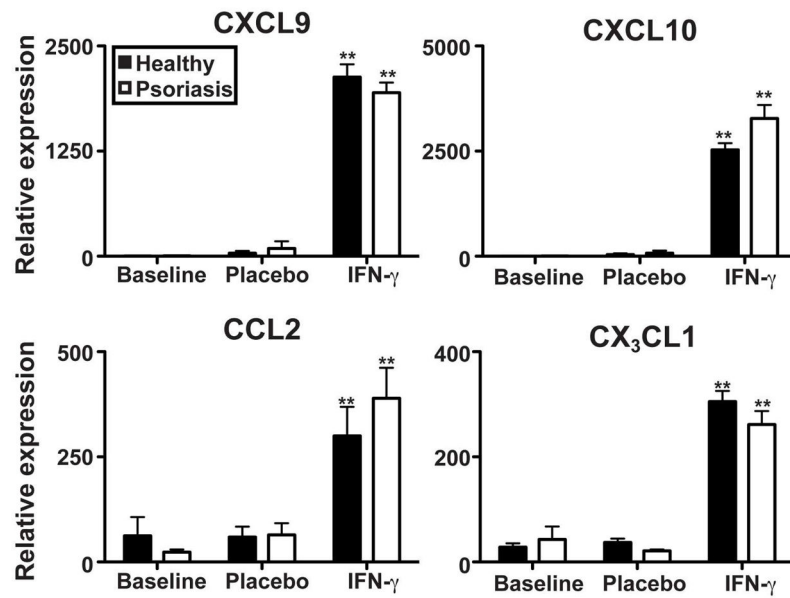
Fig. 1. IFN-γ injection induces genomic expression of known IFN-γ-regulated chemokines
mRNA expression levels normalized to hARP for the T cell (CXCL9 and CXCL10) and DC (CCL2 and CX_3CL_1) chemokines in baseline, placebo and IFN-γ-injected healthy (black bars, n=10) and psoriatic skin (white bars, n=10). Error bars represent the mean ± SEM. IFN-γ-injected skin was compared to placebo. (**) P<0.01.
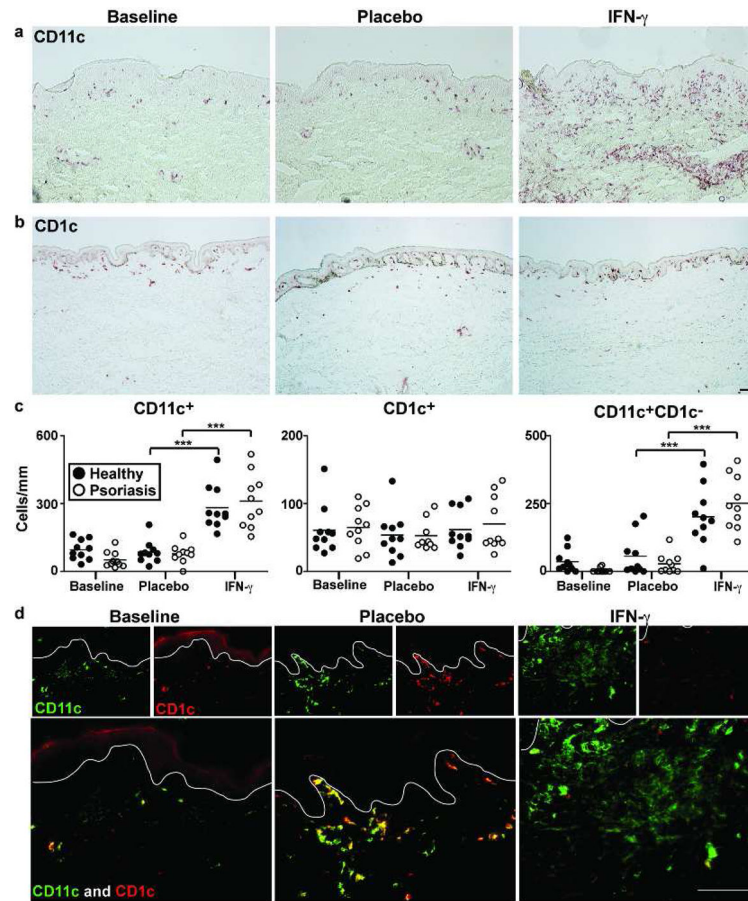
Fig. 2. IFN-γ injection induces dermal inflammatory myeloid DCs

Representative immunohistochemistry of (a) CD11c$^+$ and (b) CD1c$^+$ cells in psoriasis skin. (c) Quantification of CD11c$^+$, CD1c$^+$, and inflammatory DCs (CD11c$^+$ minus CD1c$^+$ cells) in healthy (black circles) and psoriasis skin (white circles). Each circle represents a different patient. IFN-γ-injected skin is compared to placebo. (***) P<0.001. (d) Two-color immunofluorescence of CD1c$^+$ DCs (red) and CD11c$^+$ DCs (green). The white line delineates the dermal epidermal junction. Scale bar = 100μm.
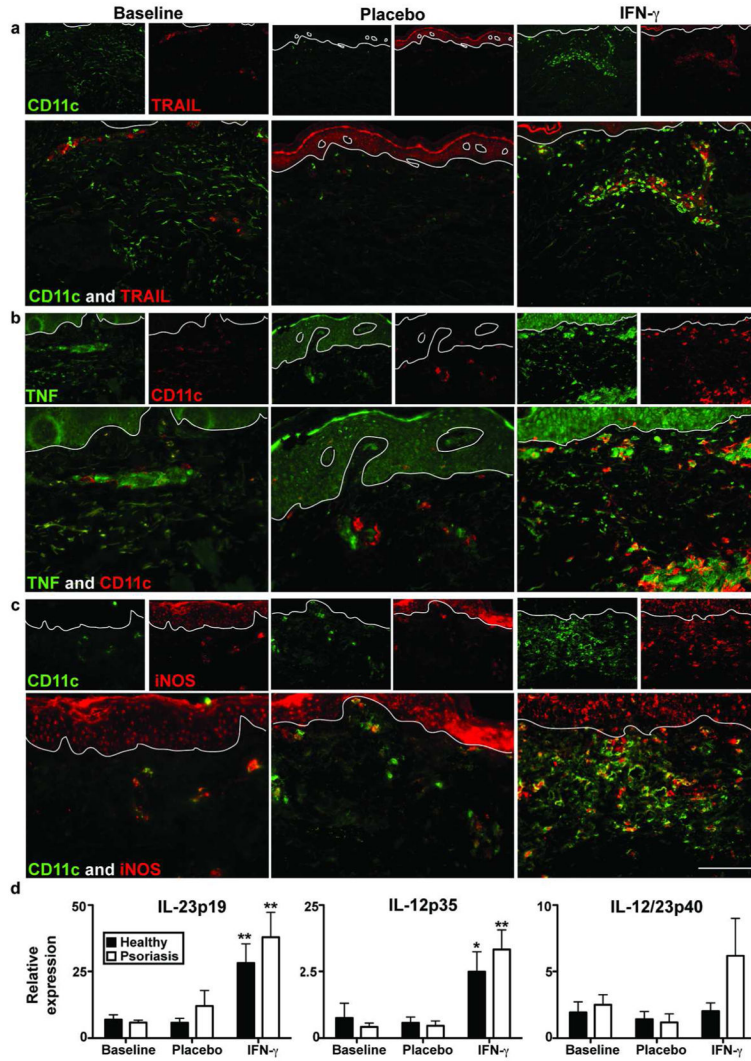
Fig. 3. IFN-γ injection induces expression of known products of inflammatory myeloid DCs
(a–c) Two-color immunofluorescence of CD11c^{+} DCs with inflammatory products: (a)
TRAIL, (b) TNF, and (c) iNOS in baseline, placebo and IFN-γ-injected psoriasis skin. The
white line delineates the dermal epidermal junction. Scale bar = 100μm. (d) mRNA
expression levels normalized to hARP for additional DC cytokines in healthy (black bars,
n=10) and psoriatic skin (white bars, n=10). Error bars represent the mean ± SEM. IFN-γ-
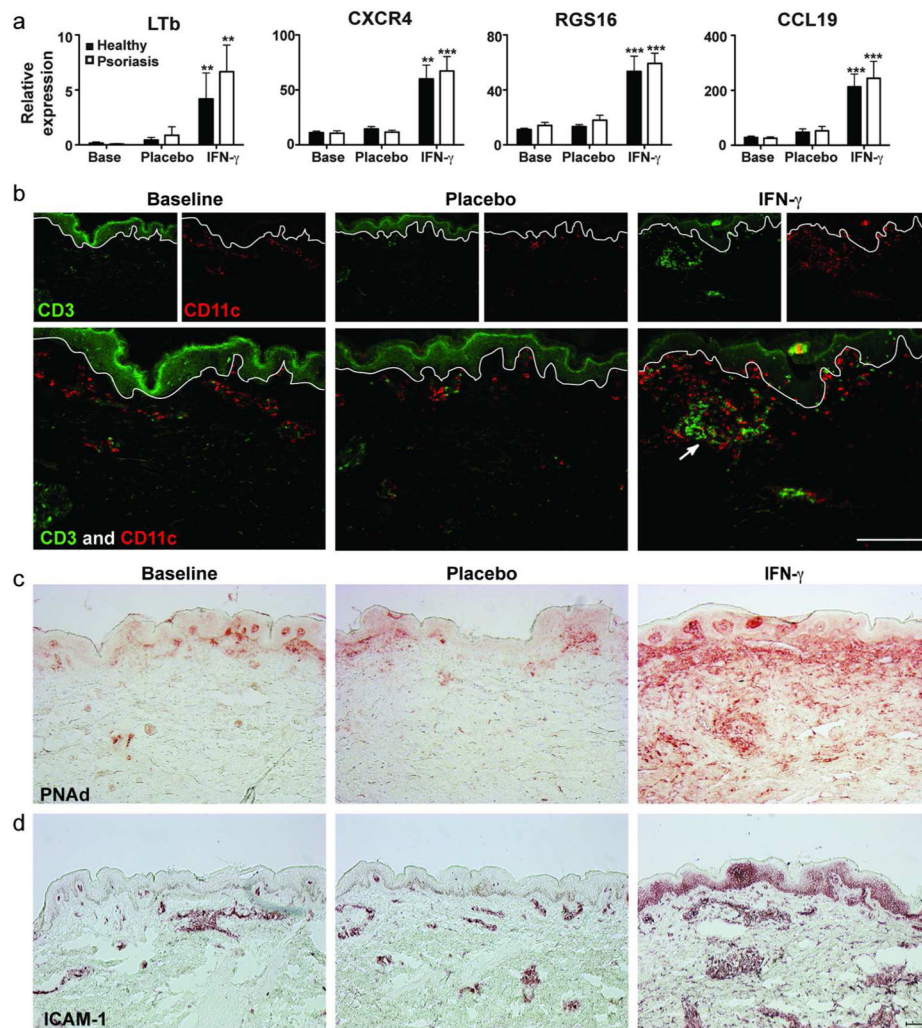injected skin is compared to placebo. (*) P<0.05, (**) P<0.01.

Fig. 4. IFN-γ injection induces lymphoid-like structures and expression of known lymphoid genes and proteins

(a) mRNA expression levels normalized to hARP for the lymphoid genes in healthy (black bars, n=10) and psoriatic skin (white bars, n=10). Error bars represent the mean ± SEM. IFN-γ-injected skin is compared to placebo. (**) P<0.01, (***) P<0.001. (b) Two-color immunofluorescence of CD11c+ DCs (red) and CD3+ T cells (green), in psoriasis skin. White arrows denote DC/T cell clusters. The white line delineates the dermal epidermal junction. (c–d) Representative immunohistochemistry of (c) peripheral node addressin (PNAd) and (d) ICAM-1 in psoriasis skin. Scale bar = 100μm.
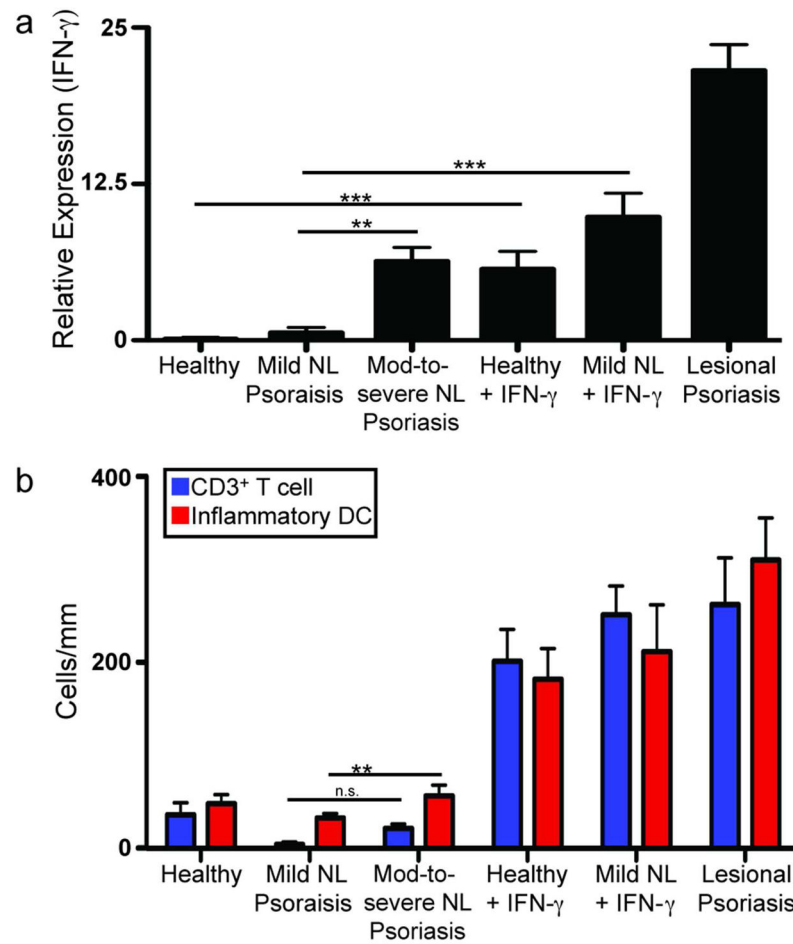
Fig. 5. The gradient of IFN-γ mRNA expression parallels T cell and DC infiltration as psoriasis becomes more severe

(a) IFN-γ mRNA expression in healthy (n=10), NL skin from mild (n=10, BSA<10%) versus moderate-to-severe (n=9, BSA>10%) psoriasis patients, IFN-γ-injected healthy (n=10) or mild NL psoriasis (n=10) skin and psoriasis LS (n=9) skin. Error bars represent the mean ± SEM. (**) P<0.01. (***) P<0.001. (b) Quantification of CD3^{+} T cells (blue bars) and CD11c^{+}CD1c^{−} inflammatory DCs (red bars) counts per mm of skin in patients in (a).

Table 1

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

Symbol	Description	IFN-γ Response (Fold change)[*]	
		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

Color code: blue=chemokines, yellow = cytokines, orange = inflammatory molecules, green = adhesion molecules.

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