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Adult Human Fibroblasts Are Potent Immunoregulatory Cells and Functionally Equivalent to Mesenchymal Stem Cells[1]

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Bone marrow mesenchymal stem cells (MSC) have potent immunosuppressive properties and have been advocated for therapeutic use in humans. The nature of their suppressive capacity is poorly understood but is said to be a primitive stem cell function. Demonstration that adult stromal cells such as fibroblasts (Fb) can modulate T cells would have important implications for immunoregulation and cellular therapy. In this report, we show that dermal Fb inhibit allogeneic T cell activation by autologously derived cutaneous APCs and other stimulators. Fb mediate suppression through soluble factors, but this is critically dependent on IFN-γ from activated T cells. IFN-γ induces IDO in Fb, and accelerated tryptophan metabolism is at least partly responsible for suppression of T cell proliferation. T cell suppression is reversible, and transient exposure to Fb during activation reprograms T cells, increasing IL-4 and IL-10 secretion upon restimulation. Increased Th2 polarization by stromal cells is associated with amelioration of pathological changes in a human model of graft-vs-host disease. Dermal Fb are highly clonogenic in vitro, suggesting that Fb-mediated immunosuppression is not due to outgrowth of rare MSC, although dermal Fb remain difficult to distinguish from MSC by phenotype or transdifferentiation capacity. These results suggest that immunosuppression is a general property of stromal cells and that dermal Fb may provide an alternative and accessible source of cellular therapy. ***The Journal of Immunology,*** **2007, 179: 1595–1604.**

Stromal cells in the bone marrow (BM)[4] were first described in 1968 (1) as the primitive mesenchymal companions of hemopoietic stem cells. Mesenchymal stem cells (MSC) from the BM were subsequently shown to be multipotential by their ability to differentiate into bone, cartilage, and fat (2). More recently, MSC were demonstrated to have pleiotropic immunomodulatory effects in vitro including direct suppression of allogeneic and mitogenic T cell proliferation (3–6), induction of T cell anergy (7) or apoptosis (8), modulation of cytokine production (9), and inhibition of dendritic cell (DC) maturation (10, 11). Immunomodulation is not Ag specific and is promoted by close contact but reported to be mediated by a number of soluble factors including TGF-β, tryptophan metabolites, and PGE_2 (4, 6, 7, 9, 12). Several animal models lend further credence to the therapeutic potential of MSC (13–16), although this is judiciously tempered by reports of increased graft rejection (17), sarcoma formation (18), and low efficacy in one graft-vs-host disease (GVHD) model (19). Recent case reports and early-phase clinical trials have suggested that i.v. cellular therapy with MSC isolated from BM by tissue culture plastic adherence followed by in vitro expansion can induce striking remissions in patients with severe GVHD after hemopoietic stem cell transplantation (Refs. 20–23; reviewed in Refs. 24 and 25).

It is widely believed that generic stromal cells such as fibroblasts (Fb) do not share the immunosuppressive effects of MSC (26). In practice, it is difficult to distinguish MSC from Fb by surface phenotype; indeed, the Ags used to define MSC for clinical trial are all found on Fb (25, 27). Transdifferentiation is said to be a key characteristic of MSC (1, 2, 28, 29) and the immunosuppressive properties of MSC are inferred to be a specialized function related to their primitive or multipotential nature (26, 30, 31). However, classical literature (32) and recent ontological studies (33) suggest that Fb are among the most primitive cells of adult tissues. In addition, Fb have previously been shown to interact with the immune system as alternative APC, either activating or down-regulating T cells (34–36) and mediating indirect antiproliferative effects on lymphocytes (37, 38). A potent effect of Fb on DC differentiation has also been observed (39). The relationship between the immune interactions of MSC and Fb has not been directly addressed.

The physiological function of MSC-mediated immunosuppression is difficult to resolve. Expansion in vitro and subsequent intravascular injection may create completely artificial interactions with the immune system. Alternatively, it is possible to hypothesize that stromal cells may have fundamental immunomodulatory functions, which in vivo might prevent inappropriate T cell activation or terminate immune responses during regeneration and healing. It is now recognized that T cell activation by APC is conditioned by a number of third parties such as B cells and NK cells (40, 41) and similar regulation by stromal cells would have far-reaching consequences, given their ubiquitous presence at sites of lymphocyte priming and restimulation.

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[4] Abbreviations used in this paper: BM, bone marrow; MSC, mesenchymal stem cell; DC, dendritic cell; moDC, monocyte-derived DC; GVHD, graft-vs-host disease; Fb, fibroblast; CFU-F, fibroblast CFU; LC, Langerhans cell; 1-MT, 1-methyl-L-tryptophan.

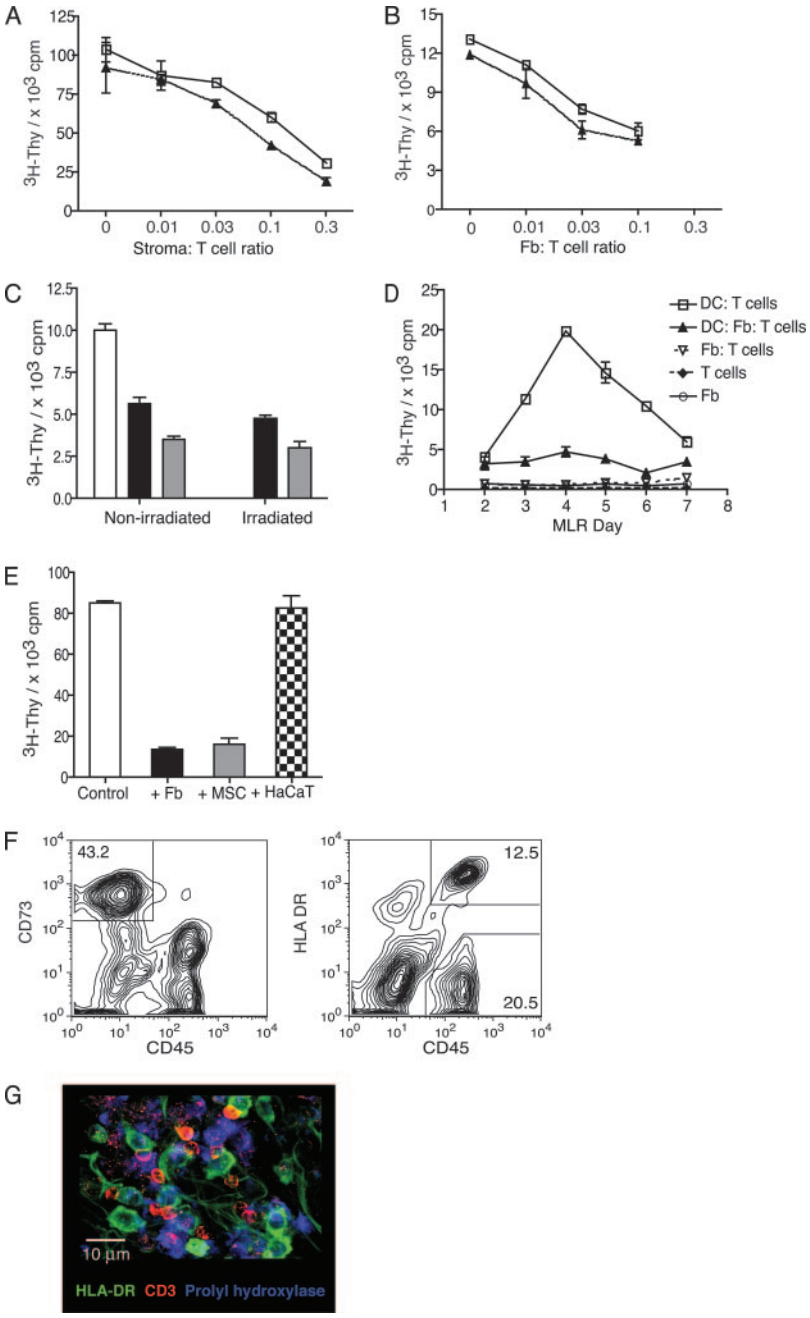

FIGURE 1. Suppression of allogeneic T cell proliferation by Fb. *A*, Allogeneic T cell proliferation stimulated by moDC in the absence and presence of increasing ratios of stromal cells (□, Fb; ▲, MSC). Results show mean ± SEM and were reproduced with at least five different donors of Fb, MSC, and leukocytes. Stromal cells were added to 10^5 T cells and 10^4 APC, and proliferation was measured by liquid scintillation counter with 40–50% counting efficiency. *B*, Allogeneic T cell proliferation stimulated by primary LC (□) and dermal DC (▲) in the absence and presence of increasing ratios of autologous Fb derived from the same skin specimen as the APC. The experiment was conducted as described in *A* except that a direct beta counter with 3–5% counting efficiency was used. Results are representative of at least six separate experiments. *C*, Gamma irradiation (20 Gy) of stromal cells did not affect their suppressive action on T cell proliferation in moDC-stimulated alloreactions. Cultures contained 10^4 moDC and 10^5 T cells (control) and 10^4 stromal cells where indicated (■, +Fb; ▨, +MSC). Results (mean ± SEM) are representative of at least three experiments. *D*, Suppression of T cell proliferation in primary alloreactions stimulated by moDC in the presence of dermal Fb is not due to kinetic distortion as demonstrated by consistently inhibited T cell proliferation over 7 days. Cultures contained 10^4 moDC and 10^5 T cells (□, control) and 10^4 Fb (▲). T cell proliferation was undetectable in the presence of third party dermal Fb (△) or alone (♦). Cultured Fb also showed similarly low proliferation (○). Results (mean ± SEM) are representative of four experiments. *E*, Stromal cell-mediated T cell proliferation suppression in moDC-stimulated alloreactions is specific and not observed when a third-party immortalized keratinocyte cell line (HaCaT) was added to culture. Cultures contained 10^4 moDC and 10^5 T cells (control) and 10^4 stromal cells or HaCaT where indicated (■, +Fb; ▨, +MSC; ▩, +HaCaT). Results (mean ± SEM) are representative of at least three separate experiments. *F*, Flow cytometric analysis of collagenase-digested dermis showing the proportions of $CD45^-CD73^+$ Fb, $CD45^+HLA\ DR^+$ APC, and $CD45^+HLA\ DR^-$ lymphocytes. Results are representative of three separate experiments. *G*, Maximal projection image of 10-μm dermal section analyzed by confocal laser-scanning microscopy. Pseudocolored image showing HLA-DR^+ dermal DC (FITC; green); $CD3^+$ T cells (Alexa 555; red), and prolyl hydroxylase$^+$ Fb (Alexa 633; blue).

In this study, we aimed to shed light on the phenomenon of MSC-mediated immunosuppression by determining whether generic stromal cells such as dermal Fb were capable of modulating T cell responses in the same fashion. Our findings show that Fb are indeed potent immunoregulatory cells with closely related functional properties. The potential physiological and therapeutic implications are discussed.

Materials and Methods

Cell isolation and culture

Dermal Fb were isolated from 4-mm punch biopsies of skin or from 300-μm dermatome sections of skin recovered from mammoplasty surgery. Apical dermis was digested for 2–12 h in collagenase (1–2 mg/ml collagenase D; Roche). A single-cell suspension obtained by pipetting, and seeded at 10^6 cells per 25-cm^2 flask. Passage 0 to passage 1 Fb were obtained by two rounds of adherence to tissue culture plastic and were >90% $CD73^+CD45^-$. MSC were obtained from BM mononuclear cells isolated over Lymphoprep (Fresenius Kabi) according to established protocols (42). Human samples were obtained following informed consent and in accordance with a favorable ethical opinion from North Tyneside Research Ethics Committee. Fb and MSC were passaged in RPMI 1640 with 20% FCS, glutamine, penicillin, and streptomycin (Invitrogen Life Technologies) or Poietics MSC medium with MSC growth supplements, glutamine, penicillin, and streptomycin (Cambrex). In fibroblast CFU (CFU-F) assays, 10^3 cells from collagenase-digested dermis or 10^7 mononuclear cells from BM aspirate were cultured undisturbed in 25-cm^2 flasks for 2 wk. Colonies were methanol fixed, stained with Giemsa, and counted manually.

For transdifferentiation assays, passage 1–6 Fb were cultivated in RPMI 1640 with 20% FCS and the following supplements: osteogenic medium, 100 nM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 0.05 mM ascorbic acid (Sigma-Aldrich); chondrogenic medium, 6.25 μg/ml insulin (Sigma-Aldrich), 50 nM ascorbic acid, and 10 ng/ml TGFβ_1 (PeproTech); adipogenic medium, 1 μM dexamethasone, 10 μg/ml insulin, 0.5 mM methylisobutylxanthine (Sigma-Aldrich), and 100 μM indomethacin (Sigma-Aldrich). Chondrogenesis was detected by staining for sulfated proteoglycans with 1% Alcian blue in 0.1 N hydrochloric acid, osteoblasts stained for alkaline phosphatase with red violet,

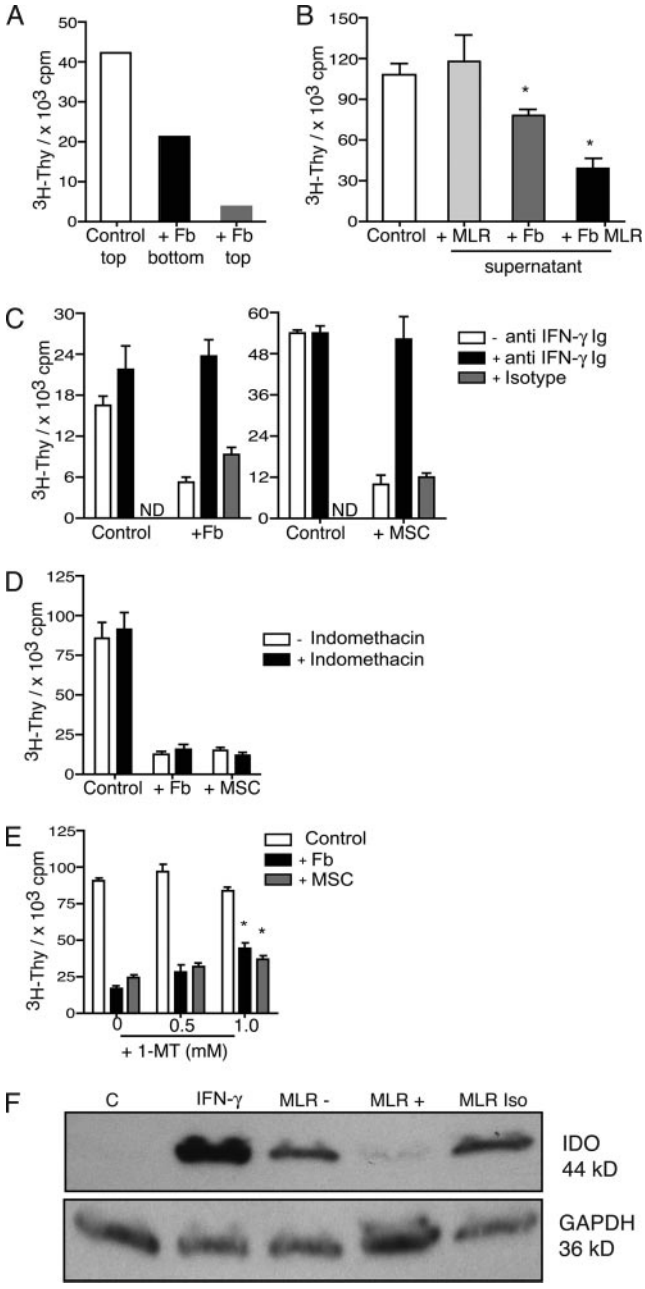

FIGURE 2. Mechanism of suppression of T cell proliferation. *A*, Fb-mediated suppression of T cell proliferation was observed across Transwell membranes. Control, moDC and T cells in the top chamber; +Fb bottom, dermal Fb separated across the Transwell membrane; +Fb top, dermal Fb cocultured in the top chamber with moDC and T cells. Results show mean ± SEM. Cultures contained the following: 5×10^4 moDC, 5×10^5 T cells, and 5×10^4 Fb. Data shown are one of two experiments performed in entirety; all sections of the experiment were repeated at least six times in total. *B*, T cell proliferation suppression was also observed using day 4 Fb culture supernatant (+Fb) and Fb stimulated by MLR (+Fb MLR) but not MLR supernatant (+MLR). Supernatants were used at 50:50 with normal medium; *, $p < 0.05$ with respect to control. Data represent at least three experiments. *C*, Abrogation of stromal cell suppression of T cell proliferation with blocking Ab to IFN-γ (20 μg/ml). □, minus anti-IFN-γ Ig; ■, plus anti-IFN-γ Ig; ▨, plus isotype control. Cultures contained 10^4 moDC and 10^5 T cells (control) and 10^4 stromal cells where indicated (+Fb; +MSC). Results are representative of at least three experiments. *D*, Stromal cell suppression of T cell proliferation is unaffected by 20 μM indomethacin; □, minus indomethacin; ■, plus indomethacin. Experimental conditions were as in *C*. Results are representative of at least four experiments. *E*, Partial reversal of stromal cell suppression of T cell proliferation by increasing concentrations of 1-MT. □, Control; ■, +Fb; ▨, +MSC. Experimental conditions were as in *C*. *, $p < 0.05$ with respect to suppressed cultures with no 1-MT. Results representative of at least five experiments. *F*, Immunoblots probed with anti-IDO and anti-GAPDH Ab of lysates from Fb cultured under the following conditions. C, Untreated cells; IFN-γ, treated with IFN-γ (1000 IU/ml); MLR −, exposed to MLR alone; MLR +, exposed to MLR plus neutralizing anti IFN-γ Ab; MLR Iso, isotype control. Result from one of two experiments is shown.

and adipocytes stained for lipid with Oil Red-O and visualized using laser confocal microscopy.

Monocyte-derived DC (moDC) were generated from magnetically isolated $CD14^+$ monocytes and cultured for 6 days with 50 ng/ml rGM-CSF and IL-4 (R&D Systems) followed by 24-h activation with 1 μg/ml LPS (Sigma-Aldrich), 10 ng/ml IL-1β (PeproTech), and 10 ng/ml TNF-α (PeproTech) as previously described (43). Langerhans cells (LC) and dermal DC were isolated by spontaneous migration from 1 mg/ml dispase (Roche) separated epidermal and dermal sheets cultured with 50 ng/ml GM-CSF over 60 h as previously described (44). $CD3^+$ T cells (purity, >90%) were isolated using Rosette-Sep according to manufacturer's instructions (StemCell Technologies) and the nontumorigenic keratinocyte cell line, HaCaT, was a gift from Dr. N. E. Fusenig (German Cancer Research Centre, Heidelberg, Germany).

Proliferation assays and cytokine production

T cell proliferation was measured in flat-bottom 96-well plates using a 16-h pulse of 0.548 MBq/ml [^{3}H]thymidine (TRA310; Amersham Biosciences) on day 5 unless otherwise stated. Thymidine incorporation was measured by direct scintillation counting (Matrix 9600; Packard Instrument) or luminescence counter (Microbeta TriLux; PerkinElmer).

Transwell experiments used 0.4-μm pore size inserts in 24-well format (Corning). In restimulation assays, allogeneic moDC and $CD3^+$ T cells were cocultured for 6 days in the presence or absence of dermal Fb or MSC. The nonadherent T cells (>90% $CD3^+$ T cells) were harvested and rested for a further 4–6 days in the presence of 0.1 ng/ml IL-2 (Glaxo) before restimulation with the same donor-derived moDC or CD3/CD28-coated beads (1 bead:3 T cells; Dynabeads T cell expander; Invitrogen Life Technologies). Neutralizing anti-IFN-γ Ab (B27) was obtained from BD Biosciences; 1-methyl-L-tryptophan (1-MT) and indomethacin were from Sigma-Aldrich. Supernatants were collected and stored frozen for cytokine assays using the BD Biosciences cytometric bead array (CBA-Flex) and analyzed with the BD-FCAP Array software, version 1.0.

DC modulation by dermal Fb

A total of 2.5×10^5 LPS-, TNF-α-, and IL-1β-matured moDC was cocultured with and without 2.5×10^5 dermal Fb in 24-well plates in the presence of 50 ng/ml GM-CSF and IL-4. After 2 days, the mature moDC were washed three times and assessed by flow cytometry. A total of 4×10^4 mature moDC (>96% DC) cocultured with and without dermal Fb was stimulated with 4×10^4 CD40L-transfected J558L mouse cells line (provided by P. Lane, Birmingham University, Birmingham, U.K.) in 96-well flat-bottom plates for 24 hs. Supernatants were collected and stored frozen for cytokine analysis.

Flow cytometry

Abs were obtained from BD Biosciences unless stated otherwise. Ag (clone), CD3 (Abcam; polyclonal ab5690); CD73 PE (AD2); CD90 FITC (5E10); CD105 FITC (Immunokontact; 8E11); MHC class I PE (Immunotools; W6/32); CD31 PE (WM59); CD34 FITC (581); CD45 allophycocyanin (H130); HLA DR allophycocyanin (L243); CD86 PE (2331: FUN-1); CD83 FITC (HB15e); CD80 PE (L307.4); CD19 PE (HIB19); CD14 PE (M5E2); CD271 FITC (LNGFR) (Miltenyi Biotec; ME20.4-1.H4); IDO (Upstate/Millipore; 10.1); GAPDH (Chemicon; 6C5); and prolyl hydroxylase (Abcam; 5B5) were used. Secondary detection was achieved with Alexa 555-conjugated goat anti-rabbit IgG2a and Alexa 633-conjugated goat anti-mouse IgG1 (Invitrogen Life Technologies). Flow cytometry was performed using FACSCalibur (BD Biosciences) and analyzed with FlowJo (Tree Star).

Protein immunostaining

Protein lysates from day 4 Fb (1×10^5) from allogeneic reactions (>88% $CD45^-$ Fb) and recombinant human IFN-γ (R&D Systems; 0–1000 IU/ml)-treated culture were separated by gel electrophoresis and electroblotted

onto nitrocellulose filters (Hybond C extra, Amersham Biosciences). The membranes were probed with mouse monoclonal anti-IDO (Upstate/Millipore; clone 10.1) and anti-GAPDH Abs (Chemicon; clone 6C5), and detected using ECL system (Amersham Biosciences) according to manufacturer's instructions.

Skin explant assay

A total of 10^6 donor $CD3^+$ T cells was added to 10^5 recipient mature moDC with and without third-party dermal Fb or MSC and cultured in 24-well plates in 1 ml of RPMI 1640 with 10% FCS for 6 days. $CD3^+$ T cells (>90% pure) were harvested and rested for a further 4–6 days in the presence of 0.1 ng/ml IL-2. A total of 5×10^5 of rested $CD3^+$ T cells was added to recipient skin obtained from a 4-mm punch biopsy or shave biopsy and cultured for 3 days. Skin sections cultured with $CD3^+$ T cells were used as background controls. The skin samples were subsequently fixed in formalin, paraffin embedded, and stained with H&E. The skin sections were assessed and scored according to the grading system of Lerner (45) by two independent blinded assessors. The histopathological grading system is as follows: grade 0, normal skin; grade I, mild vacuolar degeneration of basal epidermal layer; grade II, diffuse vacuolar degeneration of basal cells with scattered dyskeratotic bodies; grade III, subepidermal cleft formation; and grade IV, complete epidermal separation.

Microscopy

Phase contrast images were taken with an Olympus inverted microscope fitted with a Pentax digital camera. Bright-field/dark-field images were acquired with a Leica microscope fitted with a Leica imaging system and software. Laser confocal microscopy was performed using Leica TCS SP2 UV confocal microscope and analyzed using LCS V2.51 imaging software (Leica).

Statistical analyses

Unpaired *t* test and ANOVA for parametric and Mann-Whitney *U* test and Kruskal-Wallis test for nonparametric data were performed using Prism 4.0 (GraphPad Software). All *p* values are two-tailed.

Results

Fb suppress allogeneic T cell proliferation

Dermal Fb were found to be potent suppressors of allogeneic T cell responses to mature moDC. Suppression of proliferation was observed at cell ratios of 1 Fb to 10 T cells, comparable with the effect of BM MSC (Fig. 1*A*) and occurred using passaged stromal cells that were third party to both T cells and DC. We also observed that allogeneic T cell responses to epidermal LC and dermal DC were inhibited by freshly isolated dermal Fb from the same skin sample (Fig. 1*B*). The finding that fresh Fb suppress T cell proliferation primed by cutaneous DC from the same skin sample with equal potency as passaged Fb demonstrates that immunosuppression is not a property restricted to rare subpopulations of stromal cells that only expand in vitro. Irradiation, which rendered Fb vegetative, did not abrogate suppression (Fig. 1*C*), and Fb did not merely skew the kinetics of T cell stimulation (Fig. 1*D*). Other components of the skin, such as keratinocytes, did not suppress T cell proliferation at similar cell ratios (Fig. 1*E*). The specificity and potency of Fb-mediated suppression in vitro is hard to put in a physiological context. Examination of the frequency of Fb, T cells, and APC in dermis by collagenase digestion and flow cytometry showed an in situ ratio of 43.2:21.5:12.5 (Fig. 1*F*), i.e., a Fb:T cell ratio of 2:1, greatly in excess of that used in the experiments described. Confocal microscopy also confirmed that Fb were closely apposed to dermal T cells and DC in situ (Fig. 1*G*).

T cells induce IFN-γ-dependent IDO expression in Fb

T cell proliferation was inhibited across a Transwell membrane by stromal cells in the lower chamber and APC and T cells in the upper chamber, although coculture frequently caused more potent suppression (Fig. 2*A*). This result shows that soluble factors mediate at least part of the suppressive effect but that close contact either favors short-acting soluble mediators or provides an additional

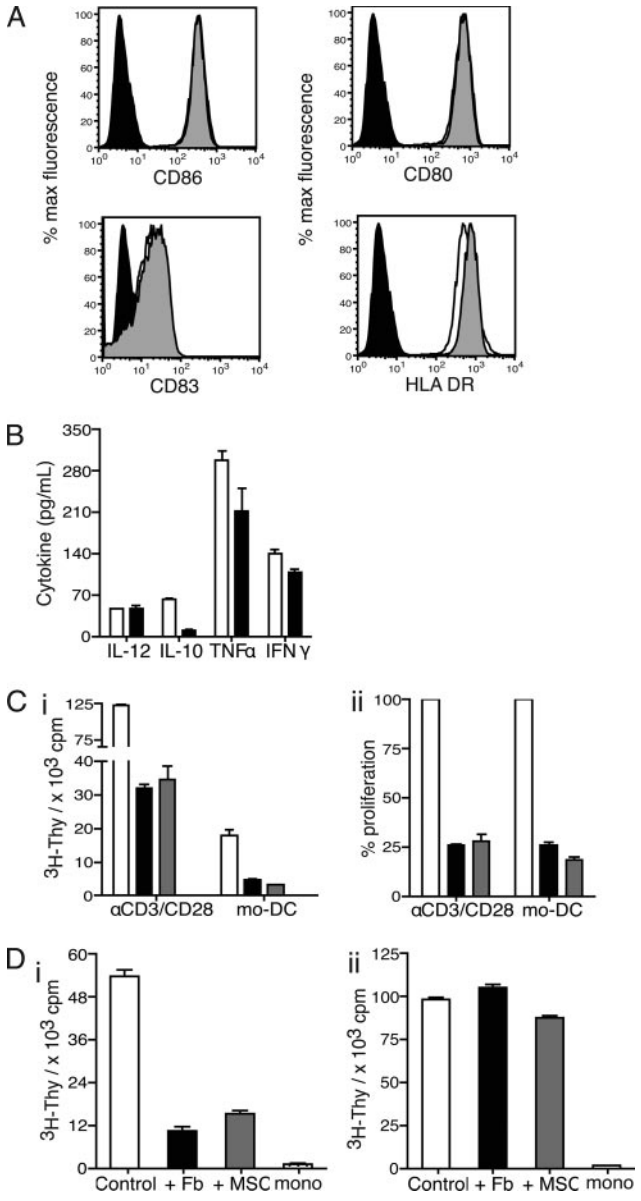

FIGURE 3. Inhibition of proliferation by Fb does not depend on APC and is reversible. *A*, MoDC (2×10^5) cocultured for 48 h with dermal Fb (2×10^5) retain their phenotype. Solid black, Isotype control; solid gray, moDC alone; continuous line, moDC with Fb. Median fluorescence intensity for CD86 FITC (moDC, 348; moDC with Fb, 353); CD80 PE (moDC, 697; moDC with Fb, 686), CD83 FITC (moDC, 17.8; moDC with Fb, 15.7); HLA DR PE (moDC, 782; moDC with Fb, 569). Results were representative of three separate experiments. *B*, Cytokine profile of moDC cultured with (■) and without (□) dermal Fb. MoDC were cultured at a ratio of 1:1 with Fb for 48 h. Cytokine secretion (mean ± SEM) was measured 24 h after stimulation with CD40L-transfected J558L mouse cells. Cultures contained 4×10^4 moDC and 4×104 CD40L-transfected cells. Results were representative of three separate experiments. *C*, T cell proliferation stimulated by CD3/CD28-coated beads and moDC is suppressed by stromal cells. *i*, Absolute cpm; *ii*, suppression relative to control. □, T cells; ■, +Fb; ▨, +MSC. Mean ± SEM is shown. A total of 10^5 T cells, 10^4 moDC, 3.3×10^4 CD3/CD28 stimulator beads, and 10^4 stromal were used as indicated. Each result represents at least three experiments. *D*, *i*, T cell proliferation upon primary stimulation by moDC (control), moDC with stromal cells (+Fb; +MSC), or with freshly isolated $CD14^+$ peripheral blood monocytes (without Fb) as stimulators (mono). A total of 10^6 T cells, 10^5 APC, and 10^5 stromal cells used as indicated. *ii*, Proliferation of T cells recovered from the same primary cultures and rested for 4–6 days with 0.1 ng/ml IL-2 before secondary stimulation for a further 3 days by CD3/CD28-coated beads. Mean ± SEM is shown. Results were reproduced up to six times.

Table I. *Modulation of T cell phenotype by Fb and MSC*[a]

	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean Fold Induction
IFN-γ (pg/ml)					
Control	8,008	8,491	20,292	23,613	1.00
+Fb	9,917	9,312	28,221	24,068	1.18
+MSC	9,004	9,793	28,917	25,866	1.22
TNF-α (pg/ml)					
Control	1,814	1,212	433	1,945	1.00
+Fb	2,246	1,989	883	2,101	1.34
+MSC	1,386	2,139	1,442	3,385	1.54
IL-10 (pg/ml)					
Control	65	34	12	83	1.00
+Fb	494	192	25	128	4.32*
+MSC	598	521	245	408	9.13*
IL-4 (pg/ml)					
Control	202	50	65	83	1.00
+Fb	307	165	207	253	2.33*
+MSC	1,036	384	493	424	5.84*

[a] Cytokine secretion by restimulated T cells of the experiment described in Fig. 3*D*. In the left column, Control, +Fb, and +MSC refer to the conditions of primary sensitization prior to resting and restimulation of T cells. Supernatants were analyzed on day 3.

*, $p < 0.05$ using ANOVA with a statistically significant positive trend comparing control, +Fb, and +MSC for IL-10 and IL-4, respectively.

direct signal. Supernatants from Fb/MLR cocultures suppressed T cell proliferation to a similar extent to Transwell experiments and much more than Fb monoculture supernatant (Fig. 2*B*). Together, these results suggest that stimulated T cells induce Fb to elaborate inhibitory mediators, creating a negative-feedback loop. Prompted by previous work using MSC (6, 12), we investigated the role of IFN-γ and found that blocking Abs to IFN-γ completely reversed the suppressive effect of Fb and MSC, compared with no treatment or isotype control (Fig. 2*C*). The downstream effects of IFN-γ on stromal cells are likely to be complex but may include stimulation of PG synthesis and acceleration of tryptophan metabolism through induction of IDO. In our hands, there was no evidence for increased PG synthesis in either Fb or MSC because indomethacin did not restore proliferation (Fig. 2*D*) but 1-MT at least partially reversed the suppressive effect of stromal cells indicating a potential role for IDO (Fig. 2*E*). In support of this, immunoblot confirmed that coculture with activated T cells induced IFN-γ-dependent IDO expression in Fb (Fig. 2*F*).

Fb do not modulate mature moDC

Stromal cells are known to alter the differentiation and maturation of DC from monocytes (10, 11, 39), and we investigated whether Fb modulated the activation and cytokine secretion of mature moDC used in these experiments. Expression of CD80, CD83, CD86, and HLA-DR was not significantly altered by coculture with Fb (Fig. 3*A*) nor was release of IL-10, IL-12, TNF-α, and IFN-γ (Fig. 3*B*). These results are in keeping with a direct action of Fb on T cells rather than through modulation of mature APC, at least under these experimental conditions.

Suppression of T cell proliferation is reversible

Suppression of T cell proliferation was observed when T cells were stimulated with CD3/CD28-coated beads or moDC, again consistent with a direct effect of Fb on T cells. Much higher rates of DNA synthesis were recorded with CD3/CD28-coated beads but relative inhibition was comparable (Fig. 3*C*). Previous reports have shown T cell anergy after exposure to murine MSC (7). In our hands, CD3/CD28-coated beads stimulated vigorous proliferation comparable with controls, when T cells were rested for 4–6 days after initial sensitization in the presence of Fb or MSC (Fig. 3*D*). The reversible proliferation of T cells suppressed by stromal cells was in marked contrast to the profound hyporesponsiveness of T cells primed directly by suboptimal or immature APC such as freshly isolated $CD14^+$ peripheral blood monocytes (without Fb).

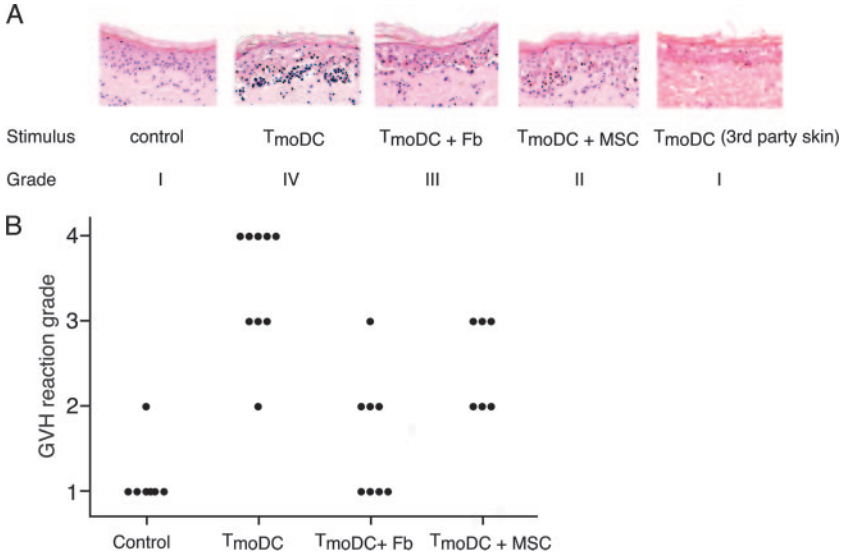

FIGURE 4. Inhibition of in vitro graft vs host (GVH) reaction by stromal cells. *A*, Histological appearance and GVH reaction pathological grading of skin explants according to the Lerner scoring system (45). Control, T cells only; TmoDC, T cells primed by moDC; TmoDC + Fb, T cells primed by moDC in the presence of Fb; TmoDC + MSC, T cells primed by moDC in the presence of MSC. T cells primed by mature moDC (TmoDC) do not react to third party skin. *B*, Summary of pathological grades from five independent experiments, performed in duplicate, showing individual data points. $p < 0.05$ using the Kruskal-Wallis test comparing the three groups: TmoDC, TmoDC + Fb, and TmoDC + MSC. T cells were prepared for the assay by an initial sensitization using moDC derived from the skin donor with or without stromal cells. After a 4- to 6-day rest period with 0.1 ng/ml IL-2, sensitized T cells were incubated with skin for 72 h. T cells alone (control) or T cells sensitized with moDC (TmoDC) or moDC in the presence of Fb (TmoDC + Fb) or MSC (TmoDC + MSC) were used as indicated. Skin sections were fixed in formalin and stained with H&E before analysis.

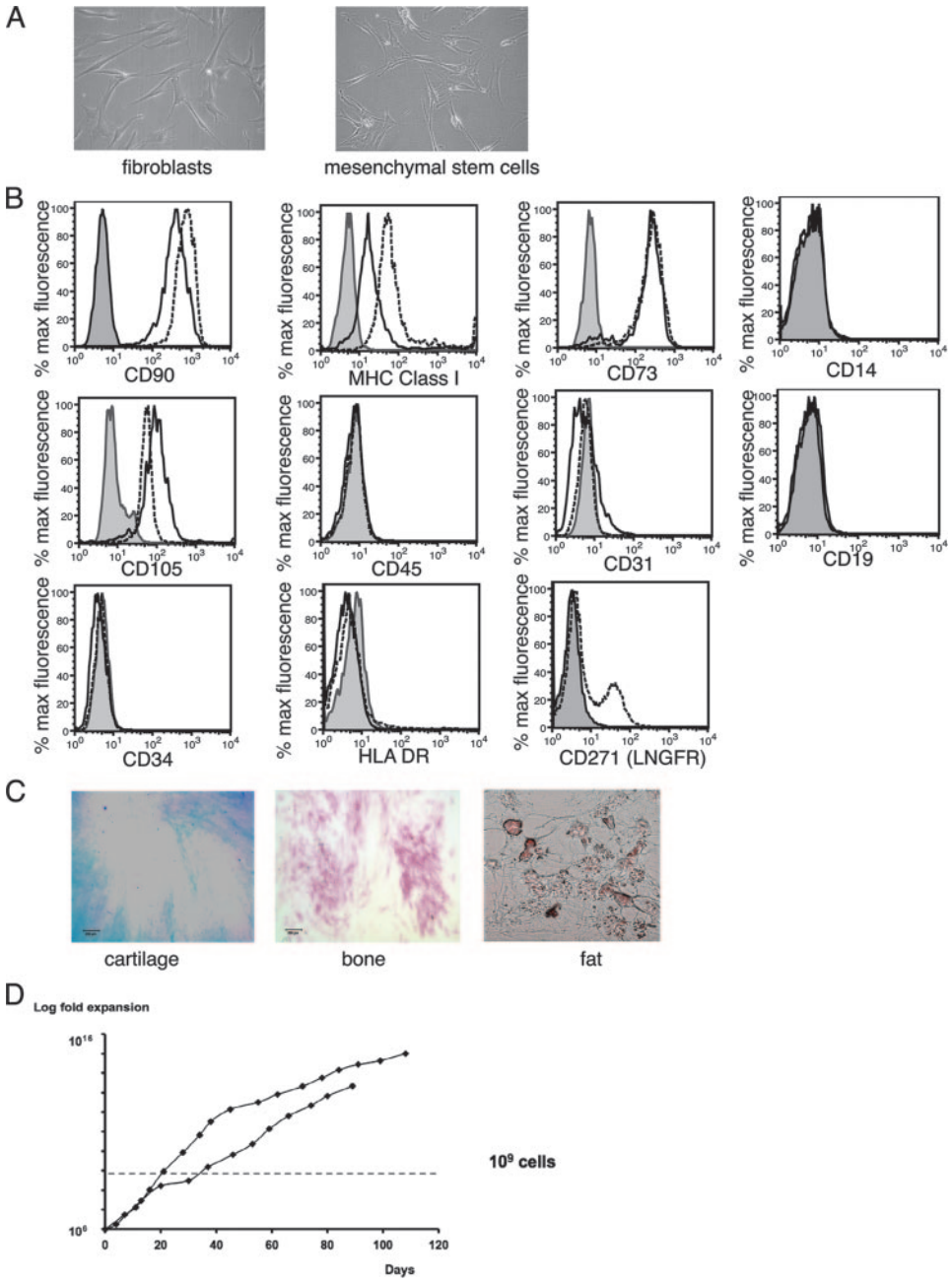

FIGURE 5. Characterization of Fb and MSC. *A*, Phase contrast microscopy of Fb (*left panel*) and MSC (*right panel*). Original magnification, ×400. *B*, Flow cytometry of Fb (broken line) and mesenchymal cells (solid line). Isotype controls are shown for comparison (filled). Results are representative of three experiments. *C*, Differentiation of Fb into chondrocytes, stained for sulfated proteoglycans with 1% Alcian blue in 0.1 N hydrochloric acid (*left*), osteoblasts stained for alkaline phosphatase with red violet (*center*), and adipocytes stained for lipid with Oil Red-O (*right*). Results are representative of four separate experiments. *D*, Dermal Fb from a 5-mm skin biopsy could be expanded to 10^9 cells (MSC dose for an average 70-kg adult) in 3 wk. Results shown are of two separate experiments.

Reprogramming of T cell cytokines by stromal cell conditioning

Although T cells previously exposed to stromal cells were able to proliferate normally on restimulation, we investigated whether there had been additional functional consequences such as the modulation of cytokine production. In these experiments, T cells were removed from the initial coculture with stromal cells, rested and then restimulated with CD3/CD28-coated beads in the absence of stromal cells. We were surprised to discover that there was marked induction of Th2 cytokines, IL-4 and IL-10, in cells exposed to stromal cells, but no increase in Th1 cytokines, IFN-γ and TNF-α (Table I). MSC appeared more potent in this regard, inducing ~2-fold greater levels of Th2 cytokines than Fb.

In contrast, there was little evidence of cytokine modulation by stromal cells during the initial sensitization phase, although it was notable that high levels of IFN-γ were produced in all cultures in keeping with the IFN-γ dependence of suppression described above (data not shown).

Amelioration of graft-vs-host reaction by exposure to stromal cells

Although MSC are reported to ameliorate clinical GVHD, it is difficult to correlate these observations directly with immunomodulatory effects measured in vitro. In an attempt to resolve this, we adapted an in vitro model of human GVHD (46, 47) to determine whether there was any association between the modulation of cytokine production by stromal cells and histopathological reactions in human GVHD target tissue. This model would allow detection of direct host tissue damage upon host Ag-specific restimulation of stromal cell modulated-donor T cells to assess potential therapeutic effect. In this experiment, a skin fragment is exposed for 72 h to allogeneic T cells previously sensitized by moDC from the skin donor. The resulting pathological reaction in the skin is then graded according to the Lerner system (Fig. 4*A*) (45). This model has been used in mechanistic studies or prediction of human clinical GVHD (47, 48). T cells stimulated with mismatched skin

Table II. *Clonogenic potential of Fb and MSC*[a]

	BM Mononuclear Cells		Digested Dermal Cells		
	Expt.1	Expt.2	Expt.1	Expt.2	Expt.3
CFU-F/cell	0.000014	0.000015	0.130	0.074	0.157
$CD45^-CD73^+$/cell	0.00006	0.00005	0.380	0.370	0.340
CFU-F/$CD45^-CD73^+$	0.23	0.30	0.34	0.20	0.46

[a] Estimates of the clonogenic potential (the ratio of CFU-F to $CD73^+CD45^-$ cells) from individual specimens of BM mononuclear cells compared with digested dermis.

donor moDC mediated high grades of graft-vs-host reaction when cultured with skin, as predicted. However, when T cells were sensitized in the presence of Fb or MSC, pathological damage was ameliorated by at least one grade, consistent with the observed modulation of cytokine production (Fig. 4*B*). MoDC-primed T cells cultured with third-party skin showed changes indistinguishable to that of unprimed T cell control (Fig. 4*A*) demonstrating the host Ag-specific nature of this assay.

Phenotype and growth characteristics of dermal Fb and BM MSC

Having compared the functional properties of MSC and dermal Fb in detail and found them to be closely related, it was necessary to examine the phenotype of the cells used in these experiments. Both dermal Fb and BM MSC were similar in appearance (Fig. 5*A*) and had identical surface phenotype: MHC class I^+, $CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD19^-$, $CD31^-$, $CD34^-$, $CD45^-$, and HLA DR^- (Fig. 5*B*). Up to 30% of freshly isolated Fb expressed CD271 (Fig. 5*B*), a marker of BM MSC reported to be associated with higher proliferative and transdifferentiation potential (49). In our hands, primary Fb readily differentiated into osteoblasts, chondrocytes, and adipocytes when cultured in appropriate medium (Fig. 5*C*). Dermal Fb were also easily expanded to high numbers in vitro; up to 10^9 dermal Fb were grown from a single 5-mm punch biopsy of the skin in 3 wk (Fig. 5*D*). Dermal Fb displayed a very high clonogenic potential as estimated by the fraction of $CD73^+CD45^-$ cells capable of initiating CFU-F in vitro (Table II). This measurement confirms that many Fb (up to 46%) are progenitor cells in vitro and that the immunosuppressive effects of Fb cultures are not due to the expansion of rare stem cells present in dermal cell suspensions. The prior observation that MSC are rare in BM cultures, as determined by the CFU-F measurements (50), is consistent with the rarity of $CD73^+CD45^-$ cells in BM (Table II). In fact the clonogenicity (ratio of CFU-F to $CD73^+CD45^-$ cells) is similar in both tissues (Table II).

Discussion

In this study, we have attempted to address whether MSC-mediated immunosuppression is shared by dermal Fb, an easily accessible and alternative stromal cell. In addition to demonstrating a similar magnitude of suppressive effects, we have also provided evidence that there are many mechanistic similarities between Fb and MSC using assays previously reported for MSC and presented new data on the modulation of cytokines and amelioration of in vitro GVHD by both types of cell. We have presented a phenotypic description of dermal Fb and have attempted to shed some light on the possible physiological relevance of stromal cell-mediated immunosuppression.

Our results show that dermal Fb inhibit T cell proliferation in vitro in response to allogeneic moDC with similar potency to BM-derived MSC. In addition, freshly isolated Fb inhibit alloresponses to autogenous cutaneous APC at cell ratios that are consistent with the frequency of Fb, T cells, and APC occurring in situ. Suppression was not observed with use of a nonstromal cell line. Previous studies have shown that Fb are not immunologically inert (34–36). More recently, they have been shown to regulate T cell development (51, 52) and survival (53). Immunosuppressive effects of Fb have been observed in response to mitogens and other stimuli (37, 38). We have now shown that these properties relate very closely to the phenomenon of MSC-mediated T cell suppression in vitro. Although we did not see any modulation of APC function by Fb using mature moDC, it is known that Fb and MSC are able to influence the earlier stages of DC differentiation in other systems (10, 11, 39, 54, 55). Together, these data suggest that stromal cells have pleiotropic immunoregulatory functions.

Our studies suggest that Fb use similar mechanisms to those reported previously for MSC to suppress T cell proliferation. Using Transwells and conditioned supernatants, we found evidence for soluble mediators. Similar results have been reported using MSC (12, 16, 56–58), although there is some variation due to different experimental designs. Transwell experiments with stromal cells in the lower chamber and stimulated T cells in the smaller upper chamber result in more pronounced suppression than the converse, which has led some investigators to conclude that contact effects are paramount (5, 10). We do not dispute that proximity augments the suppressive effect, because the greatest inhibition is always seen within cocultures, but true contact-mediated suppression has not been demonstrated in the literature. Failure to test MSC-stimulated T cell coculture medium for suppressive effects (rather than MSC-conditioned medium alone) has also lead some investigators to conclude that soluble mediators are not produced by MSC (5, 10). Indeed, inhibition by conditioned medium from Fb or stimulated T cells alone is much less potent than from Fb cocultured with stimulated T cells. Stromal cells appear to require induction by activated T cells to mediate their effects. This is supported by our observation that neutralizing Abs to IFN-γ abrogate Fb-mediated suppression, as has been observed in MSC (12).

A role for IFN-γ in promoting immunosuppression mediated by stromal cells has been suspected for some time (37, 38) and appears to form the efferent limb of a negative-feedback loop between T cells and stroma. Induction of IDO and the acceleration of tryptophan degradation may be at least one mechanism of the afferent limb. In common with MSC studies (6, 12), we have shown that IFN-γ induces IDO in Fb and that suppression is at least partly reversed by the addition of L-tryptophan. The lack of complete reversal with this maneuver is likely to be explained by the continued accumulation of tryptophan metabolites kynurenine, 3-hydroxykyrnurenine and 3-hydroxyanthranillic acid, which suppress T cell proliferation, although we did not formally test this (59). Supporting the relevance of this mechanism in vivo, up-regulated expression of IFN-γ, IDO, and increased tryptophan degradation have been found in psoriatic skin lesions (60) and in the intrinsic resistance of Fb to *Toxoplasma gondii* infection (61). We did not find any evidence for the role of PG in T cell-suppressive effects

by dermal Fb or BM MSC. PG synthesis and action has been recorded previously using PBMC rather than purified T cells mixed with MSC in a shorter 3-day coculture (9) and in mitogen rather than allostimulation of T cells (62).

In common with several reports using MSC (4, 58), we found that Fb-mediated suppression of T cells was reversible, in contrast to direct allostimulation using immature population of APC, such as peripheral blood monocytes (without Fb). Priming with monocytes alone induces anergy due to TCR ligation in the absence of adequate costimulation, as previously described (63). A murine study also described anergy but using a shorter 48-h exposure to MSC (7). In addition to suppression of T cell proliferation, previous reports have examined the potential of MSC to modulate cytokine secretion (7, 9, 10, 58). These have usually examined the primary exposure to MSC and demonstrated variable modulation of IFN-γ, IL-10, and TNF-α (7, 10, 58). One study also demonstrated enhanced skewing toward Th2 cytokine production when MSC were added to Th2-promoting culture conditions (9). Our results are the first to show a reprogramming of T cell cytokine production after transient exposure to both MSC and Fb, by using a rest period before T cells are restimulated. Both IL-4 and IL-10 are up-regulated. Interestingly, MSC are ~2-fold more potent than dermal Fb, illustrating the subtle differences between these two cells. It is difficult to ascertain the significance of changes in in vitro cytokine secretion in terms of potential therapeutic effects in humans, especially given the continued production of IFN-γ and TNF-α that we observed. The skin explant model of GVHD reflects the summation of cytokine production and cell-mediated injury in a relevant tissue and has been successfully used to dissect the antigenic specificity of human GVHD (47). T cells that had been modulated by Fb and MSC caused significantly less cytopathic effect in the skin explant than unmodulated cells with improvements in pathological scores similar to that previously observed between nonreactive and cutaneous Ag-specific T cell clones (47). This is in keeping with the low pathological damage observed when moDC-primed T cells were cultured with third-party skin in our assay.

Our results suggest that there are many similarities between immunosuppression mediated by MSC and Fb. We found little to separate the Fb and MSC by plastic adherence, immunophenotype, and transdifferentiation, according to the minimal criteria for defining MSC expounded by the International Society for Cellular Therapy (25). Our findings are at odds with previous assertions that markers such as CD271 are restricted to MSC (64) or that only MSC retain the capacity to differentiate into adipocytes, chondrocytes, and osteoblasts (64, 65). Previous studies may have used commercial Fb lines, with altered biological characteristics, but in our hands, ~30% of dermal Fb express CD271 and dermal Fb are easily transdifferentiated, at least up to the sixth passage. We also examined the clonogenicity of dermal Fb and found it to approach 50%. This argues that the properties we describe are a common feature of Fb and not related to the expansion of rare dermal stem cell populations in vitro. Interestingly, the clonogenicity of dermal and BM $CD73^+CD45^-$ cells is almost identical.

In our hands, there are subtle functional differences between Fb and MSC, but our data support the concept that immunosuppression is certainly not restricted to MSC and may even be an intrinsic function of many different stromal or Fb-like cells. Indeed, the isolation of MSC with immunosuppressive properties from many different tissues is revealing (16, 66–68). The potential significance of this relates not only to the potency, durability, and unrestricted nature of Fb-mediated T cell suppression but also to the ubiquitous presence of Fb in lymphoid organs and sites of lymphocyte trafficking. It is conceivable that stromal cell immunosuppressive tone regulates lymphocyte activation in different anatomical sites such as the lymph nodes (69, 70). The similarity between in vitro culture of Fb and wound healing has been noted previously (71), and a physiological role of stromal cells may be to promote a state of tolerance during wound repair. Stromal cell regulation may also be brought into play during chronic immune-mediated tissue damage in diseases such as rheumatoid arthritis, and it is perhaps significant that T cells isolated from the synovium of rheumatoid arthritis patients show poor proliferative potential or anergy (72) and often maintain or up-regulate IL-10-secreting Th2 phenotype (73–76).

Although these results extend the notion of cross talk between stromal cells and the immune system, the demonstration that i.v. delivery of MSC appears to modulate acute GVHD in humans remains striking and poorly understood. We have shown that the in vitro phenomenon of MSC-mediated immunosuppression is shared by dermal Fb, but whether the in vivo efficacy of MSC can also be emulated by Fb remains undetermined. Because sufficient Fb for cellular therapy can be grown from a single punch biopsy in 3 wk, the opportunity to use autologous or directed donor therapy without recourse to more invasive procedures such as BM aspiration warrants further exploration.

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Disclosures

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