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Prominent Production of IL-20 by CD68$^+$/CD11c$^+$ Myeloid-Derived Cells in Psoriasis: Gene Regulation and Cellular Effects

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We assessed expression of IL-20 and its receptors in psoriasis, given the recent implication of IL-20 in epidermal hyperplasia. Psoriatic lesional (LS) skin consistently expressed more IL-20 mRNA than nonlesional (NL) skin. Immunoreactivity to IL-20 protein was greater in LS tissue and mainly localized to infiltrating CD68$^+$/CD11c$^+$ (myeloid-derived) dermal leukocytes. Because this contrasted with earlier reports of a keratinocyte source, we assessed IL-20 mRNA expression in a variety of cells *in vitro,* and confirmed a myeloid-derived cellular source (monocytes). Plastic adhesion, activation of β2 integrins, and incubation with tumor necrosis factor-α stimulated expression in these cells. IL-20 receptor (IL-20R)α and IL-20Rβ mRNA was decreased in LS *versus* NL skin, which also contrasted with earlier findings. To investigate the relationship between IL-20 and disease activity, we examined psoriasis patients treated with the CD2-targeted agent alefacept. In therapeutic responders, lesional IL-20 mRNA decreased to NL levels, suggesting that CD2$^+$ leukocytes may proximally regulate IL-20. Finally, to assess IL-20 function, we used microarrays to screen IL-20-treated keratinocytes, which demonstrated upregulation of disease-related and IFN-γ-induced genes. Hence, IL-20 may influence inflammation through IFN-like effects. Together, these data indicate that IL-20 may be an important effector cytokine in psoriasis, and that its inhibition may represent a potential therapeutic target.

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INTRODUCTION

IL-20 is a recently discovered cytokine implicated in psoriasis. IL-20 belongs to the IL-10 cytokine family (Blumberg *et al.*, 2001), which also includes IL-19 (Gallagher *et al.*, 2000), IL-22 (Dumoutier *et al.*, 2000), IL-24 (MDA-7) (Jiang *et al.*, 1996), and IL-26 (AK155) (Knappe *et al.*, 2000). In contrast to IL-10, which suppresses psoriasis and other T-cell-mediated diseases (Moore *et al.*, 2001), IL-20 exhibits proinflammatory properties. For instance, keratinocytes stimulated with IL-20 (in combination with IL-1) upregulate a variety of inflammatory genes (Blumberg *et al.*, 2001), including tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), and myeloid-related protein-14 (MRP-14) (Rich and Kupper, 2001).

In transgenic mice, overexpression of IL-20 induces neonatal lethality and skin abnormalities, including a wrinkled, shiny appearance (Blumberg *et al.*, 2001). Histologically, these mice lack the immune infiltrates found in human psoriatic skin, but display hyperproliferative skin features, including a thickened epidermis and elevated markers of hyperproliferation (e.g. keratin 6). The authors have suggested that this is consistent with a psoriasis-like phenotype.

In humans, lesional (LS) psoriatic skin contains elevated IL-20 mRNA, which diminishes after short-term treatment with cyclosporine A or calcipotriol (Romer *et al.*, 2003; Wei *et al.*, 2005). To date, IL-20 protein has been localized to basal and suprabasal keratinocytes (Wei *et al.*, 2005). Gene and protein expression for the receptor subunits IL-20 receptor (IL-20R)α and IL-20Rβ are also increased in psoriasis (Blumberg *et al.*, 2001; Romer *et al.*, 2003; Wei *et al.*, 2005). IL-20 binds two heterodimeric receptors, Type I IL-20R consisting of IL-20Rα/IL-20Rβ and Type II IL-20R consisting of IL-20Rβ/IL-22R (Dumoutier *et al.*, 2001; Parrish-Novak *et al.*, 2002). Type I IL-20R also binds IL-19 and IL-24, whereas Type II IL-20R also binds IL-24. All of the subunits are highly expressed in the epidermis (Blumberg *et al.*, 2001; Dumoutier

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Abbreviations: DC, dendritic cell; hARP, human acidic ribosomal protein IL-20R, IL-20 receptor; iNOS, inducible nitric oxide synthase; K16, keratin 16; LS, lesional; NL, nonlesional; PBMC, peripheral blood mononuclear cell RT-PCR, reverse transcriptase-PCR; STAT1, signal transducer and activator of transcription 1; TNF-α, tumor necrosis factor-α

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et al., 2001), suggesting that IL-20 affects keratinocyte function.

Given the potential importance of this cytokine, we assessed the expression of IL-20 and IL-20R in psoriatic skin and attempted to identify the cell types and regulatory factors responsible for IL-20 synthesis. We found IL-20 mRNA, but not IL-20R mRNA, to be tremendously increased in psoriasis. Myeloid-derived ($CD68^+/CD11c^+$) cells at the dermo-epidermal junction and within the epidermis appear to be an important source of IL-20. Synthesis of the cytokine is regulated by cell adhesion and exposure to TNF-α. In keratinocytes, exposure to IL-20 upregulates the expression of inflammatory genes, including IFN-responsive elements. Lastly, we report that IL-20 expression is highly correlated with disease activity and suppressed by treatment with alefacept (LFA-3TIP, anti-CD2, Amevive, Biogen Idec), implying that IL-20 is a product of leukocyte-driven inflammation.

RESULTS

IL-20 mRNA is consistently upregulated in psoriasis

We obtained mRNA from matched LS and nonlesional (NL) psoriatic skin and performed real-time, reverse transcriptase-PCR (RT-PCR) to quantify IL-20 mRNA. Values were normalized to the housekeeping gene human acidic ribosomal protein (hARP). In all psoriasis patients (34/34), we found IL-20 mRNA to be elevated in LS skin compared to NL skin (Figure 1a). Mean expression in NL skin was 16.1 (arbitrary scale), whereas expression in LS skin was 726.2 and elevated 45-fold (range 3- to 565-fold, $P<0.0001$). In comparison, mean expression of IL-20 in 11 control patients without psoriasis was 8.62, which was significantly less than LS and NL skin ($P<0.0001$ and $P=0.039$, respectively).

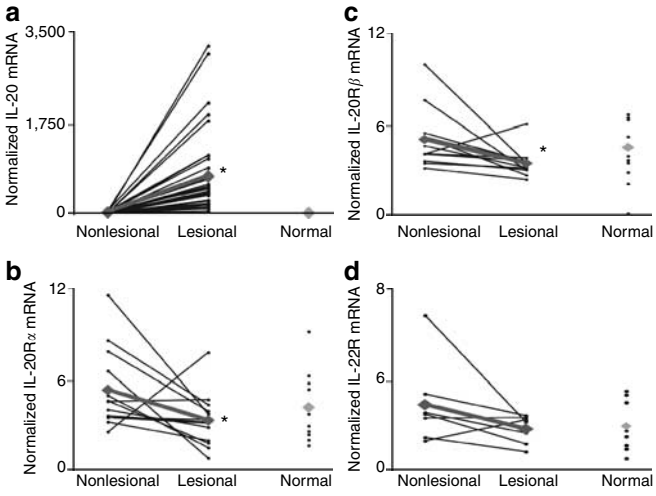


Figure 1. Gene expression of IL-20 and its receptor subunits in NL psoriatic skin, LS psoriatic skin, and normal skin. RT-PCR quantification of (**a**) IL-20, (**b**) IL-20Rα, (**c**) IL-20Rβ, and (**d**) IL-22R, normalized to hARP. A thin black line connects paired samples from an individual psoriasis patient's NL and LS skin. For normal skin, each dot represents one patient. ◆ Indicates the mean for each tissue type, and this symbol obscures individual dots in (**a**). Asterisk (*) indicates statistical significance ($P<0.05$) between NL and LS psoriatic skin.

IL-20 receptor mRNA is decreased in psoriasis

We next characterized the expression of IL-20Rα, IL-20Rβ, and IL-22R. We found mRNA for all subunits to be decreased in LS skin (Figure 1b–d). Using hARP to normalize values, mean expression in NL *versus* LS skin, respectively, was 5.8 *vs* 3.8 for IL-20Rα ($P=0.046$, $n=13$), 5.0 *vs* 3.4 for IL-20Rβ ($P=0.019$, $n=13$), and 3.3 *vs* 2.3 for IL-22R ($P=0.157$, $n=7$). Difference in expression was significant for IL-20Rα and IL-20Rβ, but not for IL-22R. In contrast to published data (Blumberg *et al.*, 2001), we found mRNA for the subunits to be expressed in unaffected, normal skin (Figure 1b–d). Mean expression was 4.7 for IL-20Rα ($n=11$), 4.5 for IL-20Rβ ($n=11$), and 2.5 for IL-22R ($n=8$). These values were not significantly different from those of LS and NL skin (both $P>0.05$).

Protein expression of IL-20 and IL-20Rα is consistent with mRNA expression

To confirm the above findings, we stained skin sections for IL-20 and IL-20Rα. At the time of manuscript preparation, no commercial antibodies for IL-20Rβ or IL-22R were available. We found that staining for IL-20 and IL-20Rα matched mRNA expression. For IL-20, we found little staining in normal and NL skin (Figure 2a and b). In contrast, staining in LS skin occurred in many cells and with greater intensity (Figure 2c). Although weak staining of keratinocytes was sometimes noticed, positive staining appeared greatest in mononuclear leukocytes in the papillary dermis and along the dermo-epidermal junction (Figure 2d). In some LS skin biopsies, strong staining also appeared in leukocytes in the mid-epidermis and along dermal capillary endothelium (Figure 2c). Immunostaining of IL-20Rα demonstrated two major findings (Figure 2e and f). Firstly, staining was restricted to keratinocytes in both NL and LS skin. Secondly, whereas staining was diffuse and uniform throughout the epidermis of NL skin, staining was decreased in LS skin and, specifically, less intense in the lower and middle epidermis, suggesting that keratinocytes in these areas had decreased expression of IL-20Rα protein.

IL-20 mRNA is expressed by monocytes *in vitro*

Having found immunostaining in leukocyte-like cells, we further investigated the source of IL-20 by examining mRNA expression in keratinocytes (HaCaT cells) and various cells isolated from normal blood (Figure 3a). We detected low expression in unfractionated peripheral blood mononuclear cells (PBMCs), but almost no expression in lymphocytes (consisting of natural killer, T, and B cells), unactivated T cells, T cells activated by CD3 and CD28 ligation, unactivated HaCaT cells, and HaCaT cells activated by IFN-γ. As PBMCs produced low quantities of IL-20 mRNA, whereas lymphocytes produced almost none, we fractioned PBMCs to specifically examine monocytes. Monocytes in suspension produced substantial amounts of IL-20 mRNA. More dramatically, monocytes adherent to polystyrene tissue culture plastic surface produced the highest quantities and nearly four times more IL-20 mRNA than nonadherent monocytes.

We then tested whether *in vitro* monocyte-derived cells produced IL-20 mRNA. Monocytes were differentiated into

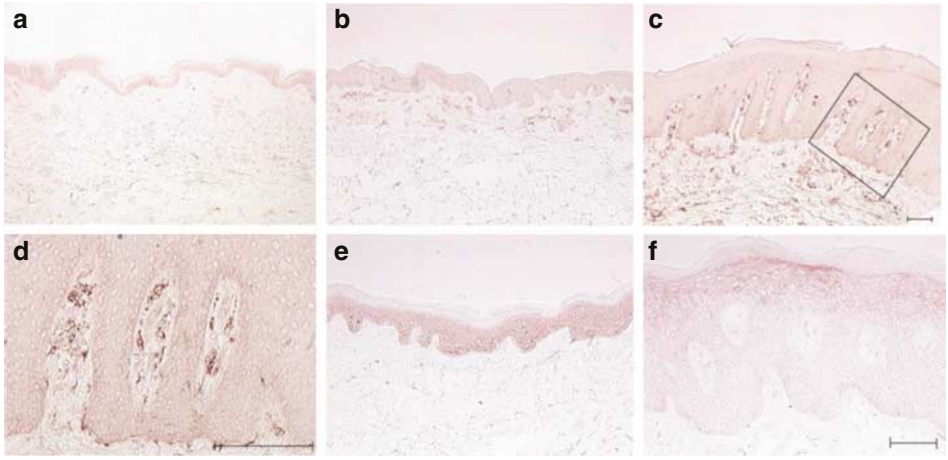


Figure 2. Immunohistochemical analysis of IL-20 and IL-20Rα in psoriasis. IL-20 antibody staining of (**a**) normal skin, (**b**) NL psoriatic skin, and (**c**) LS psoriatic skin (original magnification ×10). (**d**) ×40 magnification of the region outlined in (**c**). IL-20Rα antibody staining of (**e**) NL and (**f**) LS psoriatic skin (original magnification ×20). Positive cells appear brown (3-amino-9-ethylcarbazole staining). Bar = 100 μm.

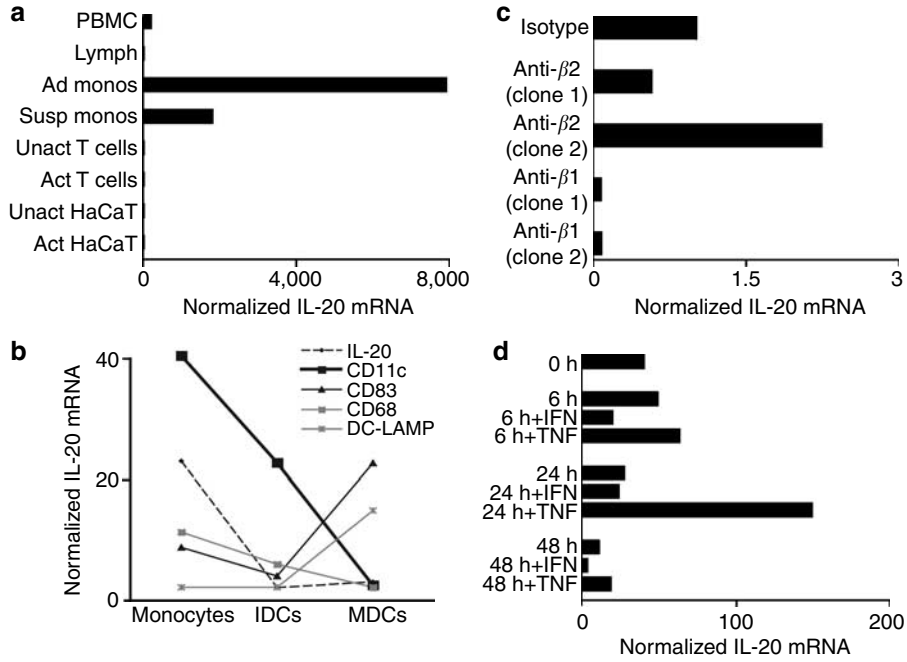


Figure 3. Production of IL-20 mRNA by adherent monocytes *in vitro*, and induction of IL-20 by β2 integrin or TNF-α. (**a**) IL-20 mRNA was measured in PBMCs, unfractionated lymphocytes (Lymph) (comprised of T, B, and natural killer cells), monocytes adherent to plastic surface (Ad monos), monocytes in suspension (Susp monos), unactivated T cells (Unact T cells), T cells activated by CD3 and CD28 ligation (Act T cells), unactivated HaCaT cells (Unact HaCaT), and HaCaT cells activated by IFN-γ (Act HaCaT). (**b**) Gene expression of IL-20 and cellular/maturational markers was measured in pure monocytes and monocyte-derived immature DCs (IDCs) and mature DCs (MDCs). Lines demonstrate progression of mRNA expression with maturation. (**c**) IL-20 mRNA was analyzed in monocytes activated by integrin ligation. Two different antibody clones against either β1 or β2 integrin were incubated with monocytes in suspension. (**d**) IL-20 mRNA was measured in monocytes at baseline (0 hour) and monocytes stimulated with no cytokine, IFN-γ, or TNF-α for 6, 24, or 48 hours. All gene results were normalized to hARP expression.

immature dendritic cells (DCs) by incubation with GM-CSF and IL-4 (Dhodapkar *et al.*, 2002). Mature DCs were then generated by adding a cocktail of TNF-α, IL-1β, IL-6, and prostaglandin E_2. We found that only monocytes, but not immature or mature DCs, produced substantial IL-20 mRNA (Figure 3b). To verify the phenotype of these cells, we also measured various cellular markers. Consistent with phenotype, monocytes expressed CD11c and CD68 mRNA, mature DCs produced high levels of CD83 and DC-LAMP mRNA, and immature DCs produced low levels of CD83 mRNA.

Monocytes produce IL-20 in response to β2-integrin activation or TNF-α

Having found that adherence elicited IL-20 mRNA expression, we suspected that integrins may stimulate IL-20 production. Indeed, monocytes produce inflammatory molecules,

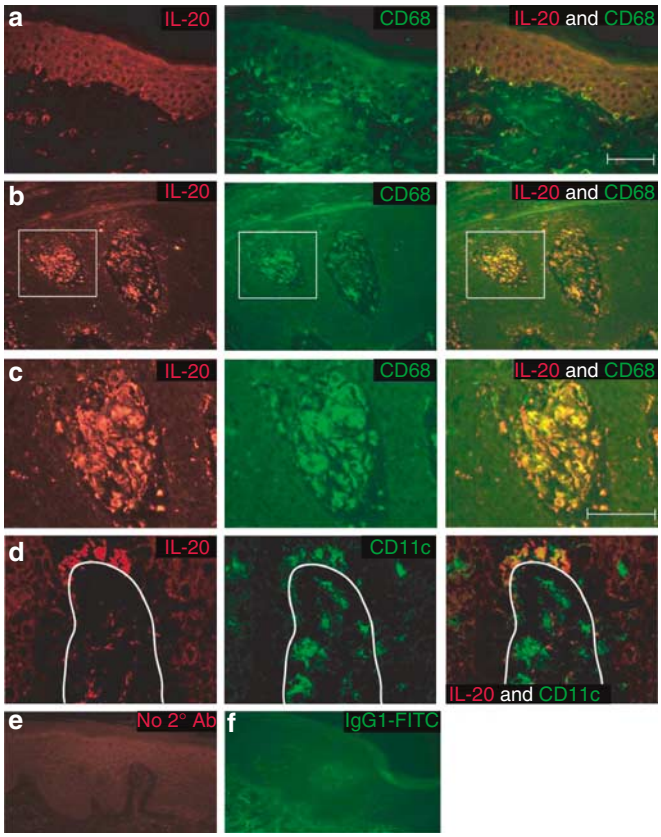


Figure 4. Immunofluorescent staining of IL-20, CD68, and CD11c in psoriatic skin. (**a**–**c**) Two-color immunofluorescence for IL-20 (red, Cy3-labeled) and CD68 (green, FITC-labeled) in (**a**) NL and (**b** and **c**) LS psoriatic skin. The boxed region in (**b**) is enlarged in (**c**). (**d**) Two-color staining for IL-20 (red, Alexa 568-labeled) and CD11c (green, Alexa 488-labeled) of LS skin is also presented. In all panels, double positive cells appear yellow. (**e**) As a control, LS skin was stained with IL-20 antibody, but no Cy3-conjugated secondary antibody. (**f**) Isotype control (IgG-FITC) staining of LS skin. Original magnification: (**a**, **b**, **e**, and **f**) ×20; (**c** and **d**) ×40. Bar = 100 μm.

such as TNF-α, following integrin ligation to surfaces or extracellular matrix. This process is known as ''outside-in'' integrin-mediated signaling (de Fougerolles and Koteliansky, 2002). We found that nonadherent monocytes incubated with a β2 integrin (CD18) antibody upregulated IL-20 mRNA more than two-fold *versus* control (mouse IgG) (Figure 3c). In contrast, a different β2 integrin antibody (from a different clone) and two separate β1 integrin antibodies did not upregulate IL-20.

As cytokines are produced in response to integrin ligation and may themselves be stimulatory, we considered that IL-20 may be induced by specific cytokines. To investigate this, we incubated monocytes with IFN-γ or TNF-α. Monocytes incubated with TNF-α for 24 hours upregulated IL-20 mRNA maximally (>5-fold *vs* time-matched control) (Figure 3d). Monocytes incubated with TNF-α for 6 or 48 hours also upregulated IL-20 mRNA, but to a lesser degree. Monocytes incubated with IFN-γ for 6, 24, or 48 hours downregulated IL-20 production by a small degree compared to time-matched controls.

IL-20 is primarily produced by CD68$^+$/CD11c$^+$ leukocytes *in vivo*

To determine the source of IL-20 *in vivo*, we double-stained tissue with fluorescent antibodies against IL-20 (Cy3-labeled) and CD68 (FITC-labeled) (Figure 4a–c). Based on our findings that monocytes produce IL-20 *in vitro*, we chose CD68 because it identifies myeloid-derived leukocytes, including monocytes/macrophages and some DCs (Pulford *et al.*, 1990; Nakamura *et al.*, 1998). In NL skin, we found very little IL-20 or CD68 staining (Figure 4a). In contrast, we found intensely positive staining for IL-20 and CD68 in LS skin (Figure 4b and c). Although we noticed some diffuse staining in keratinocytes, IL-20-Cy3 appeared most strongly in mononuclear cells in the papillary dermis near the dermo-epidermal junction, as well as in sporadic mononuclear cells in the epidermis. The staining pattern for CD68-FITC appeared very similar, and when both fluorochromes were co-visualized, there was a high degree of overlap, indicating that IL-20 protein was present in many CD68$^+$ leukocytes in psoriatic lesions. To confirm these findings, we also stained sections with CD11c and found that IL-20$^+$ cells partially overlapped with cells expressing this marker (Figure 4d). CD11c identifies myeloid-derived cells, including monocytes, macrophages, myeloid DCs, and a recently identified subset of inflammatory DCs that produce TNF-α and inducible nitric oxide synthase (iNOS) (TNF- and iNOS-producing DCs) (Lowes *et al.*, 2005). Of note, we found that CD11c$^+$ cells were localized in the same region of psoriatic skin as CD68$^+$ cells (Figure 4d and Figure S1b).

As the most intense staining for CD68 and IL-20 demonstrated marked overlap, we attempted to identify potential myeloid sources of IL-20 by characterizing CD68$^+$ cells in psoriasis. Firstly, by using flow cytometry, we confirmed that CD68 identified myeloid cell populations (CD11c$^+$), including monocytes, *in vitro*-derived macrophages, and *in vitro*-generated mature DCs (Figure S1a). Secondly, additional double staining of LS skin demonstrated that CD68 partially overlapped with inflammatory DC markers (CD11c$^+$, HLA-DR$^+$, DC-SIGN$^+$), but not markers of mature DCs (CD1b/c$^+$ or CD83$^+$) (Figure S1b–f). Little overlap was seen with the classic monocyte/macrophage marker CD14, which was minimally expressed in psoriatic skin (Figure S1g). These findings suggest that the markers of CD68$^+$ leukocytes in psoriasis may be consistent with immature DCs and, furthermore, with inflammatory TNF- and iNOS-producing DCs. Thus, these cell types may be important, but not exclusive, sources of IL-20 *in vivo*.

IL-20 expression is modulated by alefacept and correlates with disease activity

To relate IL-20 to disease activity, we examined patients treated with alefacept, a biological agent that preferentially reduces T cells in psoriatic plaques (Chamian *et al.*, 2005). Based on histology, including epidermal hyperplasia and keratin 16 (K16) immunostaining, patients were categorized as responders ($n = 12$) or nonresponders ($n = 8$) after 13 weeks of treatment. In general, responders demonstrated decreased T cells in LS skin at the end of treatment, as well as normalization of epidermal thickness and K16 staining. In

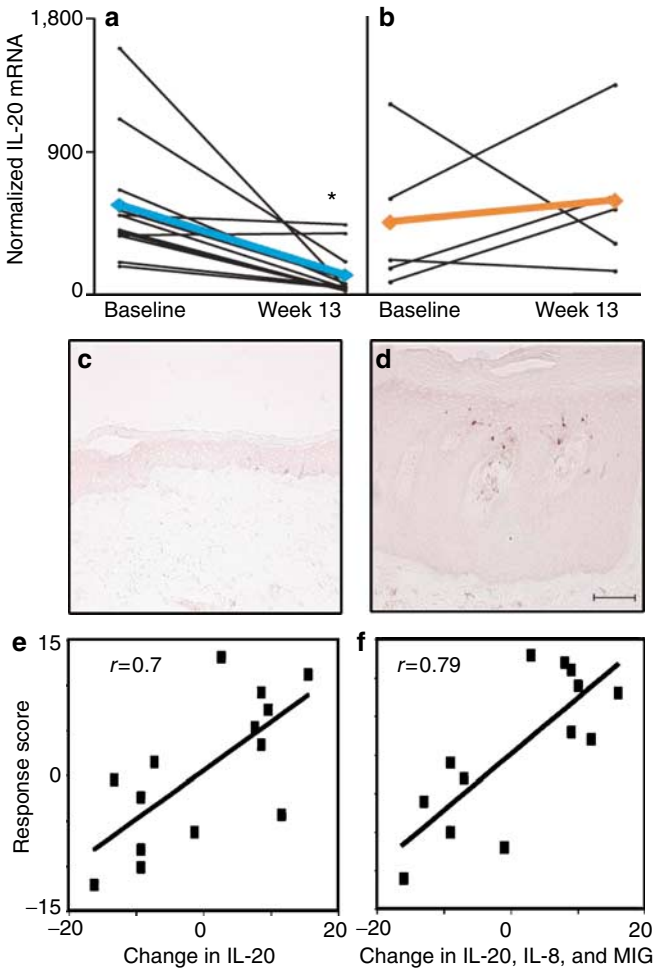


Figure 5. IL-20 mRNA is modulated by alefacept treatment and correlates with disease activity. RT-PCR quantification of IL-20 mRNA (normalized to hARP) in (**a**) alefacept responders and (**b**) nonresponders. A black line connects samples taken from an individual psoriasis patient's LS skin before and after treatment with alefacept. ♦ Indicates the mean for each type of specimen. Asterisk (*) indicates statistical significance ($P<0.05$) between baseline and post-treatment results. IL-20 staining of skin at the end of treatment from (**c**) a responder and (**d**) a nonresponder (original magnification $\times 20$). Bar = 100 μm. Correlation plots with linear regression (line of best fit) were used to compare response score (disease activity) with change in gene expression for (**e**) IL-20 or (**f**) a set of disease-related genes including IL-20. Black squares represent an individual psoriasis patient's response score plotted against change in mRNA (pre- *vs* post-treatment). Response score was determined by change in epidermal thickness, K16 mRNA, and K16 staining with treatment. Higher response score values indicate improvement in these disease parameters with treatment.

11/12 responders, IL-20 mRNA decreased by the end of therapy (Figure 5a), with mean expression being 571.9 before treatment and 102.8 after treatment ($P<0.004$). This represented a decrease of 82% and an effective normalization of IL-20 mRNA to NL quantities. In contrast, nonresponders demonstrated sustained T-cell numbers, thickened epidermis, and persistence of K16 staining in LS skin. In nonresponders, IL-20 mRNA remained unchanged (Figure 5b). Whereas mean expression in LS skin was 441.2 before treatment, it was 557.2 after treatment ($P=0.66$).

To verify our results, we performed immunostaining for IL-20 protein. Before treatment, IL-20 staining in LS skin of both responders and nonresponders was similar and localized mostly to mononuclear leukocytes in the papillary dermis and lower-to-mid epidermis (e.g. as in Figure 2). At the end of treatment, staining for IL-20 was virtually nonexistent in responders, but persistent in nonresponders (Figure 5c and d).

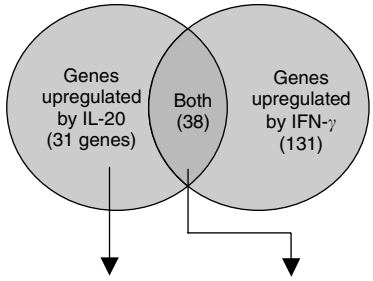
To correlate changes in IL-20 gene expression with clinical response, we used a previously described multivariate u-score method that considers multiple parameters to measure disease improvement in both responders and nonresponders (Wittkowski *et al.*, 2004; Gottlieb *et al.*, 2005). For our parameters, we used epidermal thickness, K16 mRNA expression, and K16 staining at week 13 of alefacept treatment and calculated a response score using these criteria. We then correlated response score to changes in IL-20 expression. IL-20 mRNA expression was highly correlated ($r=0.70$) with objective parameters of disease activity (i.e. response score) (Figure 5e), suggesting that IL-20 expression closely paralleled clinical response to alefacept. Using u-statistics for multivariate data, we took into account changes in the expression of other genes and found that IL-20 expression had the highest correlation to response score when IL-8 and monokine induced by IFN-γ (MIG) expression was also considered ($r=0.79$) (Figure 5f). This suggested that IL-20 expression was additionally correlated with known disease-related pathways.

IL-20 induces expression of IFN-regulated and disease-related genes in keratinocytes

Lastly, we examined the effects of IL-20 on keratinocytes. Previously, it was reported that keratinocytes possess IL-20 receptors and therefore are a target for IL-20 (Blumberg *et al.*, 2001; Romer *et al.*, 2003). We verified that one of the receptor subunits, IL-20Rα, was present on keratinocytes *in vivo* (Figure 2e and f). In addition, we found that keratinocytes in culture (HaCaT cells) expressed substantial IL-20Rα, IL-20Rβ, and IL-22R mRNA (data not shown). Therefore, we postulated that IL-20 might induce important responses in keratinocytes.

To investigate this, we used Affymetrix U95 microarrays to analyze HaCaT cells stimulated with recombinant IL-20. We found that IL-20 induced genes involved in regulation of proliferation, apoptosis, or epidermal differentiation. These include PI3/SKALP, BIRC3, TNFAIP3, PTHLH, SPRR1A, RAI3, NINJ1, TGM1, and SPRR1B (Figure 6a). In addition, IL-20 upregulated various genes involved in immune response (SERPINB4, SERPINB3, IL-1R2, RELB, and IKBKE), chemotaxis (CXCL1, IL-8, CXCL6, and CCL20), angiogenesis (TNFAIP2), and collagen metabolism (MMP-7 and MMP-13). Based on previous microarray studies of psoriasis (Bowcock *et al.*, 2001; Oestreicher *et al.*, 2001; Zhou *et al.*, 2003), many of these are considered to be disease-associated genes. Figure 6a lists genes that were most highly upregulated by IL-20.

IL-20 also stimulated the expression of many IFN-regulated genes. Among these were signal transducer and activator of transcription 1 (STAT1), IFN-γ-inducible protein



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Fold	Symbol	Gene product
4.5	SERPINB4*	Serine proteinase inhibitor, clade B (ovalbumin), member 4
4.0	CXCL1*	GRO-α
3.1	PI3*	SKALP/elafin
3.1	BIRC3	Baculoviral IAP repeat-containing 3
2.5	IL-8*	Interleukin-8
2.5	CXCL6	Granulocyte chemotactic protein 2
2.4	CCL20*	MIP-3α
2.4	SERPINB3*	Serine proteinase inhibitor, clade B (ovalbumin), member 3
2.2	TNFAIP3	TNF-α-induced protein 3
2.2	MMP7	Matrilysin
2.1	PTHLH	Parathyroid hormone-like hormone
1.9	SPRR1A	Small proline-rich protein 1A
1.8	TNFAIP2	TNF-α-induced protein 2
1.8	MMP13	Collagenase 3
1.7	IL-1R2	Interleukin-1 receptor, type II
1.6	HRASLS3	HRAS-like suppressor 3
1.6	ABTB2	Ankyrin repeat and BTB (POZ) domain containing 2
1.6	NINJ1	Ninjurin 1
1.5	TGM1*	Transglutaminase
1.5	RELB	V-rel reticuloendotheliosis viral oncogene homolog B
1.5	KIAA0133	KIAA0133 gene product
1.4	AMD1*	Adenosylmethionine decarboxylase 1
1.4	FHL2	Four and a half LIM domains 2
1.4	PLAUR	Six transmembrane epithelial antigen of the prostate 1
1.4	STEAP1	BAC clone CTB-41D11 from 7
1.3	RAI3	Retinoic acid induced 3
1.3	DTR	Heparin-binding EGF-like growth factor
1.3	IKBKE	Inhibitor of NF-κB kinase epsilon subunit
1.3	uPA	Urokinase-type plasminogen activator
1.3	CD83*	CD83 antigen
1.3	SPRR1B*	Small proline-rich protein 1B (cornifin)

b

Fold	Symbol	Gene product
3.1	PLAT	Tissue plasminogen activator
2.6	SERPINB1	Serine proteinase inhibitor, clade B (ovalbumin), member 1
2.4	CCL2*	Monocyte chemotactic protein-1
2.3	CCL17	TARC
2.3	STOM	Stomatin
1.9	CD47*	CD47 antigen
1.7	TRB2	Tribbles homolog 2
1.7	BF	B-factor, properdin
1.7	CXCL3	GRO-γ
1.6	UBD	Ubiquitin D
1.6	STAT3*	Signal transducer and activator of transcription 3
1.6	WARS	Tryptophanyl-tRNA synthetase
1.6	SECTM1	Secreted and transmembrane 1
1.5	CX3CL1	Fractalkine
1.5	IL15RA	Interleukin-15 receptor-α
1.5	PML	Promyelocytic leukemia
1.5	UBE2L6	Ubiquitin-conjugating enzyme E2L 6
1.4	P2RY6	Pyrimidinergic receptor P2Y, G-protein coupled, 6
1.4	TAP1	Transporter associated with antigen processing 1
1.4	ECGF1	Endothelial cell growth factor 1
1.4	STAT1*	Signal transducer and activator of transcription 1
1.4	XDH	Xanthine dehydrogenase
1.4	SP110	IFN-induced human nuclear phosphoprotein
1.4	PSMB9	Proteasome subunit β9
1.4	IFI16	Interferon-γ-inducible protein 16
1.4	OAS1	Homo sapiens 2′-5′ oligoadenylate synthetase
1.4	CASP10	Caspase 10
1.3	INPP1	Inositol polyphosphate-1-phosphatase
1.3	CASP1	Caspase 1
1.3	BAZ1A	Bromodomain adjacent to zinc finger domain 1A
1.3	PSME2	Proteasome activator subunit 2 (PA28β)
1.3	MYD88	Myeloid differentiation primary response gene 88
1.3	SP100	Nuclear antigen Sp100
1.3	TMSB10	Thymosin β10
1.2	PSMB10	Proteasome subunit β10
1.2	SSA1	Sjogren syndrome antigen A1
1.2	NMI	N-myc (and STAT) interactor
1.2	IRF7*	Interferon regulatory factor 7

Figure 6. IL-20-induced upregulation of IFN-γ-regulated and psoriasis disease-associated genes in keratinocytes. (**a**) Keratinocytes (HaCaT cells) were treated with recombinant IL-20 and their gene expression profile analyzed using microarrays. Genes that were upregulated at least 1.2-fold are listed. (**b**) For comparison, a group of HaCaT cells was treated separately with IFN-γ and analyzed with microarray screening. Overlapping genes that were upregulated by IFN-γ and IL-20 (at least 1.2-fold) are listed. Asterisk (*) indicates a known psoriasis disease-associated gene, based on three previous microarray reports of psoriasis.

16 (IFI-16), and IFN regulatory factor 7 (IRF-7). IL-20 has not been previously reported to possess IFN-like properties. As such, we treated a separate group of HaCaT cells with IFN-γ alone and examined their expression profile. Comparing this to the group of genes upregulated by IL-20, we found at least 38 overlapping genes. Thus, IL-20 appeared to induce a subset of genes that also respond to IFN-γ. Besides those already mentioned, these include genes involved in transcription (STAT3, SP110, NMI), protein synthesis (WARS), Th1 polarization (IL-15Rα), antigen presentation (TAP1, PSMB9, PSBM10, and PSME2), leukocyte trafficking (CCL2/MCP-1, CD47, CCL17/TARC, CX3CL1, and CXCL1/GRO-α), innate immunity (OAS1, BF, and MYD88), growth (ECGF1), and apoptosis (CASP1, CASP10, and PLAT). Many of these genes, including STAT1, CCL2, CD47, PSME2, STAT3, and IRF-7, have been previously described as disease-related, based on microarrays of psoriatic lesions. Figure 6b lists overlapping genes upregulated by IL-20 and IFN-γ.

DISCUSSION

To explain cytokine interactions in the pathogenesis of psoriasis, we have previously proposed a working hypothesis called the ''Type 1 pathway'' (Lew *et al.*, 2004a, b; Lowes *et al.*, 2004; Wang *et al.*, 2004; Bowcock and Krueger, 2005). In this model, upstream cytokines such as IL-12 and IL-23 stimulate T cells to produce IFN-γ and TNF-α, resulting in activation of relevant transcription factors (STAT1 and NF-κB, respectively). This induces inflammatory mediators such as iNOS, IL-8, and MIG, which may activate keratinocytes and endothelial cells, ultimately leading to the psoriatic phenotype. IL-20 is consistently elevated in psoriatic skin, indicating that it is highly correlated with disease. However, IL-20 was not regulated by IFN-γ, but instead by cell adhesion and TNF-α. Thus, the production of this cytokine potentially introduces a new pathogenic inflammatory pathway that is independent of the Type 1 pathway. Alternatively, as TNF-α induces IL-20 and is produced by Type 1 T cells, the induction of IL-20 may represent a branch point in the Type 1 pathway. With regard to its effector functions, IL-20 induces a pattern of genes that define psoriasis and that are usually considered to be IFN-γ-regulated. This broadens the definition of cytokines that are able to induce ''IFN-γ-responsive genes'' and other disease-related genes.

Our observations of IL-20 receptors in psoriasis contrast with previous reports (Blumberg *et al.*, 2001; Romer *et al.*, 2003; Wei *et al.*, 2005). We found gene expression for IL-20Rα/IL-20Rβ and protein expression for IL-20Rα to be decreased in LS skin. Based on immunostaining, IL-20Rα appeared to be diminished in the middle and lower epidermis. Given the limitations of cytokine diffusion, IL-20 derived from leukocytes near the dermo-epidermal junction may act on more proximal keratinocytes and induce transcriptional downregulation or internalization of the receptors. We also observed that gene expression of the receptor subunits in normal skin was not significantly different from LS or NL psoriatic skin. Previously, it was suggested that upregulation of IL-20 receptors implied a role for IL-20 in psoriasis (Blumberg *et al.*, 2001). However, our finding suggests that the effects of IL-20 are mediated by differential expression of the cytokine itself, rather than by significant changes in its receptors.

Although diffuse IL-20$^+$ staining was sometimes observed in keratinocytes, we identified a major *in vivo* source of IL-20 to be infiltrating CD68$^+$ and CD11c$^+$ leukocytes (Figure 4). This result extends previously published studies implicating a keratinocyte origin (Romer *et al.*, 2003; Wei *et al.*, 2005). CD68 identifies myelomonocytic cells (Nakamura *et al.*, 1998) that localize to the papillary dermis of psoriasis lesions (Gillitzer *et al.*, 1993). CD68 also overlaps with a subset of cells expressing CD11c (Figure S1b), a marker of monocytes/macrophages and certain DCs that produce TNF-α and iNOS in psoriasis (Lowes *et al.*, 2005). Blood-derived myeloid leukocytes clearly have the capacity to synthesize IL-20 (Figure 3a). Together with *in vivo* data indicating that CD68$^+$ cells do not overlap with monocytes, or mature DCs (Figure S1), we suggest that myeloid-derived leukocytes, in particular immature DCs and/or inflammatory TNF- and iNOS-producing DCs, may be important, but not exclusive, sources of IL-20 in psoriasis.

Production of IL-20 mRNA in myeloid-derived cells (monocytes) after plastic adherence suggested that ''outside-in'' cellular activation may induce IL-20 expression. Indeed, we found that IL-20 mRNA was increased in cells after β2 integrin ligation (Figure 3c). However, as this induction was modest, the role of cytokines may potentially be more important for IL-20 induction. In particular, TNF-α appeared to increase IL-20 production (Figure 3d), demonstrating that multiple mechanisms may activate IL-20 synthesis. Given that myeloid-derived leukocytes in psoriasis may produce IL-20 and TNF-α (Wang *et al.*, 2004), it is possible that TNF-α acts in an autocrine fashion to upregulate IL-20. TNF-α derived from T cells, keratinocytes, and other cells may contribute to this process. Interestingly, we found that IFN-γ did not promote IL-20 production, which supports our suggestion that IL-20 is not directly regulated by IFN and that it may be induced outside the Type 1 pathway. The stimulation of IL-20 by TNF-α does not contradict this, as the induction of certain inflammatory molecules by TNF-α is independent of Type 1 (STAT1- or NF-κB-mediated) pathways. Thus, the stimulation of IL-20 by TNF-α may alternatively indicate a branch point in the Type 1 signaling cascade.

To better relate IL-20 to disease activity, we studied patients treated with alefacept, an LFA-3Ig fusion protein that targets CD2 on T cells. Using response score as an objective measure of outcome, we found that IL-20 mRNA expression closely paralleled clinical disease activity (Figure 5e). Response score combined epidermal thickness, K16 mRNA, and K16 staining (Wittkowski *et al.*, 2004; Chamian *et al.*, 2005; Gottlieb *et al.*, 2005). Moreover, when changes in the disease-associated genes IL-8 and MIG were included in the analysis, IL-20 became even more highly correlated with response score (Figure 5f). As a single gene product, IL-20 mRNA exhibited an extremely high correlation with disease activity, and overall, this is similar to disease relationships seen with products like iNOS and IL-12p40 (IL-23p40) (Wittkowski *et al.*, 2004; Gottlieb *et al.*, 2005). Considering that alefacept preferentially targets T cells, this may also argue that T cells are involved in the proximal regulation of IL-20, possibly via TNF-α elaboration.

To investigate downstream effects of IL-20, we used microarrays to screen keratinocytes stimulated with IL-20. We found three groups of induced genes: known psoriasis disease-associated genes, certain IFN-inducible genes, and a group of genes with potentially important disease regulatory activity. Firstly, by comparing IL-20-induced genes with three published microarray studies of psoriasis (Bowcock *et al.*, 2001; Oestreicher *et al.*, 2001; Zhou *et al.*, 2003), we found that IL-20 promoted the expression of previously described disease-associated genes. These include the neutrophil chemoattractants CXCL1 (GRO-α) and IL-8, as well as the DC/T-cell chemoattractant CCL20 (MIP-3α). SERPIN B4 and SERPIN B3 are two highly upregulated genes in psoriasis (Zhou *et al.*, 2003), and these were two of the top genes induced by IL-20. Interestingly, these two genes were not upregulated in IFN-stimulated keratinocytes, which suggests that IL-20 can exert

some of its effects through IFN-independent pathways. IL-20 also influenced disease-related genes associated with epidermal differentiation (TGM1, SPRR1B, and SKALP).

Secondly, by comparing IFN-γ- and IL-20-treated keratinocytes, we found that IL-20 upregulated at least 38 IFN-inducible genes. These include several prototypical IFN-inducible transcriptional modulators (STAT1, IFI-16, and IRF-7), as well as other disease-defining genes (CCL2/MCP-1, CD47, and PSME2). Given the time course of our experiments (exposure to IL-20 for 4 hours), it is likely that IL-20 achieves this by directly activating IFN-response elements. Considering that IL-20 may be derived mostly from myelomonocytic cells, this potentially suggests a greater role for innate immune mechanisms in augmenting psoriatic inflammation through IFN-like effects.

Thirdly, we found that IL-20 induced additional genes that have not been previously implicated in psoriasis, but potentially have important regulatory activity. For instance, IL-20 may influence keratinocyte hyperproliferation by inducing genes that are cell growth promoting (RAI3) or antiapoptotic (BIRC3 and TNFAIP3). IL-20 may play a role in neovascularization by upregulating the angiogenic factor TNFAIP2 and by inducing metalloproteinase synthesis (MMP-7 and MMP-13). In addition, IL-20 may influence inflammation by upregulating CXCL6 (GCP-2), a neutrophil chemotactic factor.

Overall, our results potentially define an important source and role for IL-20. Currently, a working hypothesis is that IFN-γ and TNF-α are the major cytokines driving Type 1 inflammation in psoriasis. Our studies demonstrate that IL-20 specifically induces IFN-like effects in keratinocytes. Moreover, it is possible that the effects of IL-20 may overlap with TNF-α. Our microarray studies demonstrate that IL-20 can activate the TNF-inducible transcription factor RELB, which is a part of the NF-κB2 complex. Therefore, we believe that IL-20 is an effector cytokine with important functions in psoriasis. Although IL-20 may not directly induce inflammation (i.e. leukocyte activation), the net effect of this cytokine may be inflammatory through its actions on keratinocytes. Furthermore, the inhibition of IL-20 or its receptors may represent potential therapy for patients. Preliminary studies indicate that anti-IL-20 antibodies reverse phenotype in a psoriatic skin-severe combined immunodeficient mouse transplant model (Kjeldsen *et al.*, 2005). As such, it will be interesting to further define the role of IL-20 in psoriasis by using specific antagonists.

MATERIALS AND METHODS

Psoriasis subjects

In a Rockefeller University Hospital Institutional Review Board-approved protocol, 34 adults with psoriasis (affecting 5–10% of body surface area) provided written consent for LS and NL skin biopsies. The study was conducted according to the Declaration of Helsinki Principles. Before this, patients had no systemic and topical treatment for 4 and 2 weeks, respectively. Additionally, 11 normal volunteers had biopsies. Half of each biopsy was frozen in OCT (Sakura Finetechnical, Tokyo, Japan) and stored at -80°C for immunohistochemistry, and the remaining section stored in liquid nitrogen until analysis.

TaqMan RT-PCR quantitation of mRNA

RNA extraction and TaqMan RT-PCR assays were performed as described previously (Chamian *et al.*, 2005). To prevent false negative results due to absent mRNA, we measured hARP and found appropriate expression in all cells/tissue samples (data not shown). Owing to inadequate mRNA, data were obtained in only 5/8 alefacept nonresponders. Sequences of primers and probes can be found as Supplementary material.

Statistical analysis

Paired Student's *t*-test was used to compare LS, NL, and normal skin gene expression levels. Significance was accepted as $P<0.05$.

Immunohistochemistry

Tissue sections were stained with monoclonal mouse anti-human IL-20 antibody (R&D Systems, Minneapolis, MN) followed by biotin-labeled horse anti-mouse antibody (Vector Laboratories, Burlingame, CA), or polyclonal goat anti-human IL-20Rα antibody (R&D Systems) followed by biotin-labeled rabbit anti-goat antibody (Vector Laboratories). Secondary antibodies were amplified with avidin–biotin complex (Vector Laboratories) and developed with 3-amino-9-ethylcarbazole chromogen (Sigma-Aldrich, St Louis, MO).

In vitro cell studies

PBMCs were isolated from buffy coats using Ficoll–Paque PLUS (Amersham Biosciences, Piscataway, NJ). Lymphocytes were isolated from nonadherent PBMCs following incubation on polystyrene culture dishes for 2 hours at 37°C. T cells were isolated from whole blood using a T-Cell Negative Isolation Kit (Dynal Biotech, Brown Deer, WI) and activated for 24 hours using the Dynabeads CD3/CD28 T-Cell Expander Kit (Dynal Biotech). HaCaT cells were grown in DMEM (Gibco-BRL Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and activated with 10 μg/ml recombinant IFN-γ (R&D Systems) for 24 hours at 37°C. Monocytes were isolated from buffy coats using a Monocyte Negative Isolation Kit (Dynal Biotech). For adherence, monocytes were incubated in RPMI-1640 (Gibco-BRL Life Technologies) on 100 mm $\times$ 20 mm polystyrene culture plates (BD Falcon, San Jose, CA) for 2 hours at 37°C. For integrin experiments, monocytes (0.5–1 million cells/ml) were incubated in 15 ml conical tubes (BD Falcon) in RPMI-1640 with 10% normal human serum (Labquip Ltd, Niagara Falls, NY) for 2 hours at 37°C with β1 integrin antibodies (Chemicon, Temecula, CA), β2 integrin antibodies (Chemicon/Cymbus Biotech and Calbiochem, San Diego, CA), or mouse IgG1 control (Chemicon). For cytokine activation experiments, monocytes were isolated as described previously (Dhodapkar *et al.*, 2005) and incubated for 6, 24, or 48 hours at 37°C with 10 μg/ml of IFN-γ (R&D Systems) or 10 ng/ml of TNF-α (R&D Systems). For DC generation, monocytes (purity >95%) were cultured to mature DCs as described previously (Dhodapkar *et al.*, 2002).

Immunofluorescence

After acetone fixation and treatment with 10% normal horse serum (Vector Laboratories), tissue sections were incubated with IL-20

antibody (R&D Systems) (1:10), then with Cy3-labeled secondary antibody (Jackson Immunoresearch, West Grove, PA) (1:100), followed by CD68-FITC antibody (Dako, Carpinteria, CA) (1:200). Controls were mouse IgG1-FITC (BD Biosciences), or IL-20 antibody without a secondary antibody. For CD11c staining, sections were fixed in acetone and treated with 10% normal goat serum (Vector Laboratories). Sections were incubated overnight with IL-20 antibody (R&D Systems) (1:10), then IgG1 goat anti-mouse secondary antibody conjugated to Alexa 568 (Molecular Probes, Eugene, OR) (1:250) for 30 minutes, followed by CD11c (BD Pharmingen) custom conjugated to Alexa 488 (MSKCC mAb Core Facility, New York, NY) (1:10) for 2 hours. Images were acquired as described previously (Chamian *et al.*, 2005; Gottlieb *et al.*, 2005).

Alefacept studies

Twenty-two patients with moderate to severe psoriasis were treated with alefacept (7.5 mg/kg/week for 12 weeks) and determined to be ''responders'' or ''nonresponders'' as described previously (Chamian *et al.*, 2005). Gene expression analysis and statistical correlation with disease activity (u-statistics, response score) were performed as described previously (Wittkowski *et al.*, 2004; Chamian *et al.*, 2005; Gottlieb *et al.*, 2005).

Gene arrays

Confluent HaCaT cells grown in DMEM with 10% fetal bovine serum were changed to serum-free media overnight. Cells were treated in triplicate for 4 hours with 10 nM human IL-20 (Biosource International, Camarillo, CA), 100 ng/ml IFN-γ (R&D Systems), or no cytokine. RNA was obtained as above and hydridized to Affymetrix (Santa Clara, CA) HuGeneU95 Av2 microarrays, according to the manufacturer's protocol. Raw fluorescent intensity values were analyzed using GeneChip Operating software version 1.2 (Affymetrix) and GeneSpring™. Data for triplicates were averaged.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Characterization of CD68 cells by flow cytometry and dual-color immunofluorescence.

Primers and probes.

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