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Dermal Fibroblasts Sustain Proliferation of Activated T Cells via Membrane-Bound Interleukin-15 upon Long-Term Stimulation with Tumor Necrosis Factor-α

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In chronic inflammatory conditions, mononuclear cells infiltrate the connective tissue attracted by fibroblast-secreted chemokines. The role of fibroblasts in sustaining the lymphocyte immune response upon cellular infiltration is so far unresolved. We here report that, upon prolonged stimulation with tumor necrosis factor-α, dermal fibroblasts enhance proliferation of activated T cells whereas unstimulated fibroblasts do not. T cell growth stimulation requires cell contact of tumor necrosis factor-α stimulated fibroblasts to T cells and is not due to soluble factors. Growth stimulation is substantially blocked by neutralizing antibodies to interleukin-15. Fluorescence-activated cell sorter analyses revealed that tumor necrosis factor α stimulated fibroblasts expose interleukin-15 in a membrane-bound form on the cell surface whereas nonstimulated fibroblasts and interferon-γ treated fibroblasts do not. The amount of membrane interleukin-15 increases with the duration of tumor necrosis factor-α stimulation for at least 3 d. Unstimulated fibroblasts, however, accumulate interleukin-15 in the cytoplasm. No interleukin-15 could be detected in the culture supernatant. Immunohistochemical analyses confirmed membrane interleukin-15 on dermal fibroblasts in discoid lupus erythematosus skin lesions whereas no membrane interleukin-15 was found on the surface of fibroblasts in healthy skin. We conclude that dermal fibroblasts upon long-term tumor necrosis factor-α stimulation during chronic inflammation are involved via membrane-bound interleukin-15 in stimulating proliferation of accumulated, activated T cells. ***Key words: cell-to-cell interactions/human fibroblasts/inflammation. J Invest Dermatol 116:102–109, 2001***

Chronic inflammation is characterized by accumulation of mononuclear cells, i.e., lymphocytes and monocytes, in the connective tissue. The inflammatory process is driven by a complex series of interactions between cells, the extracellular matrix, and secreted factors of various cell types. Inflammatory processes, although triggered by a variety of events, are largely driven by the expression of cytokines and their receptors by infiltrating as well as resident cells of the tissue. Upon migration across vascular endothelium, leukocytes reach the extravascular space and interact with connective tissue cells such as fibroblasts as well as with extracellular matrix proteins such as fibronectin, laminin, and collagen. Leucocyte–fibroblast interactions are mediated by a panel of specific adhesion molecules induced by proinflammatory cytokines on both leukocytes and fibroblasts (Cabanas and Hogg, 1991; Morzycki and Issekutz, 1991; Bombara *et al*, 1993; Meng *et al*, 1995). Interleukin-1 (IL-1) and transforming growth factor-α (TGF-α) act synergistically to elicit a number of phenotypic responses in dermal fibroblasts, including proliferation, which are thought to contribute to chronic inflammation (Unemori *et al*, 1994). Tumor necrosis factor-α (TNF-α), a potent mediator of inflammatory functions, induces fibroblasts to express matrix metalloproteinases, chemokines, including RANTES (Sticherling *et al*, 1995) and eotaxin (Bartels *et al*, 1996), and adhesion molecules, e.g., ICAM-1 and VCAM-1 (for review see Barker, 1995). Lymphocytes are attracted by secreted chemokines and adhere to TNF-α-stimulated fibroblasts within the inflammatory lesion via LFA-1 and VLA-4. Numerous actions of TNF-α, however, occur in combination with other cytokines within a cytokine network as part of a cascade of inflammatory factors.

Whereas the mechanisms of retention of mononuclear cells at sites of chronic inflammation by adhesion to fibroblasts are more and more solved, the role of fibroblasts in sustaining proliferation of attracted, activated leukocytes is poorly understood. This process is potentially important because the cytokine crosstalk of mononuclear cells and dermal fibroblasts probably contributes to the chronicity of inflammatory skin diseases. We asked whether dermal fibroblasts upon chronic inflammatory stimulation are involved in sustaining proliferation of activated T cells. We here report that upon prolonged incubation with TNF-α human dermal fibroblasts stimulate proliferation of activated T cells. The growth-promoting activity of TNF-α-treated fibroblasts requires cell-cell contact and is substantially blocked by neutralizing antibodies to IL-15. We demonstrate that TNF-α-treated fibroblasts express membrane-

Manuscript received July 15, 2000; revised October 23, 2000; accepted for publication October 31, 2000.

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Abbreviation: PBMC, peripheral blood mononuclear cells.

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bound IL-15 on the cell surface whereas unstimulated fibroblasts do not. Biopsies from chronic inflammatory lesions confirm membrane expression of IL-15 of fibroblasts within the lesion whereas fibroblasts in healthy skin do not express membrane IL-15. Growth stimulation of activated T cells by membrane IL-15 exposed on fibroblasts in chronic inflammatory lesions implies a crucial role of TNF-α-stimulated fibroblasts not only in cell attraction but moreover in sustaining proliferation of attracted, activated T cells.

MATERIALS AND METHODS

Cytokines, cells, and cell lines Human recombinant interferon-γ (IFN-γ) and TNF-α were purchased from R&D Systems (Wiesbaden, Germany). Fibroblasts were isolated from skin biopsies of healthy donors and grown in Dulbecco's modified Eagle's medium, 10% (vol/vol) fetal bovine serum, 50 μg per ml sodium ascorbate, and 300 μg per ml glutamine. Monocytes and peripheral blood mononuclear cells (PBMC) were purified from peripheral blood by Percoll gradient centrifugation according to standard procedures. The purity of the monocyte preparations used in this study was 90% ± 4% as assessed by morphology of Giemsa-stained preparations. T cells were isolated from PBMC by nylon wool adherence (purity of T cells 98% ± 2% CD3+ as assessed by flow cytometry). Jurkat is a T leukemia line (obtained from ATCC; ATCC TIB 152), HuT102 is a T cell line (obtained from ATCC; ATCC TIB162), HL60 is a human promyeloid cell line (obtained from DZM; DSM ACC3), and U937 is a monoblastoid cell line (obtained from ATCC; ATCC CRL-1593.2). All cells were cultured in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) containing 100 U per ml penicillin, 100 mg per ml streptomycin, 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated fetal bovine serum (Life Technologies).

Proliferation assay Human fibroblasts were seeded at 5×10^4 cells per well in 200 μl RPMI 1640 medium in a microtiter plate. [^{3}H]TdR incorporation assays were performed in triplicate. T cells were seeded at 5×10^4 cells per well, [^{3}H]TdR was added (37 kBq [^{3}H]TdR per well) for 16 h, and proliferation was assessed by β-scintillation spectroscopy.

Flow cytometric analysis To monitor membrane-bound IL-15, cells were washed twice in phosphate-buffered saline (PBS), incubated in PBS, 2% (vol/vol) rabbit serum for 30 min at 4°C, washed in PBS, 1% (wt/vol) bovine serum albumin (BSA), and incubated with the mouse antihuman IL-15 antibody (clone M112; IgG2b; Genzyme, Wiesbaden, Germany) or an isotype-matched control antibody (IgG2b; Becton Dickinson, Heidelberg, Germany), respectively, for 30 min at 4°C. Essentially the same data were obtained when using anti-IL-15 monoclonal antibody (clone 34505.11) (Preprotech, Rocky Hill) for monitoring IL-15. To monitor cytoplasmic IL-15, cell membranes were permeabilized by incubation with PermeaFix (1:2 dilution; Ortho, Raritan, NJ) prior to antibody incubation. Cells were washed twice in PBS, 1% (wt/vol) BSA, and incubated with the rabbit phycoerythrin-conjugated antimouse antibody (Dako, Hamburg, Germany) for 30 min at 4°C. To monitor IL-15 receptor α-chain, cells were incubated with the goat antihuman IL-15 Rα antibody (R&D Systems). Cells were analyzed by flow cytometry utilizing an EPICS XL (Beckman-Coulter, Krefeld, Germany) equipped with WinMDI software. Human monocytes and Jurkat cells served as positive and negative controls, respectively. Statistical analysis was performed by z test (*p < 0.001).

Western blot analysis Fibroblasts were lyzed in the presence of 1% (vol/vol) Nonidet-P40, 3 mM $MgCl_2$, 10 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris-HCl, pH 7.5, and the protein lysate was electrophoretically separated through a 12.5% polyacrylamide gel and blotted onto nitrocellulose. Loading of equal amounts of protein was recorded by Ponceau S staining of the membrane. Western blot analysis was performed according to standard methods utilizing rabbit antihuman IL-15 antiserum (BioSource International, Camarillo, CA), swine peroxidase-labeled antirabbit Ig (Dako), and the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

IL-15 enzyme-linked immunosorbent assay (ELISA) Supernatants from confluent fibroblast cultures (10^6 cells per ml supernatant) were filtered through a 0.22 μm filter (Millipore, Molsheim, France) and assayed for human IL-15 by ELISA (BioSource International; assay sensitivity 11 pg IL-15 per ml). Alternatively, the supernatant was 10-fold concentrated using a Centricon Concentrator (Amicon, Beverley, MA) prior to ELISA testing. To monitor intracellular IL-15, fibroblasts were lyzed (10^6 cells in 1 ml) by repeated freezing and thawing in the presence of 3 mM $MgCl_2$, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5. Nuclei and cellular debris were sedimented and the supernatant was subjected to IL-15 ELISA.

Reverse transcription polymerase chain reaction (RT-PCR) analysis RNA was purified utilizing High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), cDNA was synthesized according to standard procedures, and a cDNA aliquot (5 μl) was amplified in a 50 μl PCR sample. As control, each sample was subjected to PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The following oligonucleotides were used as primers in PCR reactions (Onu *et al*, 1997): IL-15 SSP, 5′ATGGTATT-GGGAACCATAGA3′; IL-15 LSP, 5′ATGAGAATTTCGAAACCA-CAT3′; IL-15 MP, 5′CTCCAAGAGAAAGCACTTC3′ (cycling conditions: 1 min 94°C, 1 min 55°C, and 1 min 72°C; 40 cycles); GAPDH sense, 5′GGTCGGAGTCAACGGATTTG3′; and GAPDH antisense 5′ATGAGCCCCAGCCTTCTCCAT3′ (cycling conditions: 1 min 94°C, 1 min 60°C, and 1 min 72°C; 35 cycles).

Immunohistochemistry Membrane-bound IL-15 within cells of a skin biopsy was monitored using the following staining technique. One half of a fresh punch biopsy (4 mm in diameter) was incubated overnight at 4°C in PBS containing anti-IL-15 monoclonal antibody M112 (Genzyme) (1 μg per μl); the other half of the biopsy was incubated in PBS containing mouse IgG2b (Becton Dickinson) as isotype control. Non-binding antibody was washed out by incubating in PBS, 1% (wt/vol) BSA for 1 h at 37°C. Biopsies were subsequently snap frozen in liquid nitrogen, processed for cryostat sections, and stained according to standard techniques. Bound antibody within the section was detected by incubation with a biotin-labeled goat antimouse F(ab′)$_2$ antibody (Dako) for 30 min at room temperature. Subsequently the section was incubated with an avidin-biotin-peroxidase complex for 30 min at room temperature, washed, and the staining reaction was developed using 3-amino-9-ethylcarbazol and H_2O_2. Nuclei were stained with Mayer's hematoxylin.

RESULTS

We explored whether normal human skin fibroblasts after prolonged stimulation with TNF-α stimulate proliferation of T cells *in vitro*. Therefore, fibroblasts were incubated with TNF-α (20 ng per ml) for 72 h, thoroughly washed, and subsequently coincubated with ConA preactivated, CD3$^+$ T cells. Induction of lymphocyte proliferation was tested by [^{3}H]TdR incorporation. As summarized in **Fig 1(*A*)**, fibroblasts preincubated with TNF-α stimulated proliferation of coincubated T cells whereas untreated fibroblasts did not. Coincubation of TNF-α-pretreated fibroblasts in Transwell plates without cell contact to T cells did not stimulate lymphocyte proliferation nor did the fibroblast supernatant (data not shown). This indicates that the lymphocyte stimulatory activity of TNF-α-pretreated fibroblasts requires cell contact with lymphocytes. Preactivated but not unstimulated CD3$^+$ T cells were induced to proliferate upon contact with TNF-α-treated fibroblasts (data not shown). The effect is specific and not due to residual contamination with TNF-α because the supernatant did not stimulate proliferation of T cells. We conclude that, upon stimulation with TNF-α, dermal fibroblasts enhance proliferation of activated T lymphocytes by cell-to-cell contact.

To identify the fibroblast surface molecule responsible for increasing T cell proliferation, we incubated TNF-α-stimulated fibroblasts with neutralizing antibodies to various cytokines prior to coincubation with activated T cells. As summarized in **Fig 1(*A*)**, lymphocyte proliferation is substantially, although not completely, repressed upon blocking with a neutralizing antibody to IL-15 (10 μg per ml) whereas an isotype-matched antibody is ineffective. This indicates that the growth stimulatory effect of TNF-α-treated fibroblasts is substantially due to IL-15. In contrast, unstimulated fibroblasts did not significantly increase T cell proliferation (**Fig 1*A***). As controls, T cell proliferation in the absence of fibroblasts was stimulated by recombinant IL-15 and blocked by addition of the neutralizing antibody to IL-15 but not by addition of an isotype-matched antibody (**Fig 1*B***). Taken together, the data imply that TNF-α-treated fibroblasts stimulate proliferation of activated lymphocytes by cell-to-cell contact by a process that involves IL-15 exposed on the cell surface of stimulated fibroblasts.

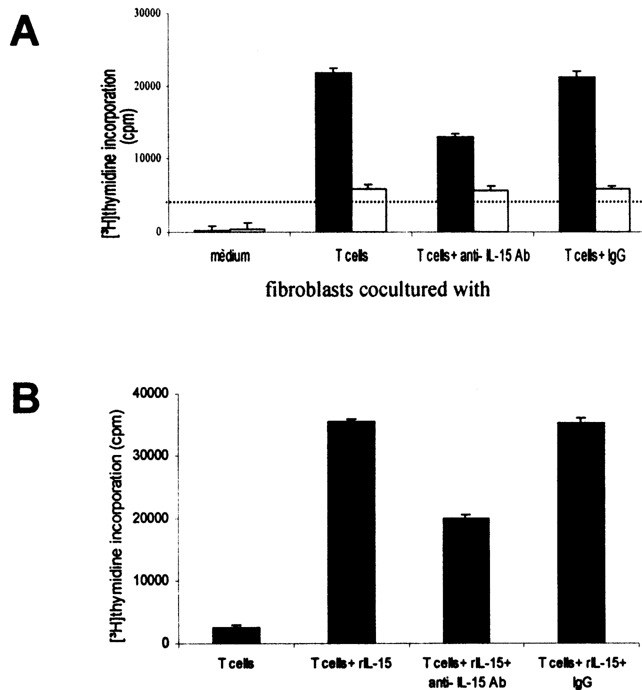

Figure 1. Fibroblasts stimulate proliferation of activated T cells. (*A*) Dermal fibroblasts were seeded in microwell plates (5×10^4 cells per well) and cultured without stimulation (□) or with TNF-α stimulation (20 ng per ml) for 96 h (■). Subsequently, cells were arrested by incubation with mitomycin C (100 μg per ml, 30 min), washed, and CD3+ T cells preactivated with ConA (10 μg per ml, 72 h) were added (5×10^4 cells per well). In addition, antihuman IL-15 antibody (BioSource) (10 μg per ml) or rabbit IgG (10 μg per ml), respectively, was added. As control, fibroblasts were cultured without T cells (medium). Incorporation of [^{3}H]TdR was monitored after 72 h of coculture. The dotted line indicates [^{3}H]TdR incorporation of T cells without cocultured fibroblasts. (*B*) CD3+ T cells were prestimulated with ConA (10 μg per ml, 72 h) and subsequently plated in microwell plates (5×10^4 cells per well). Cells were incubated without additives or with human recombinant IL-15 (10 ng per ml), rIL-15 plus antihuman IL-15 antibody (BioSource) (10 μg per ml), or rIL-15 plus rabbit IgG (10 μg per ml), respectively. After 72 h, incorporation of [^{3}H]TdR was monitored. Data represent the means (± SEM) of triplicates.

To monitor expression of membrane-bound IL-15, fibroblasts were cultured for 72 h in the presence of TNF-α (20 ng per ml) and, as control, with IFN-γ (2000 U per ml), respectively, and analyzed by fluorescence-activated cell sorter (FACS) analysis utilizing the anti-IL-15 antibody M112. Staining of nonpermeabilized cells revealed IL-15 on the surface of TNF-α-stimulated fibroblasts but not on the surface of unstimulated or IFN-γ-incubated fibroblasts (**Fig 2*A***). Monocyte cells, which constitutively express membrane-bound IL-15 (Musso *et al*, 1999), were used as positive controls and Jurkat cells as negative controls for monitoring IL-15 (**Fig 2*B*, *C***). Essentially the same data were obtained using anti-IL-15 monoclonal antibody clone 34505.11 (Preprotech). IFN-γ used in the assay is functionally active because incubation of fibroblasts with IFN-γ (2000 U per ml) induced ICAM-1 expression (data not shown). Time course analyses indicate that prolonged incubation with TNF-α gradually increases IL-15 expression on the surface of fibroblasts during an incubation time as long as 72 h (**Fig 3**). In unstimulated fibroblasts, we detected IL-15 after permeabilization but not without permeabilization of the cell membrane (**Fig 2*A***). This indicates that IL-15 is constitutively expressed in unstimulated fibroblasts and stored in the cytoplasm. Upon TNF-α stimulation, however, IL-15 is additionally exposed on the cell surface of fibroblasts. As controls, membrane IL-15 was recorded in monocytes but not in Jurkat cells in accordance with previous reports (Musso *et al*, 1999).

Dermal fibroblasts express IL-15 receptor α-chain, the expression of which is 3-fold increased after stimulation with TNF-α (20 ng per ml, 72 h) (data not shown). Human endothelial cells were used as positive controls, HL60 cells as negative controls in the FACS analysis. Membrane IL-15 on the surface of TNF-α-stimulated fibroblasts, however, did not elute on treatment with acetate (pH 4.4, 10 min incubation) suggesting that IL-15 is expressed as an integral membrane protein and is not associated with the IL-15 receptor complex. This property is in accordance with membrane IL-15 exposed on the surface of monocytes (Musso *et al*, 1999).

ELISA tests of cellular lysates confirmed expression of IL-15 in unstimulated fibroblasts. Fibroblasts treated with various concentrations of TNF-α (2–200 ng per ml) for 72 h express nearly similar amounts of IL-15 as unstimulated fibroblasts (**Fig 4**). This observation was confirmed by Western blot analysis of cellular lysates of unstimulated and TNF-α-stimulated fibroblasts, and, as control, of IFN-γ-stimulated fibroblasts (**Fig 5**). Western blot analysis moreover revealed that IL-15 in dermal fibroblasts is expressed in two isoforms, i.e., with an apparent molecular mass of 14 kDa and 16 kDa, respectively. Because IL-15 has two potential N-glycosylation sites, the two IL-15 isoforms are likely to present two different glycosylation patterns as recently reported (Gaggero *et al*, 1999). No IL-15 was detected in the culture supernatant conditioned by proliferating fibroblasts during 2 d of culture *in vitro* with and without TNF-α stimulation (2–200 ng per ml) (detection limit of the ELISA is 11 pg IL-15 per ml) (**Fig 4**). Even in fibroblast culture supernatants that had been concentrated about 10-fold, no detectable amounts of IL-15 were monitored. As controls and consistent with previous reports (Satoh *et al*, 1998), IL-15 was detected in the supernatant of HuT102 cells but not in the supernatant of K562 cells.

We assayed dermal fibroblasts for expression of IL-15-specific mRNA by RT-PCR techniques utilizing primer oligonucleotides specific for the short and the long leader peptide isoform, respectively (Onu *et al*, 1997). As shown in **Fig 6**, both isoforms of IL-15 RNA were found to be expressed in unstimulated and in TNF-α (20 ng per ml) stimulated fibroblasts. Monocytes served as positive control and HL60 cells as negative control of the assay.

Taken together, we conclude that proliferating dermal fibroblasts *in vitro* synthesize and store IL-15 intracellularly without secretion of detectable amounts of IL-15 into the supernatant. After prolonged incubation with TNF-α, fibroblasts expose IL-15 on the cell surface that is functionally active in stimulating proliferation of activated, but not of nonactivated, $CD3^+$ T cells. These observations imply a functional role of dermal fibroblasts in sustaining proliferation of accumulated T cells during chronic inflammatory diseases.

We therefore asked whether fibroblasts in chronic inflammatory lesions expose membrane-bound IL-15. Tissue samples derived from discoid lupus erythematosus skin lesions and, as control, from healthy skin were stained *in toto* with the anti-IL-15 antibody M112 as described in *Materials and Methods*. Cryostat sections were subsequently obtained, stained with an antimouse $F(ab)_2$ antibody, and visualized by peroxidase reaction. Incubation of biopsies *in toto* with the primary antibody prior to making thin sections allows preferential staining of membrane-bound molecules within a tissue specimen. As shown exemplarily in **Fig 7**, fibroblasts within lesions expose IL-15 on the cell membrane whereas fibroblasts in healthy skin do not. In accordance with previous reports (Agostini *et al*, 1996; Musso *et al*, 1999), monocytes within the same section show membrane staining with the IL-15 antibody. Staining is specific as an isotype control antibody does not stain fibroblasts or monocytes. This analysis exemplarily demonstrates that fibroblasts in chronic inflammatory conditions *in vivo* expose membrane-bound IL-15 whereas dermal fibroblasts in healthy skin do not. Taken together the data suggest the model that fibroblasts upon TNF-α stimulation in inflammatory skin

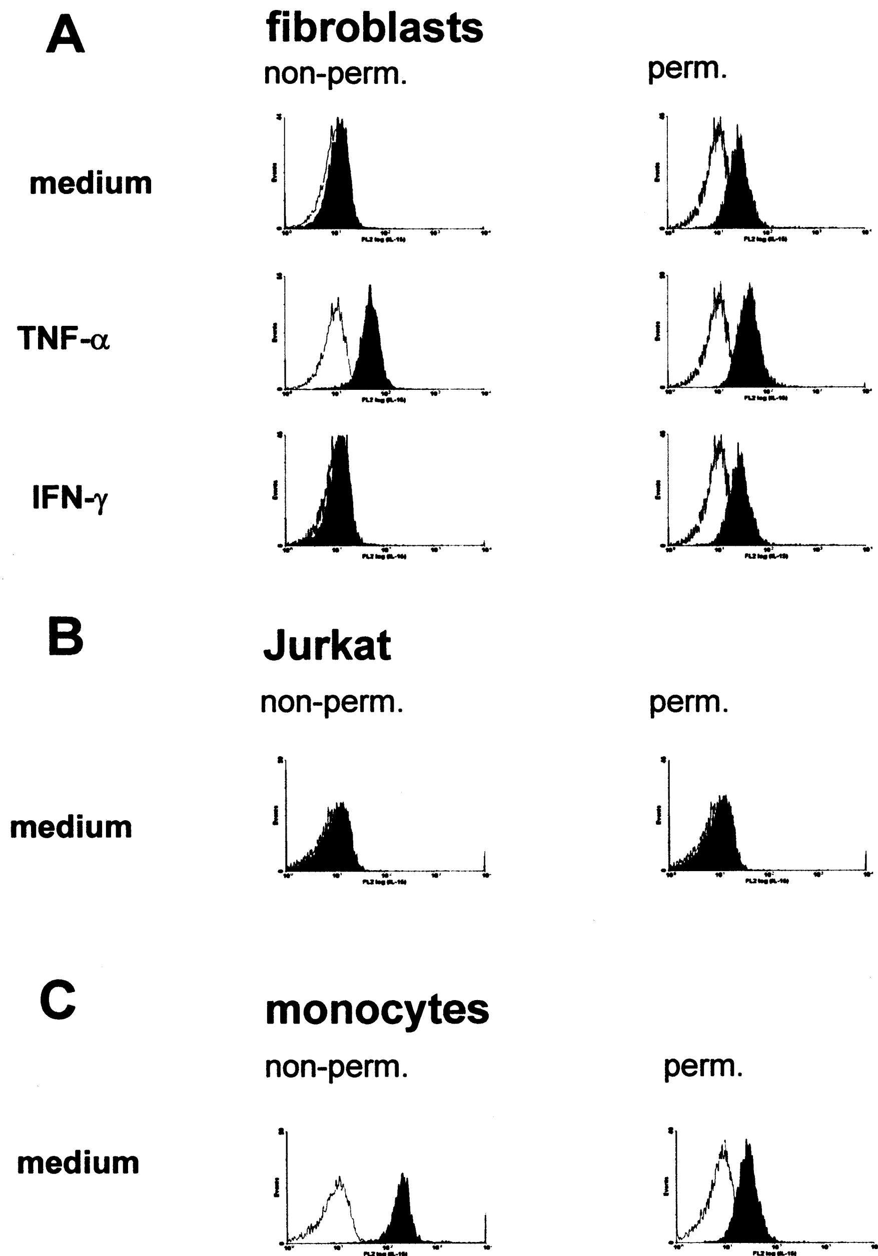

Figure 2. Dermal fibroblasts express IL-15 on the cell surface after TNF-α stimulation. Dermal fibroblasts were grown in medium or incubated with TNF-α (20 ng per ml) or IFN-γ (2000 U per ml), respectively, for 72 h. Cells were analyzed nonpermeabilized (non-perm.) or after permeabilization (perm.) as described in *Materials and Methods*. Cells were stained with the anti-IL-15 monoclonal antibody M112 (*closed histograms*) or as control with an isotype-matched control antibody (IgG2b) (*open histograms*) and subjected to FACS analysis. Nonpermeabilized cells indicate expression of IL-15 on the cell surface, permeabilized cells indicate the total amount of IL-15 expression, i.e., intracellular plus membrane IL-15. Jurkat cells ($IL-15^-$) and monocytes ($IL-15^+$) served as controls. Monocytes constitutively express membrane IL-15 as reported (Musso *et al*, 1999). Essentially the same data were obtained using anti-IL-15 monoclonal antibody (clone 34505.11) (Preprotech). IFN-γ used in this assay is functionally active because it induces ICAM-1 expression on fibroblasts (data not shown).

lesions expose membrane-bound IL-15 that is capable of stimulating proliferation of infiltrating, activated T cells.

DISCUSSION

An inflammatory process can be initiated by a variety of events that are followed by cell migration, extracellular matrix degradation, and extensive activation and proliferation of infiltrating mononuclear cells. These processes are believed to be driven by the expression of cytokines and their receptors by infiltrating macrophages and lymphocytes and by resident stromal cells. Here we demonstrate that, upon prolonged incubation with TNF-α, dermal fibroblasts stimulate proliferation of activated T cells by cell-cell contact. A fibroblast-secreted factor seems not to be involved in this process because the conditioned culture supernatant is ineffective in the assay. The growth-promoting activity towards activated T cells can be partially blocked by addition of a neutralizing antibody to IL-15 (**Fig 1*A***). The inability of the anti-IL-15 antibody to block the growth-promoting activity completely may be due to additional T cell stimulatory molecules expressed in increased amounts on the surface of TNF-α-treated fibroblasts compared with unstimulated fibroblasts, e.g., ICAM-1 or VLA-4. Taken together, the data suggest that IL-15 is localized on cell membranes of TNF-α-treated fibroblasts. FACS analysis confirmed expression of IL-15 on the cell surface of TNF-α-stimulated fibroblasts (**Fig 2*A***) whereas nonstimulated and IFN-γ-treated fibroblasts do not expose IL-15 on the cell membrane. Because membrane IL-15 is not eluted at pH 4.4, we conclude that IL-15 is exposed as an integral membrane protein on the surface of TNF-α-stimulated fibroblasts and is not associated with the IL-15 receptor. Expression on the cell surface assigns IL-15 to the family of cytokines with membrane-bound activities, as for example TGF-α, TNF-α, IL-1, and IL-10 (Kurt-Jones *et al*, 1985; Luettig *et al*, 1989; Noso *et al*, 1998; Musso *et al*, 1999). The same conclusion was recently drawn from the analysis of membrane IL-15 in monocytes (Musso *et al*, 1999). Expression of membrane IL-15 on fibroblasts, however,

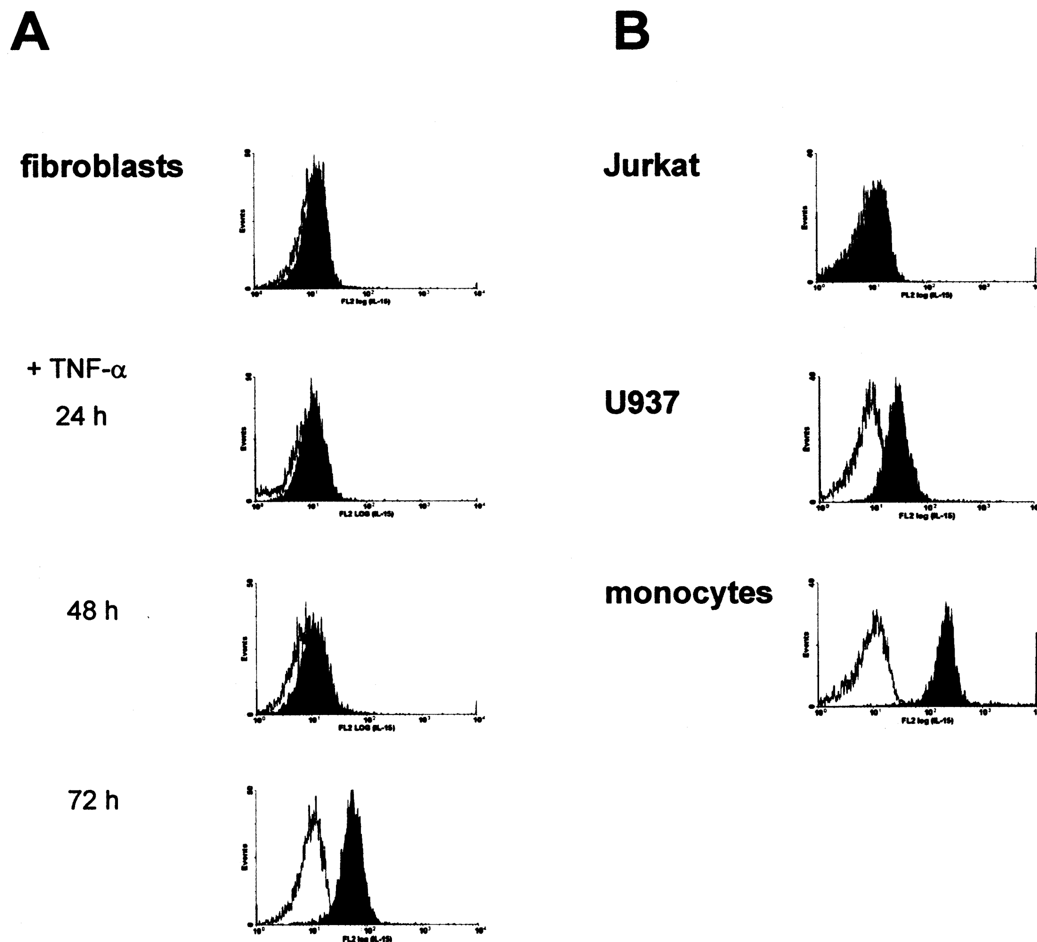

Figure 3. Time course of the induction of membrane IL-15 on the surface of fibroblasts after TNF-α stimulation. (*A*) Dermal fibroblasts were grown *in vitro* without TNF-α for 72 h or incubated with TNF-α (20 ng per ml) for 24 h, 48 h, or 72 h, respectively, washed with PBS, and subjected to FACS analysis of membrane IL-15 utilizing anti-IL-15 monoclonal antibody M112 (*closed histograms*) or as control an isotype-matched control antibody (IgG2b) (*open histograms*). (*B*) Jurkat cells (IL-15⁻), U937 cells (membrane IL-15⁺), and monocytes (membrane IL-15⁺) served as controls.

differs from that on monocytes because unstimulated fibroblasts, in contrast to monocytes, do not express detectable amounts of IL-15 on the cell surface (cf. **Fig 2**). Induction of membrane IL-15 in dermal fibroblasts by stimulation with TNF-α is specific because IFN-γ did not induce membrane IL-15. This is in contrast to monocytes, which constitutively express membrane-bound IL-15 in substantial amounts that are furthermore increased by stimulation with IFN-γ (Musso *et al*, 1999).

The conditioned supernatant of TNF-α-stimulated fibroblasts did not stimulate proliferation of activated T cells. This is in accordance with our observation that TNF-α-stimulated fibroblasts do not secrete IL-15 in detectable amounts into the culture medium as monitored by ELISA (**Fig 4**). Unstimulated fibroblasts, however, store intracellular IL-15 that can be monitored by FACS analysis of permeabilized cells and by Western blot analysis and ELISA of fibroblast lysates. Because IL-15 is present in cellular lysates and detectable in permeabilized cells by FACS, we conclude that unstimulated dermal fibroblasts constitutively synthesize IL-15 that is stored intracellularly and not secreted in detectable amounts. This conclusion is in contrast to the result of the RT-PCR analysis indicating the long leader IL-15 mRNA isoform suggested to code for the secreted form of IL-15 (Onu *et al*, 1997; Tagaya *et al*, 1997; Kurys *et al*, 2000). Recent results, however, assign differential roles to the IL-15 mRNA isoforms (Kurys *et al*, 2000; Nishimura *et al*, 2000); their role in human fibroblasts, however, remains to be elucidated. Lack of IL-15 secretion by normal dermal fibroblasts moreover stands in contrast to

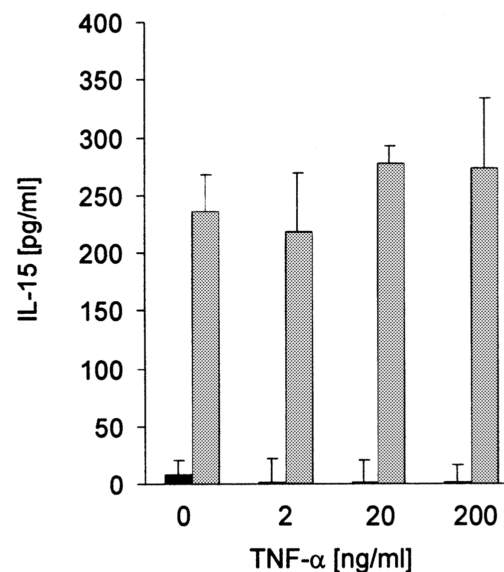

Figure 4. Dermal fibroblasts express IL-15 protein. Fibroblasts were cultured *in vitro* (10^6 cells per ml medium) without stimulation or with incubation with TNF-α (2–200 ng per ml) for 72 h. Cytoplasmic lysates (□) from unstimulated and TNF-α-stimulated fibroblasts (10^6 cells in 1 ml lysis buffer) and the corresponding culture supernatants (1 ml medium conditioned by 10^6 cells) (■) were subjected to IL-15-specific ELISA. Detection limit of the ELISA is 11 pg IL-15 per ml.

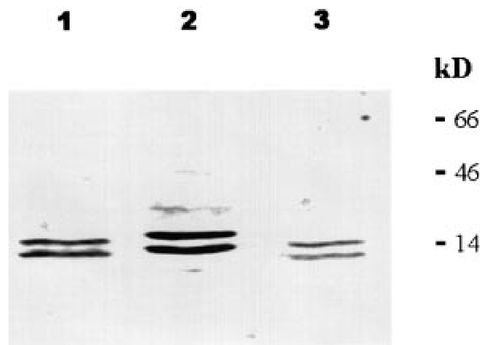

Figure 5. Western blot analysis of IL-15 expression. Dermal fibroblasts were mock-treated (*lane 1*), stimulated with TNF-α (20 ng per ml) (*lane 2*), or treated with IFN-γ (2000 U per ml) for 72 h. Total cell lysates were obtained by treatment with Nonidet P-40 (1% vol/vol), subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (about 10 μg protein per lane), blotted onto Nylon membrane, and probed with rabbit anti-human IL-15 antiserum (Biosource).

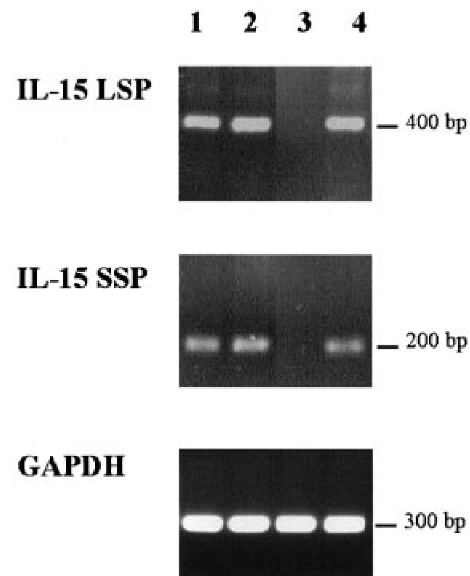

Figure 6. RT-PCR analysis of IL-15 RNA. Dermal fibroblasts were cultured *in vitro* (*lane 1*) or stimulated with TNF-α (20 ng per ml) (*lane 2*) for 72 h. cDNA was derived from these cells and subjected to PCR utilizing primer oligonucleotides specific for IL-15 RNA with the short-signal peptide (SSP) and long-signal peptide (LSP), respectively. As controls, cDNA from HL60 cells (IL-15⁻) (*lane 3*) and monocytes (IL-15⁺) (*lane 4*), respectively, were analyzed for IL-15 RNA expression. Moreover, cDNA specific for GAPDH was amplified in each sample. PCR products were separated by electrophoresis through a 2% agarose gel.

fibroblast-like synoviocytes from rheumatoid arthritis specimens that were recently reported to secrete IL-15 (Harada *et al*, 1999). On the other hand, a variety of cells of different cell type and tissue origin express IL-15 mRNA, including placenta, skeletal muscle, kidney, lung, heart, and keratinocytes (Waldmann and Tagaya, 1999); none of these cells secretes detectable amounts of IL-15 into the culture supernatant.

Continuous TNF-α stimulation of dermal fibroblasts for prolonged time, i.e., up to 72 h, gradually increases the amount of membrane IL-15 exposed on the cell surface, whereas short-term stimulation is less effective (**Fig 3**). The time kinetic of induction of membrane IL-15 is unexpected for TNF-α-induced processes, which unusually require less than 24 h. We speculate that the extended time needed to induce membrane IL-15 indicates either a delayed mechanism of IL-15 translocation from cytoplasmic stores to the cell membrane or an indirect stimulation pathway by mediators initially induced by TNF-α. A similarly delayed mechanism in dermal fibroblasts was reported for TNF-α-initiated secretion of eotaxin, an eosinophil-chemotactic protein (Noso *et al*, 1998). It is tempting to speculate that the delayed response of fibroblasts to TNF-α stimulation may be of biologic significance, particularly in the context of chronic *versus* acute inflammatory diseases, in order to initiate the cascade of inflammatory reactions preferentially upon prolonged, but not after short-term stimulation with TNF-α. In accordance with this model, prolonged exposure to TNF-α induces IL-15 expression in various tissues, i.e., in alveolar macrophages of patients with active sarcoidosis (Agostini *et al*, 1996), in fibroblast-like synoviocytes from patients with rheumatoid arthitis (Harada *et al*, 1999), in keratinocytes under chronic inflammatory conditions (Reinecker *et al*, 1996; Han *et al*, 1999), and in active hypertrophic scars (Castagnoli *et al*, 1999). Only the analysis of Agostini *et al* (1996), however, discriminates between IL-15 accumulated in cytoplasmic stores and IL-15 expressed in a membrane-bound form on the cell surface.

IL-15 on the cell membrane of stimulated fibroblasts is functionally active in enhancing proliferation of activated T cells. Within the inflammatory lesion, membrane-bound IL-15 is likely to act as a powerful and cooperative multiplier in the local T cell response upon prolonged TNF-α stimulation. Accordingly, IL-15 is reported to induce proinflammatory and antifungal activities in phagocytic cells (Musso *et al*, 1998) or chemokines and their receptors in T cells (Perera *et al*, 1999). Within the cellular architecture of the skin, IL-15 exposed on the surface of stimulated fibroblasts may play a unique role in the maintenance of particular interepithelial and intraepidermal lymphocyte pools in the absence of antigen and in the survival and expansion of these cells upon specific antigen stimulation. In agreement with this model, Lai *et al* (1999) recently reported that IL-15 promotes survival of CD8+ TCRαβ+ intraepithelial lymphocytes. IL-15-mediated stimulation of lymphocytes upon viral infections (Seder *et al*, 1995), the induction of natural killer cell activation (Fawaz *et al*, 1999), and the exposition of membrane IL-15 on the surface of fibroblasts after prolonged TNF-α stimulation may in concert represent an important effector mechanism against infections and a potent mediator of local inflammatory reactions.

We draw the model that dermal fibroblasts under chronic inflammatory conditions, such as discoid lupus erythematosus, are substantially involved in at least three pathophysiologically essential mechanisms: (i) attraction of activated lymphocytes by secreted chemokines, e.g., RANTES and eotaxin that are induced by TNF-α (Sticherling *et al*, 1995; Bartels *et al*, 1996); (ii) adherence of lymphocytes via the LFA-1/ICAM-1 pathway (Cabanas and Hogg, 1991) that is upregulated by TNF-α and IFN-γ (Rothlein *et al*, 1991), or via the VLA-4/VCAM-1 pathway that is upregulated by TNF-α and IL-1 (Gao and Issekutz, 1996); and (iii) stimulation of proliferation of activated T cells via membrane IL-15 as shown in this study. The evidence implies that IL-15 plays an important role in these processes, in particular because IL-15 acts as a potent chemoattractant for T cells, natural killer cells, and neutrophils (Wilkinson and Liew, 1995; McInnes *et al*, 1996; Perera *et al*, 1999) and, moreover, induces monocytes and polymorphonuclear cells to secrete proinflammatory and chemotactic cytokines (Musso *et al*, 1998). Accordingly, abnormalities in IL-15 expression were found in patients with inflammatory autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and psoriatic arthritis (Reinecker *et al*, 1996; McInnes *et al*, 1997; Perera *et al*, 1999; Danning *et al*, 2000). IL-15 activates proinflammatory and antimicrobial functions in polymorphonuclear blood cells suggesting that IL-15 may additionally play a crucial role in the activation of phagocytic cells against pathogens (Musso *et al*, 1998).

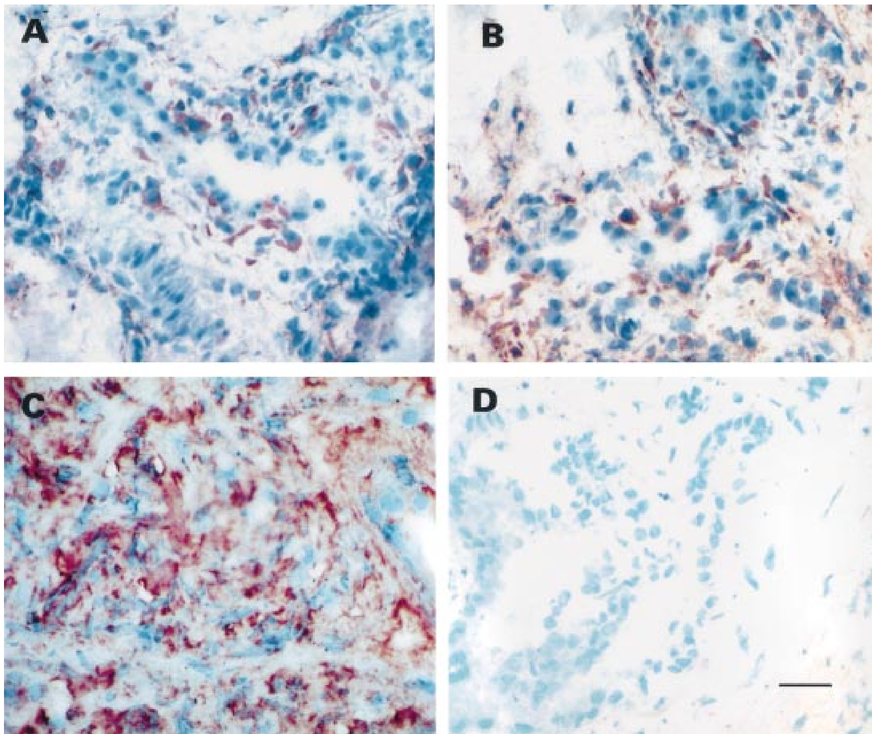

Figure 7. Expression of membrane IL-15 by fibroblasts in chronic inflammatory lesions but not in healthy skin. Punch biopsies were obtained from chronic eczema (*A*, *B*), from a discoid lupus erythematosus lesion (*C*), and from healthy skin (*D*) and stained *in toto* with the anti-IL-15 antibody as described in *Materials and Methods*. Subsequently, cryostat sections were obtained, stained with a biotin-labeled antimouse antibody, and processed by reaction with an avidin-biotin-peroxidase complex. Utilizing this procedure, the anti-IL-15 antibody preferentially detects IL-15 bound on the cell surface. Cell nuclei were stained with Mayer's hematoxylin. *Scale bar:* 50 μm.

We would like to thank Mrs. P. Hofmann, K. Hilgert, and A. Cremer for technical assistance and preparation of dermal fibroblasts, Dr. S. Bulfone-Paus (Berlin) for supplying us with the anti-IL-15 antibody, and Dr. R. Grassmann (Erlangen) for supplying us with HuT102 cells. This work was supported by the Fritz-Bender-Stiftung, Munich (H.A.), the Rudolf-Bartling-Stiftung, Hannover (H.A.), the Fortune-Program of the University of Cologne (H.A.), and the Deutsche Forschungsgemeinschaft, Bonn (Re690/4–2, U.R.).

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