Keratinocyte CDw60 Expression is Modulated by Both a Th-1 Type Cytokine IFN-γ and Th-2 Cytokines IL-4 and IL-13: Relevance to Psoriasis

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Psoriasis is a chronic skin disease with an immunocytic infiltrate, including activated T lymphocytes, producing multiple cytokines that can influence the phenotype of epidermal keratinocytes. In these studies we examined the effect of the cytokines interferon-γ and interleukin-13 or interleukin-4 on keratinocytes, alone and in combination, on surface levels of HLA-DR, intercellular adhesion molecule 1, and CDw60, as well as the transcription factors STAT1, STAT6, and BCL-6. As CDw60 is an acetylated form of the G_{D3} ganglioside and may function as a T cell costimulatory molecule, the modulation of CDw60 expression by keratinocytes in psoriatic lesions was highlighted to gain insight into potentially important T cell-keratinocyte interactions. Interferon-γ was observed to block the interleukin-4- or interleukin-13-mediated induction of CDw60 on cultured keratinocytes, but not induction of the transcription factor STAT6. Interleukin-13 and interleukin-4 were unable to block interferon-γ-mediated induction of STAT1 or BCL-6, however, or the upregulation of intercellular adhesion molecule 1 and HLA-DR. In psoriatic plaques, CDw60 was not consistently detected on keratinocytes in acute lesions, but was detected predominantly on basal layer keratinocytes in chronic lesions. In addition we found that BCL-6 levels were increased in psoriatic lesions; in acute lesions BCL-6 was primarily localized in the basal layer keratinocytes, whereas in chronic plaques nuclear BCL-6 was predominantly expressed by keratinocytes in the suprabasal cell layers. These studies highlight the complex modulation of the keratinocyte phenotype by immunocyte-derived cytokines, in which induction of CDw60 involving interleukin-4, or interleukin-13 was antagonized by interferon-γ. We suggest in psoriatic plaques that the presence or absence of CDw60 expression by keratinocytes may reflect the dynamic interplay between Th-1-type cytokines such as interferon-γ and Th-2-type cytokines such as interleukin-4 and interleukin-13. The ability of interferon-γ to induce the transcription repressor BCL-6 may also contribute to the overall immunologic events in skin, including suppression of the intermediates in the synthetic pathway leading to expression of the T cell costimulatory ganglioside CDw60. ***Key words: BCL-6/ganglioside/immunocyte/STAT1/STAT6. J Invest Dermatol 116:305–312, 2001***

Psoriasis is a chronic inflammatory skin disease (Valdimarsson *et al*, 1986; Gottlieb *et al*, 1995; Bos and DeRie, 1999) characterized by hyperproliferation of basal and suprabasal keratinocytes. The biochemical mechanism or mediators responsible for this hyperproliferation remains unclear, but immunocytes and cytokines have long been suspected of playing a pathogenic role (Nickoloff, 1999). This hypothesis is supported by the findings that a T cell infiltrate and cytokines, such as interferon-γ (IFN-γ), can be identified in psoriatic lesions, and that these factors can induce expression of a variety of cell surface activation molecules associated with psoriasis (Fierlbeck *et al*, 1990; Barker *et al*, 1993). In chronic psoriatic lesions, many immunologically relevant proteins such as intercellular adhesion molecule 1 (ICAM-1), major histocompatibility class I (MHC class I) and MHC class II antigens, as well as carbohydrate-bearing molecules such as the acetylated G_{D3} ganglioside CDw60, have been shown to be upregulated on the surface of lesional keratinocytes (Cooper, 1992; Skov *et al*, 1997). The signaling pathways responsible for the modulation of these important cell surface molecules on keratinocytes is poorly understood, however, in psoriasis. Moreover, although the synthetic pathway leading to the synthesis and expression of gangliosides such as G_{D3} has been established for keratinocytes, exactly which transcriptional factors contribute to CDw60 has not been elucidated (Paller *et al*, 1993, 1995).

Recently, our group has shown that a pathogenic T cell line, which is CD161 positive, can induce an acute psoriatic lesion when injected into symptomless autologous human skin engrafted onto a severe combined immunodeficiency (SCID) mouse (Nickoloff *et al*, 2000). Further characterization of this T cell line revealed that it produced both Th-1 (IFN-γ) and Th-2 [interleukin-13 (IL-13)] cytokines upon stimulation *in vitro*. RNase protection assays in an acute psoriatic lesion produced after *in vivo* injection, however,

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Abbreviation: SCID, severe combined immunodeficiency.

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demonstrated that there was a cytokine switch to a predominantly Th-1 IFN-γ profile, with only barely detectable levels of IL-13 and IL-4 mRNA. Interestingly, this correlates with an absence of CDw60 expression in keratinocytes in this acute lesion. Whether an imbalance of Th-1 *versus* Th-2 cytokines has an effect on the pattern of activation of keratinocytes may be pertinent to the pathogenesis of psoriasis (Nickoloff, 1991; Gottlieb, 1994;Schlaak *et al*, 1994).

IFN-γ and IL-13 are cytokines that have demonstrated opposing functions in human monocytes (Dickensheets and Donnelly, 1999). It is known that IFN-γ is a potent inducer of the transcription of many genes involved in inflammatory reactions. Genes that are upregulated by IFN-γ include both MHC class I and class II molecules, in addition to adhesion molecules such as ICAM-1 (Tessitore *et al*, 1998; Piskurich *et al*, 1999). It has also been previously demonstrated that CDw60 expression is not upregulated by treatment with IFN-γ, but is induced when cultured keratinocytes are exposed to IL-13 (Skov *et al*, 1997). In chronic psoriatic lesions, CDw60 expression has been localized to the basal and suprabasal keratinocytes, although considerable variation in the pattern of expression was observed in 12 different plaques (Skov *et al*, 1997). This acetylated ganglioside CDw60 is also an activation marker for both T and B lymphocytes (Vater *et al*, 1997). A very high frequency of CDw60 positive T cells is found in the synovial fluid of normal and arthritic patients and in cutaneous psoriatic lesions (Baadsgaard *et al*, 1990; Fox *et al*, 1990; Carr *et al*, 1995). Whether CDw60 is an activation marker for keratinocytes and the functional role of this molecule is still unclear, however, although keratinocyte CDw60 expression may serve as a costimulatory molecule for T cell activation (ibid). Currently it is also unknown whether IFN-γ and IL-13 signals can mediate biochemical reactions that impact each other in keratinocytes, or reciprocally influence cell surface marker expression.

In order to investigate possible antagonistic responses between Th-1- *versus* Th-2-type cytokine signals in keratinocytes, we initially investigated the cell surface marker expression of keratinocytes upon stimulation with IFN-γ *versus* IL-4 or IL-13, both alone and in combination. We used a panel of surface markers consisting of MHC class II (HLA-DR), ICAM-1, and CDw60. To examine the molecular pathways in keratinocytes for IFN-γ, IL-4, and IL-13, as well as their potential interactions, we also examined the downstream signaling pathways of these cytokines via STAT1, STAT6, and BCL-6 transcription factors. IL-4 and IL-13 have been shown to function through the regulatory protein STAT6 (Palmer-Crocker *et al*, 1996). IFN-γ has been shown to activate genes through the transcription factor STAT1. BCL-6 is a known transcriptional regulator for Th-2-type cytokines. BCL-6 protein is a nuclear phosphoprotein belonging to the POZ/zinc finger family of transcription factors (Onizuka *et al*, 1995). Interestingly, BCL-6-deficient mice develop a Th-2-type inflammation, suggesting that BCL-6 may be an important regulator of Th-2 response (Dent *et al*, 1999). Recently, it has been demonstrated that BCL-6 can repress IL-4-induced transcription mediated by STAT6 (Harris *et al*, 1999). Whether IFN-γ can alter BCL-6 levels in keratinocyte nuclei, however, is unknown, and whether BCL-6 plays a role in psoriatic plaques remains unexplored.

In this report it is demonstrated that IFN-γ can completely inhibit IL-4- or IL-13-mediated induction of CDw60 by keratinocytes. Moreover, IFN-γ can induce both STAT1 and BCL-6 in cultured keratinocytes, and psoriatic plaques overexpress the transcriptional repressor BCL-6. Taken together, these preliminary immunohistochemical results indicated that the lack of CDw60 expression by keratinocytes in acute psoriatic lesions may be due to the ability of IFN-γ to suppress, via BCL-6, the IL-4- or IL-13-mediated induction of CDw60. It remains to be determined exactly how IFN-γ blocked surface expression of CDw60, however, with one possibility being the involvement of BCL-6 suppressing one or more transcriptional factors that regulate the complex multistep process in which the acetylated form of the G_{D3} ganglioside is expressed by the keratinocyte. Furthermore, as the chronic plaque evolves, a dynamic interplay develops between cytokines, their respective signaling pathways, and transcription factors (Nickoloff, 2000). With this dynamic system, it can be envisioned that CDw60 expression is induced on the basal layer, but not above in more superficial layers, of lesional keratinocytes. Such reciprocal and antagonistic interactions between Th-1- and Th-2-type cytokines highlights the complexity of locally produced cytokines, and suggests that the cytokine network in acute psoriatic lesions may be different from the cytokine network in chronic psoriatic plaques.

MATERIALS AND METHODS

Patient samples Four millimeter punch biopsies of normal adult skin, symptomless psoriatic skin, and active untreated psoriatic plaques were obtained after local anesthesia and informed consent, using a protocol approved by the Institutional Review Board. Samples were then snap frozen in isopentane chilled in liquid nitrogen and stored at –80°C, or fresh tissue samples had their proteins extracted for western blot analysis as described below. Five different normal and psoriatic patients were included in this study (three female, two male). All psoriatic patients had active untreated acute ($n = 2$) or chronic ($n = 3$) psoriatic lesions.

Human skin/SCID mouse chimera animal model Acute psoriatic lesions were generated using the human skin/SCID mouse chimera model as previously described (Nickoloff *et al*, 1999). Briefly, symptomless psoriatic human skin was orthotopically transplanted onto CB17-SCID mice. Three weeks following transplantation, bacterially derived superantigen activated autologous immunocytes (2×10^6) were injected intradermally into the xenograft. Upon development of an acute psoriatic lesion at 2 wk, the animal was sacrificed and a 4 mm punch biopsy was snap frozen in liquid nitrogen chilled isopentane. Biopsies used in this study involving SCID mice were obtained during the course of earlier published experimental protocols (Nickoloff *et al*, 1999, 2000).

Cell culture Human neonatal foreskin derived keratinocytes were cultured in keratinocyte growth medium (KGM; Clonetics, San Diego, CA) containing 0.07 mM Ca^{2+} as previously described (Stoof *et al*, 1992). In some experiments, the KGM was supplemented with additional calcium to raise the concentration to 2 mM. For cytokine stimulation, keratinocytes were switched to KGM containing 0.15 mM Ca^{2+} supplemented with cytokines as indicated. Based on preliminary studies to determine optimal concentrations, the actual dosage of cytokines used were as follows: human recombinant IL-4, 50 ng per ml; human recombinant IL-13, 100 ng per ml; and IFN-γ, 1000 U per ml (all cytokines purchased from R&D Systems, Minneapolis, MN).

Flow cytometric analysis After 48 h stimulation with different cytokines, keratinocytes were harvested by using 0.03% trypsin/0.01% ethylenediamine tetraacetic acid. Then 2×10^5 cells were incubated with the following antibodies at 4°C for 45 min: 10 μg per ml of phycoerythrin-conjugated anti-HLA-DR antibody (Becton Dickinson, Sunnyvale, CA); 10 μg per ml of anti-ICAM-1 antibody (Pharmingen, San Diego, CA); anti-CDw60 antibody UM4D4 ascites was used at 1:400 dilution (generous gift from Dr. D.A. Fox, University of Michigan, Ann Arbor, MI). Samples were washed with flow buffer (phosphate-buffered saline, 5% fetal bovine serum, 0.02% sodium azide) at 4°C for 5 min and centrifuged. After removing the supernatant, the secondary antibody was added to the cell pellets and incubated at 4°C for 30 min. Fluorescein-isothiocyanate-labeled goat antimouse antibody (Biosource International, Camarillo, CA) was used as the secondary antibody for anti-ICAM-1 antibody and anti-CDw60 antibody UM4D4. Primary antibody concentrations were UM4D4 (IgM ascites) diluted 1:100 and BCL-6 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25. After incubation, cells were washed with flow cytometry buffer at 4°C for 5 min and centrifuged. Cells were fixed in 2% paraformaldehyde and analyzed with Coulter Epics XL Flow Cytometer (Miami, FL). To detect intracellular levels of CDw60, keratinocytes were fixed with paraformaldehyde and then were permeabilized using 0.3% saponin (Wrone-Smith *et al*, 1995), washed, and processed as described above.

Western blot analysis Nuclear extracts of keratinocytes were prepared by washing cells with ice-cold phosphate-buffered saline and then harvesting by scraping from the plates. Buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgC1_2$, 0.2 mM ethylenediamine tetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) was added to the cell pellets, they were incubated on ice for 15 min, and then a 10% Nonidet P-40 solution was added. After vortexing and centrifugation for

30 s at 4°C, buffer C [20 mM HEPES, 0.4 M NaCl, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride] was added to the pellets, and they were incubated at 4°C for 15 min on a shaking platform. The supernatant was collected after centrifugation at 4°C in a microfuge for 5 min.

Nuclear protein concentrations were determined by BioRad Assay in which 30 μg of protein from each sample was loaded onto 8% sodium dodecyl sulfate polyacrylamide gels, transferred to Immobilon-p (Millipore, Bedford, MA) membranes, and blocked with 5% nonfat milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). Membranes were incubated with the primary antibody in 2.5% milk in TBST for 2 h and then washed three times for 10 min each and incubated with 1:1500 diluted antirabbit horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were visualized with ECL reagents (Amersham Pharmacia Biotech) and exposure to Biomax X-ray film (Kodak, Rochester, NY). Primary polyclonal rabbit antibodies against STAT1, phospho-STAT1, STAT6, and BCL-6 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Sigma, St. Louis, MO) were used at 10 μg per ml.

Densitometric analysis Radiographic films were scanned with a top lit HP ScanJet Scanner and analyzed with NIH Image software. Relative values were determined by integrative average value. The lowest value was arbitrarily set to 1.0, and higher levels were calculated as fold increases over this value.

Electrophoretic mobility shift assay In brief, 1 μg of poly-(dI-dC) (Amersham Pharmacia Biotech) and 10^5 cpm of ^{32}P-labeled double-stranded oligonucleotide were incubated with 5 μg of nuclear protein on ice for 30 min. The reaction mixture was separated on 4% native polyacrylamide gel, dried, and exposed to film. The STAT1 oligonucleotide has the sequence 5′-CATGTTATGCATATTCCTGT-AAGTG-3′. The STAT6 oligonucleotide has the sequence 5′-AGA-TTTCTAGGAATTCAATCC-3′. The origin of the STAT1 and STAT6 oligonucleotide sequences was derived from Santa Cruz Biotechnology. Specificity for these binding assays was confirmed by use of a specific unlabeled oligonucleotide in a competition analysis for STAT1, and by performing supershift analysis using antibodies directed against STAT6 (data not shown).

Immunohistochemical staining Cryostat sections were fixed in cold acetone and the avidin-biotin peroxidase staining procedure was followed as previously described (Nickoloff *et al*, 1995). Briefly, primary antibodies were incubated for 1 h at room temperature followed by subsequent steps as per the manufacturer's instructions (Vectastain; Vector Laboratories, Burlingame, CA). Positive detection was accomplished using 3-amino-4-ethylcarbazole as the chromagen with 1% hematoxylin as the counterstain.

RESULTS

IL-13 treatment of keratinocytes upregulates CDw60 expression and IFN-γ pretreatment or simultaneous treatment blocks induction of CDw60 expression by IL-13 on keratinocytes Previous studies have shown that, in normal epidermis, basal keratinocytes are negative for CDw60 expression (Skov *et al*, 1997). In chronic psoriatic plaques, focal and confluent basal and suprabasal keratinocytes express CDw60 (Skov *et al*, 1997). The expression of CDw60 is highly variable, however. In preliminary studies, we observed that the expression pattern of keratinocyte CDw60 appeared to correlate with the stage of psoriasis. In the acute phases of psoriasis, there was little to no CDw60 expression detectable on keratinocytes (data not shown). In chronic plaques, however, there was more consistent expression of CDw60 in the basal layer keratinocytes (**Fig 1**). In searching for a possible mechanism to explain these different staining patterns, we were intrigued by the observation from our SCID/human skin model in which an acute psoriatic-like lesion was created by injecting a subset of T lymphocytes, the NK T cells (Nickoloff *et al*, 2000), directly into symptomless skin engrafted on the mouse. In this study, we also found that, in the acute psoriatic lesion that was rapidly generated, there was an absence of CDw60 expression by the epidermal keratinocytes (**Fig 1**). Probing this acute lesion, we observed that there were elevated levels of the Th-1-type cytokine (i.e., IFN-γ) mRNA and low to absent Th-2-type cytokines (e.g., absent IL-4 and low IL-13 mRNA production) (Nickoloff *et al*, 2000). As it has been shown that IL-4 and IL-13 upregulate the surface expression of CDw60 on keratinocytes, whereas IFN-γ decreases its constitutive expression (Skov *et al*, 1997), this led us to speculate that the expression of CDw60 by keratinocytes may be modulated by an antagonistic interaction involving Th-1- *versus* Th-2-type cytokines.

In order to explore the cell surface expression of the acetylated ganglioside G_{D3} (i.e., CDw60) and its regulation, normal keratinocytes were stimulated with different cytokines, and CDw60 surface expression was examined by flow cytometric analysis. Cells were maintained in a proliferative state at low density, and stimulated for 48 h before harvesting and staining. As shown in **Fig 2(*A*)**, normal cultured keratinocytes grown in low calcium (0.07 mM) KGM express a relatively low level of CDw60 constitutively on a minority of cells (30%). When grown in KGM supplemented with an elevated level of Ca^{2+} (2.0 mM), however, a greater number of keratinocytes (54%) express higher levels of CDw60 (**Fig 2*B***), whereas other surface molecules such as MHC class I, class II molecules and adhesion molecules remain unaltered (data not shown). Furthermore, stimulation of keratinocytes with IL-13 for 48 h produces a 2–3 log shift of the surface expression of CDw60 by almost all of the cells (97%) (**Fig 2*C***). Although IL-13 can cause a significant upregulation of CDw60 expression, other cell surface markers remained unchanged, namely ICAM-1, MHC class I, and MHC class II molecules (HLA-DR) (data not shown).

IFN-γ is a type 1 cytokine with biologic functions antagonistic to the type 2 cytokines in many cellular systems. The effect of IFN-γ on keratinocytes with regard to CDw60 expression, however, has not been thoroughly investigated. In our *in vitro* system, we demonstrate that treatment of keratinocytes with IFN-γ failed to induce CDw60 expression; on the contrary, we confirmed an earlier report in which IFN-γ could suppress constitutively low expression (**Fig 2*D***). Moreover, IFN-γ can antagonize the effect of IL-13 on keratinocytes by inhibiting the upregulation of CDw60 expression. This has been demonstrated by either pretreating keratinocytes with IFN-γ for 6 h before incubating with IL-13 for 48 h, or treating the cells with both IFN-γ and IL-13 simultaneously for 48 h (**Fig 2*E***, ***H***, respectively).

IFN-γ alone can induce MHC class I, MHC class II, and the adhesion molecule ICAM-1 protein expression by keratinocytes (data not shown). Moreover, in the presence of both IFN-γ and IL-13, the induction of some IFN-γ inducible genes such as HLA-DR and ICAM-1 is still prominent (**Figs 1*F***, ***G***, ***I***, ***J***). Thus, IFN-γ can inhibit IL-13-induced CDw60 expression on keratinocytes, but IL-13 has no reciprocal inhibition on the expression of IFN-γ-induced genes.

IL-4 is a type 2 cytokine that shares many functional similarities to IL-13. When keratinocytes were treated with IL-4 for 48 h, similar results regarding the upregulation of CDw60 expression as the IL-13 treatment were obtained. Moreover, pretreatment or simultaneous treatment with IL-4 at the same time as IFN-γ treatment does not block the keratinocytes from down-modulating their CDw60 expression in response to the IFN-γ (data not shown).

To determine if IFN-γ was preventing IL-4 or IL-13 upregulation of CDw60 secondary to inhibition of intracellular levels, keratinocytes were permeabilized and then examined by flow cytometry. **Figure 3** reveals that there are constitutive intracellular levels of CDw60 in keratinocytes, but that IFN-γ does not inhibit these intracellular levels, despite the complete blockage of IL-4 induction of cell surface CDw60 expression. Given the known complexity in the biochemical pathway required for synthesis of the acetylated form of the ganglioside G_{M3}, including transport from intracellular to extracellular sites, we decided to initially focus on transcriptional events that may impact various signaling pathways that could also influence CDw60 expression.

Induction of STAT1 nuclear protein by IFN-γ In order to characterize the biochemical pathways utilized by IFN-γ or IL-4/IL-13, and especially to elucidate the mechanism responsible for the IFN-γ-mediated inhibition of keratinocyte CDw60 expression

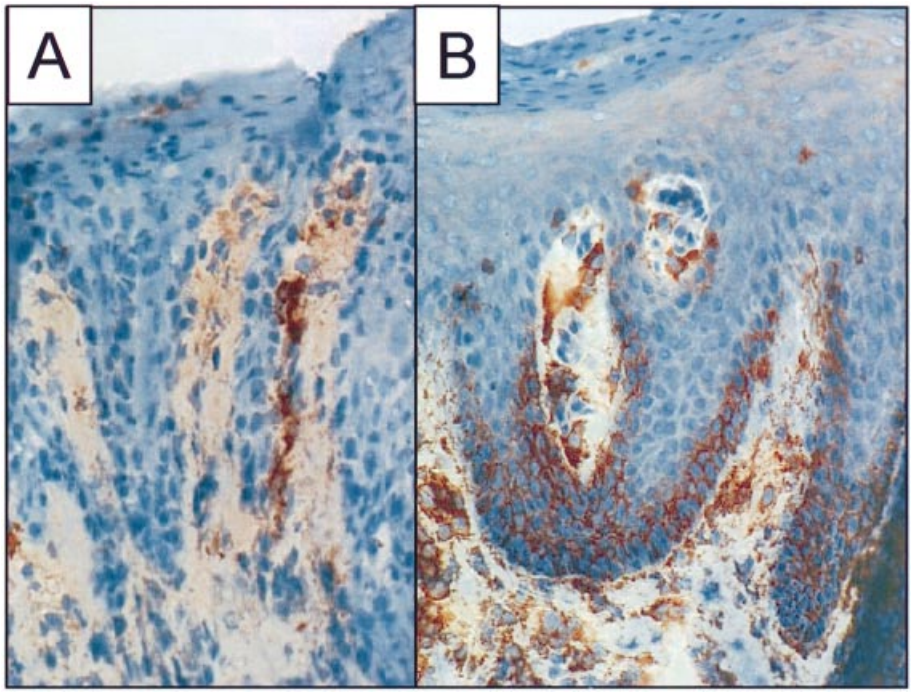

Figure 1. Immunohistochemical staining for CDw60 expression in acute and chronic psoriatic plaques. (*A*) An acute psoriatic lesion generated using the SCID/human skin model reveals no detectable CDw60 staining on keratinocytes, but some on mononuclear cells in the dermis. Note the typical histologic appearance for a psoriatic lesion including parakeratosis, loss of the granular cell layer, and elongated rete pegs. (*B*) A chronic psoriatic plaque with overexpression of CDw60 primarily confined to the basal and lower epidermal layers of keratinocytes, but absent from almost all suprabasal layer keratinocytes.

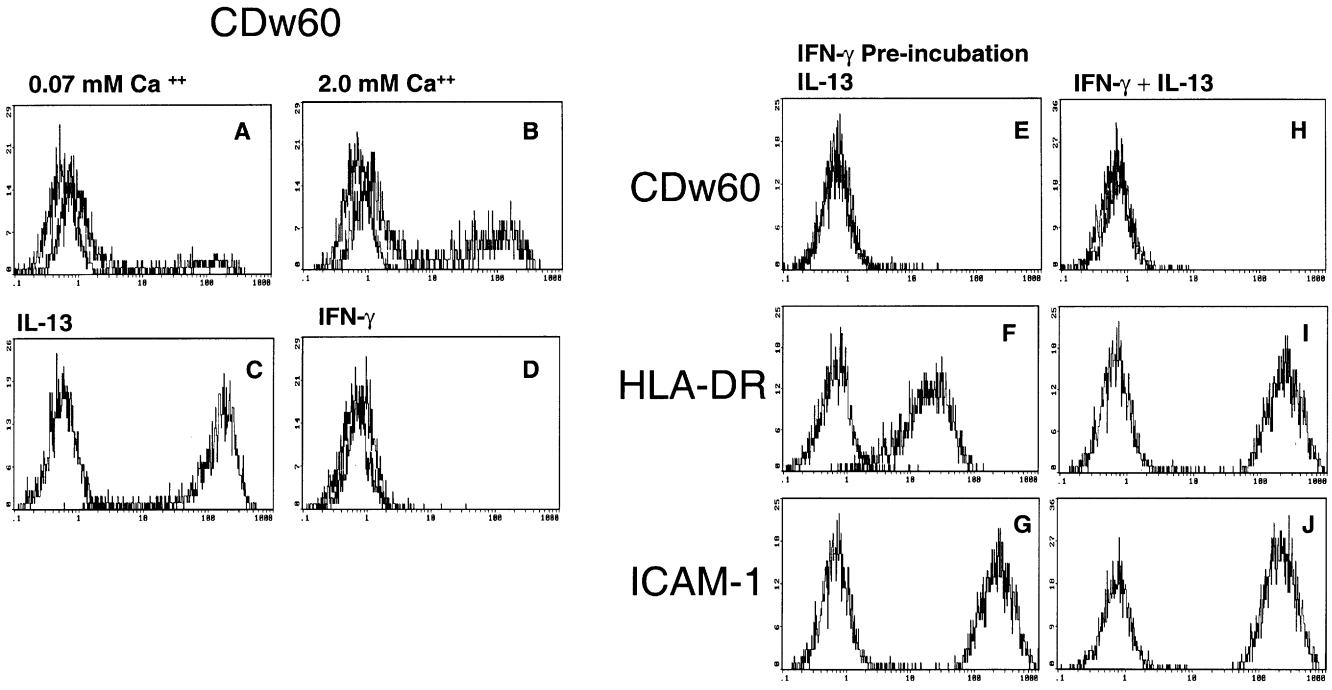

Figure 2. Analysis of the cell surface expression of CDw60, HLA-DR, and ICAM-1 on normal cultured keratinocytes by flow cytometry. (*A–E, H*) CDw60; (*F, I*) HLA-DR; (*G, I*) ICAM-1. Whereas only rare keratinocytes constitutively express CDw60 in low calcium (0.07 mM) medium (*A*), raising the extracellular calcium to 2.0 mM levels enhanced the number and intensity of CDw60 by keratinocytes (*B*). Addition of IL-13 (100 ng per ml, 48 h) triggers significantly greater CDw60 expression (*C*), but IFN-γ inhibits all constitutive CDw60 expression (*D*). Keratinocytes pretreated with IFN-γ (1000 U per ml, 6 h) followed by continuous exposure to IL-13 results in no keratinocyte CDw60 expression (*E*), nor does simultaneous addition of both IL-13 and IFN-γ (*H*). Induction of keratinocyte expression of HLA-DR by IFN-γ is not influenced by the presence of IL-13 (*F, I*). Induction of keratinocyte expression of ICAM-1 by IFN-γ is also not influenced by the presence of IL-13 (*G, I*).

mediated by IL-13, we investigated downstream signaling events. It has been shown in other cellular systems that IFN-γ can signal via STAT1 (Tessitore *et al*, 1998; Piskurich *et al*, 1999). Upon activation, STAT1 becomes phosphorylated, forms a dimer and translocates into the nucleus, and then binds to the STAT1-specific site in the promoter region, inducing IFN-γ-responsive genes. As keratinocytes still respond to IFN-γ even in the presence of Th-2 cytokines (**Fig 2**), we initially explored STAT1 signaling in keratinocytes.

In proliferating keratinocytes, a basal level of STAT1 is detectable in the nucleus (**Fig 4*A***). Upon IFN-γ stimulation (10^3 U per ml, 1 h), there is a significant induction of STAT1 translocation to the nucleus (**Fig 4*A***). Accumulation of STAT1 in the nucleus peaks at 1 h poststimulation, and then decreases but

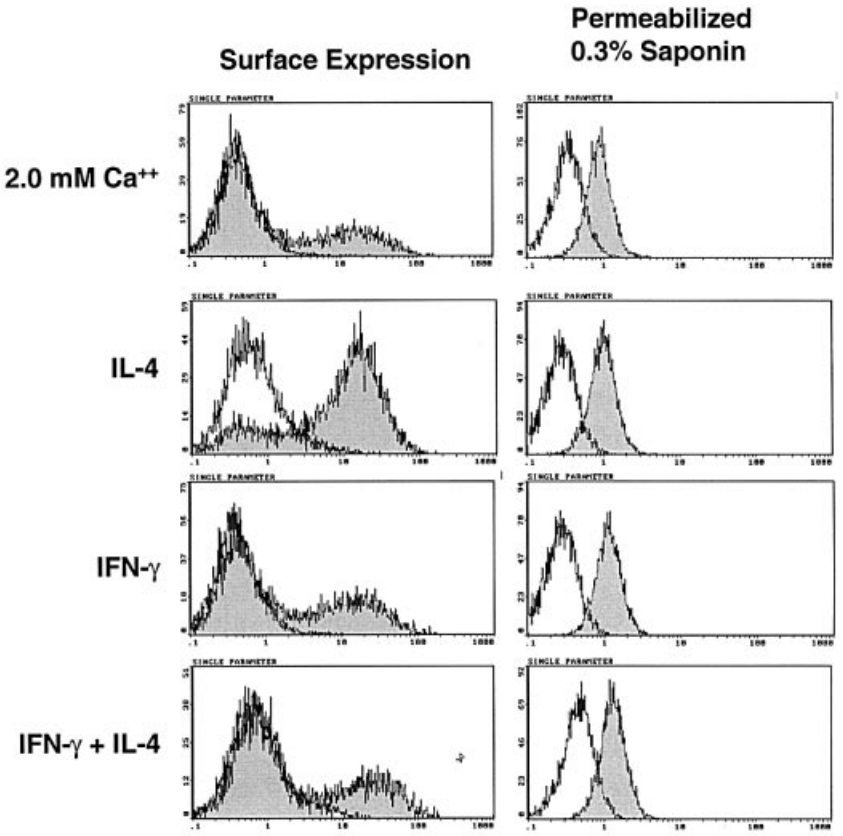

Figure 3. Determination of intracellular and cell surface levels of CDw60 in keratinocytes, before and after exposure to cytokines. Intracellular levels were measured by permeabilizing cells using 0.3% saponin (*right side panel*), whereas cell surface levels were assessed without permeabilization (*left side panels*). Keratinocytes maintained in 0.15 mM Ca^{2+}-containing KGM constitutively express low surface levels of CDw60 as well as intracellular levels compared with isotype control staining (*open curves*). IL-4 (48 h) increases surface expression of CDw60 but does not alter intracellular levels. IFN-γ (48 h) does not influence either intracellular or extracellular levels of CDw60. Addition of IFN-γ plus IL-4 inhibits induction of surface levels of CDw60, but has no effect on intracellular amounts of CDw60 in cultured keratinocytes.

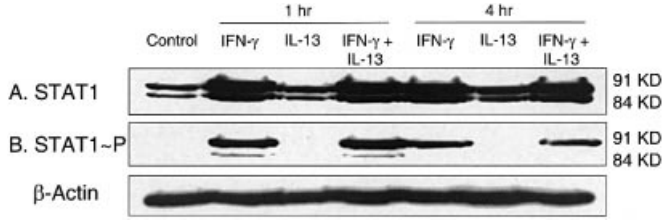

Figure 4. Western blot analysis of transcription factors STAT1 and phosphorylated STAT1 in the nuclei of keratinocytes before and after cytokine stimulation. STAT1 is a heterodimer consisting of a 91 kDa and an 84 kDa protein complex. Phosphorylated STAT1 protein detected with a phosphorylation-specific antibody to the phosphorylated 91 kDa subunit. (*A*) Constitutive level of STAT1 in the nuclei of proliferating keratinocytes. (*B*) The level of phosphorylated STAT1. *Lane 1*, proliferating keratinocytes; *lane 2*, keratinocytes following IFN-γ treatment for 1 h; *lane 3*, keratinocytes treated with IL-13 for 1 h; *lane 4*, keratinocytes stimulated with IFN-γ and IL-13 simultaneously for 1 h; *lane 5*, keratinocytes stimulated with IFN-γ for 4 h; *lane 6*, keratinocytes stimulated with IL-13 for 4 h; *lane 7*, keratinocytes stimulated simultaneously with IFN-γ and IL-13 for 4 h. β-actin levels are used as a control to verify equal protein loading of the lanes.

can still be detected at 4 h. There is no detectable induction of STAT1 translocation upon IL-13 stimulation. Interestingly, when keratinocytes are exposed to simultaneous stimulation with both IFN-γ and IL-13, there is still induction of STAT1. This suggests that, even though both cytokines IFN-γ and IL-13 are present, IFN-γ signaling via STAT1 proceeds unaffected by the Th-2 cytokine.

In order to confirm that the STAT1 accumulation is due to signaling events such as phosphorylation and translocation into the nuclei, we used a monoclonal antibody that recognizes only the phosphorylated form of STAT1. As demonstrated by the western blot analysis of phospho-STAT1 level (**Fig 4*B***), untreated proliferating keratinocytes, as well as IL-13-stimulated keratinocytes (1 h or 4 h after stimulation), do not show any detectable level of phospho-STAT1. Significant induction of phospho-STAT1 level was detected upon IFN-γ stimulation at 1 h, however. This phospho-STAT1 level was still detectable after 4 h stimulation, though at a reduced level. For keratinocytes that were stimulated with IFN-γ and IL-13 simultaneously, similar results to IFN-γ alone at the same time points were observed, again indicating that when both IFN-γ and IL-13 are present the IFN-γ signaling pathway through STAT1 is functional.

Induction of STAT6 by IL-13 stimulation In order to determine the IL-13 signaling pathway in keratinocytes, STAT6 phosphorylation and DNA binding activity were analyzed. As shown in **Fig 5**, the level of STAT6 in untreated keratinocytes is barely detectable, as is the STAT6 level in keratinocytes stimulated with IFN-γ for 1 h. IL-13 exposure of keratinocytes for 1 h increases the nuclear STAT6 levels significantly, however, although the STAT6 levels after 4 h of IL-13 stimulation subsides. Interestingly, for keratinocytes that underwent treatment with both IFN-γ and IL-13, comparable levels of induction of STAT6 as IL-13 alone were observed. This indicates that, even though IFN-γ is present, STAT6 translocation to the nucleus can still occur, suggesting that IL-13 signaling blockage by IFN-γ is downstream of STAT6 translocation.

Increased DNA binding activity of STAT1 protein in keratinocytes after IFN-γ stimulation Because there is an increased level of STAT1 protein in the nuclei of keratinocytes upon IFN-γ stimulation, including phospho-STAT1, we next examined if STAT1 from cultured keratinocytes can actually bind to the promoter region of an IFN-γ gene element. As shown in **Fig 6**, in proliferating keratinocytes there is no detectable binding activity of the transcription factor to a STAT1-specific labeled oligo, but binding increases upon IFN-γ stimulation as shown at both 30 min and 60 min poststimulation.

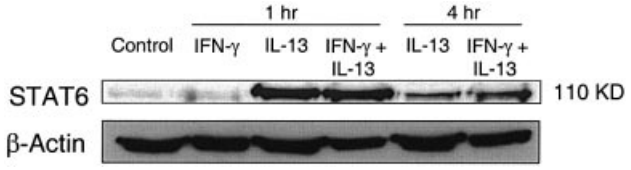

Figure 5. Western blot analysis of transcription factor STAT6 in the nuclei of keratinocytes upon cytokine stimulation. STAT6 has a molecular weight of 110 kDa. *Lane 1*, proliferating keratinocytes; *lane 2*, keratinocytes treated with IFN-γ for 1 h; *lane 3*, keratinocytes treated with IL-13 for 1 h; *lane 4*, keratinocytes stimulated with IFN-γ and IL-13 simultaneously for 1 h; *lane 5*, keratinocytes stimulated with IL-13 for 1 h; *lane 6*, keratinocytes stimulated with IFN-γ and IL-13 simultaneously for 4 h. β-actin levels are used as a control to verify the equal protein loading of the lanes.

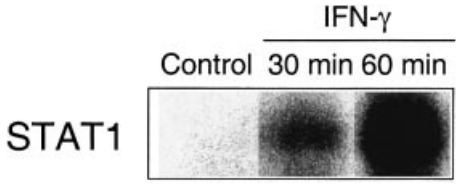

Figure 6. Mobility shift assay of STAT1 protein in keratinocytes stimulated with IFN-γ. *Lane 1*, unstimulated keratinocytes; *lane 2*, keratinocytes treated with IFN-γ (10^3 U per ml) for 30 min; *lane 3*, keratinocytes treated with IFN-γ (10^3 U per ml) for 1 h. Note the induction of binding of STAT1 protein for its DNA target sequence following treatment with IFN-γ at 30 min and 1 h.

Induction of increased BCL-6 levels in the nucleus of keratinocytes after IFN-γ stimulation In order to investigate the possible mechanisms of the differential effects of the various cytokines on keratinocyte expression of the acetylated form of the G_{M3} ganglioside (i.e., CDw60), we examined a downstream signaling pathway, focusing on the intranuclear level of BCL-6 in keratinocytes.

The BCL-6 protein is a nuclear phosphoprotein belonging to the POZ/zinc finger family of transcription factors. Interestingly, BCL-6-deficient mice develop a Th-2-type inflammation, suggesting that BCL-6 may be an important regulator of Th-2 response. Recently, it has been demonstrated that BCL-6 can repress IL-4-induced transcription mediated by STAT6 (Harris *et al*, 1999). Whether IFN-γ can influence the levels of BCL-6 in the keratinocyte nucleus, however, was not previously known.

Normal keratinocytes were stimulated with cytokines IFN-γ, IL-4, or IL-13 for 4 h. In addition, cells were also pretreated with either IFN-γ or IL-4 for 1 h before the addition of a second cytokine, IL-4 for IFN-γ and IFN-γ for IL-4, for an additional 4 h. As shown in **Fig 7**, proliferating keratinocytes have constitutive nuclear BCL-6 levels detectable by western blot analysis (*panel A*); however, treatment with IFN-γ can increase the BCL-6 level significantly, whereas IL-4 (IL-13, data not shown) treatment had no effect on the BCL-6 level. Moreover, these results also indicate that IFN-γ is a strong inducer of BCL-6, as pretreatment of keratinocytes with IL-4 (or IL-13, data not shown) could not prevent the BCL-6 induction by IFN-γ. These results indicate that IFN-γ can induce increased nuclear levels of the transcriptional repressor BCL-6, and this induction is not affected by these Th-2-type cytokines (i.e., IL-4 or IL-13).

Distribution pattern and overexpression of BCL-6 in normal and psoriatic skin To determine if these *in vitro* results could be relevant to the *in vivo* setting, skin biopsies were examined for BCL-6 expression. By immunohistochemistry, intranuclear expression of BCL-6 can be detected in keratinocytes in psoriatic plaques, but not in symptomless psoriatic skin (**Fig 7*B***). Within psoriatic skin, there were differences in the pattern of expression of

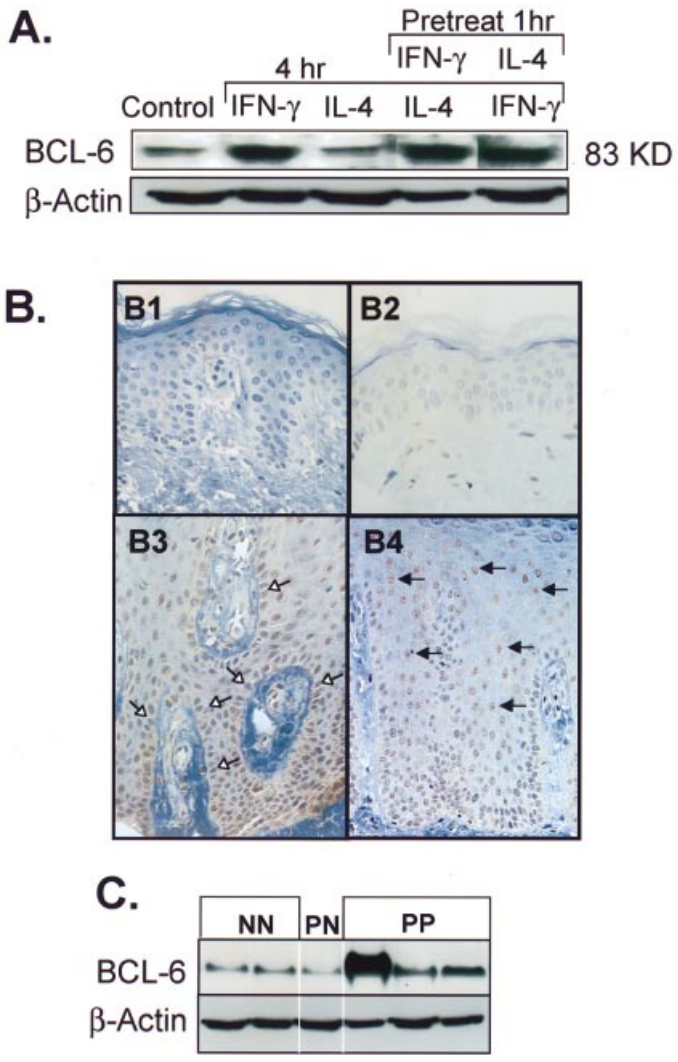

Figure 7. Western blot analysis of BCL-6 level in the nuclei of normal cultured keratinocytes. (*A*) Induction of BCL-6 in the nuclei of keratinocytes by IFN-γ. *Lane 1*, proliferating keratinocytes; *lane 2*, keratinocytes stimulated with IFN-γ for 4 h; *lane 3*, keratinocytes stimulated with IL-4 for 4 h; *lane 4*, keratinocytes pretreated with IFN-γ for 1 h followed by the addition of IL-4 for 4 h; *lane 5*, keratinocytes pretreated with IL-4 for 1 h followed by the addition of IFN-γ for 4 h. (*B*) Immunohistochemical analysis of BCL-6 in the nuclei of keratinocytes in normal skin (NN, *B1*), symptomless prepsoriatic skin (PN, *B2*), acute psoriatic lesion (*B3*), and chronic psoriatic lesion (*B4*). Note the absence of BCL-6 staining in NN and PN skin, but the BCL-6 positive staining and nuclear localization is confined primarily to basal layer keratinocytes in acute lesions (*panel B3*, *open arrows*). In contrast, in chronic psoriatic plaques (PP, *panel B4*), BCL-6 intranuclear staining is predominantly seen on suprabasal layer keratinocytes (*closed arrows*) (*C*) Western blot analysis of BCL-6 levels present in protein extracts from NN skin (*lanes 1*, *2*), PN skin (*lane 3*), PP skin (*lanes 4–6*). Note the enhanced levels of BCL-6 in psoriatic lesions. β-actin levels are used as a control to verify equal protein loading of the lanes.

BCL-6 depending on the stage of the lesion. In the acute lesion there was more prominent localization in the basal cell layer, whereas in chronic plaques the nuclear BCL-6 appears predominantly in the suprabasal layers of keratinocytes (**Figs 7*B3*, 7*B4***). This preliminary determination of BCL-6 overexpression in psoriatic plaques was confirmed by western blot analysis (**Fig 7*C***). In normal tissue from healthy individuals (NN skin, *lanes 1*, *2*), and symptomless skin from a psoriatic patient (PN skin, *lane 3*), low levels of BCL-6 expression were detected. In psoriatic plaques (PP skin, *lanes 4*, *5*), however, enhanced expression of BCL-6 levels can be observed. By densitometry, this overexpression in psoriatic

lesions ranged from 2.9- to 8.8-fold compared with symptomless skin.

DISCUSSION

Although it has been established that activated T cells can trigger the onset of psoriatic lesions, the precise biochemical pathways and transcriptional regulators mediating this phenotypic conversion are largely unknown (Valdimarsson *et al*, 1986; Schlaak *et al*, 1994; Gottlieb *et al*, 1995; Boehncke *et al*, 1996; Gilhar *et al*, 1997; Yamamoto *et al*, 1998; Nickoloff, 1999). Furthermore, whereas individual cytokines are often the focus of *in vitro* studies dealing with the interactions between T cells and keratinocytes, the actual disease state represents a mixture of multiple cytokines that produce a so-called cytokine network (Nickoloff, 1991). Even though psoriatic plaques contain an abundance of Th-1-type cytokines such as IFN-γ, the pathologic role for Th-2-type cytokines is generally disregarded, although some investigators have begun using cytokines such as IL-10 to treat psoriatic patients (Uyemura *et al*, 1993; Nickoloff *et al*, 1994; Asadullah *et al*, 1999). In this report, we investigated the complex interactions by which a Th-1-type cytokine (i.e., IFN-γ) could antagonize two different Th-2-type cytokines (IL-4 and IL-13), with regard to keratinocyte expression of CDw60.

CDw60 was selected for this study because it has been implicated in mediating T cell activation in psoriasis, and because its variable pattern of expression in lesional skin suggested a dynamic level of regulation (Skov *et al*, 1997). As CDw60 represents an acetylated form of the G_{D3} ganglioside, however, rather than a protein, it is likely that many enzymes and biochemical pathways are involved in its ultimate cell surface expression. This presents several challenges to elucidate molecular events that contribute to the surface expression of CDw60 (Paller *et al*, 1993; 1995). Nonetheless, we attempted to begin probing potentially important pathways both at the cytokine level and the transcriptional level. The current results extend these earlier findings by demonstrating that IFN-γ can completely block the ability of either IL-4 or IL-13 to induce CDw60 expression in cultured keratinocytes. Such a molecular based antagonistic effect by which IFN-γ suppressed IL-4/IL-13 induction of CDw60 parallels the cellular immune reaction in which the Th-1-type and Th-2-type T cells are observed to be mutually antagonistic and self-reinforcing (Kroemer *et al*, 1996). To begin to elucidate the mechanism by which IFN-γ could suppress IL-4/IL-13 induction of keratinocyte CDw60, several different studies were performed. First, it was demonstrated that, whereas IFN-γ antagonizes the biologic effects of IL-4/IL-13, there was no reciprocal reaction, as neither IL-4 nor IL-13 could inhibit the ability of IFN-γ to induce either HLA-DR or ICAM-1.

Second, as no previous study had addressed the potential crosstalk amongst transcription factors mediating these cytokine effects, we determined the patterns of expression for several key transcription factors in keratinocytes. It was observed that IL-4/IL-13 could induce STAT6 but not STAT1, whereas IFN-γ induced STAT1 and not STAT6. As IFN-γ could not block the IL-4/IL-13 mediated activation of STAT6, we explored a more distal signaling pathway involving the transcription repressor BCL-6. As shown in **Fig 7**, IFN-γ was documented to induce BCL-6 intranuclear levels in the keratinocytes. Moving from the *in vitro* studies, it became clear that, whereas normal or symptomless skin had low to nondetectable levels of BCL-6 by western blot analysis and immunostaining assays, there was overexpression of BCL-6 in psoriatic plaques. Moreover, the pattern of intranuclear BCL-6-positive keratinocytes was inversely correlated to the CDw60 expression in these preliminary findings. Thus in acute psoriatic plaques, high intranuclear BCL-6 levels were detected in basal layer keratinocytes in which there was no CDw60 expression, whereas in chronic plaques, the keratinocytes in the suprabasal layers that had high levels of BCL-6 failed to express CDw60. Taken together, these *in vitro* and *in vivo* studies suggest that the transcriptional repressor BCL-6 modulates the keratinocyte CDw60 expression. Obviously, considerable work is required to extend these preliminary findings to more precisely determine which transcription factors modulate CDw60 expression, and whether such transcription factors can be directly or indirectly suppressed by BCL-6.

Finally, these studies support the notion that psoriasis arises as a consequence of complex cellular and molecular interactions involving cytokines produced by various T cell subsets. Whereas it is generally appreciated that IFN-γ can function to trigger the appearance of many important immunologically relevant molecules such as HLA-DR and ICAM-1, it is now clear that IFN-γ can also have antagonistic biologic effects. One recent report documented the failure of IFN-γ-treated epithelial cells to stimulate proliferation of CD4+ T cells, whereas the same cells transfected with a full-length MHC class II transactivation gene did promote T cell proliferation (Lawson *et al*, 2000). These results, together with our data, highlight the many unresolved issues still surrounding the complexities underlying T cell-keratinocyte interactions, and the biochemical basis responsible for the modulation of expression of critically important immunologically active surface molecules.

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