Cellular Fibronectin is Induced in Ultraviolet-Exposed Human Skin and Induces IL-10 Production by Monocytes/Macrophages

Yuichi Yoshida,*† Kefei Kang,* Guofen Chen,* Anita C. Gilliam,* and Kevin D. Cooper*‡

*Department of Dermatology, Case Western Reserve University and University Hospitals of Cleveland, Ohio, U.S.A.; †Department of Dermatology, Kyushu University, Fukuoka, Japan; ‡VA Medical Center, Cleveland, Ohio, U.S.A.

CD11b$^+$ monocytic/macrophagic cells that infiltrate human skin after *in vivo* ultraviolet exposure potently produce interleukin-10. We hypothesized that binding of monocyte β1 integrins to ultraviolet-induced extracellular matrix ligands, such as fibronectin, after entry of blood monocytes into the dermis, is involved in the modulation of immunoregulatory monocytic cytokines. Immunostaining of human skin and reverse transcriptase–polymerase chain reaction studies revealed that the embryonic isoform of cellular fibronectin, in which the extra domain A (EDA) segment is spliced in (EDA$^+$ cellular fibronectin), and confers enhanced binding to β1 integrins, is newly induced and is associated with infiltrating CD11b$^+$ cells post *in vivo* ultraviolet exposure. We then tested the effect of fibronectin on resting purified peripheral monocytes *in vitro*. We found that monocyte interleukin-10, but not interleukin-12, was significantly induced in a concentration-dependent manner by *in vitro* binding to cellular fibronectin (n = 6), but not plasma fibronectin. Tumor necrosis factor-α was also induced in a concentration-dependent manner, but to a lesser extent. Monoclonal antibodies to β1 integrins β-subunit (CD29) also strongly induced tumor necrosis factor-α and interleukin-10 production, but not interleukin-12. Neutralization of tumor necrosis factor-α reduced by 54% the interleukin-10 production that was induced by monocytes binding to cellular fibronectin, indicating that interleukin-10 induction is at least in part dependent upon concomitant autocrine tumor necrosis factor-α release. In conclusion, ultraviolet skin injury results in increased production and deposition of EDA$^+$ cellular fibronectin in the papillary dermis, which may be one of the key signals capable of inducing interleukin-10 but not interleukin-12 in monocytes that infiltrate micromilieu of human skin after ultraviolet exposure. *Key words: adhesion molecules/cytokines/monocytes/macrophages. J Invest Dermatol 113: 49–55, 1999*

Ultraviolet (UV) induced immunosuppression is a well recognized phenomenon with relevance to skin cancer tumor immunity, response to infectious disease, and therapy of immunologically mediated skin diseases. The modulation by UV of immunoregulatory cytokines is one of the critical events relevant to this immunosuppression, with interleukin (IL)-10 upregulation and IL-12 downregulation being critical for UV-induced immunosuppression of delayed-type hypersensitivity and contact sensitivity (Schwarz *et al*, 1994, 1996; Shimizu and Streilein, 1994; Ullrich, 1994; Riemann *et al*, 1996). We have previously shown that CD11b$^+$ monocytic/macrophagic cells, which infiltrate into the dermis (Kang *et al*, 1998), and epidermis (Kang *et al*, 1994) after UV exposure of the skin potently produce IL-10, a well known immunosuppressive cytokine. The mechanism of induction of IL-10 production in UV-irradiated skin, however, is not fully explored.

Tissue macrophages derived from circulating monocytes play an important part in host defense involving innate and specific immunity and tissue repair. Their functions are regulated in large part through receptors for soluble and insoluble signals (Celada and Nathan, 1994; Gordon, 1995). β1 and β2 integrins are found on monocytes and macrophages (Wright *et al*, 1984; Wright and Meyer, 1985; Brown *et al*, 1989; Kohn and Klingemann, 1991; Owen *et al*, 1992; Ross and Vetvicka, 1993; Takizawa *et al*, 1995), and serve to facilitate adhesion, activation, or differentiation in response to both cellular and extracellular matrix. In skin, fibronectin (Fn) is a component of the extracellular matrix which is an important ligand for β1 integrins on monocytes entering the skin. Cellular Fn (cFn) is thought to be synthesized and secreted locally in the dermis by fibroblasts, endothelial cells and monocytes as well as by keratinocytes in the epidermis. Liver cells also synthesize Fn and secrete it into the plasma and are responsible for the majority of plasma Fn (pFn), which could also be present in skin (Clark, 1983). Multiple Fn isoforms are generated from a single pre-mRNA by alternative splicing at three distinct regions: EDA, EDB (which are present in cFn, but not in pFn), and IIICS (which is present in cFn and pFn) (Oyama *et al*, 1993; Tavian *et al*, 1994). With the discovery of EDA, it has become clear that its inclusion is distinctive of cFn. Although *in vivo*, EDA$^+$ Fn is poorly represented in the extracellular matrix of adult normal tissue, it increases in specific pathologic circumstances such as wound healing (Ffrench-Constant *et al*, 1989; Brown *et al*, 1993), epithelial fibrosis

Manuscript received January 12, 1999; revised March 23, 1999; accepted for publication March 25, 1999.

Reprint requests to: Dr. Kefei Kang, Department of Dermatology, Case Western Reserve University and University Hospitals of Cleveland, 11100 Euclid Avenue, Cleveland, OH 44106, U.S.A.

Abbreviations: Fn, fibronectin; cFn, cellular Fn; pFn, plasma Fn

(Barnes *et al*, 1994), and vascular proliferation (Glukhova *et al*, 1989). It has been speculated that this change in the splicing pattern provides an extracellular matrix that facilitates wound repair, perhaps by promoting cell migration.

In normal skin, Fn is mainly deposited along the dermal–epidermal junction, the walls of blood vessels immediately underlying the endothelium and as amorphous granules, fibrils, or patches in dermis as well (Clark, 1983). Fn is increased at sites of inflammation in the skin, such as in psoriasis and lichen planus (Clark, 1983), and it has been documented that UVA and UVB can induce Fn biosynthesis in hairless mice studied by indirect immunofluorescence and by weighing extracted Fn from irradiated mouse skin (Schwartz, 1988; Boyer *et al*, 1992). It is unknown, however, whether UV exposure of normal human skin can alter Fn expression, and which type of Fn is deposited. We hypothesized the alteration of Fn after UV injury to skin may be a regulatory factor for monocytes entering into UV-exposed skin. Because Fn fragments could also be chemotactic for peripheral blood monocytes (Norris *et al*, 1982), and because cytokines such as IL-1β can be rapidly induced by monocyte adherence to extracellular matrix components or ligation of β1 integrin with antibodies (Yurochko *et al*, 1992; Lin *et al*, 1995), we asked whether UV induces Fn expression or alteration in its splice variants in human skin. If so, could the increased Fn component be involved in regulating the unique cytokine status of monocytes that enter UV-exposed skin from the blood compartment? We report here that Fn, specifically the EDA^+ splice variant of cFn, are induced in human dermis after UV. Confirming findings of others using β1 integrin ligands, Fn induced tumor necrosis factor (TNF)-α and IL-1β production in monocytes. We found, however, that cFn and β1 integrin ligation is also a potent inducer of monocyte IL-10, but not IL-12. These findings implicate UV induction of EDA^+ Fn and β1 integrin-ligand binding as one of the mechanisms of generating the $IL\text{-}10^{high}$ monocytic/macrophagic cell phenotype in the micromilieu of human skin after UV exposure.

MATERIALS AND METHODS

Subjects Healthy adult volunteers participated in the study after Institutional Review Board approval of the protocol and informed consent. Keratomes or punch biopsies were performed on the buttocks or hips of volunteers at different time points after four minimal erythemal doses of UV irradiation from a bank of six FS40 bulbs, and nonirradiated sites were used as controls.

Immunohistofluorescence staining The samples were frozen on dry ice and stored at –80°C. After embedding in OCT Embedding Medium (Miles; Elkhart, IN), 6 μm sections were cut and thaw mounted on Probe-On slides (Fisher; Fair Lawn, NJ), fixed with acetone, air dried, and stored at –80°C. The slides were thawed, hydrated in phosphate-buffered saline (Amresco, Solon, OH), blocked with 10% goat serum (Accurate Chemical & Scientific Corporation, Westbury, NY), and treated with primary mouse monoclonal antibody (MoAb) specific to the extra domain contained in cFn (EDA sequence) and thus recognizing only cFn (Clone, DH1, IgG1; ICN Biomedicals, Costa Mesa, CA). For double staining, OKM1 (IgG2b, anti-CD11b, ATCC No.CRL 8026 cell line; American Type Culture Collection, Rockville, MD) was used as the other primary antibody. Isotype controls; IgG1 (Sigma, St. Louis, MO) and IgG2b (Sigma) were diluted to 1 μg per ml in 10% goat serum phosphate-buffered saline, then followed by secondary antibodies: fluorescein isothiocyanate-conjugated goat anti-mouse IgG1 (Boehringer Mannheim, Indianapolis, IN), and biotinylated goat anti-mouse IgG2b (Caltag, San Francisco, CA), followed by Rhodamine Red-X conjugated avidin (Molecular Probes, Eugene, OR). Coverslips were mounted on glass slides with Vectashield Mounting Medium (Vector, Burlingame, CA). Slides were viewed and photographed by fluorescence microscopy (Axiophot; Carl Zeiss, Thornwood, NY), using a triple filter which allowed visualization of multiple fluorescent markers simultaneously.

Reverse transcriptase–polymerase chain reaction (reverse transcriptase–PCR) Keratomes of skin were incubated in Dispase (50 units per ml) (Collaborative Biochem, Bedford, MA) at 4°C overnight. Dermis was separated from epidermis, snap frozen and pulverized with liquid nitrogen, and subjected to RNA extraction by Qiagen RNA/DNA Maxi kit (QIAGEN, Chatworth, CA) and the RNA was quantitated by spectrophotometric measurement. cDNA was synthesized from 500 to 1000 ng (from tissue) of total RNA, and PCR was performed as previously described (Kang *et al*, 1994), with some modifications. The cDNA mixture was mixed with a 60 μl PCR reaction mixture which contained human Fn EDA primers (primer 1: nucleotides 4775–4798 of the sense strand, 5′-GGAGAGAGTCAGCCTCTGGTTCAG-3′; primer 2: nucleotides 5149–5125 of the anti-sense strand, 5′-TGTCCACTGGGCGCTCAGG-CTTGTG-3′, EDA^+ bp 374) (Tavian *et al*, 1994). Parallel samples were primed and amplified for β-actin. 30 cycles were conducted in QuarterBath Immersion Thermal Cycler (INOTECH, Lansing, MI) with denaturation at 94°C for 1 min, annealing at 55°C (60°C for β-actin) for 1 min and extension at 72°C for 2 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide. The density of PCR products was quantitated by Optimas 6.1 software (Optimas Corporation, Bothell, WA). The densitometric values for EDA^+ cFn were normalized to that of β-actin for relative quantitation.

Isolation of monocytes Buffy coat was obtained from peripheral blood of normal volunteers after Histopaque-1077 (Sigma) centrifugation. After three washes with Hank's balanced salt solution plus 1% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), mononuclear cells were resuspended in RPMI medium 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco BRL), and L-glutamine (Irvine Scientific, Santa Ana, CA). The cells were plated in 100 mm tissue culture Petri dishes at a concentration of $35–45 \times 10^6$ in 5 ml 10% FBS-RPMI and incubated at 5% CO_2, 95% humidity, 37°C for 1 h. After aspirating off nonadherent cells, adherent cells were scraped off and washed with 1% FBS–Hank's balanced salt solution. For negative selection of human monocytes, blocking MoAb (FcγR2, CD32) and MoAb Cocktail (CD2, CD3, CD19, and CD56) were used, followed by Dextran Colloid Iron Cocktail, according to manufacturer's instructions (Stem Cell Tech., Vancouver, Canada). The treated cells were washed, resuspended in 5% FBS–Hank's balanced salt solution, and then applied to a MidiMACS Separation Column against a magnet (Miltenyi Biotec, Auburn, CA). The purity of monocytes was defined by flow cytometry (>90%). The monocytes were resuspended in 10% FBS-RPMI and kept on ice until use.

Cytokine production and neutralization: engagement of Fn or anti-β1 integrin β-subunit with monocytes Six-well tissue culture plates were coated with human cFn (Upstate Biotech., Lake Placid, NY) or pFn (Becton Dickinson, Bedford, MA) in phosphate-buffered saline, at varying concentrations as indicated, and incubated at 4°C overnight. The plates were washed with phosphate-buffered saline and blocked with 0.05% heated (65°C for 1 h) bovine serum albumin (Sigma) for 1 h at 37°C, and washed again. The monocytes were added at $1.5–2 \times 10^6$ per ml; after 1 h incubation at 37°C, TNF-α (R&D Systems, Minneapolis, MN) or *Staphylococcus aureus* cells (Calbiochem-Novabiochem, La Jolla, CA) were added in some experiments. For engagement of the β-subunit receptor of β1 integrin on monocytes via antibodies, two clones of MoAb to CD29 (Lia1/2 and K20, Coulter, Westbrook, ME) were used. Prior to plating monocytes, Lia1/2 (25 μg per ml) and K20 (1, 10, 25 μg per ml) were incubated with the cells (2×10^6 per ml) on ice for 45 min and then the monocytes were plated and blocked with bovine serum albumin (no cFn present). The supernatants were harvested after 18–22 h incubation at 37°C in a humidified incubator with 5% CO_2. Lipopolysaccharide (*Escherichia coli*, 0111:B4, Sigma) was used as a positive control.

Neutralization of TNF-α MoAb antihuman TNF-α (M-301, Endogen, Boston, MA) was used to neutralize TNF-α endogenously produced by monocytes binding to cFn. Before plating monocytes, MoAb anti-human TNF-α (10 μg per ml) was mixed with monocytes and then plated into wells coated with cFn. Cells then were incubated and the supernatants were harvested after incubation.

Cytokine assay by enzyme-linked immunosorbent assay (ELISA) After 20 h incubation, supernatants were harvested from incubated monocytes and IL-10, IL-12 p70, and TNF-α protein were determined by ELISA. The pairs of MoAb to human IL-10 (purified, 18551D; biotinylated, 18562D, PharMingen, San Diego, CA), human IL-12 p70 (purified, M-122; biotinylated, M-121-B, Endogen), and human TNF-α (purified, 18631D; biotinylated, 18642D, PharMingen) were used as capture (100 ng per 100 μl per well) and detection antibodies (100 ng per 100 μl per well), respectively. After first coating capture antibodies, 96-well plates were incubated at 4°C overnight. The plates were washed and blocked with 3% bovine serum albumin–phosphate-buffered saline. Recombinant human IL-10 (PharMingen), IL-12 (R&D Systems), and TNF-α were used as

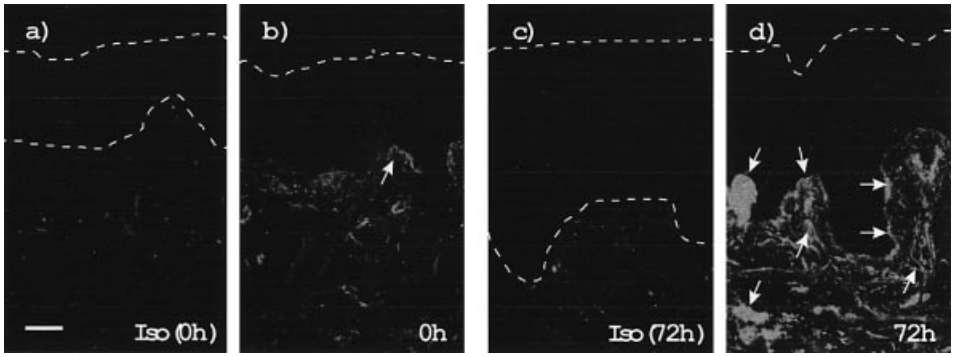

Figure 1. Upregulation of EDA$^+$ cFn in the dermis after UV exposure. Fluorescence immunostaining of skin biopsies of non-UV-irradiated control (0 h) and 72 h after UV exposure. fluorescein isothiocyanate-immunostaining with anti-cFn MoAb (clone DH1) shows that EDA$^+$ cFn is mainly deposited at the dermal–epidermal junction in control skin (*b*, 0 h, *arrow*; *a*, isotype control, *scale bar*, 50 μm); in contrast, cFn was apparently upregulated and is seen as patches and thick fibrillar deposits (*c*, isotype control, *d*, 72 h, *short arrows*) at 72 h after UV exposure. The broken lines separate dermis and epidermis. n = 3.

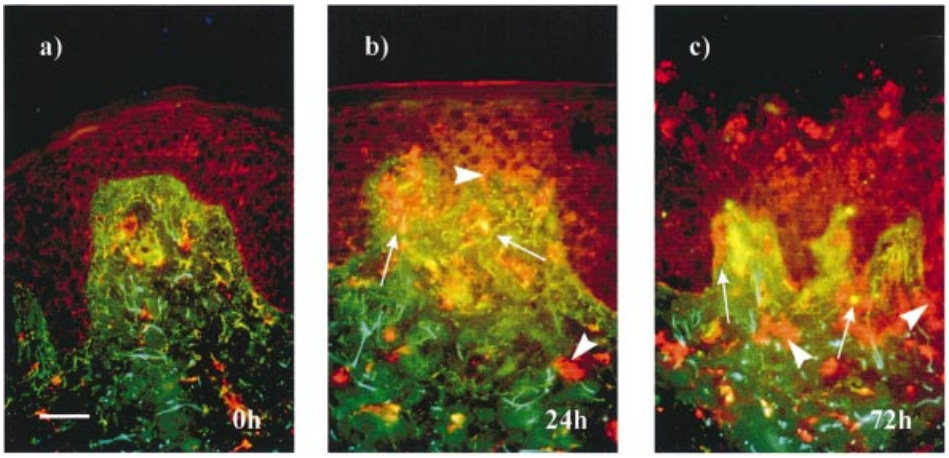

Figure 2. Upregulation of EDA$^+$ cFn in the dermis and association with induced CD11b$^+$ monocytic/macrophagic cells after UV exposure. Double staining was performed with fluorescein isothiocyanate (cFn) and Rhodamine Red-X (CD11b+ cells). A few CD11b$^+$ cells are seen in the control dermis with scattered cFn deposits (*a*, 0 h, *scale bar*, 50 μm). In contrast, at 24 h (*b*) and 72 h (*c*) after UV exposure, evident monocytic/macrophagic cells (*b*, *c*, arrowhead) infiltrate into dermis and towards or throughout the epidermis; aggregates and coarse dot deposits of cFn are surrounded or in apposition with CD11b$^+$ cells (*b*, *c*, *long arrows*). n = 3.

standards, respectively, and samples were simultaneously incubated at room temperature for 2 h. After washes, detection antibodies were added, and the plates were washed again. Peroxidase-labeled streptavidin (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD) 1:1000 was used to develop o-phenylenediamine dihydrochloride (0.02%) (Sigma) with added hydrogen peroxide (30%, 1:2000). Fifty percent H_2SO_4 was used to stop the reaction, and the plates were read at 492 nm.

Statistical analysis Statistical significance of differences was determined by Student's t-test where indicated.

RESULTS

Increased EDA$^+$ cFn in human dermis and dermal–epidermal junction after *in vivo* UV exposure: colocalization with CD11b$^+$ monocytic/macrophagic cells In order to correlate UV-induced alterations with temporal and spatial changes in immune cells such as monocytic/macrophagic cells trafficking to the dermis and epidermis and with cFn production, anti-cFn (specific for the EDA splice variant) MoAb was used alone (**Fig 1**, fluorescein isothiocyanate-labeled) and with anti-CD11b MoAb (**Fig 2**, rhodamine-labeled) for double fluorescence immunostaining on tissue sections.

In unirradiated control skin, cFn deposits faintly and discontinuously in the dermis under the dermal–epidermal junction (**Fig 1*a***, isotype *versus* ***b***, 0 h, *short arrow*). By 72 h, cFn is prominently upregulated with continuous staining in the dermal–epidermal junction. Large dermal patches, and dense and thick fibrillar deposits are also seen in the papillary dermis and perivasculature (**Fig 1*c***, isotype control *versus* ***d***, 72 h, *short arrows*, photographed with matched time of exposure as **Fig 1*a–c***). This increase was observed in three of three skin biopsy specimens from three individuals. Biopsies at 24 h and 48 h revealed gradually increasing diffuse deposition (data not shown), with maximal staining at 72 h.

In control unirradiated skin with double staining, only a few CD11b$^+$ large cells (red) are present in papillary dermis and perivasculature (**Fig 2*a***), which have previously been demonstrated to represent monocytic/macrophagic cells (Meunier *et al*, 1995; Yoshida *et al*, 1998); in addition, cFn is visualized (as in **Fig 1*b***) as modest papillary dermal staining and perivascular staining. By 24 h after UV, CD11b$^+$ cells (**Figs 2*b***, 24 h, *arrowhead*) are clearly increased in numbers and intensity and appear not only in the dermis but also at the dermal–epidermal junction and in the epidermis. Concomitantly, cFn is increased in intensity throughout the papillary dermis. Moreover, the fine deposits of cFn become aggregates and coarse dots, as well as fibrils. Interestingly, some larger aggregates are in apposition to or appear to be surrounded by CD11b$^+$ monocytic/macrophagic cells (**Figs 2*b***, 24 h, *long arrows*). By 72 h, dermal infiltration by CD11b$^+$ monocytic/macrophagic cells is prominent (**Fig 2*c***, *arrowhead*) and the upper epidermis is disrupted and edematous. CD11b$^+$ cells are also present in the epidermis. The interaction of monocytic/macrophagic cells with aggregates and dots of cFn in the dermis (**Figs 2*c***, 72 h, *long arrows*) is localized to the dermis and the dermal–epidermal junction.

EDA$^+$ cFn mRNA is upregulated after UV exposure To determine whether EDA$^+$ Fn is produced as a result of local cellular synthesis, we assayed for the EDA splice variant of cFn by reverse transcriptase–PCR using EDA-specific oligonucleotide primers (see *Materials and Methods*). In agreement with the immunostaining findings, EDA$^+$ mRNA was upregulated in the dermis after UV exposure at 72 h relative to anatomically matched unirradiated skin from the same volunteer, with β-actin as controls (n = 2) (**Fig 3**). EDA$^+$ mRNA was also detected in UV-exposed epidermis, but to a much lesser extent (data not shown). Thus, both immunostaining and reverse transcriptase–PCR provide evidence that EDA$^+$ cFn is induced locally post-UV exposure.

IL-10, but not IL-12, is markedly upregulated by monocyte binding to cFn After UV injury, we hypothesized that circulating leukocytes, such as monocytes trafficking from activated blood vessels into the dermis and epidermis, undergo modulation of their immunoregulatory cytokines via binding to the encountered

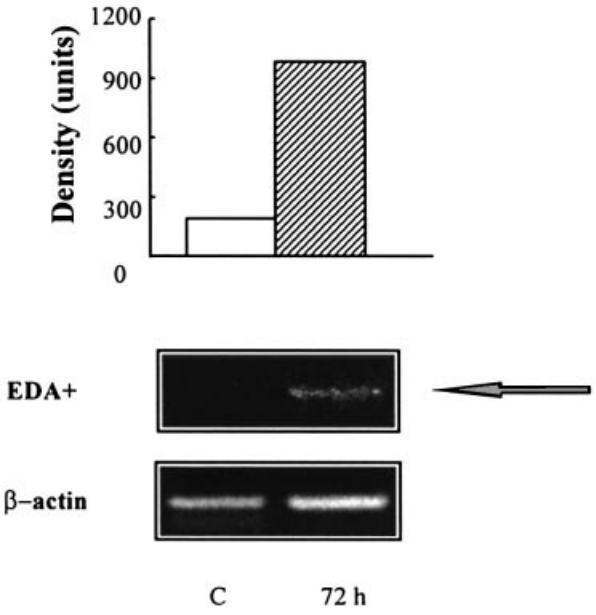

Figure 3. EDA$^+$ Fn mRNA expression is upregulated in skin 72 h post-UV (four minimal erythemal doses) exposure. Dermis was separated from epidermis by dispase, and ground with liquid nitrogen. Total RNA was prepared and subjected to reverse transcriptase–PCR. β-actin was used as a control gene (*lower panel*). The relative density of PCR products was quantitated as shown (*bars in the upper graph*) after normalization to that of β-actin. *Arrow* indicates EDA$^+$ band of PCR product. C, non-UV irradiated control skin. 72 h, post-UV (four minimal erythemal doses) irradiated skin. n = 2.

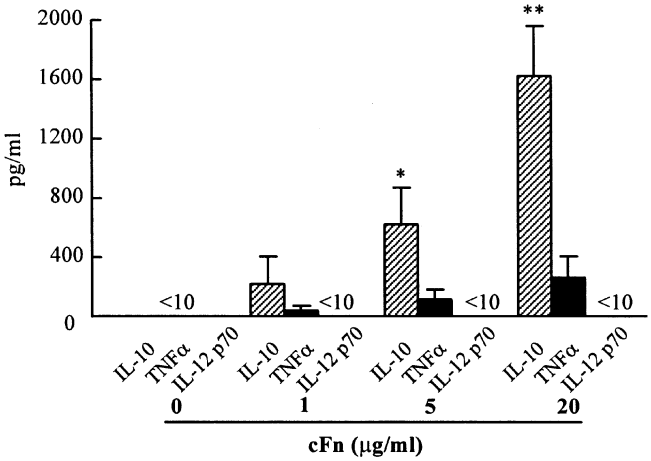

Figure 4. IL-10 and TNF-α protein, but not IL-12 p70 production, are enhanced in a concentration-dependent manner by monocyte binding to cFn. Cytokines were measured by ELISA from supernatants harvested after 20 h incubation of purified monocytes (1.5–2 × 10^6 per ml) on cFn-coated plates. *p = 0.045 and **p = 0.001 relative to cFn 0 μg per ml, respectively. *Error bars*, SEM. n = 6.

extracellular matrix molecules, particularly when the EDA$^+$ splice variant is abundant, because of its enhanced ability to bind via β1 integrins. In order to clarify the effects of increased exposure to dermal cFn on immigrating monocytes, different concentrations of cFn (1, 5, 20 μg per ml) and pFn (20 μg per ml) were employed in *in vitro* studies. IL-10 was dramatically induced by purified monocytes binding to cFn in a concentration-dependent manner (**Fig 4**, *hatched*), but not by pFn (data not shown). IL-12 p70 was not detectable. TNF-α production was also enhanced by monocytes binding to cFn by ELISA (**Fig 4**), but not by pFn (data not shown); however, the magnitude of induction was less robust than that of IL-10. IL-1β induction was similar to TNF-α (data not shown). The baseline of monocyte production of TNF-α and IL-10 were not detectable (<10 pg per ml) implying that monocytes postpurification were in a resting state.

Table I. The production of IL-12 p70 protein by monocyte binding to cellular fibronectin and stimulation with SAC

cFN (μg per ml)	IL-12 p70 (pg per ml)[a]	
	no SAC[b]	+SAC
0	<10	351 ± 164
20	40 ± 39	344 ± 75[c]

[a]Results are expressed as the mean ± SEM (n = 3).
[b]SAC concentration, 0.001%.
[c]p>0.05 relative to monocytes with no cFn binding.

Because IL-12 production by monocytes can be downregulated by complement receptor 3 (Marth and Kelsall, 1997; Sutterwala *et al*, 1997; Yoshida *et al*, 1998), we asked if β1 integrin signaling via Fn can also be involved in the regulation of IL-12. Monocytes were stimulated with *Staphylococcus aureus* cells (a potent stimulator for IL-12 production by monocytes) after placement on dishes coated with/without cFn. cFn had no effect on IL-12 production (**Table I**), however, indicating that, although cFn may play a part in inducing the IL-10^{high} phenotype of UV-induced monocytic/macrophagic cells *in vivo*, the inhibition of IL-12 production of *in vivo* cells cannot be attributed to β1 ligation/signaling. It is clear, however, that cFn can induce IL-10 without concomitantly inducing IL-12.

β1 integrin β-subunit (CD29) alone can strongly induce TNF-α and IL-10 production by blood monocytes The principal Fn receptors expressed by blood monocytes are integrins α5β1 and α4β1 (Hemler, 1990). Therefore, if Fn is acting through β1 signaling, antibodies to the β1 β-subunit (CD29), common to β1 integrins, should also affect TNF-α and IL-10 production by monocytes. Two different MoAb clones of CD29 (Lia1/2 and K20) were used. Both TNF-α (2596 ± 192 and 1854 ± 451 pg per ml) and IL-10 (2906 ± 365 and 3080 ± 682 pg per ml) protein were dramatically induced (n = 2) with Lia1/2 and K20 [which was concentration-dependent (data not shown)] by monocytes, respectively, in culture without cFn coated substrate (**Fig 5**). The baseline of the isotype mouse IgG2a (K20) was undetectable (<10 pg per ml) and IgG1 (Lia 1/2) was 39 ± 39 for TNF-α and 246 ± 246 pg per ml for IL-10, respectively. TNF-α and IL-10 production by lipopolysaccharide-stimulated monocytes as positive controls were 3491 ± 111 and 3881 ± 1045 pg per ml, respectively. The marked induction of TNF-α by β1 cross-linking using antibodies is more than 10-fold greater than the TNF-α response to cFn, indicating somewhat differing signaling responses for TNF-α; by contrast, the IL-10 response was quite similar. Neither antibodies nor isotype controls could stimulate significant amounts of IL-12 (data not shown), suggesting distinct signaling patterns through β1 integrin, and confirming the lack of endotoxin in the antibodies.

Induction of IL-10 production in monocytes by cFn is partly mediated by endogenous TNF-α Because TNF-α plays an important part in human monocyte IL-10 synthesis (Wanidworanun and Strober, 1993) and because cFn can also induce TNF-α production by monocytes, we asked whether induced endogenous TNF-α or exogenous TNF-α is important for the induction of IL-10 production by monocytes. First, recombinant human TNF-α was added after monocytes were plated on different concentrations of cFn (5, 20 μg per ml) coated wells. TNF-α exerted a consistent enhancement of IL-10 production by monocytes at each tested concentration of cFn (**Table II**); however, the magnitude of the effect was modest as compared with Fn of β1 signaling.

Despite modest effects during exogenous administration in the presence of cFn, TNF-α appears to be an important endogenous enhancing mediator of IL-10 in this system (**Fig 6**). For neutraliz-

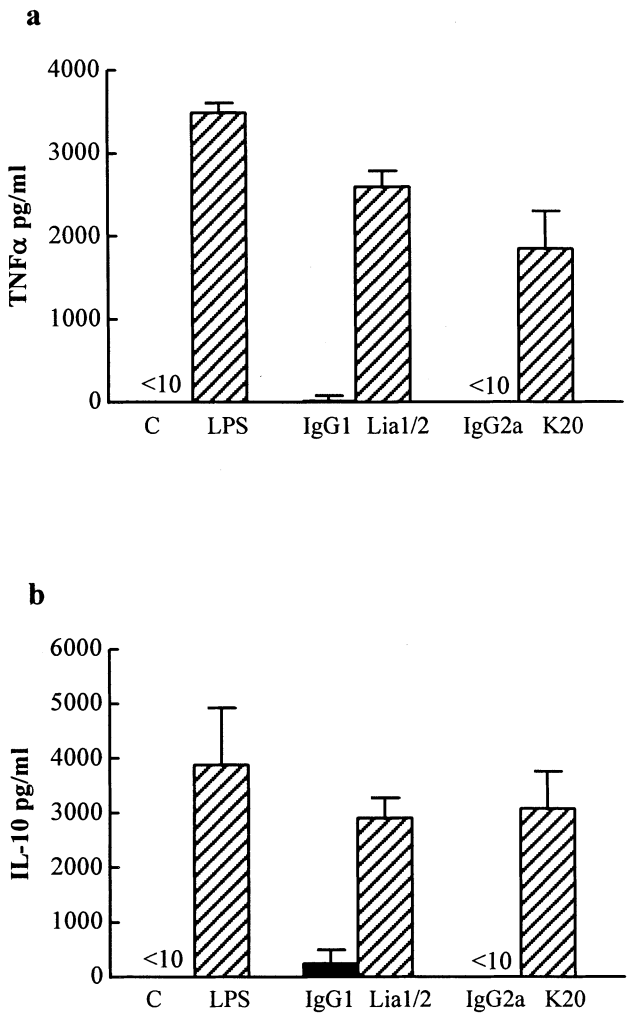

Figure 5. TNF-α and IL-10 production by monocytes is strongly stimulated by the β chain of β1 integrins (CD29). Two clones of MoAb, Lia1/2 (IgG1) and K20 (IgG2a), were used. The MoAb or isotype control antibodies (IgG1, IgG2a) were first incubated with monocytes, respectively, on ice for 45 min and then the monocytes were plated on bovine serum albumin coated wells. Cytokines were measured by ELISA from supernatants harvested after incubation of monocytes for 20 h. C, controls. Lipopolysaccharide-stimulated monocytes are shown for comparison. (*a*) and (*b*) show TNF-α and IL-10 production, respectively. *Error bars*, SEM. n = 2.

ation of TNF-α, monocyte activation by Fn was performed by adding MoAb anti-TNF-α to the cultures prior to and continuously during monocyte binding to cFn. IL-10 production was significantly reduced after induced endogenous TNF-α was neutralized; 1519 ± 115 pg per ml IL-10 in cultures with cFn plus isotype *versus* 694 ± 66 pg per ml in cultures with MoAb anti-human TNF-α (n = 4, p = 0.0008, **Fig 6**). The mean inhibition was 54%, implying that cFn-induced endogenous TNF-α is an important, but not the sole, mediator of IL-10 induction by cFn in monocytes.

DISCUSSION

Previous studies of the skin microenvironment after UV irradiation in both mice and humans showed that inflammatory leukocytes migrate into the dermis and epidermis (Cooper *et al*, 1986, 1993; Meunier *et al*, 1995), with preferential infiltration of monocytes characterized by IL-10^{high} and IL-12^{low} phenotype (Kang *et al*, 1994, 1998). This study demonstrates one of the possible mechanisms of this induced monocyte phenotype.

Monocyte/macrophagic cells are rich in receptors for immunoglobulins, complement, and extracellular matrix components such

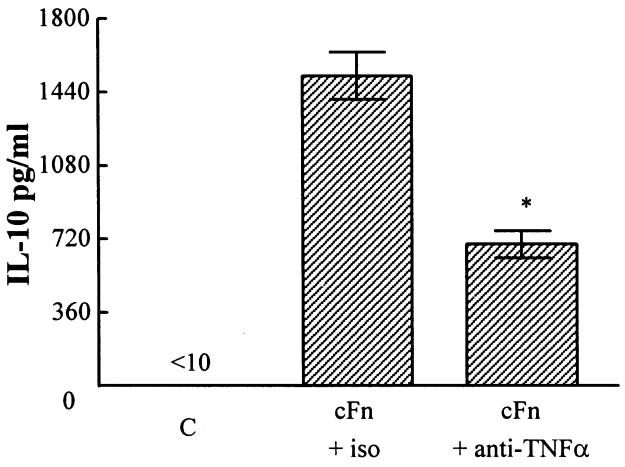

Figure 6. IL-10 production by monocytes binding to cFn is markedly reduced after neutralization of TNF-α. Anti-human TNF-α (10 μg per ml) or IgG1 isotype (+ iso) was added to purified monocytes prior to incubation on cFn-coated (20 μg per ml) plates. IL-10 was measured by ELISA from supernatants harvested after 20 h incubation. C, control. *p = 0.0008 relative to iso. *Error bars*, SEM. n = 4.

Table II. Minimal additional effect of exogenous TNFα on cellular fibronectin-stimulated IL-10 production

cFn (μg per ml)	IL-10 (pg per ml)[a]		
	No TNFα	+TNFα	p value[b]
0	<10	60 ± 38	
5	621 ± 249	846 ± 249	0.017
20	1622 ± 339	1852 ± 368	0.025

[a]Results are expressed as the mean ± SEM (n = 6).
[b]Paired t-test (n = 6).

as Fn. Fn is one of the most important ligands for β1 integrin that is apparently expressed on the surface of blood monocytes such as α5β1 and α4β1. After UV injury, Fn deposition in skin can be enhanced by leakage of pFn from blood vessels, or can be a novel synthetic product from the cells in the skin. Upon influx of blood monocytes into the skin, monocyte/macrophage cell binding to Fn can take place. Therefore, we have sought to link Fn upregulation with monocytic/macrophagic cells function in skin post-UV irradiation.

After UV exposure, immunofluorescence staining of human skin with MoAb to cFn (clone DH1), which is specific to the extra domain (EDA sequence) of cFn, shows that EDA^+ cFn is upregulated at 72 h with large patches and dense, abundant thick fibrillar deposits (**Fig 1*d***, *short arrows*). In order to query the upregulation of EDA^+ cFn at the mRNA level, the primers for the EDA splice variant of cFn were used for reverse transcriptase–PCR. EDA^+ bands are present in the dermis of UV-exposed skin, but not control skin (**Fig 3**). EDA^+ Fn is an isoform known to be expressed in carcinoma (Oyama *et al*, 1993) as well as in wound healing (Brown *et al*, 1993). The upregulation of EDA^+ Fn in UV-exposed skin might be directly caused by an UV carcinogenic effect resulting in tissue specific alternative splicing or due to wound healing after UV injury. To date, increased UVA-and UVB-induced Fn biosynthesis has been reported only in hairless mice (Schwartz, 1988; Boyer *et al*, 1992). We confirm Fn upregulation in human skin and further identify the deposited Fn as the functionally important EDA^+ cFn splice variant.

In addition, we examined the spatial relationship of infiltrating $CD11b^+$ monocytic/macrophagic cells to UV-induced cFn. The numbers of $CD11b^+$ monocytic/macrophagic cells were clearly increased at 24 h in the dermis (**Fig 2*b***, red cell, *arrowhead*) and near epidermis, and at 72 h, they were found throughout the skin

(**Fig 2*c***, *arrowhead*). UV-induced monocytic/macrophagic cells and cFn were closely associated physically, suggesting that signaling may well have occurred *in vivo* via the Fn–monocytic/macrophagic cell interaction, perhaps resulting in the *in vivo* phenotype induced in monocytes as they enter and encounter UV-modified skin. To address the latter question, we used purified monocytes from peripheral blood to model the proposed *in vivo* sequence of resting monocyte entry and Fn exposure in UV-exposed skin. Because tissue macrophages have already been stimulated in this altered skin milieu, they would not model well the resting monocyte as it exits the blood and encounters the dermal perivasculature and papillary dermis. That *in vivo* signals in UV-exposed skin in monocytes can be modeled *in vitro* to induce *in vivo* phenotype has been demonstrated to be valid in studying ligation to the β2 integrin of blood monocytes (Yoshida *et al*, 1998; Hammerberg *et al*, 1998).

To determine cytokine production by monocyte binding to Fn, monocytes were incubated with cFn. We found that IL-10 upregulation was cFn concentration-dependent, whereas changes in IL-12 regulation were not observed. TNF-α was also but relatively slightly upregulated in parallel with IL-10 production (**Fig 4**). In contrast, neither IL-10 nor TNF-α production were upregulated by monocyte binding to pFn (data not shown). These data indicate clearly that cFn containing EDA plays an important part in the upregulation of IL-10 and TNF-α production by monocytes, suggesting that the close physical association of monocytic/macrophagic cells with cFn *in vivo* by immunofluorescence staining of skin is likely an active process, involving alteration of Fn conformation or change of the spacing between well-established binding sites.

To examine whether the β chain, which is common to β1 integrins, may be responsible for the induction of IL-10 and TNF-α by monocyte binding to cFn, two clones of MoAb to CD29 were used to stimulate cytokine production. Indeed, the β chain of β1 integrin (both Lia1/2 and K20) did consistently and markedly induce IL-10 and TNF-α production (**Fig 5**), showing an important role of β1 integrin signaling. It has been reported that in monocytes, signaling through the VLA integrins for IL-1 induction requires only divalent cross-linking, which is consistent with our results, and does not always need an additional secondary antibody to cross-link for signaling (Yurochko *et al*, 1992). It is known that β1 integrin signaling induces tyrosine phosphorylation (Lin *et al*, 1995) and induces activation of mitogen-activated protein kinase (Chen *et al*, 1994). Using lipopolysaccharide stimulation, mitogen-activated protein kinase has been shown to play a key role in the induction of IL-10 and TNF-α by monocytes (Meisel *et al*, 1996; Foey *et al*, 1998). We presume that these mechanisms are involved in Fn-mediated production of IL-10 and TNF-α by monocytes.

In addition to being a responding element, monocytes may also be a source of cFn in UV-exposed skin. There are several potential candidates for the cellular source of the enhanced cFn found after UV, including keratinocytes, fibroblasts, endothelial cells, and monocytes (Clark, 1983; Brown *et al*, 1993).

Although $CD11b^+$ cells are present at 24 h after UV, this actually precedes the time at which the most considerable amounts of cFn were found, raising the possibility that other factors are involved in the infiltration of monocytes and their IL-10 production. The early induction of IL-10, prior to papillary dermal and dermal–epidermal junction Fn deposition after UV, may be through signals distinct from Fn. We recently found that complement degradation products (iC3b) are involved in monocyte induction of IL-10 within 24 h after UV (Yoshida *et al*, 1998). We hypothesize that monocytic/macrophagic cell IL-10 is initially stimulated by both iC3b and perivascular cFn and then is sequentially stimulated by increasing amounts of cFn in the 72 h period after UV, thus sustaining the IL-10 production as iC3b wanes.

TNF-α can also be induced and released by keratinocytes and dermal mast cells after UV irradiation (Kock *et al*, 1990; Walsh, 1995). To model *in vivo* conditions further in UV-exposed skin, recombinant TNF-α was added, and resulted in further enhancement of IL-10 production (**Table II**), although this was a modest effect. Thus TNF-α could potentiate IL-10 production; TNF-α could act either via endogenous (Foey *et al*, 1998) or exogenous sources. To clarify that TNF-α induced by monocytes after binding to cFn strongly participates in the induction of IL-10, the neutralization of induced endogenous TNF-α was performed. Our data showed that after neutralization of TNF-α, the IL-10 production was greatly reduced (>50%). Thus, the endogenous TNF-α here does play an important, perhaps autocrine, role (Foey *et al*, 1998) and may desensitize the effect of further exogenous TNF-α on IL-10 production by monocytes, as shown in this study.

Although it is known that Fn is increased in psoriasis (Fyrand, 1979; Clark, 1983) and that pFn plays an important part in the regulation of keratinocyte cell cycling in psoriasis (Bata-Csorgo *et al*, 1998), a role for cFn after UV therapy for psoriasis has not been explored. One potential mechanism is that UV can upregulate cFn in the dermis, and cFn, but not pFn, interacts with UV-induced monocytic/macrophagic cells after UV exposure of the skin, resulting in the enhancement of IL-10 production as shown in this study. It has recently been demonstrated that IL-10 is very useful for psoriasis treatment (Asadullah *et al*, 1998).

In summary, the oncofetal isoform EDA of cFn can be induced in skin post-UV irradiation. IL-10 and TNF-α, but not IL-12, are significantly upregulated by blood monocytes binding to cFn, but not pFn. Endogenous/autocrine TNF-α induced by monocytes binding to cFn plays an important part in the upregulation of IL-10. Thus, β1 integrin binding provides signals to monocytes for generating the $IL\text{-}10^{high}$ monocytic/macrophagic cell phenotype in the micromilieu of the skin after UV injury.

This work is supported in part by grants from the NIH (NIADM AI-41766) (KDC), the VA Medical Research Service (KDC), the NIAMS Skin Diseases Research Center (AR-39750) (KDC, ACG), the Dermatology Foundation: Mary Kay Cosmetic Grant and Novartis Clinical Career Development Award Grants (ACG), and Ohio Reagents Pilot Study Grant (ACG).

REFERENCES

Asadullah K, Sterry W, Stephanek K, *et al*: IL-10 is a key cytokine in psoriasis: Proof of principle by IL-10 therapy: a new therapeutic approach. *J Clin Invest* 101:783–794, 1998

Barnes JL, Hastings RR, De la Garza MA: Sequential expression of cellular fibronectin by platelets, macrophages, and mesangial cells in proliferative glomerulonephritis. *Am J Pathol* 145:585–597, 1994

Bata-Csorgo Z, Cooper KD, Ting KM, Voorhees JJ, Hammerberg C: Fibronectin and α5 integrin regulate keratinocyte cell cycling: a mechanism for increased fibronectin potentiation of T cell lymphokine-driven keratinocyte hyperproliferation in psoriasis. *J Clin Invest* 101:1509–1518, 1998

Boyer B, Fourtanier A, Kern P, Labat-Robert J: UVA- and UVB-induced changes in collagen and fibronectin biosynthesis in the skin of hairless mice. *J Photochem Photobiol B* 14:247–259, 1992

Brown DL, Phillips DR, Damsky CH, Charo IF: Synthesis and expression of the fibroblast fibronectin receptor in human monocytes. *J Clin Invest* 84:366–370, 1989

Brown LF, Dubin D, Lavigne L, Logan B, Dvorak HF, Van De Water L: Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. *Am J Pathol* 142:793–801, 1993

Celada A, Nathan C: Macrophage activation revisited. *Immunol Today* 15:100–102, 1994

Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL: Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* 269:26602–26605, 1994

Clark RAF: Fibronectin in the skin. *J Invest Dermatol* 81:475–479, 1983

Cooper KD, Neises GR, Katz SI: Antigen-presenting OKM5+ melanophages appear in human epidermis after ultraviolet radiation. *J Invest Dermatol* 86:363–370, 1986

Cooper KD, Duraiswamy N, Hammerberg C, Allen E, Kimbrough-Green C, Dillon W, Thomas D: Neutrophils, differentiated macrophages, and monocyte/macrophage antigen presenting cells infiltrate murine epidermis after UV injury. *J Invest Dermatol* 101:155–163, 1993

Ffrench-Constant C, Van de Walter L, Dvorak HF, Hynes RO: Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J Cell Biol* 109:903–914, 1989

Foey AD, Parry SL, Williams LM, Feldmann M, Foxwell BMJ, Brennan FM: Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-α: role of the p38 and p42/44 mitogen-activated protein kinases. *J Immunol* 160:920–928, 1998

Fyrand O: Studies on fibronectin in the skin: II. Indirect immunofluorescence studies in psoriasis vulgaris. *Arch Dermatol Res* 266:33–41, 1979

Glukhova MA, Frid MG, Shekhonin BV, Vasilevskaya TD, Grunwald J, Saginati M, Koteliansky VE: Expression of extra domain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. *J Cell Biol* 109:357–366, 1989

Gordon S: The macrophage. *Bioessays* 17:977–986, 1995

Hammerberg C, Katiyar SK, Carroll MC, Cooper KD: Activated complement 3 (C3) is required for UV induction of immunosuppression and antigenic tolerance. *J Exp Med* 187:1133–1138, 1998

Hemler ME: VLA proteins in the integrin family: Structures, functions, and their role on leukocytes. *Annu Rev Immunol* 8:365–400, 1990

Kang K, Hammerberg C, Meunier L, Cooper KD: CD11b+ macrophages that infiltrate human epidermis after in vivo ultraviolet exposure potently produce IL-10 and represent the major secretory source of epidermal IL-10 protein. *J Immunol* 153:5256–5264, 1994

Kang K, Gilliam AC, Chen G, Tootell E, Cooper KD: In human skin, UVB initiates early induction of IL-10 over IL-12 preferentially in the expanding dermal monocytic/macrophagic population. *J Invest Dermatol* 111:31–38, 1998

Kock A, Schwarz T, Kirnbauer R, Urbanski A, Perry P, Ansel JC, Luger TA: Human keratinocytes are a source for tumor necrosis factor alpha: Evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. *J Exp Med* 172:1609–1614, 1990

Kohn FR, Klingemann HG: Regulation of fibronectin receptor (alpha 5 beta 1) mRNA expression in human-monocytes and monocyte-derived macrophages by activation/differentiation signals. *Exp Hematol* 19:653–658, 1991

Lin TH, Rosales C, Mondal K, Bolen JB, Haskill S, Juliano RL: Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. *J Biol Chem* 270:16189–16197, 1995

Marth T, Kelsall BL: Regulation of interleukin-12 by complement receptor 3 signaling. *J Exp Med* 185:1987–1995, 1997

Meisel C, Vogt K, Platzer C, Randow F, Liebenthal C, Volk HD: Differential regulation of monocytic tumor necrosis factor-α and interleukin-10 expression. *Eur J Immunol* 26:1580–1586, 1996

Meunier L, Bata-Csorgo Z, Cooper KD: In human dermis, UV induces expansion of a CD36+CD11b+CD1− macrophage subset by infiltration and proliferation; CD1+ Langerhans-like dendritic antigen-presenting cells are concomitantly depleted. *J Invest Dermatol* 105:782–788, 1995

Norris DA, Clark RAF, Swigart LM, Huff JC, Weston WL, Howell SE: Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. *J Immunol* 129:1612–1618, 1982

Owen CA, Campbell EJ, Stockley RA: Monocyte adherence to fibronectin: role of CD11/CD18 integrins and relationship to other monocyte functions. *J Leukocyte Biol* 51:400–408, 1992

Oyama F, Hirohashi S, Sakamoto M, Titani K, Sekiguchi K: Coordinate oncodevelopmental modulation of alternative splicing of fibronectin pre-messenger RNA at ED-A, ED-B, and CS1 regions in human liver tumors. *Cancer Res* 53:2005–2011, 1993

Riemann H, Schwarz A, Grabbe S, *et al*: Neutralization of IL-12 in vivo prevents induction of contact hypersensitivity and induces hapten-specific tolerance. *J Immunol* 156:1799–1803, 1996

Ross GD, Vetvicka V: CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. *Clin Exp Immunol* 92:181–184, 1993

Schwartz E: Connective tissue alterations in the skin of ultraviolet irradiated hairless mice. *J Invest Dermatol* 91:158–161, 1988

Schwarz A, Grabbe S, Riemann H, *et al*: In vivo effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. *J Invest Dermatol* 103:211–216, 1994

Schwarz A, Grabbe S, Aragane Y, *et al*: Interleukin-12 prevents ultraviolet B-induced local immunosuppression and overcomes UVB-induced tolerance. *J Invest Dermatol* 106:1187–1191, 1996

Shimizu T, Streilein JW: Evidence that ultraviolet B radiation induces tolerance and impairs induction of contact hypersensitivity by different mechanisms. *Immunology* 82:140–148, 1994

Sutterwala FS, Noel GJ, Clynes R, Mosser DM: Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med* 185:1977–1985, 1997

Takizawa T, Nishinarita S, Kitamura N, Hayakawa J, Kang H, Tomita Y: Interaction of the cell-binding domain of fibronectin with VLA-5 integrin induces monokine production in cultured human monocytes. *Clin Exp Immunol* 101:376–382, 1995

Tavian D, DePetro G, Colombi M, Portolani N, Giulini SM, Gardella R, Barlati S: RT-PCR detection of fibronectin EDA^+ and EDB^+ mRNA isoforms: Molecular markers for hepatocellular carcinoma. *Int J Cancer* 56:820–825, 1994

Ullrich SE: Mechanism involved in the systemic suppression of antigen-presenting cell function by UV irradiation. Keratinocyte-derived IL-10 modulates antigen-presenting cell function of splenic adherent cells. *J Immunol* 152:3410–3416, 1994

Walsh LJ: Ultraviolet B irradiation of skin induces mast cell degranulation and release of tumor necrosis factor-alpha. *Immunol Cell Biol* 73:226–233, 1995

Wanidworanun C, Strober W: Predominant role of tumor necrosis factor-α in human monocyte IL-10 synthesis. *J Immunol* 151:6853–6861, 1993

Wright SD, Meyer BC: Fibronectin receptor of human macrophages recognizes the sequence Arg-Gly-Asp-Ser. *J Exp Med* 162:762–767, 1985

Wright SD, Licht MR, Craigmyle LS, Silverstein SC: Communication between receptors for different ligands on a single cell: ligation of fibronectin receptors induces a reversible alteration in the function of complement receptors on cultured human monocytes. *J Cell Biol* 99:336–339, 1984

Yoshida Y, Kang K, Berger M, *et al*: Monocyte induction of IL-10 and down-regulation of IL-12 by iC3b deposited in ultraviolet-exposed human skin. *J Immunol* 161:5873–5879, 1998

Yurochko AD, Liu DY, Eierman D, Haskill S: Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. *Proc Natl Acad Sci USA* 89:9034–9038, 1992