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Dermal Fibroblasts Induce Maturation of Dendritic Cells

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To trigger an effective T cell-mediated immune response in the skin, cutaneous dendritic cells (DC) migrate into locally draining lymph nodes, where they present Ag to naive T cells. Little is known about the interaction of DC with the various cellular microenvironments they encounter during their migration from the skin to lymphoid tissues. In this study, we show that human DC generated from peripheral blood monocytes specifically interact with human dermal fibroblasts via the interaction of $\beta_2$ integrins on DC with Thy-1 (CD90) and ICAM-1 on fibroblasts. This induced the phenotypic maturation of DC reflected by expression of CD83, CD86, CD80, and HLA-DR in a TNF-$\alpha$- and ICAM-1-dependent manner. Moreover, fibroblast-matured DC potently induced T cell activation reflected by CD25 expression and enhanced T cell proliferation. Together these data demonstrate that dermal fibroblasts that DC can encounter during their trafficking from skin to lymph node can act as potent regulators of DC differentiation and function, and thus actively participate in the regulation and outcome of DC-driven cutaneous immune responses. The Journal of Immunology, 2007, 178: 4966–4974.

Dendritic cells (DC) are specialized APCs that are pivotal in initiating immune responses. As sentinel cells of peripheral tissues, DC reside in and traffic through non-lymphoid peripheral tissues, continuously surveying the environment for invading microorganisms. Under steady-state conditions and in the absence of microbial stimulation or inflammation, apparently immature DC capture Ags from apoptotic cells, transport them to secondary lymphoid organs, and are involved in the maintenance of tolerance (1, 2). In contrast, the contact of DC with danger signals such as microbial agents or inflammatory mediators induces the maturation of DC, resulting in an increase in their expression of MHC molecules, costimulatory molecules, and cytokines, and an enhancement of their migratory capacity (3). DC subsequently migrate from the periphery to the T cell zone of secondary lymphoid organs (4). Thus, activated DC are specialized cells that obtain information from the periphery and transfer it to lymphoid organs, where they display Ags to naive Ag-specific T cells and thereby initiate primary T cell immune responses.

When traveling from peripheral to lymphoid tissues, DC interact with the stromal microenvironment that is composed of the extracellular matrix, soluble mediators, and a variety of different cell types. There is accumulating evidence that the stromal microenvironment plays an important role in the regulation of DC functions and in the priming of DC. Dudda et al. (5, 6) demonstrated that intracutaneously injected bone marrow-derived DC can induce skin-homing CD8$^+$ T cells that express the cutaneous vascular entry code (cutaneous leukocyte Ag$^*$). T cells induced after i.p. injection of the same bone marrow-derived DC, however, expressed the intestinal entry code (integrin $\alpha_4\beta_7$). These experiments prove that DC possess enough plasticity to acquire tissue-specific signals from their local microenvironment and subsequently to instruct T cells for tissue-selective homing. Stromal cells such as fibroblasts, endothelial cells, and macrophages display different functional properties, including migratory capacity, contractility, and extracellular matrix production and degradation that are dependent on the anatomical site or disease status (7). Thus, it seems plausible that DC may receive instructions from stromal cells and/or their secreted products. Furthermore, recent evidence suggests that the stromal microenvironment directly affects the behavior and differentiation of DC. For example, endothelial cells are able to influence the differentiation and activation of DC (8, 9).

Fibroblasts are an extremely heterogeneous multifunctional cell population, and their role in wound healing, developmental processes, and tumor development is well established (10). Nevertheless, immunologists have regarded fibroblast activation as relatively unimportant in regulating immune responses and have focused on cellular interactions between lymphocytes, macrophages, and dendritic cells, all of which generate Ag-specific responses (11). However, fibroblasts are capable of producing various paracrine immune modulators, such as peptide growth factors, cytokines, chemokines, and inflammatory mediators (12). Additionally, they express cell surface adhesion molecules and costimulatory molecules such as CD40 (13). Furthermore, there is abundant evidence that fibroblasts taken from diseased tissues display a fundamentally different phenotype compared with fibroblasts taken from normal tissues of the same anatomical site (14, 15). Fibroblasts might therefore not only be purely structural elements, but potentially could also actively participate in the regulation of inflammation and immune responses.
In this study, we found human DC in close proximity to dermal fibroblasts in vivo. We also report that monocyte-derived DC have the capacity to adhere specifically to human dermal fibroblasts in vitro. This interaction of immature DC with fibroblasts induced the maturation of DC in a manner that was dependent on cell-cell contact as well as on soluble mediators. Fibroblast-induced DC maturation was accompanied by up-regulation of HLA, costimulatory molecules and cytokines, and the acquisition of an allostimulatory capacity to activate T cells. In summary, we demonstrate for the first time that fibroblasts actively participate in the regulation of DC function. This results in the maturation of DC and subsequent effective T cell activation, demonstrating that fibroblasts can be involved in the control of DC-driven immune responses.

Materials and Methods

Antibodies

CD1a PE, CD83 FITC, CD80 FITC, HLA-DR PE, CD86 FITC, and CD154 FITC Abs were purchased from BD Biosciences. CD1c PE and CD3 PE Abs were purchased from Caltag Laboratories. CD40 FITC Ab was provided by Dr. S. Hausschildt (Institute of Biology, University Leipzig, Leipzig, Germany). Anti-ICAM-1 Ab (clone R6.5D6) was purchased from American Type Culture Collection.

Cell culture

Primary fibroblast cultures were obtained by outgrowth from skin biopsies. The fibroblasts were cultured at 37°C, 5% CO2 in DMEM medium (Biochrom) containing 10% FCS (Promo Cell), 1% penicillin/streptomycin (Biochrom). After reaching confluence, cells were passaged using 0.05% trypsin and 0.02% EDTA (Biochrom). Thy-1-, ICAM-1-, and vector-transfected Chinese hamster ovary (CHO) cells were cultured similarly. Thy-1- and vector-transfected CHO cells were generated, as described before (16–18), and cultured in the presence of 7.5 μM ml basicidian (Invitrogen Life Technologies) to select for Thy-1-expressing cells. ICAM-1-transfected CHO cells were provided by Dr. A. E. May (German Heart Center, Munich, Germany).

Preparation of DC

PBMC were isolated using an endotoxin-free Ficoll-Paque gradient (Amersham Biosciences). CD14-positive monocytes were purified using anti-CD14 magnetic beads (Milenyi Biotec), according to manufacturer’s protocol. DC were generated by culture of 10^5/ml CD14-positive cells for 5 days in DC-RPMI 1640 (RPMI 1640 (Invitrogen Life Technologies) supplemented with 2% FCS (Promo Cell), 1% penicillin/streptomycin (Invitrogen Life Technologies), 1% L-glutamine (Invitrogen Life Technologies), 0.1 mM nonessential amino acids (Invitrogen Life Technologies), 10 mM HEPES (Invitrogen Life Technologies), 100 U/ml IL-4 (PeproTech), and 1000 U/ml GM-CSF (Leukine; Berlex). The quality of DC preparations with an immature phenotype was controlled by morphologic analysis and by verification of high expression of CD1a and CD11c. Furthermore, immature DC did not express CD83 and displayed only low expression of CD86, CD80, and HLA-DR.

Coculture of DC and fibroblasts

Fibroblasts were seeded onto 24-well plates and cultured until they became confluent. Immature day 5 DC (5 x 10^4) were directly added to fibroblast monolayers and were cocultured for 24 h in DC-RPMI 1640 supplemented with 2% FCS. In other experiments, fibroblasts and DC were separated by culture inserts (BD Biosciences; 3-μm pore size) to prevent direct cell-cell contact. As controls, immature DC were cultured for 24 h either in DC-RPMI 1640 supplemented with 2% FCS or in the presence of 1 μg/ml LPS to induce full DC maturation.

Flow cytometry

Cells were harvested and washed twice in PBS, and 2 x 10^5 cells were incubated with the requisite fluorescently labeled mAbs. Cells were analyzed by flow cytometry (Epics XL; Beckman Coulter) after washing twice with PBS/10% Gelafusel (Serumwerke Bernburg). For staining of intracellular TNF-α, cells were stimulated for 2 h in the presence of 100 ng/ml brefeldin A (Calbiochem). Cells were then harvested, fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, and permeabilized using 0.1% saponin (Sigma-Aldrich) for 10 min. Cells were subsequently incubated with 1 μg of anti-TNF-α Ab (R&D Systems) or an isotype control Ab for 60 min at 4°C. After extensive washing with PBS/10% Gelafusel, cells were incubated with a Cy2-labeled goat anti-mouse Ab (Dianova) for 60 min at 4°C. For the identification of the cell source of TNF-α, DC were identified by an anti-CD11c PE Ab.

Analysis of phagocytic ability

Immature DC, LPS-stimulated DC, or fibroblast-stimulated DC (5 x 10^4) were incubated for 45 min at 37°C with 1 x 10^5 FITC-conjugated Escherichia coli (Invitrogen Life Technologies) in RPMI 1640 medium containing 10% FCS. They were then washed twice with ice-cold PBS, resuspended in ice-cold PBS, and immediately analyzed using flow cytometry. Cells incubated at 4°C were used as negative controls. To quench the fluorescence signal from attached, but not internalized E. coli, cells were incubated with 0.4% trypan blue in 10 mM sodium citrate and 0.9% sodium chloride.

Immunohistochemistry

Thy-1 and ICAM-1 protein expression was detected in frozen sections of skin biopsies from patients with atopic dermatitis (n = 5) with an anti-Thy-1 Ab (20) or anti-ICAM-1 Abs, respectively. Bound Abs were detected using the avidin-biotin complex technique, according to the manufacturer’s protocol (supersensitive multitlink alkaline phosphatase ready-to-use detection system; Biogenex) and visualized colorimetrically using the New Fuchsin substrate system (DakoCytomation). DC were subsequently stained using a biotin-labeled anti-CD1a Ab in the same tissue sections, using streptavidin-peroxidase (Vectorstain) and dianaminobenzidine for detection. Tissue sections were extensively washed with TBS/0.3% Tween 20 after every Ab incubation step. In control sections, primary Abs were replaced with an isotype control Ab. Tissue sections were photographed using a DP70 charge-coupled device camera mounted on a light microscope (Olympus).

Adhesion assay

Thy-1-, ICAM-1-, control vector-transfected CHO cells or primary fibroblasts in the third to fifth passage were seeded onto 8-well chamber slides (Nunc). Two confluent cell monolayers were subsequently fixed with ice-cold methanol and stored at −20°C. DC were biotinylated with n-biotinoyl-e-aminoacaproic-acid-hydroxysuccinimide-ester (Roche Biochemicals), according to the manufacturer’s protocol. DC (2 x 10^5) in RPMI 1640 without serum were added to Thy-1-, ICAM-1-, control vector-transfected CHO cells or to primary fibroblasts, and then incubated for 30 min at 37°C. Unbound DC were removed by several washes with RPMI 1640. Adherent cells were fixed with ice-cold methanol for 10 min at 4°C. Tissue sections were blocked by incubation with PBS/1% BSA. Adherent biotinylated cells were subsequently visualized by incubation with CY3-conjugated streptavidin (Beckman Coulter; 1:500 in PBS/1% BSA) for 60 min at room temperature, and finally washed twice with PBS. The number of DC that adhered to each confluent monolayer was assessed microscopically in 10 independent fields (magnification X20) by two independent observers.

Ag-specific T cell activation

Immature DC were cultured alone or in the presence of LPS or fibroblasts. The DC were challenged 24 h later with 0.5 μg/ml tetanus toxoid (Calbiochem) for 4 h. Subsequently, 2 x 10^6 DC in 100 μl of proliferation medium (RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Promo Cell), 1% penicillin/streptomycin (Invitrogen Life Technologies), 1% L-glutamine (Invitrogen Life Technologies), nonessential amino acids (Invitrogen Life Technologies), and 10 mM HEPES (Invitrogen Life Technologies) were seeded into 96 round-bottom wells. To exclude the possibility that contaminating fibroblasts remaining from the DC fibroblast coculture might affect T cell function, all fibroblasts were removed using an anti-Thy-1 Ab coupled to goat anti-mouse magnetic beads (Dynal Biotech), as previously described (17, 19, 20). Autologous T cells were isolated from peripheral mononuclear cells using the negative T cell isolation kit II from Miltenyi Biotec, according to the manufacturer’s protocol. T cells (2 x 10^5) were resuspended in 100 μl proliferation medium. In preliminary experiments, an optimal DC:T ratio of 1:10 was established. Thus, DC were cocultured with purified T cells at a ratio of 1:10 in round-bottom wells for up to 5 days.

For detection of proliferation autologous, purified T cells (1 x 10^5 cells/ml PBS) were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFDA) and were resuspended in 100 μl proliferation medium, and incubated for 30 min at 37°C with 5% CO2. Proliferation was detected after 5 days of coculture by measuring the decrease in the fluorescence staining (17, 21).
T cells were identified by staining with an anti-CD3 PE Ab. Dead cells were excluded from the analyses using propidium iodide (Calbiochem) staining. The number of proliferating cells per 5000 nonproliferating cells was calculated from flow cytometry data.

Mixed leukocyte reaction

Immature DC were cultured alone or in the presence of LPS or fibroblasts. Allogeneic T cells were isolated and labeled, as described above. For MLR, 1 $\times 10^5$ allogeneic T cells were cultured with DC in different ratios for 6 days. Proliferation was detected, as described above.

Cytokine determination

TNF-α was detected in conditioned medium from DC cocultures using an ELISA (BD Biosciences). Experiments were performed in duplicate.

Statistical analysis

Data were analyzed using Student’s t test. Values of p < 0.05 were considered significant.

Results

DC interact with fibroblasts via ICAM-1 and Thy-1 and their β2 integrin counterreceptors

To study whether DC interact with stromal cells, we analyzed the relative localization of CD1a-positive DC and fibroblasts in skin. Frozen tissue sections of human atopic eczema lesions were stained with an anti-CD1a biotin Ab to identify DC. Fibroblasts were identified using the anti-Thy-1 Ab AS02 (17, 19, 20, 22). A close colocalization of CD1a-positive DC (brown) and Thy-1-positive fibroblasts (red) was found in the dermis of atopic eczema lesions (Fig. 1).

Next, the interaction of human DC and fibroblasts was investigated in vitro. DC were generated from CD14$^+$ PBMC using GM-CSF- and IL-4-containing medium. Immature (5-day) DC adhered to fibroblasts in static adhesion assays (Fig. 2a). To identify the molecules mediating this interaction, Thy-1- and ICAM-1-mediated adhesion was inhibited using blocking Abs to Thy-1 (clone BC9-G2) (16, 17) and ICAM-1 (clone R6.5D6) that bind to fibroblasts. Blocking either Thy-1 or ICAM-1 significantly reduced the adhesion of immature DC to fibroblasts (Fig. 2b). Incubation of DC with an isotype control Ab or with an anti-MHC I Ab did not significantly affect the adhesion. However, blocking the counterreceptors for Thy-1 and ICAM-1 on DC, the β2 integrins CD11a/CD18 and CD11b/CD18, respectively, also inhibited the adhesion of DC to fibroblasts (Fig. 2b).

To prove the involvement of Thy-1 and ICAM-1 in the adhesion of immature DC to fibroblasts, adhesion of immature DC to ICAM-1-, Thy-1-, and vector-transfected CHO cells was studied in static adhesion assays. As seen in Fig. 2c, DC adhered strongly to Thy-1- and ICAM-1-transfected cells, whereas very few cells bound to vector-transfected control cells. These results together suggest that the adhesion of monocyte-derived DC to fibroblasts is mediated by ICAM-1 and Thy-1.

The interaction of DC with fibroblasts induces maturation of DC in vitro

Next, we determined whether the interaction of DC with fibroblasts affects the maturation of DC. Immature monocyte-derived DC were cultured for 24 h either alone or on a confluent monolayer of fibroblasts. As a control, DC were stimulated with 1 $\mu$g/ml LPS to induce full DC maturation. DC stimulated with LPS or by contact to fibroblasts exhibited typical veils, as described for LPS-stimulated DC (data not shown). Subsequently, DC were harvested, and the expression of DC maturation markers was analyzed using flow cytometry. Stimulation of DC with LPS induced the maturation of DC, as demonstrated by the enhanced expression of CD80, CD86, CD83, and HLA-DR (Fig. 3a). Interestingly, the coculture of DC with allogeneic (data not shown) as well as syngeneic (Fig. 3a) fibroblasts also induced the expression of these maturation markers on DC (Fig. 3a). However, activation of DC by fibroblasts resulted in less CD83-positive DC compared with LPS-stimulated DC (Fig. 3a). To investigate whether soluble factors or direct cell-cell contact were responsible for this effect, we separated DC and fibroblasts using Transwell inserts. As shown in Fig. 3a, expression of CD80,
CD86, and CD83 was also induced on DC when DC and fibroblasts were cocultured in the absence of direct cell-cell contact. However, this expression was significantly lower in comparison with DC that were directly cocultured with fibroblasts, indicating that both direct cell-cell contact and soluble factors are involved in the fibroblast-induced DC maturation. Stimulation of DC maturation by coculturing with fibroblasts from the same donor excluded the possibility of an unspecific allogenic stimulation of DC by fibroblasts.

Another hallmark of DC maturation is the decrease in their phagocytic activity (23). Immature DC are highly active phagocytic cells, as detected by the ingestion of FITC-labeled \textit{E. coli} at 37°C (Fig. 3b). However, maturation induced by LPS or by coculture of DC with fibroblasts both resulted in a distinct loss of phagocytic activity (Fig. 3b). DC incubated at 4°C did not internalize FITC \textit{E. coli} (Fig. 3b). Together these data demonstrate that the interaction of DC with fibroblasts results in phenotypic DC maturation.

**FIGURE 2.** DC adhere to fibroblasts via Thy-1 and ICAM-1. Adhesion of immature DC to Thy-1-, ICAM-1-, vector-transfected CHO cells or primary fibroblasts was examined using static adhesion assays. \textit{a}, Representative picture of immature DC adhering to confluent monolayers of fibroblasts. Examples of DC are indicated by arrows. \textit{b}, Adhesion of immature DC to human dermal fibroblasts was studied after blocking of Thy-1 and ICAM-1 on fibroblasts, and CD11a, CD11b, and CD18 on DC using function-blocking Abs. Isotype control Abs (isotype ctr) and Abs that bind to the cell surface, but do not influence adhesion (anti-HLA-ABC), were used as negative controls to exclude unspecific effects due to Ab binding. Adherent cells were counted in 10 independent fields using a fluorescence microscope (magnification, \times 20). The adhesion of untreated cells was set to 100%. Data represent the mean ± SD of four independent experiments. *, \( p < 0.05 \) compared with the adhesion without Ab. \textit{c}, The attachment of immature DC to Thy-1-, ICAM-1-, and vector-transfected CHO cells (CHO.Thy-1, CHO.ICAM-1, and CHO.vec, respectively) was studied in static adhesion assays. The number of adherent cells was counted in 10 independent fields (magnification, \times 20). Data represent the mean ± SD of four independent experiments. *, \( p < 0.05 \) compared with vector-transfected CHO cells.

**FIGURE 3.** Adhesion of immature DC to dermal fibroblasts induces maturation of DC. Immature DC were cultured either alone, in the presence of 1 \( \mu \)g/ml LPS, or on a confluent monolayer of dermal fibroblasts (FB) for 24 h. Fibroblasts were obtained from the same donor as DC (FB syngeneic). In some experiments, fibroblasts and DC were separated by semipermeable culture inserts to prevent direct cell-cell contact (DC-[FB syngeneic-TW]). \textit{a}, Expression of CD80, CD83, CD86, and HLA-DR was analyzed by flow cytometry. One representative experiment of five is shown. \textit{b}, Phagocytotic capability of immature DC, LPS-stimulated DC, and fibroblast-stimulated DC (DFC) was assessed by the uptake of FITC-labeled \textit{E. coli} (black line). Cells without \textit{E. coli} incubation (gray line) or with \textit{E. coli} incubation at 4°C (black dotted line) were used as negative control. Data represent one of three experiments.
TNF-α plays a central role in the fibroblast-induced maturation of DC

Because TNF-α plays a central role in the regulation of DC maturation (23–25), we analyzed the involvement of TNF-α in the fibroblast-induced maturation of DC. TNF-α levels in the medium from DC fibroblast cocultures were increased dramatically, similar to the levels in the medium from LPS-stimulated DC (Fig. 4a). In contrast, untreated DC or fibroblasts alone did not secrete detectable amounts of TNF-α. To determine whether induction of TNF-α secretion is dependent on cell-cell contact, DC and fibroblasts were cocultivated, but separated by a semipermeable membrane to prevent direct cell-cell contact. Interestingly, virtually no TNF-α secretion could be detected, indicating that direct cell-cell contact is essential to induce TNF-α secretion in DC/fibroblast cocultures (Fig. 4a).

To identify the cells responsible for TNF-α secretion in DC fibroblast cocultures, DC were cocultured with fibroblasts in the presence of brefeldin A to block TNF-α secretion and thereby to increase intracellular TNF-α protein levels. Intracellular TNF-α in both cell populations was subsequently analyzed using flow cytometry. As shown in Fig. 4b, CD11c+ DC expressed TNF-α, whereas CD11c– fibroblasts did not.

Next, we investigated the role of TNF-α secretion on fibroblast-induced DC maturation. DC and fibroblasts were cocultured for 24 h in the presence of either a blocking anti-TNF-α Ab or an isotype control Ab. Blocking of TNF-α resulted in a substantially reduced expression of the costimulatory molecules CD80, CD83, CD86, and HLA-DR on DC (Fig. 4c), the control Ab having no effect.

The importance of the CD40/CD40L interaction for DC regulation is well established (26). We therefore studied its role in the fibroblast-induced DC maturation. CD40 is expressed on DC and fibroblasts (data not shown). However, CD40L could not be detected either on DC or on fibroblasts (data not shown). Additionally, we could not detect soluble CD40L in conditioned medium from immature DC or stimulated DC (data not shown). Finally, addition of a CD40L-blocking Ab to the coculture of DC and fibroblasts did not affect fibroblast-induced DC maturation (data not shown). Due to the absence of CD40L expression in the coculture of DC and fibroblasts, we consider it highly unlikely that CD40/CD40L interactions are involved in the fibroblast-induced maturation of DC.

Together, these data suggest that the interaction of DC with fibroblasts induces the secretion of TNF-α by DC, which in turn stimulates the maturation of DC in an autocrine manner, as reflected by the up-regulated expression of costimulatory molecules.

ICAM-1 is involved in the fibroblast-induced maturation of DC

The results above indicate that the adhesion of DC and fibroblasts is at least partially mediated by the interaction of Thy-1 and ICAM-1 with β2 integrins (Fig. 2). To investigate whether this interaction is able to directly induce TNF-α secretion, DC were cultured with ICAM-1–, Thy-1–, or vector-transfected CHO cells. As shown in Fig. 5a, DC produced high amounts of TNF-α when...
cocultured with ICAM-1-transfected cells, whereas no TNF-α secretion was induced in cocultures of DC with Thy-1- or vector-transfected CHO cells. In addition, coculture of DC with ICAM-1-transfected cells also up-regulated the expression of the costimulatory molecules such as CD80 and CD86 on DC, similar to the situation with LPS treatment or fibroblast coculture of DC (Fig. 5b). In contrast, Thy-1- and vector-transfected cells did not affect the expression of the costimulatory molecules.

FIGURE 5. Interaction of DC with ICAM-1 induces maturation of DC. Immature DC were cultured on monolayers of ICAM-1 (CHO.ICAM1), Thy-1 (CHO.Thy1), or vector-transfected CHO cells (CHO.vec), or on dermal fibroblasts (FB). As control, DC were cultured alone or in the presence of 1 μg/ml LPS. a, TNF-α was detected in conditioned medium after 24-h coculture using ELISA. *, p < 0.05 compared with CHO.vec. b, Expression of CD80 and CD86 on CD11c+ DC was detected by flow cytometry after 24 h of coculture. *, p < 0.05 compared with CHO.vec; #, p < 0.05 compared with DC without stimulation. c, Immature DC were cultured on fibroblasts (DCFB) that had been preincubated with a function-blocking anti-ICAM-1 or an anti-Thy-1 Ab. An anti-MHC-I Ab was used as a negative control. TNF-α was detected in conditioned medium using ELISA. Secretion of TNF-α in the presence of the control Ab (anti-MHC I) was set to 100%. Data represent the mean ± SD of five independent experiments. *, p < 0.05.

FIGURE 6. Fibroblast-stimulated DC (DCFB) activate T cells (TC). a, DC, LPS-stimulated DC, and FB-stimulated DC (DCFB) were pulsed with tetanus toxoid and were cocultured with autologous, purified T cells in a ratio of 1:10. Expression of CD25 on T cells was analyzed by flow cytometry. Data represent 1 of 10 independent experiments. b, Proliferation of CD3+ T cells cocultured with tetanus toxoid-pulsed DC was detected by flow cytometry as the decrease of CFDA labeling after 5 days. The number of proliferated CD3+ cells per 5000 nonproliferated cells is indicated in brackets. The data show the FACS plots of one representative of five independent experiments. c, The data represent the mean ± SD of triplicate wells. *, p < 0.05 compared with basal T cell proliferation. d, In MLR DC, LPS-stimulated DC and FB-stimulated DC (DCFB) were cocultured with allogeneic T cells in the indicated ratios. Proliferation of CD3+ T cells was detected by flow cytometry as the decrease of CFDA labeling after 6 days. Data show one representative experiment of three.
Next, we investigated the importance of ICAM-1 in the fibroblast-induced DC maturation. Cultured fibroblasts expressed ICAM-1, as detected by flow cytometry (data not shown). Treatment of fibroblast DC cocultures with a blocking anti-ICAM-1 Ab (clone R6.5D6) resulted in only a 25% reduction in the TNF-α production. Treatment of similar cocultures with function-blocking anti-Thy-1 Abs (BC9-G2) (16, 17) or anti-MHC I Abs (HLA-ABC) did not affect the TNF-α production in cocultures of DC and fibroblasts. Additionally, blocking of ICAM-1 did not affect the up-regulation of costimulatory molecules in DC that were cocultured with fibroblasts (data not shown). In conclusion, ICAM-1 is involved in the fibroblast-induced TNF-α secretion, but other molecules on DC and fibroblasts must also be engaged for full DC maturation.

**Fibroblast-matured DC activate T cells**

To characterize the fibroblast-matured DC functionally, we investigated their capacity to activate T cells. Immature DC, LPS-stimulated DC, and fibroblast-matured DC were pulsed with tetanus toxoid and cocultured with purified autologous CD3+ T cells. T cells were harvested after 3 days, and the expression of CD25 on the CD3+ T cells was detected by flow cytometry. T cells alone did not express significant amounts of CD25 (<2%) (Fig. 6a). Untreated DC only stimulated CD25 expression on <10% of the T cells (Fig. 6a). As expected, LPS-stimulated DC robustly induced CD25 expression on 30% of T cells. Importantly, fibroblast-matured DC also strongly activated T cells, as demonstrated by CD25 expression on 36% of the T cells. The activation of T cells by fibroblast-matured DC was further reflected in the enhanced T cell proliferation that was detected as a decrease in CFDA labeling after 5 days of coculture. Activation of DC by LPS or fibroblasts enhanced the T cell proliferation two times more efficiently compared with T cells cocultured with immature DC (Fig. 6, b and c). In accordance, in MLR, fibroblast-matured DC also significantly stimulated proliferation of allogeneic T cells. LPS- and fibroblast-matured DC significantly increased the proliferation up to a ratio of DC/TC of 1:40 (Fig. 6d).

**Discussion**

DC in peripheral tissues mature in response to antigenic stimulation and migrate to secondary lymphoid organs, where they activate T cells. During their migration to secondary lymphoid organs, DC have to travel through the stromal microenvironment comprised of the extracellular matrix and stromal cells, such as fibroblasts, macrophages, and endothelial cells. In this study, we show that DC are located in close proximity to stromal fibroblasts in inflamed skin. In accordance, a Thy-1- and ICAM-1-dependent adhesion of DC to fibroblast could be detected in vitro. Coculture experiments demonstrate that fibroblasts are effective in inducing both phenotypic and functional maturation of DC in a manner that is dependent on both direct cell-cell contact as well as soluble mediators. The resulting fibroblast-matured DC are able to support T cell-driven immune responses. Together, these data suggest that dermal fibroblasts can be active regulators of DC functions and of DC-driven cutaneous immune responses.

The fibroblast-dependent expression of TNF-α in DC plays a central role in the fibroblast-induced maturation of DC, because TNF-α production in DC was strongly induced as a result of the direct coculture of DC with fibroblasts, and the blocking of TNF-α activity inhibited this DC maturation. Virtually no TNF-α expression was induced in DC when they were cocultured with fibroblasts, but physically separated from them with a semipermeable membrane, indicating that direct cell-cell contact is required for fibroblasts to induce DC to express TNF-α. Nevertheless, partial maturation of DC as demonstrated by their expression of costimulatory molecules was observed in these physically separated cocultures (Fig. 3a), suggesting that TNF-α is not the only signal for fibroblast-induced DC maturation. These observations also suggest that in addition to the TNF-α produced by DC, fibroblast-derived soluble mediators play a role in fibroblast-mediated DC maturation, a notion supported by the observation that blocking anti-TNF-α Abs only partially inhibited the expression of costimulatory molecules on DC in response to direct coculture with fibroblasts (Fig. 4c).

The data we present in this study show that the direct cell-cell contact required for fibroblast-induced maturation of DC has both adhesive and inductive components. The importance of the interaction of β2 integrins (LFA-1 and Mac-1) on leukocytes for the adhesion to the endothelium via ICAM-1 and Thy-1 is well established (17), and our data suggest that these interactions also mediate the adhesion of DC to fibroblasts, at least in part. In the leukocyte-endothelium interaction, ICAM-1 acts not only as an adhesion molecule, but also as a signaling receptor that induces the reorganization of F-actin filaments and thereby regulates the affinity of LFA-1 for its receptors (27). Consistently, ICAM-1 on fibroblasts represents a signal for the initiation of DC maturation, because ICAM-1 on the cell surface induced the maturation of DC, as shown by the up-regulation of costimulatory molecules on DC and the expression of TNF-α (Fig. 5). Furthermore, blocking of ICAM-1 on fibroblasts reduced TNF-α production by DC. The role of ICAM-1 expression by fibroblasts in the maturation of DC in vivo is likely to be physiologically and pathologically relevant, as increased expression of ICAM-1 on fibroblasts during inflammatory and immune reactions has been reported in several diseases, such as renal allograft rejection, rheumatoid arthritis, and hypertrophic scars (28–30). We also found ICAM-1 expressed on endothelial cells and fibroblasts in inflamed skin, whereas in healthy skin only very few ICAM-1-positive cells were detectable (data not shown).

Although our data implicate an involvement of ICAM-1 in the fibroblast-induced maturation of DC, the ICAM-1/β2 integrin interaction is not the only mechanism involved. Ab-mediated blocking of ICAM-1 on fibroblasts only partially reduced TNF-α production by DC (Fig. 5). Moreover, this blocking had no influence on the expression of costimulatory molecules on DC. As we show that direct cell-cell contact is partially required for the fibroblast-induced maturation of DC (Fig. 3a), these data suggest that cell surface molecules in addition to ICAM-1 must also be involved in fibroblast-induced maturation of DC. However, anti-Thy-1-blocking Abs had no effect on fibroblast-induced DC maturation. Despite the importance of the CD40/CD40L interaction for DC maturation, we could find no evidence for a role of the CD40/CD40L interaction in the fibroblast-induced maturation of DC (26). CD40L could not be detected either on DC, on fibroblasts, or in the coculture of both (data not shown); thus, we consider it unlikely that CD40/CD40L play a role in the fibroblast-induced DC maturation.

We found that fibroblast-matured DC were functionally active, as they were capable of Ag-dependent activation of autologous T cells as well as of stimulation of T cell proliferation in allogeneic MLR in vitro.

Why should fibroblasts induce DC maturation and subsequent T cell activation? We suggest that fibroblasts are not activated in resting skin (e.g., no ICAM-1) and thus are not able to stimulate DC maturation. In contrast, in inflammatory conditions, in the tumor environment, or in granulation tissue, resident cells are activated (7, 11, 31–33). We suppose that under such conditions...
fibroblasts are actively participating in regulating DC maturation. Our data underline the impact of the fibroblast from the dermal stromal microenvironment for the differentiation and regulation of DC function. Several studies have reported the importance of the stromal microenvironment for immune responses in other contexts. For example, Dudda et al. (5) and Johansson-Lindbom et al. (37) have demonstrated the role of the stromal microenvironment for the imprinting of a target organ-specific homing of T cells. Furthermore, CD34+ progenitor cells differentiate into defined subsets of DC when cocultured with splenic fibroblasts in a manner that is dependent on M-CSF and CD40 (38). Consistently, CD34+ stem cells differentiated into DC in response to cell-cell contact with cutaneous fibroblasts and M-CSF (39). However, little is known about the effect of stromal cells on immature DC. Splenic endothelial-like CD106+ stromal cells have been reported to induce DC maturation (40). These stromal cells induced a defined regulatory T cell phenotype by cultured fibroblasts, suggesting this activation of cutaneous fibroblasts. Thus, we suggest that fibroblasts may act as mediators of inflammation and adaptive immunity. In contrast, we show in this study that dermal fibroblasts stimulate DC that are capable of supporting proliferation of naïve T cells through costimulation and cytokine generation. Transplantation 73: 1573–1571.

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Disclosures
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References
20. Wetzel, A., T. Wetzl, U. F. Haustein, M. Sticherling, U. Anderegg, J. C. Simon, and A. Saalbach. 2006. Increased neutrophil adherence to cultured fibroblasts, resulting in the maturation of DC and the subsequent promotion/inhibition of an immune response. Moreover, given that the stromal microenvironment can determine organ-specific T cell homing, it will be interesting in future studies to determine whether stromal fibroblasts play any role in this process.

In summary, we demonstrate in this study for the first time that dermal fibroblasts actively participate in the regulation of DC function, resulting in the maturation of DC and the subsequent T cell activation. Our data thus identify a new pathway of Ag-independent DC maturation induced by the local stromal microenvironment.


