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In Psoriasis Lesional Skin the Type I Interferon Signaling Pathway Is Activated, Whereas Interferon- α Sensitivity Is Unaltered

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The epidermal phenotype as observed in psoriatic skin results from inflammation and abnormal proliferation and terminal differentiation of keratinocytes. Mice deficient for interferon regulatory factor-2, a repressor of interferon signaling, display psoriasis-like skin inflammation. The development of this phenotype is strictly dependent on type I interferon (interferon- α/β) signaling. The aim of this study was to assess the involvement of interferon- α/β in the pathogenesis of human psoriasis. In psoriatic skin, we measured an increased expression of components that play central and crucial roles in interferon- α/β signal transduction. Culturing keratinocytes or healthy skin biopsies with recombinant interferon- α stimulated this signaling pathway; however, this did not induce the expression of markers that are generally used to define the psoriasis phenotype. Furthermore, skin from psoriasis patients responded identically to interferon- α stimulation, demonstrating that psoriatic skin does not have an aberrant sensitivity to type I interferon. We conclude that in psoriatic lesional skin the type I interferon signaling pathway is activated, despite an unaltered interferon- α sensitivity. Our data furthermore show that type I interferon, in contrast to interferon- γ , does not act directly on keratinocytes to induce a psoriatic phenotype. Thus, if the observed activated type I interferon signaling is indeed functionally involved in the pathogenesis of psoriasis, its contribution might be indirect, putatively involving other cell types besides keratinocytes.

Key words: inflammation/interferon regulatory factor/interferon- α /keratinocytes.
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Interferons (IFN) are cytokines that play a central and complex role in defending the mammalian host to pathogens. Type I IFN (IFN- α/β) are secreted by virus-infected cells, and are required to mount an effective anti-viral response. Type II IFN (IFN- γ) is produced by activated T cells and natural killer cells, and is implicated mainly in regulating adaptive immune responses. The link between type I IFN and autoimmunity is apparent in the literature, although evidence supporting this is merely circumstantial. Associations have been reported between viral infections and autoimmune disorders, such as autoimmune thyroid disease, rheumatoid arthritis, and systemic lupus erythematosus (McMurray and Elbourne, 1997). Furthermore, case reports showed that patients treated with IFN- α can develop autoimmune diseases (Unoki *et al*, 1996; Pittau *et al*, 1997; Fukuyama *et al*, 2000; Recasens *et al*, 2001).

Although the chronic inflammatory skin disease psoriasis does not meet all criteria for autoimmune diseases (Rose and Bona, 1993), it shares many characteristics with autoimmune disorders (Davidson and Diamond, 2001). Therefore, in analogy to the above-mentioned autoimmune

diseases, a role for type I IFN in the pathogenesis of psoriasis is plausible. Associations between chronic viral infections and the development of psoriasis have been reported (e.g., Yamamoto *et al*, 1995; Erkek *et al*, 2000). Furthermore, it has been observed that psoriasis can be induced or exacerbated by IFN- α treatment (e.g., Pauluzzi *et al*, 1993; Erkek *et al*, 2000). More direct evidence for the involvement of type I IFN in the pathogenesis of psoriasis comes from studies on mice deficient for interferon regulatory factor (IRF)-2, a transcriptional repressor of IFN signaling. These mice display psoriasis-like skin abnormalities, characterized by increased epidermal intercellular adhesion molecule (ICAM)-1 expression and infiltrates of CD4⁺ and CD8⁺ T cells. IFN- α/β signaling appeared to be absolutely required for the development of this skin phenotype, as mice deficient for both IRF-2 and the type I IFN receptor displayed no signs of skin inflammation (Hida *et al*, 2000).

To elucidate the role for type I IFN in the pathogenesis of psoriasis in humans, we studied the expression of type I IFN signaling components in healthy and psoriatic skin. Activation of the type I IFN receptor results in the formation of the heterotrimeric transcription factor complex interferon stimulated gene factor (ISGF)3, which is composed of phosphorylated signal transducer and activator of transcription (STAT)1, STAT2, and IRF-9. Upon translocation into the nucleus, this complex is capable of activating IFN-inducible genes (Taniguchi *et al*, 2001). IRF-7 is one of the

Abbreviations: ICAM, intercellular adhesion molecule; IRF, interferon regulatory factor; ISGF, interferon stimulated gene factor; K17, keratin 17; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription.

genes that is transcriptionally induced by, and completely dependent on, the formation and nuclear translocation of ISGF3 (Sato *et al*, 1998). IRF-7 itself also acts as a transcription factor and shares homology with other IRF family members in its DNA-binding domain (Taniguchi *et al*, 2001).

We demonstrate that the expression of components that are involved in type I IFN signal transduction is markedly increased in psoriatic skin, implying functional activation of this signaling pathway. *In vitro* activation of this pathway in cultured keratinocytes or skin biopsies did not induce the expression of markers that are characteristic for the psoriatic skin phenotype. Furthermore, nonlesional and lesional skin biopsies from psoriatic patients responded identically to IFN- α stimulation as healthy skin biopsies, demonstrating that psoriatic skin displays a normal sensitivity to type I IFN.

Results

Nuclear expression of STAT1 and IRF-9 is increased in the psoriatic epidermis The activity of the type I IFN signal transduction pathway in psoriatic skin was assayed by determining the expression patterns of several components of this signaling pathway by immunohistochemistry. STAT1

occupies a central position in both type I and type II IFN signaling. After activation of the IFN- γ receptor, homodimers of phosphorylated STAT1 are formed, which are subsequently translocated into the nucleus. On the other hand, upon stimulation with IFN- α/β , activated STAT1 forms a heterotrimeric complex with STAT2 and IRF-9. This ISGF3 complex becomes biologically active when translocated into the nucleus, and is capable of activating the transcription of many genes, among others IRF-7.

Immunohistochemical analysis of healthy skin biopsies using STAT1 antibodies showed a diffuse cytoplasmic staining (Fig 1, *upper left panel*), whereas no epidermal cells stained positive using a control antibody (normal rabbit IgG) (data not shown). We observed that in healthy skin, STAT1 was localized in the nucleus of $36 \pm 3\%$ of the epidermal keratinocytes (Table II). In psoriasis lesional skin, however, the number of STAT1-positive nuclei was significantly increased to $56 \pm 5\%$, implying functional activation of IFN signaling.

As IRF-9 only forms part of the ISGF3 complex, and is therefore more restricted to type I IFN signaling, immunohistochemical analysis of IRF-9 expression was performed (Fig 1, *middle row*). Quantification of the percentages of epidermal keratinocytes with nuclear IRF-9 expression revealed that in both nonlesional and lesional skin from psoriasis patients, the nuclear IRF-9 expression was

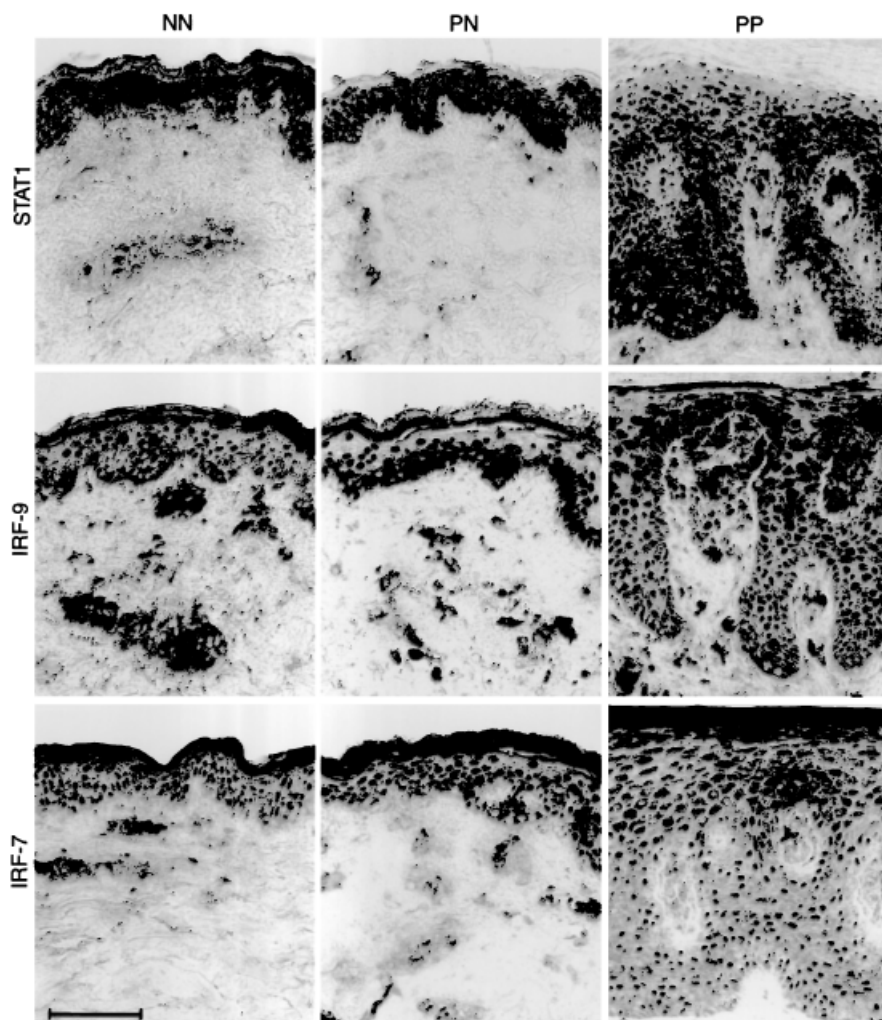


Figure 1
STAT1, IRF-9, and IRF-7 proteins are expressed in the nuclei of keratinocytes. Immunohistochemical analysis of STAT1 (*upper panel*), IRF-9 (*middle panel*), and IRF-7 (*lower panel*) proteins in healthy (NN, *left row*), nonlesional (PN, *middle row*), and lesional psoriatic (PP, *right row*) skin biopsies. Scale bar = 100 μ m.

Table I. Antibodies used in this study

Antibody	Supplier	Application ^a	Dilution
rabbit α -STAT1	Santa Cruz Biotech	IHC	1/1000
rabbit α -IRF-7	Santa Cruz Biotech	IHC	1/50
rabbit α -IRF-9 (ISGF-3 γ)	Santa Cruz Biotech	IHC	1/50
mouse α -ICAM-1	Roche Diagnostics	IHC	1/50
mouse α -ICAM-1-PE	BD Biosciences	Flow	1/4
mouse α -K17	DAKO	IHC	1/100
		Flow	1/100
mouse α -MHC-II	DAKO	IHC	1/400
mouse α -HLA-DR-PE	BD Biosciences	Flow	1/20

^aIHC = Immunohistochemistry; Flow = flowcytometric analysis.

Table II. Nuclear STAT1 and IRF-9 expression is significantly increased in psoriatic skin

	% nuclei positive for		
	STAT1 ^a	IRF-9 ^a	IRF-7 ^a
Healthy skin (n = 8)	36 \pm 3	18 \pm 5	60 \pm 6
Nonlesional psoriatic skin (n = 8)	40 \pm 3	42 \pm 5*	63 \pm 5
Lesional psoriatic skin (n = 8)	56 \pm 5*	56 \pm 4*	61 \pm 3

^aSkin sections were stained with either STAT1, IRF-9 or IRF-7 specific antibodies. Epidermal STAT1, IRF-9 and IRF-7 positive nuclei were counted and calculated as percentage of the total number of nuclei present in the epidermis as determined by haematoxylin staining. Values are expressed as mean % \pm SEM. Asterisks indicate that percentages are statistically significant higher in psoriatic skin when compared to healthy skin using the non-parametric Mann-Whitney test (p \leq 0.01).

significantly increased (Table II). IRF-7 protein expression was detectable in the nucleus of most epidermal cells (Fig 1, lower row), and the percentages of positive nuclei in healthy, nonlesional, and lesional psoriatic epidermis were comparable (Table II).

IRF-7 mRNA expression is elevated in epidermal cells from psoriasis skin lesions In addition to the analysis of IFN signaling components by immunohistochemistry, IRF-9 and IRF-7 mRNA expression was quantified. In total RNA extracted from epidermal cells, IRF-9 mRNA was detectable by quantitative RT-PCR, and the expression levels in healthy skin, nonlesional and lesional psoriatic skin were comparable (Fig 2). Quantitative analysis of IRF-7 mRNA levels, however, revealed that IRF-7 mRNA expression was significantly increased in psoriatic lesional skin, when compared with healthy and nonlesional skin. On average, a 6.2-fold increase was observed (Fig 2).

Thus the increased nuclear localization of both STAT1 and IRF-9, in combination with the increased IRF-7 mRNA expression, demonstrate that type I IFN signaling is activated in psoriatic lesional skin, when compared with both healthy skin and nonlesional skin from psoriasis patients.

IFN- α does not induce the expression of psoriasis markers in HaCaT keratinocytes The psoriatic skin phenotype is characterized by upregulation of several molecules that are related to skin inflammation, and to abnormal keratinocyte proliferation and terminal differentia-

tion (e.g., HLA-DR, ICAM-1, keratin 16, keratin 17 (K17) and keratinocyte transglutaminase). Previously, it was shown that in the human keratinocyte cell line HaCaT the expression of several of these so-called psoriasis markers could be induced by IFN- γ (Bonnekoh *et al*, 1995; Sebok *et al*, 1998; Wei *et al*, 1999) or interleukin-1 (Sebok *et al*, 1994).

To determine whether the activated IFN- α/β signaling that is observed in psoriatic skin (Table II, Fig 2) is

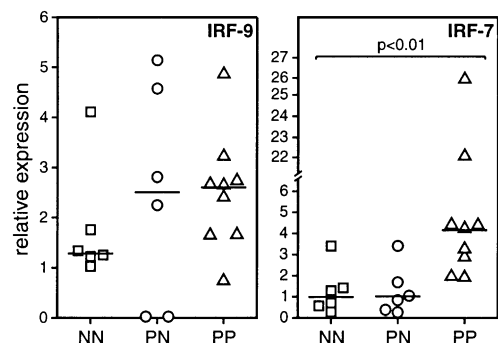


Figure 2 IRF-7 mRNA expression is significantly increased in epidermal cells from psoriasis plaques. IRF-9 and IRF-7 mRNA levels were assayed in total RNA extracts from healthy (NN, n = 6 donors), nonlesional (PN, n = 6 patients), and lesional psoriatic (PP, n = 9 patients) epidermal cells. IRF mRNA levels were calculated relative to amounts found in a standard sample, and standardized for GAPDH. Each symbol in the left and right panel represents the relative amount of IRF-9 mRNA or IRF-7 mRNA, respectively, for an individual patient. The median expression levels are indicated with horizontal lines. Statistical analysis was performed using the nonparametric Mann-Whitney test.

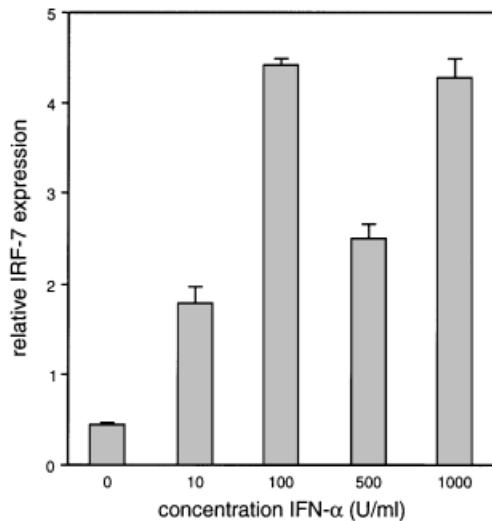


Figure 3
IFN- α induces IRF-7 mRNA expression in human keratinocytes. HaCaT cells were cultured for 24 h with the concentrations of IFN- α indicated. Total RNA preparations were assayed for IRF-7 expression by RT-PCR, calculated relative to amounts found in a standard sample, and standardized for GAPDH mRNA levels. A representative experiment is shown.

functionally involved in the early initiation of the psoriasis skin phenotype, we tested the effects of triggering this pathway on the expression of HLA-DR, ICAM-1, and K17. We demonstrated that components of the type I IFN signaling pathway are present and functional in HaCaT keratinocytes, as an increase in IRF-7 mRNA expression was observed after stimulation of HaCaT cells with IFN- α (Fig 3). Flow cytometric analysis, however, demonstrated that the expression of the psoriasis markers HLA-DR, ICAM-1, and K17 was not induced by IFN- α (Fig 4). When, as a control, HaCaT cells were treated with IFN- γ , the expression of the above-mentioned markers was increased, similarly as described previously (Fig 4).

To assess whether IFN- α was able to induce the psoriasis markers with delayed kinetics, HaCaT cells were cultured with IFN- α for 48 and 72 h. No induction of HLA-DR, ICAM-1, and K17 expression, however, was observed after these periods of time (data not shown).

It was demonstrated previously that IRF-9 expression is induced by IFN- γ (Levy *et al*, 1990; Veals *et al*, 1992), resulting in a considerably higher IFN- α response (Levy *et al*, 1990). To evaluate the effects of this IFN- γ priming on the induction of psoriasis markers by IFN- α , HaCaT cells were pretreated with low doses of IFN- γ for 6 h. Quantitative RT-PCR analysis indicated that these low IFN- γ doses induced both IRF-9 and IRF-7 mRNA expression. No synergistic effects of IFN- γ pretreatment on the IFN- α induced IRF-7 mRNA expression were observed (Fig 5a). The expression of ICAM-1 was induced to some extent by the low IFN- γ doses (Fig 5b, graph b). This IFN- γ pretreatment, however, did not have any priming effect on the IFN- α response, i.e., ICAM-1 expression was not further induced by IFN- α after IFN- γ pretreatment (Fig 5b, graph c). Identical results were observed for the other markers tested (e.g., HLA-DR and K17, data not shown). Thus, combinatorial treatment of keratinocytes with both IFN- α and IFN- γ did not result in a synergistic induction of the expression of psoriasis markers.

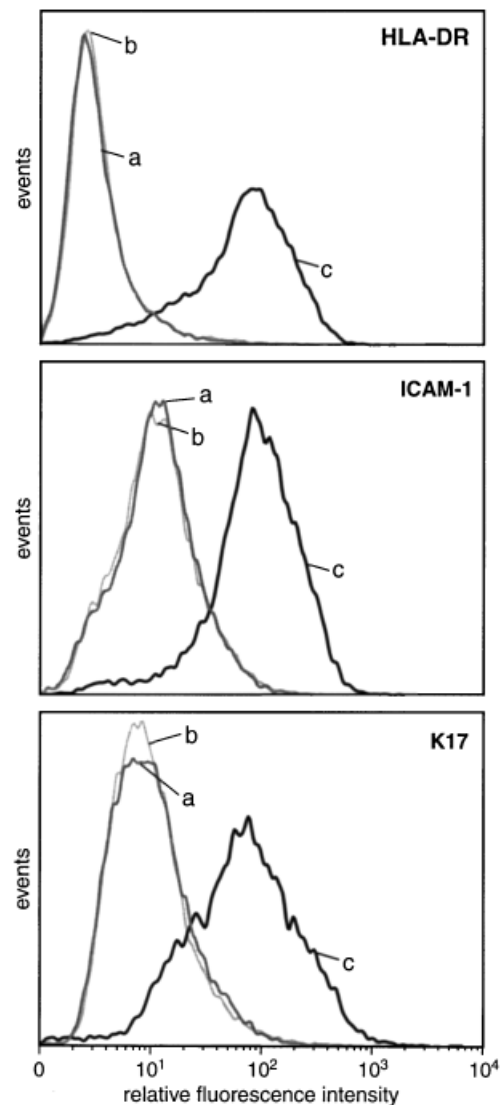


Figure 4
IFN- α does not induce the expression of psoriasis markers in HaCaT cells. HaCaT cells were stimulated for 24 h with 500 U per mL IFN- α or 500 U per mL IFN- γ . Cells were analyzed for expression of HLA-DR, ICAM-1, and K17 using flow cytometry. A comparison between cells cultured in medium alone (dark gray line, a), medium supplemented with IFN- α (light gray line, b), or medium supplemented with IFN- γ (black line, c) is shown in a representative experiment.

Stimulation of cultured healthy skin biopsies with IFN- α increases IRF-7 mRNA expression, but does not induce a psoriasis skin phenotype In addition to stimulation experiments using cultured HaCaT keratinocytes, we determined the effect of triggering type I IFN signaling in *ex vivo* cultured skin biopsies. Healthy skin biopsies were cultured for 24 h with IFN- α using a previously described skin biopsy culturing system (Companjen *et al*, 2001). To test whether in this model system IFN- α is capable of activating the type I IFN signaling pathway, we determined IRF-7 expression in RNA preparations from the epidermis of the cultured biopsies. In skin biopsies from most donors, IFN- α significantly induced epidermal IRF-7 mRNA expression (Fig 6). There was a considerable interdonor variability in the magnitude of the induction of IRF-7 expression after IFN- α stimulation (Fig 7, NN).

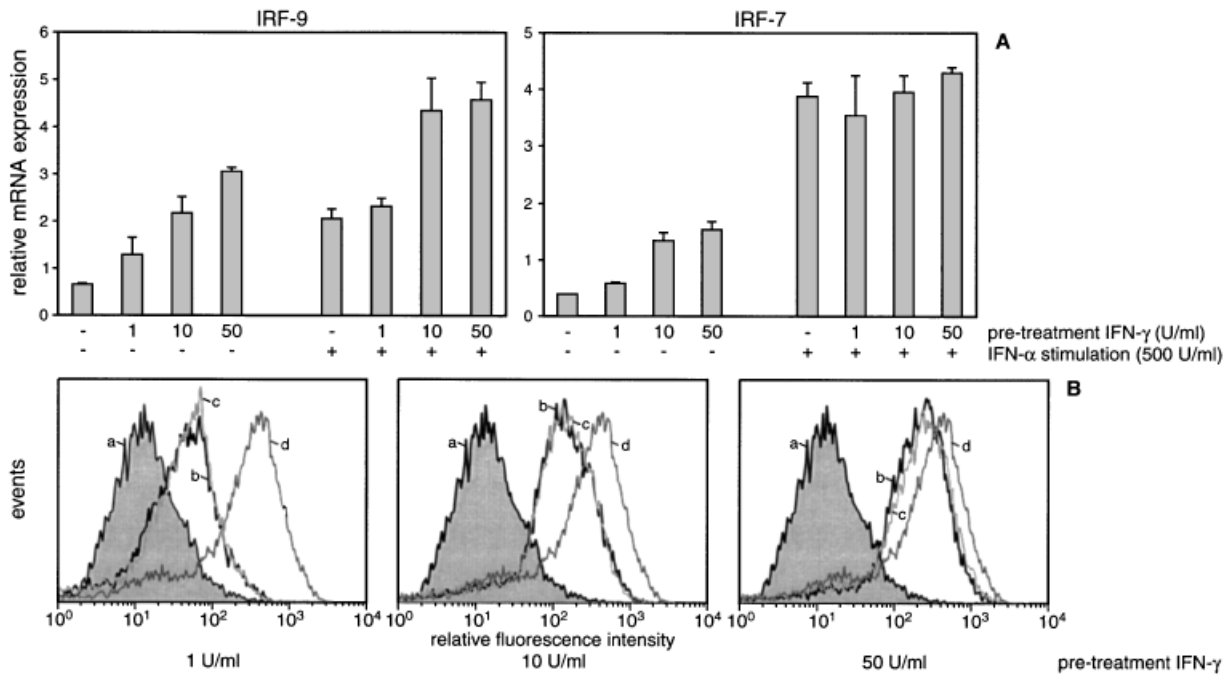


Figure 5
In HaCaT cells, pretreatment with low doses of IFN- γ does not have a priming effect on the induction of psoriasis markers by IFN- α . HaCaT cells were treated for 6 h with the indicated concentrations of IFN- γ , prior to stimulation with 500 U per mL IFN- α for 24 h. (a) IRF-9 and IRF-7 mRNA expression were determined in total RNA preparations by RT-PCR. (b) Flow cytometric analysis of ICAM-1 expression on cells cultured in medium alone (filled graph, a), medium with 1, 10, or 50 U per mL IFN- γ without (black line, b) or with additional IFN- α stimulation (light gray line, c). For comparison, ICAM-1 expression on cells treated with 500 U per mL IFN- γ (dark gray line, d) is depicted. Results from a representative experiment of three are shown.

To assess the effect of stimulation of the ISGF3/IRF-7 pathway on the expression of markers that are characteristic for the psoriasis phenotype, immunohistochemical analysis was performed on the cultured skin biopsies. The expression of the markers tested (i.e., MHC-II, ICAM-1, and K17) was increased upon culturing the skin biopsies in medium alone when compared with fresh, not cultured skin biopsies (Table III). Addition of IFN- α to the culture medium did not result in an additional increase in the expression of these markers. In contrast, addition of IFN- γ did result in a

clear and significant increase in ICAM-1 expression. For the other markers MHC-II and K17, no induction was observed by IFN- γ . This is not in agreement with previous findings that demonstrate a significant increase in HLA-DR and K17 expression upon IFN- γ stimulation of healthy skin biopsies (Wei *et al*, 1999). In this study, the basal expression of MHC-II and K17 in skin biopsies cultured in medium alone is higher when compared with previously reported data (Table III; Wei *et al*, 1999), and this expression could not be upregulated any further by type I or type II IFN.

To exclude that induction of psoriasis markers by IFN- α appears with delayed kinetics, skin biopsies were stimulated for 48 h or 72 h with IFN- α or IFN- γ . Results from immunohistochemical analysis of these biopsies were identical to the results presented in Table III (data not shown).

Our data therefore demonstrate that activation of type I IFN signaling, as accomplished by triggering healthy skin biopsies with recombinant IFN- α , is not sufficient to induce the initiation of the psoriasis skin phenotype in healthy skin biopsies.

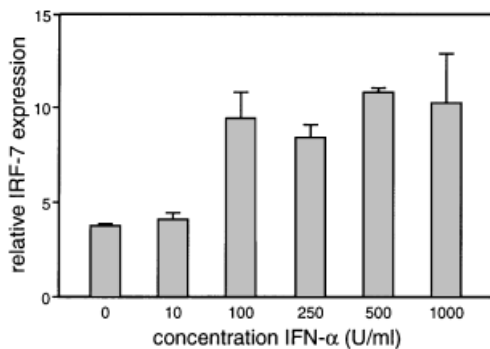


Figure 6
IRF-7 mRNA expression is increased in the epidermis of healthy skin biopsies stimulated with IFN- α . Healthy skin biopsies were cultured *ex vivo* for 24 h with the concentrations of IFN- α indicated. Dermis and epidermis were separated, and IRF-7 mRNA expression was determined by RT-PCR in total RNA isolated from the epidermis. Bars represent IRF-7 expression, calculated relative to amounts found in a standard sample, and standardized for GAPDH mRNA levels. Representative results obtained with material from one donor of eight are shown.

Non-lesional and lesional skin biopsies do not show an altered sensitivity to IFN- α stimulation To assess whether the activated ISGF3/IRF-7 signaling in lesional epidermal cells (Table II, Fig 2) is due to an altered sensitivity to IFN- α stimulation, nonlesional and lesional psoriatic skin biopsies were stimulated for 24 h with 500 U per mL IFN- α . Similarly to the healthy skin biopsies, a considerable interpatient variability in the induction of IRF-7 expression was observed (Fig 7). On average, IFN- α stimulation

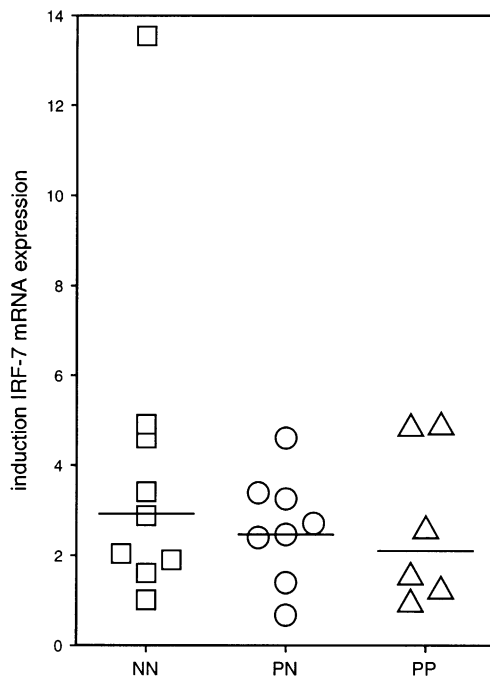


Figure 7
IFN- α stimulation of healthy, nonlesional, and lesional psoriasis skin biopsies results in comparable induction of IRF-7 mRNA expression. Biopsies from healthy donors (NN, n = 9), nonlesional (PN, n = 8), and lesional skin (PP, n = 6) from psoriasis patients were cultured for 24 h in medium alone, or medium supplemented with 500 U per mL IFN- α . Dermis and epidermis were separated, and total RNA was extracted from the epidermis. IRF-7 mRNA expression was assayed by quantitative RT-PCR, and standardized for GAPDH expression. For each individual donor or patient, the IRF-7 expression in the skin biopsy cultured in medium with IFN- α was calculated relative to the expression measured in the biopsy cultured in medium without additions. Each symbol represents the relative induction of IRF-7 expression for an individual donor/patient. The median induction levels are indicated with horizontal lines.

resulted in an induction of IRF-7 expression of 2.6-fold in nonlesional, and 2.4-fold in lesional skin biopsies (Table IV). These values were comparable with the IFN- α induced IRF-7 expression in healthy skin biopsies (Fig 7, Table IV).

In addition, healthy and nonlesional psoriatic skin biopsies were cultured with lower doses of IFN- α (10 and 100 U per mL). Also when these suboptimal IFN- α concentrations were used, the induction of IRF-7 mRNA expression was very similar for both skin types (Table IV).

Finally, the effect of IFN stimulation on the expression of the psoriasis markers in nonlesional psoriasis skin biopsies was assessed by immunohistochemistry. Similarly, as observed for healthy skin biopsies, protein expression of MHC-II, ICAM-1, and K17 was not induced in nonlesional biopsies stimulated with 500 U per mL IFN- α (Table III).

In summary, normal and psoriasis skin show identical responses to type I IFN with respect to induction of IRF-7 mRNA expression and protein expression of psoriasis markers.

Discussion

In this study we demonstrate that the type I IFN signal transduction pathway is activated in psoriatic lesional epidermis. Activation of this signaling route in cultured keratinocytes or skin biopsies can be mimicked by recombinant IFN- α . Triggering of this pathway, however, is not sufficient to induce markers that are used to define the psoriasis skin phenotype. Furthermore, we demonstrate that the sensitivity to type I IFN stimulation is not altered in psoriatic nonlesional and lesional skin.

Previously published work of Hida *et al* (2000) suggests that continuous, excessive IFN- α/β signaling, due to genetic deletion of IRF-2, is the primary cause of the development of inflammatory psoriasis-like skin lesions in mice. We here demonstrate that also in human psoriasis the IFN- α/β signaling pathway is activated. Dissimilar to the IRF-2^{-/-} mice, this is accomplished by increased expression levels and expression patterns of the type I IFN signaling components STAT1, IRF-9, and IRF-7, rather than by decreased IRF-2 expression (this study; Van Der Fits *et al*, 2003).

Our results show that nuclear localization of STAT1 and IRF-9 is increased in keratinocytes from psoriasis lesional

Table III. IFN- α does not induce the psoriatic epidermal phenotype in normal and psoriasis nonlesional skin biopsies

	MHC-II ^a	Keratin-17 ^a	ICAM-1 ^a
<i>Normal skin (n = 5)</i>			
Not cultured	0.8 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
Medium	1.5 ± 0.2	1.8 ± 0.5	0.4 ± 0.2
IFN- α	1.3 ± 0.2	1.5 ± 0.4	0.3 ± 0.1
IFN- γ	1.6 ± 0.3	1.1 ± 0.1	1.8 ± 0.4*
<i>Nonlesional psoriasis skin (n = 7)</i>			
Not cultured	0.8 ± 0.2	0.0 ± 0.0	0.1 ± 0.1
Medium	1.3 ± 0.2	1.3 ± 0.3	0.4 ± 0.1
IFN- α	1.5 ± 0.2	1.6 ± 0.4	0.4 ± 0.1
IFN- γ	1.8 ± 0.3	1.8 ± 0.4*	3.3 ± 0.2*

^aNormal or non-lesional psoriasis skin biopsies were not cultured, or cultured for 24 h in medium, or in medium supplemented with 500 U/ml IFN- α or 500 U/ml IFN- γ . Cryosections were stained for the markers indicated and semi-quantitatively scored. Values indicate the mean score ± SEM. Asterisks indicate statistically significant differences in marker expression between the biopsy cultured in the medium with IFN, when compared to be biopsy cultured in medium alone ($p < 0.05$) tested using the parametric T-test.

Table IV. IRF-7 mRNA expression is induced by IFN- α to similar levels in healthy, nonlesional and lesional skin biopsies

Concentration IFN- α	Fold induction in IRF-7 expression ^a		
	NN (n = 9)	PN (n = 6 or 8)	PP (n = 6)
10 U/ml	1.4 \pm 0.4	1.1 \pm 0.2	ND ^b
100 U/ml	2.2 \pm 0.6	1.9 \pm 0.3	ND ^b
500 U/ml	4.0 \pm 1.3	2.6 \pm 0.4	2.4 \pm 0.7

^aBiopsies from healthy donors (NN), nonlesional skin (PN) and lesional skin (PP) from psoriasis patients were cultured for 24 h with the concentrations of IFN- α indicated. The relative induction in IRF-7 mRNA expression was assayed and calculated as described in the legend of Figure 6. Values represent mean IRF-7 induction \pm SEM. Statistical analysis using the non-parametric Mann-Whitney test revealed no significant differences in IRF-7 induction between NN, PN and PP.

^bND: not determined.

skin, implying functional activation of the ISGF3 complex. This was confirmed by high levels of IRF-7 mRNA, a gene that is transcriptionally activated by nuclear ISGF3. Also in nonlesional skin, nuclear localization of IRF-9 is significantly increased (Table II). In this skin type, however, functional activation of the ISGF3 complex is not detectable, as IRF-7 mRNA expression remains unaltered (Fig 2). This discrepancy might be due to methodologic differences between the assays used. To assay the IRF-9 nuclear translocation, we counted the number of epidermal cells that express nuclear IRF-9 using immunohistochemistry, thereby making a clear distinction between cells that do or do not display nuclear IRF-9 localization. In contrast, IRF-7 mRNA expression represented the average expression level in the complete population of epidermal cells, irrespective of the nuclear IRF-9 expression. Thus the increase in IRF-9 nuclear translocation that is observed in nonlesional psoriatic skin might not be sufficient to cause measurable increases in IRF-7 mRNA expression in the total population of epidermal cells.

In this study we used a generally accepted definition of the psoriasis skin phenotype based on the epidermal expression of markers that are associated with skin inflammation (i.e., HLA-DR and ICAM-1) and altered differentiation and proliferation of keratinocytes (i.e., K17). These markers are upregulated in psoriatic keratinocytes, and widely used to monitor improvement of psoriasis upon therapy (e.g., de Jong *et al*, 1991; Rizova *et al*, 1994; Trepicchio *et al*, 1999). Here we show that expression of these markers on HaCaT keratinocytes is not induced by triggering the IFN- α/β signal transduction pathway. As the human skin is a complex tissue comprising many different cell types, results obtained in HaCaT keratinocytes might not be representative for the *in vivo* situation in skin. Experiments that were performed using skin organ cultures yielded identical results, i.e., no induction of psoriasis markers by IFN- α . Together this suggests that type I IFN does not have a direct effect on keratinocytes in the initiation phase of psoriasis. In contrast, a direct role for IFN- γ in the initiation of inflammatory skin diseases has been demonstrated by *in vivo* studies in transgenic mice (Carroll *et al*, 1997). These data are supported by results from *in vitro* experiments showing that the expression of psoriasis markers can be induced in HaCaT cells and skin biopsies cultured with IFN- γ (this study, Bonnekoh *et al*, 1995; Sebok *et al*, 1998; Wei *et al*, 1999).

Type I IFN can have several immunomodulatory effects (reviewed by Tompkins, 1999). For example, IFN- α provides an important signal for the differentiation of CD4⁺ T cells into T helper 1 cells. Furthermore, IFN- α is responsible for the amplification of the CD8⁺ T cell responses and natural killer cell activation. Both CD4⁺ T helper 1, CD8⁺ T cells and natural killer cells have been implicated to be important in the pathogenesis of psoriasis (Nickoloff, 1999). Thus, the effects of type I IFN during the initiation phase of psoriasis might involve effects exerted on T or natural killer cells, rather than directly on keratinocytes. This might explain the failure of IFN- α to induce the psoriatic epidermal phenotype in cultured skin biopsies, which are almost devoid of T cells.

Numerous previous studies demonstrated that psoriasis lesional skin displays an altered sensitivity to IFN- γ . For example, it was shown that IFN- γ -induced expression of IRF-1, a transcription factor that occupies a central role in IFN- γ signaling (Taniguchi *et al*, 2001), was decreased in cultured psoriatic keratinocytes (Jackson *et al*, 1999; Chen *et al*, 2000). We observed similar results on the expression of IRF-1 when stimulating psoriasis lesional skin biopsies with recombinant IFN- γ (data not shown), validating the experimental set-up used in this study. Here we demonstrate that the induction of IRF-7 expression by type I IFN is similar in healthy, nonlesional, and lesional psoriatic skin. Thus the observed increase in ISGF3/IRF-7 signaling in lesional psoriasis skin (Table II, Fig 2) is not caused by an increased sensitivity of psoriatic skin to type I IFN. In addition, also with respect to the induction of psoriasis markers, the sensitivity to type I IFN signaling in healthy and nonlesional psoriasis skin biopsies was identical. In both types of skin biopsies, IFN- α was not able to induce the initiation of the psoriasis skin phenotype.

Demonstration of the activated type I IFN signaling pathway in psoriatic skin in this study is in complete agreement with increased expression levels of various IFN-inducible proteins such as MxA and 2'-5' oligoadenylated synthetase that have been described previously (Schmid *et al*, 1994; Fah *et al*, 1995). These alterations might be caused by an enhanced type I IFN production in psoriatic skin. Reports on this topic are conflicting. Immunohistochemical analysis showed that IFN- α protein was not detectable in healthy skin, or in stable psoriatic skin (Livden *et al*, 1989). In contrast, Schmid *et al* (1994) demonstrated that IFN- α mRNA was expressed throughout the epidermis of stable psoriatic skin lesions, whereas no specific signals

were revealed in nonlesional skin from psoriasis patients or in skin from healthy donors. Recently, it was demonstrated that the number of plasmacytoid dendritic cells, cells that are capable of producing high levels of IFN- α , is significantly increased in psoriatic skin (Wollenberg *et al*, 2002). Thus, the observed ISGF3/IRF-7 signaling in psoriasis skin lesions might well be caused by IFN- α production by infiltrating plasmacytoid dendritic cells.

Recently, a novel type I IFN was described, IFN- κ , which is expressed in human keratinocytes (LaFleur *et al*, 2001). Whereas healthy skin only showed scattered IFN- κ -positive cells in the dermis, numerous monocytes and dendritic cells expressing IFN- κ were present in the epidermis and dermis of chronic skin lesions from psoriasis patients. Furthermore, keratinocytes in basal and suprabasal epidermal cell layers showed IFN- κ expression (Nardelli *et al*, 2002). As IFN- κ , like IFN- α , signals through the type I IFN receptor (LaFleur *et al*, 2001), it is possible that the increased ISGF3/IRF-7 signaling observed in this study is directly linked to enhanced IFN- κ expression. Elucidation of the functional role for IFN- κ in the skin immune system would shed more light on the contribution of type I IFN to the pathogenesis of psoriasis.

In summary, we demonstrate that the expression of type I IFN signaling components is markedly increased in psoriasis skin lesions. Triggering this pathway through its physiologic receptor, however, does not result in the induction of the regenerative epidermal phenotype of psoriatic lesions. Furthermore, responses to IFN- α stimulation do not differ between healthy, nonlesional, and lesional psoriatic skin. Our data might suggest that activated type I IFN signaling is dispensable during the initiation phase of psoriasis, although a functional role in the maintenance of the skin inflammation cannot be excluded. Alternatively, activation of the type I IFN system might play an indirect role in the induction of the psoriasis skin phenotype, possibly by exerting its effects on other cell types, such as IFN- γ secreting natural killer or T cells, rather than directly on keratinocytes.

Materials and methods

Patient material All human tissue samples were obtained after informed consent and approval of the medical ethics committee of the Erasmus MC, the Netherlands. Normal skin samples were obtained from 23 females (aged 17–59 y, median age 36 y), undergoing breast or abdominal plastic surgery (Sint Franciscus Hospital Rotterdam, Erasmus MC, the Netherlands). Lesional and nonlesional skin samples were taken from 29 patients (11 females, 18 males, aged 19–73 y, median age 48 y) with stable plaque-type psoriasis (Ziekenhuis Walcheren Vlissingen, the Netherlands). Prior to participation in this study, psoriasis patients received no topical treatment for a period of at least 3 wk, and no systemic treatment for at least 2 mo.

Isolation of epidermal cell suspensions Split skin specimens were obtained using a portable dermatome (Padgett Instruments Inc., Kansas City, Missouri). Epidermal cell suspensions were prepared using standard methods (Prens *et al*, 1991; Van Der Fits *et al*, 2003). Viability of the cells was checked by trypan blue exclusion.

Culturing of skin biopsies and HaCaT cells Biopsies were taken from skin of healthy individuals or psoriasis patients using a 3 mm

diameter biopsy punch (Stiefel, Leuven, Belgium). Biopsies were cultured as described previously (Companjen *et al*, 2001). Briefly, four 2 mm holes (two holes in case of culturing psoriasis skin biopsies) were punched in a Netwell filter (pore size: 0.75 μ m; Corning Costar, Corning, New York). In each hole, one biopsy was inserted. The filter was placed in a 12-well culture plate containing 1 mL IMDM medium (GibcoBRL, Paisley, UK) supplemented with 1% human serum (Sigma, St Louis, Missouri), 100 U penicillin per mL, 100 μ g streptomycin per mL, and 2 mM ultra glutamine (BioWhittaker, Walkersville, Maryland). Biopsies were stimulated with clinically approved IFN- α (IntronA IFN- α 2b, Schering-Plough, Kenilworth, New Jersey) or IFN- γ (Boehringer Ingelheim, Heidelberg, Germany) for 24 h at 37°C and 5% CO₂. After culturing, one of the biopsies was immersed in TissueTek (Bayer, Munich, Germany), snap frozen in liquid nitrogen and stored at –80°C until use. The remaining biopsies were incubated in phosphate-buffered saline (PBS) containing 5 mg thermolysin per mL (Protease type X, Sigma) for 2 h at 37°C. The epidermis was separated from the dermis using fine forceps. Epidermal cells were lysed in 250 μ L lysis buffer (GenElute Mammalian Total RNA kit, Sigma) and stored at –80°C until use.

HaCaT cells (Boukamp *et al*, 1988) were cultured in RPMI 1640 medium (GibcoBRL) supplemented with 5% heat inactivated fetal calf serum (BioWhittaker), 100 U penicillin per mL, 100 μ g streptomycin per mL, and 2 mM ultra glutamine at 37°C and 5% CO₂. The cells were passaged every 7 d.

Immunohistochemistry Six micrometer cryosections of snap frozen biopsies were cut using a cryostat (Jung Frigocut 2800 E, Leica, Rijswijk, the Netherlands) and stored until use at –80°C in a sealed box containing silica gel. Sections were fixed in acetone (Fluka Chemie AG, Buchs, Switzerland) containing 0.5% H₂O₂ for 10 min at room temperature. Sections for immunohistochemical staining for STAT1, IRF-7, and IRF-9 were fixed by dipping in acetone, air-drying, incubating in Zamboni's fixative (2% w/v paraformaldehyde, 15% v/v saturated picric acid, 1.88% w/v NaH₂PO₄·H₂O, pH 7.3) for 30 min, dipping in 80% ethanol and rinsing for 30 min in PBS in the presence of 0.5% H₂O₂ during the last 10 min. After fixation, sections were preincubated for 10 min in PBS at room temperature. Endogenous peroxidase activity was revealed with 4-chloro-1-naphthol (Sigma), resulting in a dark blue precipitate. Slides were washed with PBS containing 0.05% Tween 20 (Merck, Whitehouse Station, New Jersey) and incubated with primary antibody (Table I) overnight at 4°C, or for 1 h at room temperature. This was followed by incubation for 30 min with biotin-linked secondary rabbit-anti-mouse or donkey-anti-rabbit antibody and peroxidase-linked avidin (DAKO, Glostrup, Denmark). Biotinylated major histocompatibility complex (MHC) II (HLA-DP-DQ-DR) antibody was detected directly by streptavidin-linked peroxidase. 3-amino-9-ethylcarbazole (Sigma) was used as the chromogen, resulting in a bright red staining. Sections incubated with an antibody of the same isotype as the specific antibody but of irrelevant specificity served as controls. MHC-II, ICAM-1, and K17 expression on keratinocytes was evaluated independently by two observers using semiquantitative grading from 0 to 4, as described previously (Griffiths *et al*, 1991); 0 = negative; 1 = weak staining, 2 = moderate staining; 3 = strong staining; 4 = very strong staining. STAT1, IRF-7, and IRF-9 expression was quantified independently by two observers by counting positive nuclei in the epidermis. The total number of nuclei present in the epidermis of the different biopsies was counted in slides stained with hematoxylin.

Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) Total RNA was extracted from epidermal cell suspensions, HaCaT (1–2 \times 10⁶ cells) or epidermal sheets using RNazolB (Campro Scientific, Veenendaal, the Netherlands) or the GenElute Mammalian Total RNA kit (Sigma). Using 1 μ g of the total RNA as template, copy DNA (cDNA) was obtained using the AMV Reverse Transcription System (Promega, Madison, Wisconsin).

Real-time quantitative RT-PCR was performed as described previously (Van Der Fits *et al*, 2003). Primer sequences (forward primer and reverse primer, respectively) and their optimal concentrations were: IRF-7 (TCC CGC CGC TAT ACC ATC TAC CT (50 nM), ACA GCC AGG GTT CCA GCT T (50 nM)) and IRF-9 (GGA GCA GTC CAT TCA GAC ATT G (50 nM), ATG AAG GTG AGC AGC AGT GAG TAG T (900 nM)). The FAM-labeled probes (PE-Applied Biosystems, Foster City, California) CTT CGG GCA GGA CCT GTC AGC TG (IRF-7) and TCA GAG GTC CCT GGA GTT TCT GCT TCC T (IRF-9) were used at a concentration of 100 nM. GAPDH mRNA levels were measured as a control to normalize for RNA input. For this, a commercially available primer/probe mixture was used (PE-Applied Biosystems). There was no consistent effect of IFN treatment on the GAPDH mRNA expression, validating the use of this gene as control gene. IRF mRNA levels were calculated relative to amounts found in a standard sample, and corrected for GAPDH mRNA levels.

Flow cytometric analysis HaCaT cells were seeded into six-well culture plates (Nalgen Nunc International, Rochester, New York) at a density of 3.5×10^5 cells per well in 2 mL. The cells were allowed to adhere and to grow for 24 h. Cells were rinsed with PBS and stimulated for 24 h in fresh medium with or without IFN- α or IFN- γ . Cells were detached with PBS containing 0.025% trypsin and 0.1% EDTA, washed in PBS and resuspended at a concentration of 10×10^6 cells per mL. Forty microliters of cell suspension was mixed with 40 μ L of the primary antibody solution (Table I) and incubated for 10 min at room temperature. Prior to labeling with monoclonal antibodies against K17, cells were permeabilized using Intraprep (Immunotech, Marseille, France). Cells were washed twice with PBS/0.2% bovine serum albumin (pH 7.8), and, if the primary antibody was not directly labeled, incubated with a secondary antibody, goat-anti-mouse-PE (Sanbio, Uden, the Netherlands) for 10 min room temperature. Cells were washed twice, resuspended in FACS buffer and analyzed on a flow cytometer (FACScan, BD Biosciences, Franklin Lakes, New York).

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