Skin fibroblasts are potent suppressors of inflammation in experimental arthritis

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Accepted 24 April 2011
Published Online First 29 May 2011

ABSTRACT

Objectives Mesenchymal stromal cells (MSC) are characterised by their capacity to suppress immune reactions. This function was reported to be shared in vitro by fibroblasts but their role has been poorly investigated in vivo. This study explored whether fibroblasts isolated from skin may suppress the host immune response in a model of autoimmune disorder.

Methods and Results It was first confirmed that skin fibroblasts lack the capacity to differentiate into osteoblasts or chondrocytes but possess the capacity to inhibit in vitro the proliferation of T lymphocytes. Fibroblasts also secrete modulatory molecules, in particular prostaglandin E2 and nitric oxide, similar to MSC. To assess their role in vivo, the collagen-induced arthritis model was used, and showed that similar to MSC the intravenous injection of fibroblasts efficiently suppress clinical signs of arthritis and delay disease onset. This effect was associated with reduced inflammation as reflected by biological parameters and increased levels of IL-5, IL-10 and IL-13 in the spleens of treated mice. To characterise the mechanism of immunosuppression further, phenotypic analyses were performed and could not detect any induction of CD4 CD25 Foxp3$^+$ regulatory T (Treg) cells. A population of CD4 IL-10$^+$ T cells was, however, detected that was slightly increased after fibroblast injection and significantly upregulated after MSC administration.

Conclusions This study gives the first evidence for an immunosuppressive role of fibroblasts in vivo, and strongly suggests that fibroblasts induce a T-helper type 2 immune profile, although the possibility that IL-10-secreting Treg cells may be generated cannot be excluded.

Stromal cells are defined as connective tissue cells found in the loose connective tissue that makes up the support structure of biological tissues. Stromal cells comprise either differentiated cells such as fibroblasts, pericytes or endothelial cells and multipotent mesenchymal stromal or stem cells (MSC). Differentiated stromal cells and MSC share a large number of phenotypic markers but MSC are defined as cells able to differentiate into at least three lineages: adipocytes, osteoblasts and chondrocytes.[1] They can also differentiate into myoblasts, fibroblasts, tendinocytes or ligamentocytes. Along with their differentiation potential, MSC are reported to be immunoprivileged and to exert immunosuppressive properties by inhibiting the proliferation of virtually all immune cells.[2] This immunomodulatory effect has been shown in vitro to be exhibited by cells that have been differentiated from MSC towards osteoblasts, chondrocytes and adipocytes.[3] Likewise, mature terminally differentiated cells isolated from tissues of various origins, in particular articular chondrocytes, colonic myofibroblasts and fibroblasts from synovium, skin or lung also display immunosuppressive functions.[4–6]

The antiproliferative effects of skin fibroblasts were shown in vitro to be comparable to those of MSC. Indeed, skin fibroblasts inhibit the proliferation of T lymphocytes by inducing a G_0/G_1 cell cycle arrest and reducing the secretion of tumour necrosis factor alpha and interferon gamma (IFNγ).[4] Immune suppression is dependent on IFNγ activation and mediated by indoleamine 2,3-dioxygenase in human fibroblasts.[6–8] Moreover, fibroblasts induce a marked increase of the T-helper (Th) type 2 cytokines interleukin (IL) 4 and IL-10 by exposed T cells with no change for Th1 cytokines.[6] Other important mediators evidenced for MSC, such as IL-6, inducible nitric oxide (NO) synthase or prostaglandin E2 (PGE2) have not been investigated in detail. Moreover, fibroblast-induced immunosuppression was essentially described in vitro, whereas in-vivo experiments reported no effect in one experimental model.[9] Similarly, terminally differentiated MSC-derived osteoblasts were reported to lose their suppressive activity in vivo, probably due to the re-induction of major histocompatibility complex class II molecules.[10]

The aim of the present study was to elucidate the role of some important soluble mediators, namely NO, PGE2 or IL-6, in the immunomodulatory effect of skin fibroblasts in vitro, and to investigate whether fibroblasts may exert an immunosuppressive effect in vivo relying on the collagen-induced arthritis (CIA) model. Our findings show that skin murine fibroblasts are potent immunosuppressor cells both in vitro and in vivo exerting a suppressive effect similar to that of MSC.

MATERIALS AND METHODS

Preparation of stromal cells

Murine MSC were isolated from bone marrow as already described.[11] Cells were cultured in complete minimum essential medium alpha and used between passages 6 and 10. Dermal fibroblasts were isolated from DBA1 mice as reported elsewhere.[12] Briefly, skins were dissociated with 1 mg/ml collagenase D at 37° for 2 h. Skins were then crushed with the piston of a syringe and cell suspensions were filtered on a 100 µm filter. Cells were centrifuged (300 g, 5 min) and plated at the density of 3×10^6 cells in 75 cm^2 flasks in RPMI containing 10% bovine calf serum and antibiotics. Fibroblasts were used at passage 1 or 2. Phenotypes of isolated cells were determined by flow cytometry analysis.

Differentiation induction

Differentiation of stromal cells towards chondrogenesis, osteogenesis or adipogenesis was induced by culture under inductive conditions.[11] Expression of markers specific for each differentiated cell was assessed by reverse transcriptase quantitative PCR and histochemistry.

T-cell proliferation assay

For in-vitro experiments, splenocytes (10^5 cells/well) were stimulated with 1 µg/ml concanavalin A and 5×10^4 stromal cells in the presence or absence of inhibitors (indomethacine 5 µM; l-NAME 10 mM). For immune cells isolated from in-vivo experiments, 8×10^5 splenocytes or lymph node cells were stimulated with 5 µg/ml concanavalin A or 1, 5, 25 µg/ml bCII.

Quantification of cytokines

Cytokines and PGE2 in supernatants from T-cell proliferation assays (2×10^6 cells in the presence of 10 µg/ml bCII) were quantified by specific ELISA after 24 h (IFNγ and IL-17) or 48 h (others). IL-6 and bCII-specific IgG1 or IgG2a immunoglobulins in mouse sera were evaluated by specific ELISA and inducible NO synthase activity was quantified using colorimetric assays as reported.[11]

Flow cytometry analysis

Cells were incubated for 20 min on ice with isotypic control or specific conjugated monoclonal antibodies (BD Biosciences, Le Pont de Claix, France) as described elsewhere.[11] Detection of fluorescence was performed on a fluorescence activated cell sorter (FACSCalibur; BD Biosciences).

Arthritis induction and measurement

Adult male DBA1 mice aged 9–10 weeks were immunised by bCII injection on day 0 and recalled on day 21. MSC or fibroblasts (1×10^6 cells) were injected intravenously on days 18 and 24. Signs of arthritis were assessed by measuring the swelling of the hind paw and evaluating the clinical score using the macroscopic scale as previously described.[13] After the mice were killed, the hind limbs were collected and processed for routine histology. Histological scoring was performed on haematoxylin/eosin/safranin O sections on both ankles as follow: 0, normal; 1, inflammatory infiltrates and synovial hyperplasia; 2, pannus formation and cartilage erosion; 3, important cartilage erosion and bone destruction; 4, loss of joint integrity. The results shown are the mean of three experiments (total n=27 in fibroblast groups and 34 in control and MSC groups).

Statistical analysis

Statistics were performed using the unpaired t test and for in-vivo experiments an unpaired Mann–Whitney test to compare non-parametric data.

RESULTS

Characterisation of skin fibroblasts

Fibroblasts were isolated from dorsal and abdominal skin of DBA1 adult mice, expanded in vitro and evaluated for the expression of a number of cell surface molecules. At passage 2, they were negative for the haematopoietic markers CD45, CD11b, CD14 and positive for the MSC markers CD44, CD90, CD105 as well as Sca-1 (figure 1A). They were, however, negative for CD73. To evaluate their differentiation ability, they were cultured in specific conditions currently used for inducing

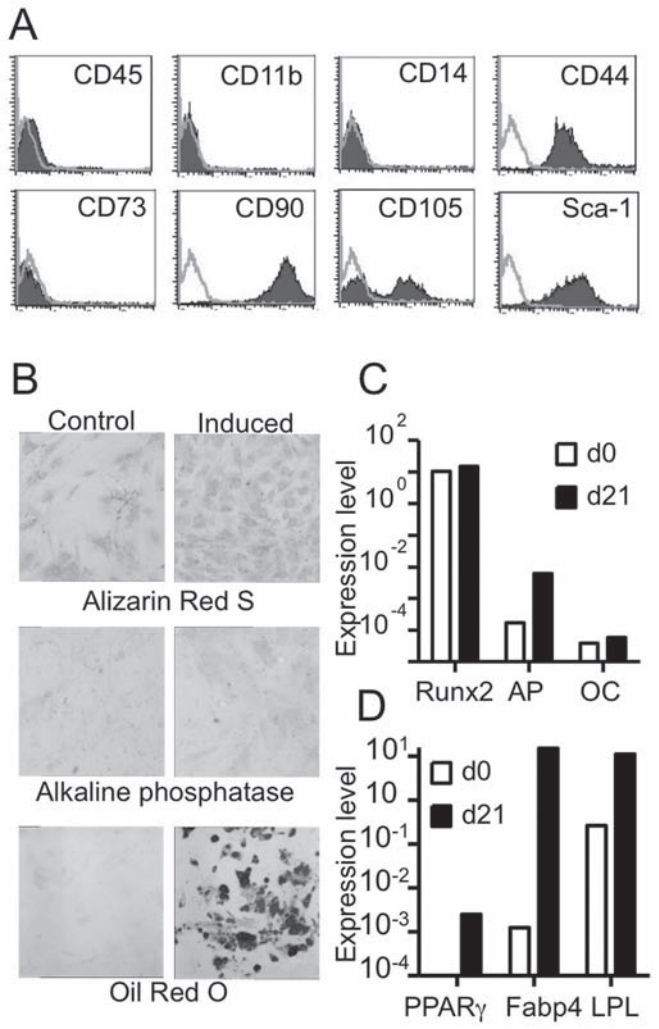

Figure 1 Phenotypic and functional characterisation of murine skin fibroblasts. (A) Immunophenotype of skin fibroblasts at passage 2 detected by flow cytometry. Grey lines correspond to isotypic controls and dark surfaces show staining with the indicated antibodies. (B–D) Differentiation potential of skin fibroblasts. (B) Osteogenesis and adipogenesis were assessed by alizarin red S or alkaline phosphatase (AP) and oil red O staining, respectively, after culture in osteogenic and adipogenic (induced) or proliferative (control) conditions for 21 days. (C) Absence of osteogenic differentiation was defined by lack of induction of specific markers (Runx2, AP and osteocalcin (OC)) at day 21 vs 0 by reverse transcriptase quantitative PCR. (D) Adipogenic differentiation was confirmed by upregulation of peroxisome proliferator-activated receptor gamma (PPARγ), fatty acid protein 4 and lipoprotein lipase (LPL) at day 21 vs 0. Results are shown as one representative experiment out of two.

MSC differentiation towards the three main mesenchymal lineages. They were unable to form a condensed pellet during the 21-day culture and to differentiate into chondrocytes. They did not differentiate to osteoblasts as shown by the absence of mineralisation (background staining with alizarin red S) and stable messenger RNA levels of Runx2 and osteocalcin markers, even if a slight increase of alkaline phosphatase could be observed both at the mRNA level and by histochemical staining (figure 1B,C). Nevertheless, fibroblasts could differentiate towards adipocytes producing lipid vacuoles stained by oil red O (figure 1B) and expressing high levels of the adipogenic markers fatty acid protein 4 and lipoprotein lipase as well as a significant but lower

level of peroxisome proliferator-activated receptor gamma (figure 1D). We therefore confirmed that the isolated cells were fibroblasts and not MSC because they were unable to display a trilineage differentiation potential.

Skin fibroblasts and MSC exert a similar immunoregulatory function in vitro

The immunosuppressive effect of fibroblasts has been tested in a proliferative assay and compared with that of bone marrow-derived MSC isolated from the same DBA1 mouse strain. We showed that skin fibroblasts inhibited the proliferation of splenic T cells to the same extent as MSC (figure 2A). Similarly to MSC, fibroblasts secreted high levels of NO. However, in the presence of the specific inhibitor l-NAME, the production of NO was significantly decreased only with fibroblasts (figure 2B). Moreover, fibroblasts secreted high levels of IL-6 and PGE2 (figure 2C,D). The secretion of PGE2 was increased by a 200-fold factor in fibroblasts versus MSC and was significantly inhibited by the addition of the specific inhibitor indomethacin. We showed that the production of IL-6 was also reduced in the presence of l-NAME. These data demonstrate that in vitro, primary dermal fibroblasts and MSC display a similar modulatory function and secrete high amounts of antiproliferative mediators, in particular NO and PGE2.

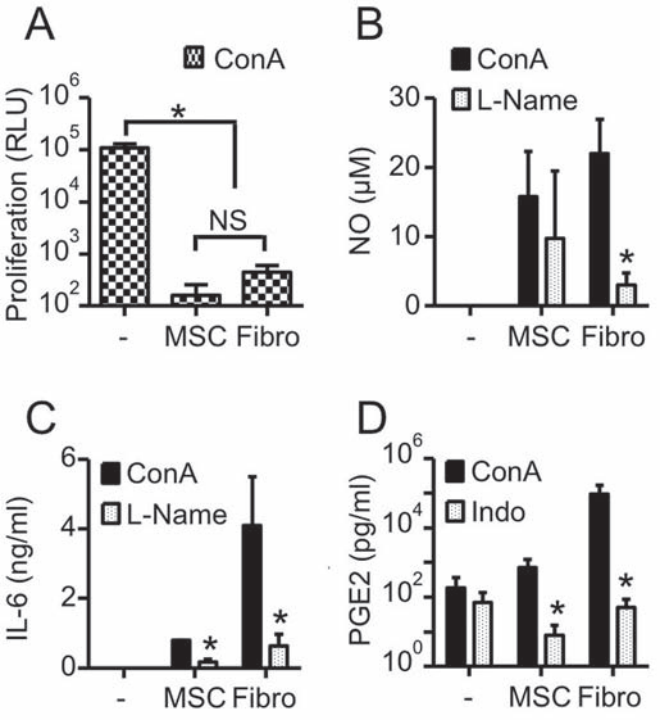

Figure 2 Immunosuppressive properties of skin fibroblasts. (A) Proliferation of splenocytes (1×10^5) stimulated with concanavalin A (con A) in the absence (−) or presence of 5×10^4 mesenchymal stromal cells or fibroblasts (Fibro) was determined after 3 days using the Cell Titer Glo assay. Results are expressed as relative luciferase units (mean RLU±SEM from three independent experiments). (B–D) Quantification of soluble mediators was assessed in the supernatant of the proliferative assays in the presence or absence of the inducible nitric oxide synthase and cyclooxygenase-2 inhibitors, l-NAME and indomethacin, respectively (n=3). (B) Secretion of nitric oxide was determined using a modified Griess reagent. (C) Quantification of interleukin 6 (IL-6) by ELISA. (D) Quantification of prostaglandin E2 by specific ELISA (*$p<0.05$ compared with control; Ctrl).

Skin fibroblasts potently inhibited the onset of clinical signs of arthritis

We then investigated whether fibroblasts may exert an immunomodulatory function in vivo. To this aim, we relied on the CIA experimental model of arthritis and evaluated the effect of the intravenous injection of fibroblasts on disease progression. Because we recently showed that primary murine MSC could efficiently inhibit arthritis when injected at days 18 and 24,[11] we determined the effect of fibroblasts or MSC on the occurrence of CIA when administered at the same time points. Fibroblast administration resulted in a significant inhibition of the clinical signs of arthritis as measured by the reduction of hind paw swelling, as well as the decrease in the clinical score (figure 3A,B). There was, however, no significant difference between the groups injected with MSC or fibroblasts. The onset day of arthritis also tended to be delayed in mice injected with fibroblasts as well as the incidence of arthritis to be decreased (figure 3C,D). Furthermore, when they were killed, histological

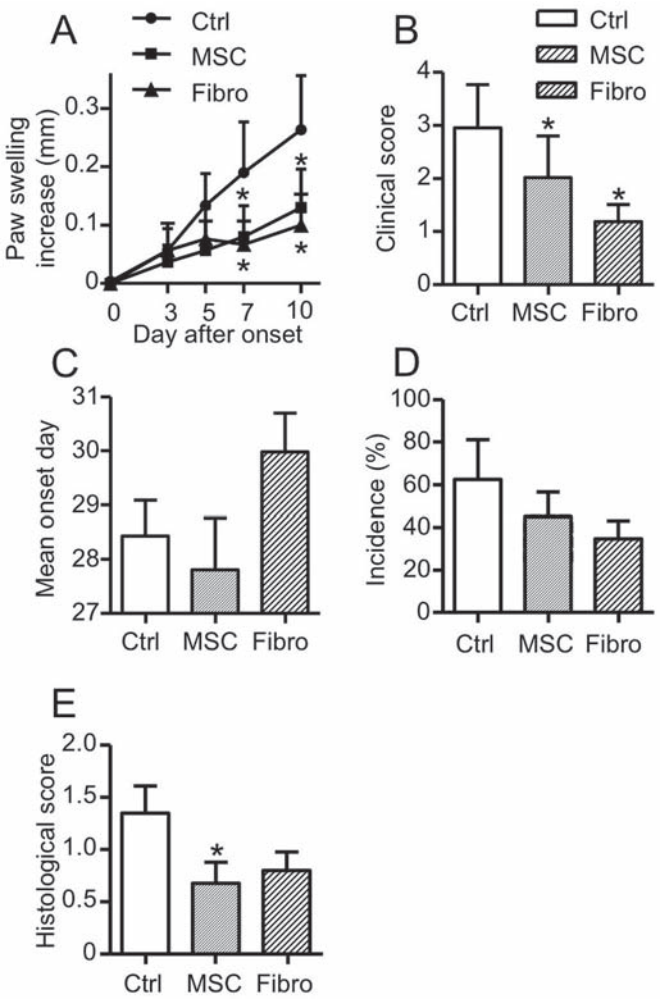

Figure 3 Immunosuppressive effect of skin fibroblasts in the collagen-induced arthritis model. (A–D) Evaluation of arthritis in DBA1 mice after immunisation with bCII (control; Ctrl) and intravenous injection of 1×10^6 mesenchymal stromal cells (MSC) or fibroblasts on days 18 and 24. (A) Evaluation of mean paw swelling of hind paws. (B) Determination of mean clinical score by measuring arthritic signs of the four paws. (C) Determination of mean day of disease onset. (D) Incidence of arthritis in each group. (E) Histological score evaluated on hind paw sections from mice killed on day 31. Results show the mean of three independent experiments (*$p<0.05$ compared with Ctrl).

analysis revealed a reduction in the histological score in both groups, which was significant in the MSC-treated group of mice (figure 3E). Altogether, the results demonstrate that skin fibroblasts behave similarly to MSC in vivo, displaying a high potential to prevent or at least delay the occurrence of arthritis.

Skin fibroblasts induced hyporesponsiveness of the host immune response

In the next experiments, we wanted to elucidate the mechanisms of suppression induced by fibroblasts. Using a lymphocyte proliferation assay, we showed a bCII dose-dependent proliferation of T cells isolated from spleen or draining lymph nodes of immunised mice (figure 4A,B). When cultured under the same conditions, T lymphocytes from mice treated with fibroblasts or MSC showed a lower proliferative activity when recalled by bCII. We then assessed in vitro the profiles of secreted cytokines in the supernatants of bCII-stimulated splenocytes isolated from fibroblast or MSC-treated mice. In those samples, a trend towards a lower expression of IFNγ and a stable production of IL-17 were observed (figure 4C). We also showed that transforming growth factor beta 1 (TGFβ1) was not induced upon recall by bCII, although it was nicely expressed on the baseline (approximately 700 pg/ml) both in control mice and mice injected with fibroblasts or MSC. On the contrary, a significant induction of all the anti-inflammatory cytokines tested (IL-5, IL-10, IL-13) was detected in groups of immunised mice that had received fibroblasts or MSC (figure 4C). Similar data were obtained in the supernatants from draining lymph nodes (data not shown). Accordingly, the bCII-specific IgG1/IgG2a profile tended to increase in the sera from fibroblast or MSC-treated mice (figure 4D). The expression level of IL-6 in the sera of treated mice also tended to decrease, reflecting a lower systemic inflammatory response (figure 4E). Overall, the administration of fibroblasts in the experimental model of arthritis allowed modifying different biological parameters, strongly suggesting a switch of the host immune response towards a regulatory or Th2 cell profile.

Induction of IL-10-producing CD4 T cells in spleens of treated mice

Because IL-10 may be a signature of the Th2 cell subset but also of regulatory T (Treg) cells, B cells, macrophages and dendritic cells (DC), we aimed at evaluating which cells were secreting IL-10 in the splenocyte population. To this aim, when mice were killed, part of the splenocytes were incubated in culture wells for 2 h and then divided into two fractions: cells in suspension were incubated in separate wells whereas adherent cells were maintained in the wells. The cell populations were then cultured for another 48 h before quantifying IL-10 secretion after bCII stimulation. Both the adherent cells and the cells in suspension secreted IL-10 (figure 5A). However, significantly higher levels of IL-10 were secreted by cells cultured in suspension from MSC and fibroblast-treated groups compared with controls. After bCII priming, secretion was detected uniquely when splenocytes were isolated from mice treated with fibroblasts or MSC. We cannot exclude the possibility that among the fraction of adherent cells, cells that were in suspension may attach to the adherent cells. In the same culture conditions, TGFβ1 was detected both in the adherent and non-adherent fraction at levels that were similar in mice treated or not with fibroblasts or MSC, suggesting the absence of natural Treg induction by both stromal cells (figure 5B). To identify more precisely the population of IL-10-secreting cells, we performed a phenotypic

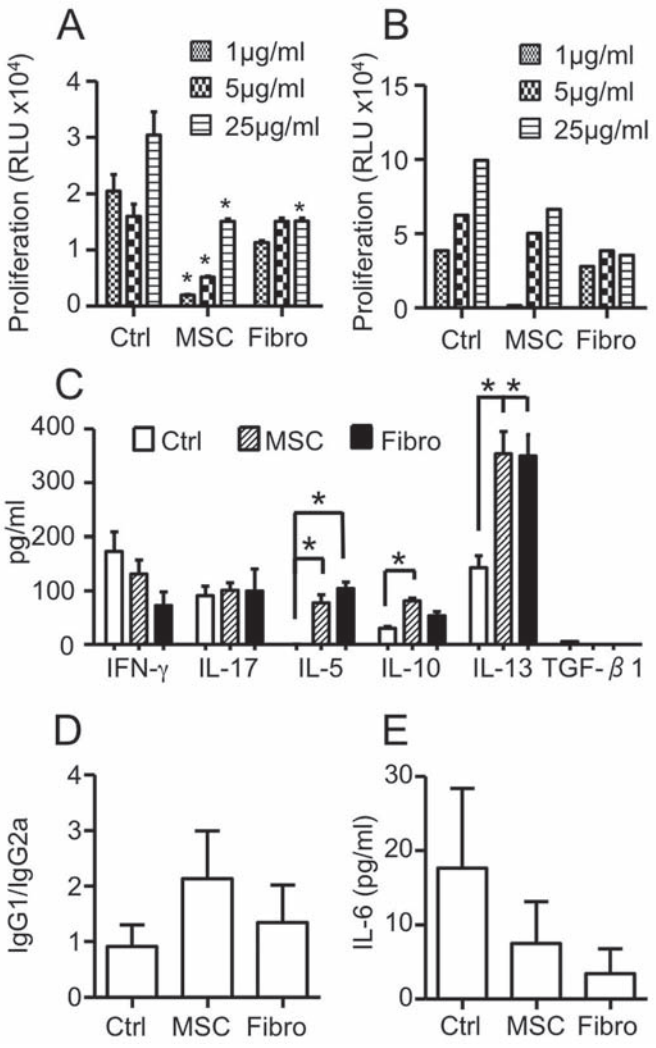

Figure 4 Evaluation of the inflammatory response in mice with collagen-induced arthritis (Ctrl) and treated with mesenchymal stromal cells (MSC) or fibroblasts. (A–B) Proliferative response of splenocytes (A) or draining lymph nodes (DLN) (B). Cells were isolated on day 31 from mice and stimulated in vitro with different concentrations of bCII. Results are expressed as the mean value of the stimulated proliferation minus basal proliferation±SEM in A (n=10) or the proliferation of the pool of DLN in B. (C) Cytokine quantification in the supernatant of splenocytes isolated from mice treated or not with fibroblasts or MSC. Results are expressed as the level of cytokines expressed after 10 µg/ml bCII stimulation minus basal level (mean±SEM, n=10). (D) Ratio of bCII-specific IgG1/IgG2a immunoglobulins detected by ELISA in sera collected on day 31 (mean±SEM, n=10). (E) Quantification of interleukin 6 (IL-6) in sera collected on day 31 from the different groups of mice (mean±SEM, n=10; *p<0.05; if not indicated, differences are not significant). TGF, transforming growth factor.

characterisation of splenocytes by flow cytometry. A stable percentage of natural CD4 CD25 Foxp3$^+$ Treg cells as well as a stable percentage of F4/80$^+$IL-10$^+$ macrophages or CD83 IL-10$^+$ DC were observed between treated and not treated mice (figure 5C). Interestingly, a significantly higher percentage of CD4 IL-10$^+$ T cells was observed in the splenocyte population from MSC-treated mice compared with control CIA mice (figure 5C). Although not significant, the same trend was observed in fibroblast-treated mice. Indeed, in vivo, MSC and likely fibroblasts exhibit immunomodulatory functions by inducing a population of induced CD4 T cells secreting high levels of IL-10.

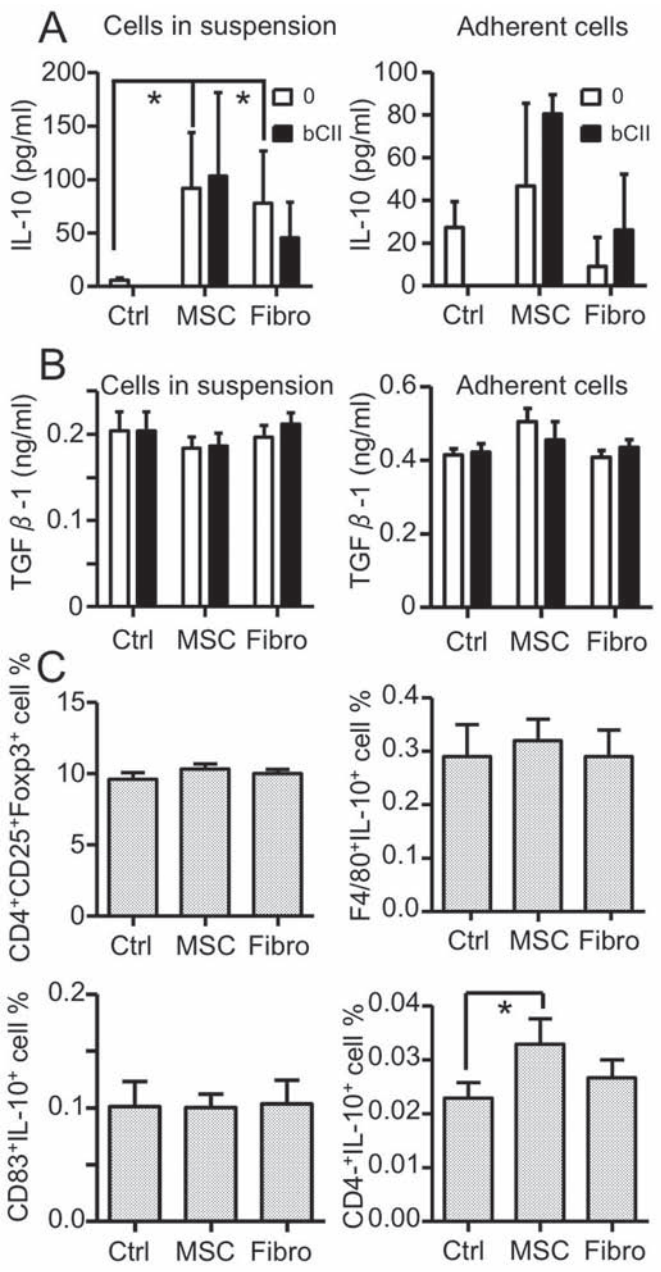

Figure 5 Determination of the subset of interleukin 10 (IL-10)-secreting immune cells involved in immunosuppression. (A) IL-10 secretion was assayed by ELISA in the supernatants of adherent and non-adherent cell fractions isolated from spleens and stimulated or not with 10 μg/ml bCII. (B) Transforming growth factor (TGF) beta 1 secretion was assayed by ELISA in the supernatants of adherent and non-adherent cell fractions isolated from spleens and stimulated or not with bCII. (C) Percentage of natural regulatory T cells (CD4 CD25 Foxp3$^+$ cells), IL-10-secreting F4/80$^+$ macrophages, IL-10-secreting CD83 dendritic cells or IL-10-secreting CD4 T lymphocytes in the spleens of the different groups of mice. IL-10 intracellular labelling was performed on splenocytes after bCII activation for 48 h. Membrane labelling was then performed using antibodies specific for each cell population and analysed by flow cytometry (mean±SEM, n=9; *p<0.05). Ctrl, control; MSC, mesenchymal stromal cell.

DISCUSSION

In the present study, we demonstrate that skin fibroblasts exert in vitro an immunosuppressive effect by secreting high levels of soluble antiproliferative mediators identical to those secreted by MSC and in particular, NO, IL-6 and PGE2. More importantly, for the first time, we provide evidence that, similar to MSC, skin fibroblasts exhibit in vivo an immunomodulatory function by inhibiting the occurrence of autoimmune arthritis. This effect was associated with the induction of a population of CD4 T cells secreting IL-10.

We showed here that fibroblasts isolated from skin are different from MSC because they do not differentiate towards the chondrocytic or osteoblastic lineage. Adipocytic differentiation was obtained after the addition of rosiglitazone, which is an agonist of peroxisome proliferator-activated receptor gamma required for murine MSC differentiation.[11] Although it was reported that MSC may be derived from skin,[14 15] our results indicate that the fibroblasts isolated from skin and used in the present study are not MSC. These observations suggest that different populations of MSC-like cells or fibroblasts may be isolated from skin. In vitro, fibroblasts behaved like MSC by inducing a potent immunosuppressive effect on T-cell proliferation. Although immunosuppressive activity has predominantly been associated with MSC, corneal and gingival fibroblasts have previously been shown to have IFNγ-activated antiproliferative functions on T lymphocytes.[7 16] More recently, dermal fibroblasts have been reported to be potent immunoregulatory cells through the secretion of soluble factors and the induction of indoleamine 2,3-dioxygenase activity.[4 6 8] We also previously showed that synovial fibroblasts that are phenotypically and functionally similar to bone marrow-derived MSC displayed the same capacity to suppress T-cell proliferation.[17] Here, we show that the suppressive effect of fibroblasts was mediated, at least partly, by the secretion of NO and PGE2, whose production was inhibited by the addition of specific inhibitors. IL-6 was also an important mediator because its production by MSC and fibroblasts was induced in proliferative assays. IL-6 production is induced by PGE2 and NO, as shown by the inhibitory effects of indomethacin and l-NAME, as already reported.[18 19] IL-6 was also reported to regulate the production of PGE2 and NO indicating a feedback loop.[20 21] This result is consistent with our recent data showing that IL-6-dependent PGE2 secretion is correlated with the immunosuppressive activity of MSC.[11]

In the present study, we demonstrate that, in vivo, fibroblasts were able to inhibit the clinical signs associated with experimental arthritis. This inhibitory effect was comparable to that observed after MSC administration. To our knowledge, this is the first study highlighting the immunosuppressive effect of fibroblasts in vivo. On the contrary, the absence of therapeutic efficacy of fibroblasts has been reported in a few cases.[9 22] Although fibroblasts are ubiquitous mesenchymal cells, they have distinct gene expression patterns depending on the anatomical sites from which they are isolated. Using large-scale analysis of gene expression patterns in primary fibroblasts, three signatures associated with anatomical divisions were found: one demarcating proximal and distal compartments of the limbs; a second demarcating fibroblasts from the anterior or posterior half of the body and a third expression distinguishing dermal fibroblasts from non-dermal origin.[23 24] The differential transcriptional pattern of fibroblasts is likely to be dependent on the expression pattern of *HOX* genes because the embryonic pattern of *HOX* gene expression appears to be retained in cultured adult fibroblasts and is related to their anatomical site of origin. There are indeed strong arguments suggesting that the *HOX* genes act as regulators of fibroblast differentiation.[23] As differentiation is also associated with specialisation and function, it may be hypothesised that skin fibroblasts isolated

from different anatomical locations may behave differently, explaining the diverse effects observed in vivo in different experimental settings.

We also showed that the suppressive effect of fibroblasts is associated with the inhibition of T-cell proliferation in secondary lymphoid organs and the induction of a suppressive immune response, independently of CD4 CD25 Foxp3$^+$ natural Treg or CD8 CD28 Treg cell expansion (data not shown). Interestingly, we found that fibroblast administration tended to increase the number of IL-10-secreting CD4 T cells in the spleens of treated animals. The increase of these cells was statistically significant after MSC injection. Until recently, it was commonly accepted that IL-10 was associated with the Th2 subset of T cells. It is now proposed that the IL-10-producing T regulatory 1 cell population is the relevant source of IL-10.[25] In the absence of higher numbers of TGFβ1-producing cells and/or CD4 CD25 Foxp3$^+$ natural Treg cells in the spleen, it may be speculated that in our experimental settings, MSC are likely to induce Tr1 cells. The induced Treg or Tr1 cells develop from conventional T cells after exposure to immature DC or IL-10 itself.[25] As PGE2 produced by MSC has been shown to promote the generation of both regulatory DC and macrophages secreting IL-10,[22 26] we investigated whether such cell subtypes were induced by fibroblasts. However, we were not able to detect a significant increase in IL-10-producing macrophages or DC in the spleens of the treated animals, making difficult to appreciate the role of PGE2 in this process. Therefore, the analysis of the biological parameters strongly suggests that fibroblast administration induced a switch of the host immune response towards a Th2 profile; we cannot, however, totally exclude the possibility that Tr1 cells may also be generated.

In summary, our results provide evidence for the first time that in vivo dermal fibroblasts are equivalent to MSC. Our data thus confirm that immunosuppression is a property shared by stromal cells, which can display in vivo the same capacity to modulate the host immune response. As a result of the easy way of isolating and expanding fibroblasts ex vivo, these findings may have important implications for future clinical applications.

Funding Work in the laboratory Inserm U844 was supported by the Inserm Institute, the University of Montpellier I and a grant from the 'Agence Nationale de la Recherche' (project ANR Physio 2006 'Immunostem').

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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