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Murine Sclerodermatous Graft-Versus-Host Disease, a Model for Human Scleroderma: Cutaneous Cytokines, Chemokines, and Immune Cell Activation¹

Yan Zhang,² Laura L. McCormick,² Snehal R. Desai, Caiyun Wu, and Anita C. Gilliam³

Murine sclerodermatous graft-vs-host disease (Scl GVHD) models human scleroderma, with prominent skin thickening, lung fibrosis, and up-regulation of cutaneous collagen mRNA. Fibrosis in Scl GVHD may be driven by infiltrating TGF-β1-producing mononuclear cells. Here we characterize the origin and types of those cutaneous effector cells, the cytokine and chemokine environments, and the effects of anti-TGF-β Ab on skin fibrosis, immune cell activation markers, and collagen and cytokine synthesis. Donor cells infiltrating skin in Scl GVHD increase significantly at early time points post-transplantation and are detectable by PCR analysis of Y-chromosome sequences when female mice are transplanted with male cells. Cutaneous monocyte/macrophages and T cells are the most numerous cells in Scl GVHD compared with syngeneic controls. These immune cells up-regulate activation markers (MHC class II-A-D molecules and class A scavenger receptors), suggesting Ag presentation by cutaneous macrophages in early fibrosing disease. Early elevated cutaneous mRNA expression of TGF-β1, but not TGF-β2 or TGF-β3, and elevated C-C chemokines macrophage chemoattractant protein-1, macrophage inflammatory protein-1α, and RANTES precede subsequent skin and lung fibrosis. Therefore, TGF-β1-producing donor mononuclear cells may be critical effector cells, and C-C chemokines may play important roles in the initiation of Scl GVHD. Abs to TGF-β prevent Scl GVHD by effectively blocking the influx of monocyte/macrophages and T cells into skin and by abrogating up-regulation of TGF-β1, thereby preventing new collagen synthesis. The Scl GVHD model is valuable for testing new interventions in early fibrosing diseases, and chemokines may be new potential targets in scleroderma. The Journal of Immunology, 2002, 168: 3088–3098.

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Scleroderma is a progressive acquired connective tissue disease characterized by skin thickening and visceral fibrosis. There is no effective treatment for this chronic autoimmune disease. We have previously characterized a murine sclerodermatous graft-vs-host disease (Scl GVHD) model that recapitulates important features of human scleroderma. Our model appears to represent rapidly progressive early cutaneous scleroderma that evolves over months rather than years. We used BALB/c mice transplanted with B10.D2 bone marrow and spleen cells, which differ at minor histocompatibility loci, to generate Scl GVHD. We have previously shown that infiltrating cutaneous mononuclear cells and increased TGF-β1 mRNA expression precede up-regulation of collagen mRNA and protein synthesis, subsequent skin thickening, and lung fibrosis (1). This form of GVHD shows mainly fibrosis, with minimal cytotoxic injury to epithelia (2) and without evidence for vascular injury or autoantibodies at early time points. In contrast, cytotoxic GVHD shows epithelial injury that predominates over dermal fibrosis, and effector cells are thought to be cytotoxic T cells and NK cells (2). The effector cells and their corresponding activation markers in Scl GVHD have not been previously identified. We show here that many donor cells are present in skin of mice with Scl GVHD, but not in syngeneic bone marrow-transplanted controls. During characterization of our model, we noted that the initial cutaneous inflammation consisted primarily of CD11b⁺ monocyte/macrophages and T cells. Although T cells are critical cells in initiating immune reactions in skin and are probably the initiating effector cells in Scl GVHD, monocytes are the predominant cell population infiltrating skin and lungs in patients with early, rapidly progressive scleroderma (3) and in murine Scl GVHD.

The migration and recruitment of leukocytes to a specific tissue site is a multistep process involving the sequential activation of various adhesion molecules on immune cells and on the vascular endothelium as well as a vast array of chemokines (4, 5). These chemokines are capable of attracting and activating various resident and inflammatory cutaneous immune cells (6). The involvement of C-C chemokines in inflammation is an area of active investigation, but the role of chemokines in the progression of fibrosing diseases is not completely understood. Chemokines may be involved in accumulation of inflammatory immune cells that induce matrix synthesis in scleroderma skin lesions (7–9). Specifically, macrophage chemoattractant protein-1 (MCP-1) and RANTES might play important roles in early pathogenesis of scleroderma, both
by chemotraction of immunocompetent cells and/or by modulation of collagen production via TGF-β1 in skin (10–12).

To fully characterize the types of cells infiltrating skin during early Scl GVHD and their activation status, we performed immunostaining of skin sections, and flow cytometric analysis of single-cell suspensions from skin of mice on days 14 and 21 post-bone marrow transplantation (post-BMT), time points when skin thickening is detectable. We also examined the up-regulation of cutaneous C-C chemokines, MCP-1, macrophage inflammatory protein-1α (MIP-1α), and RANTES, as well as TGF-β isoform mRNAs in murine Scl GVHD at time points after bone marrow transplantation, and integrated these findings into a dynamic model of sclerodermatous fibrosis. Our goals were to understand the early events preceding skin thickening to devise effective therapies to inhibit fibrosis in scleroderma and Scl GVHD and to understand the pathophysiologic of cutaneous fibrosis disease.

Materials and Methods

Bone marrow transplantation

In a typical transplantation experiment, 7- to 8-week-old male and female B10.D2 (H-2d) and BALB/c (H-2d, The Jackson Laboratory, Bar Harbor, ME) mice are used as donors and recipients, respectively, for BMT to produce Scl GVHD in a standard method using added spleen cells as a source of mature T cells (1, 13, 14). Briefly, female recipient mice were lethally irradiated with 700 Gy from a Gammacel 173Cs source (J. L. Shepherd & Associates, San Fernando, CA). Approximately 6 h later they were injected by tail vein with male donor bone marrow (1 × 10^7/mouse) and spleen (2 × 10^7/mouse) cells suspended in RPMI 1640 (BioWhittaker, Walkersville, MD) with 20 U/ml heparin (Fisher Scientific, Pittsburgh, PA). The dose of cells was a standard one used routinely in the generation of GVHD (13, 14). A control group of female BALB/c recipient mice were injected with male BALB/c spleen and bone marrow cells (syngeneic BMT, referred to as control animals). Animals that did not engraft died within 7–10 days. Transplanted animals were maintained in sterile Microisolator cages (Lab Products, Seaford, DE) and supplied with autoclaved food and acidified water. Three to five animals per group (Scl GVHD or control) and per time point were studied in each experiment.

Anti-TGF-β Ab treatment

Mice were given two doses of 150 µg anti-pan TGF-β Abs (rabbit polyclonal IgG; Sigma, St. Louis, MO) by tail vein injection on days 1 and 6 post-BMT as previously described (1). The dose was selected as a standard one used for in vivo inhibition of fibrosis in other mouse models (15, 16).

Collection of tissue

For these experiments three to five transplanted animals per group were sacrificed via cervical dislocation on days 7, 14, and 21 post-BMT. Days 14 and 21 were chosen because they are the earliest time points at which there are reliable changes in skin thickening and inflammation. Back skin was depilated and harvested for RNA extraction (snap-frozen in liquid nitrogen and stored at −80°C), flow cytometry (see below), immunostaining (frozen on dry ice and stored at −80°C), and routine histologic staining (fixed in 10% buffered formalin (Surupgiph Medical Industries, Richmond, IL) and paraffin-embedded). Tongue and lung were also collected for routine histology to confirm the development of Scl GVHD.

Histologic and morphometric analysis

Formalin-fixed, paraffin-embedded sections of tissue were stained by H&E (Surupgiph Medical Industries, Richmond, IL). Frozen skin was embedded in OCT embedding medium (Miles, Elkhart, IN) and sectioned by cryostat (Leica CM1800, Nussloch, Germany) for immunostaining (described below). For morphometric analysis, histologic sections of lung or back skin were deparaffinized and stained with a PAM camera (Optimas, Goleta, CA) using an Axioptroph photomicroscope system (Zeiss, Oberkochen, Germany), stored as tiff files, and subjected to image analysis (Optimas 6.1, Bothell, WA). Skin thickening was evaluated for each animal as previously described (1). Quantification of immunostaining by image analysis was performed on slides stained with specific Abs and corresponding isotypes. Isotype control Ab staining was always tested on the same slide as specific Ab staining and subtracted in the analysis. Areas were calculated in arbitrary square units by outlining the dermis on a ×10 view for each microscopic image. The same threshold settings were used on the set of slides stained with the same Ab. The density of positive immune cell staining within the outlined areas was plotted as the percentage of positive area. A minimum of six measurements was taken from two or more skin sections from each animal, and the variation among animals was expressed as the SE.

Antibodies

Immunostaining. Abs and corresponding isotype controls were as follows. Anti-CD11b (anti-Mac-1, mAb M1/70, rat IgG2b; BD Pharmingen, San Diego, CA) was used to identify monocyte/macrophages, and anti-class A scavenger receptor type I and II Ab (2F8, IgG2b, Serotec, Raleigh, NC) was used to identify mature monocyte/macrophages. Anti-CD3 (mAb 17A2, rat IgG2b; BD Pharmingen) was used to identify T cells. Anti-I-A^d (mAb AMS-32.1, mouse IgG2b) was used to detect immune cell activation status (MHC II). Mouse IgG2a (1 rabbit anti-mouse polyclonal anti-serum, Chemicon, Temecula, CA) was used to identify type I collagen protein in tissue. Goat anti-rat IgG-biotin or goat anti-rabbit IgG-biotin (Vector Laboratories, Burlingame, CA) followed by peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and diaminobenzidine (Kirkegaard & Perry Laboratories) was used for detection. Diaminobenzidine orange enhancer (Kirkegaard & Perry Laboratories) was used for type I collagen staining. Isotype control Abs were rat IgG2b (R35-38; BD Pharmingen), mouse IgG2b and normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Flow cytometry. Direct single stains of CD3 were performed for T cells (mAb 17A2, rat IgG2b-PE; Anti-CD45 (mAb 30-F11, rat IgG2b-FTC; BD Pharmingen) was used to identify all leukocytes of hemopoietic origin. Indirect two-color stains were performed for anti-CD11b (anti-Mac-1, mAb M1/70, rat IgG2b-PE), and MIP-1α (mAb 2H5, hamster IgG; BD Pharmingen) using F(ab’)2, anti-rat IgG-FITC (Jackson Immunoresearch Laboratories, West Grove, PA) to detect bound Ab. The corresponding isotype controls were used for instrument set-up. All specific and isotype control Abs for two-color flow cytometry were obtained from BD Pharmingen. Indirect single-color stains were performed for VLA-4 (CD49d, mAb 9C10, rat IgG2b; BD Pharmingen), type A I/II scavenger receptors (mAb 2F8), and macrophage receptor with collagenous structure (MARCO) (mAb ED31, rat IgG1; Serotec) using F(ab’)2, anti-rat IgG-FTC (Jackson Immunoresearch Laboratories) to detect bound Ab. NK cells were detected by single stains using an anti-pan NK Ab (mAb DX5, rat IgM; BD Pharmingen) and F(ab’)2, anti-rat IgG-M-PE (Jackson Immunoresearch Laboratories) as the detecting Ab. Direct two-color stains were performed for anti-I-A^d (mAb AMS-32.1, mouse IgG2b-FITC) and anti-CD11b (anti-Mac-1, mAb M1/70, rat IgG2b-PE) and for anti-I-A^d and anti-CD45 (mAb LY-5, rat IgG2b-FTC) using the corresponding isotype controls and dermal cells from a normal mouse for instrument set-up. All specific and isotype controls for two-color flow cytometry were obtained from BD Pharmingen. Blocking steps included purified murine IgG to inhibit nonspecific staining.

Preparation of skin cell suspensions for flow cytometry

As described previously (1), small pieces of depilated skin were digested in RPMI containing 10 nM HEPES (Irvine Scientific, Santa Anna, CA), 0.01% DNase (Sigma), 0.27% collagenase (Sigma), and 1000 U hyaluronidase (Sigma) at 37°C for 2 h. The digested skin was filtered through 100-µm pore size nylon meshes to generate a single-cell suspension of skin cells containing resident cells (keratinocytes, fibroblasts, endothelial cells, and perivascular cells such as mast cells) and infiltrating cells (lymphocytes, monocytes, and NK cells). This is a standard method for analysis of cutaneous immune cells (17). Approximately 4 × 10^6 cells were typically obtained from a 1 × 2-cm^2 piece of skin for control mice, and 8 × 10^6 cells were obtained from mice with Scl GVHD on day 21. Before specific Ab staining, all isolated skin cells were blocked with purified murine IgG (1 µg/10^6 cells; Sigma) for 5 min on ice. For MCP-1 staining, permeabilization buffer containing 1% saponin was used as washing and staining buffer. Abs and isotype-matched controls were used in 1:100 dilutions. The appropriate goat or rabbit secondary Abs were used at 1:100 dilution for staining. For additional controls, cells were stained with untreated secondary Ab. The density of positive immune cell staining within the outlined areas was plotted as the percentage of positive area. A minimum of six measurements was taken from two or more skin sections from each animal, and the variation among animals was expressed as the SE.

Immunohistochemistry

Immunohistochemistry was performed on acetone-fixed frozen sections. Staining was performed at least three times on each specimen by standard methods (18). In all immunostaining experiments, specific Ab staining was always compared with isotype control-stained sections on the same slide (see histologic and morphometric analysis).
Isolation of monocytes and T cells by magnetic bead separation

Single-cell suspensions of skin cells from the backs of experimental animals with Scl GVHD were prepared as described above. For positive selection of monocyte/macrophages and T cells, MACS CD11b microbeads (Miltenyi Biotec, Auburn, CA) or Thy-1.2 beads (Miltenyi Biotec) were incubated with the skin cells and then applied to a MidiMACS separation column (Miltenyi Biotec). The purity of monocytes and T cells was determined by flow cytometry (>90%) for each population. The isolated monocyte/macrophages, T cells, and residual cells (remaining skin cells after monocyte and T cell separation) were then used for RNA purification and RT-PCR analysis.

RNA and genomic DNA purification

Animals were sacrificed by cervical dislocation at each time point. Dissected depilated back skin was snap-frozen in liquid N2 and stored at −80°C until used for RNA or DNA isolation. RNA was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD) as described previously (1). Genomic DNA was extracted using standard methods (19).

Semiquantitative RT-PCR and PCR analysis

As previously described for analysis of other cutaneous cytokine mRNAs (20), PCR reactions contained RT reaction products; specific oligonucleotide primers for TGF-β1 (Clontech, Palo Alto, CA), TGF-β2, TGF-β3, MCP-1, MIP-1α, RANTES, pro(α1)I collagen, and β-actin (Table I); 10× PCR buffer (Perkin-Elmer, Norwalk, CT); nucleotide mix (Promega, Madison, WI); and 2 U Taq DNA platinum polymerase (Life Technologies) in a volume of 50 μl. Y-chromosome sequence analysis was performed on extracted genomic DNA using a PCR primer set specific for both X- and Y-chromosome copies of the supernumerary marker chromosome (SMC) gene (21). Reactions were heated to 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min using GeneAmp 9600 PCR System (Perkin-Elmer). Reactions were also performed in the absence of reverse transcriptase and were always negative. The cycle number for each system was chosen (cytokines and chemokines, 36; SMC, 40), so that all signals were in the linear range of detection, determined by ethidium bromide-stained gels, which were photographed and acquired via GelDoc (Bio-Rad, Hercules, CA). The bands were then analyzed by image analysis using Optimas 6.1 software, and the results were expressed as relative density for each PCR product following normalization for the DNA loading amount based on the β-actin band.

Data analysis and statistics

All data were expressed as the mean ± SE, and unpaired t test (two-tailed) was used for statistical significance to determine differences among means of treatment, experimental, and control groups. Differences with p < 0.05 were considered significant.

Results

To understand early events in skin fibrosis and to devise effective therapies for scleroderma, we undertook experiments to characterize the cutaneous cytokine and chemokine environments in murine Scl GVHD, a model for scleroderma. In this model we always compare results from animals with Scl GVHD with those from control animals subjected to the same regimen of irradiation and to transplantation with syngeneic BALB/c bone marrow and spleen cells (syngeneic BMT controls). Three to five animals per group and per time point are examined. We concentrated on early events within days 7–21 post-BMT. Previously, we showed that TGF-β1 mRNA up-regulation occurs as early as day 7, and skin thickening is detectable by days 14–21 (1). Here we report that up-regulation of mRNA for cutaneous TGF-β1 (not TGF-β2 or -β3) and C-C chemokines precedes influx of donor immune cells (mainly monocyte/macrophages and T cells) and skin thickening. Both monocyte/macrophages and T cells, but not fibroblasts, appear to be the source of TGF-β1. We also document up-regulation of scavenger receptor molecules, VLA-4 and I-A, on immune cells, suggesting activation and Ag presentation.

Donor cells infiltrate skin in early Scl GVHD

We observed numerous mononuclear cells infiltrating thickened skin at early time points in Scl GVHD, but not in control animals (Fig. 1). By routine histology these were a mixture of large pale oval monocyte/macrophages (arrowhead) and smaller, darker, more compact T cells (arrow). Immunostaining and flow cytometric analysis confirmed the histologic impressions. This histology can also be seen in human early morphea and early scleroderma, which can both be highly inflammatory. To determine whether these cells infiltrating skin are of donor or host origin, we transplanted bone marrow and spleen cells from male mice into female recipient mice and detected Y-chromosome sequences by PCR analysis of total cutaneous cellular DNA. We used a PCR primer pair that amplifies a gene (SMC) on both X- and Y-chromosomes (Table I) (21). On days 14 and 21 post-transplantation, the smaller SMCY band was seen when DNA from the skin of female experimental animals with Scl GVHD was analyzed on ethidium bromide-stained gels, but was absent in the DNA from skin of female control mice (Fig. 2; the day 21 control is not shown, but was identical with the day 14 control). We did not test day 7 in this experiment. Therefore, male donor cells infiltrate the skin of female recipient mice with Scl GVHD by day 14 post-BMT and may play a role in the resulting skin fibrosis. The analysis of Y-chromosome sequences by PCR analysis and ethidium bromide staining in a gel is a relatively insensitive assay for donor cells, because many resident recipient skin cells express SMCY sequences (keratinocytes, fibroblasts, and endothelial cells). Therefore, our evaluation of donor cells infiltrating skin may be an underestimate, and quantification of donor cells by this method was not useful. Our experiments demonstrate that donor cells are present and presumably functional.
Early marked increase in CD45<sup>+</sup> cells accompanies skin thickening, in which monocyte/macrophages and T cells predominate in the dermal infiltrates

To further evaluate cells infiltrating skin in Scl GVHD, we prepared single-cell preparations from back skin on days 14 and 21 post-BMT when skin thickening is evident (see Materials and Methods). This technique provides ample numbers of cells for flow cytometric analysis with staining for several different surface markers, reliable from experiment to experiment. The total number of cells in skin is not as critical as the relative numbers of skin cells of each type (T cell, monocyte/macrophage, NK cell), and we made no attempt to quantify cells per square millimeter of skin.

Therefore, we expressed the data as a percentage of the total skin cells. On day 14 post-BMT there was a 2-fold increase (8–18%) in CD45<sup>+</sup> cells in the skin of animals with Scl GVHD (E) that was further increased (22 to 43%) on day 21 post-BMT compared with control animals (Fig. 3A plot). The increase in CD45<sup>+</sup> cutaneous immune cells in controls from days 14 to 21 may represent non-
specific effects of irradiation and transplantation that later disappear by immunostaining (data not shown) and do not result in skin fibrosis. The percentage of CD45<sup>+</sup> cells in control animals on day 21 seemed abnormally high compared with the low values for CD11b<sup>+</sup>, CD3<sup>+</sup>, and NK cells at the same time, but the results were consistent in all control and experimental animals in each group (n = 3). The infiltrating cells in Scl GVHD were primarily CD11b<sup>+</sup> or CD3<sup>+</sup>. By day 21 post-BMT, a small number of NK cells (<10% of total skin cells) appeared in the inflamed skin (flow histogram not shown). In these flow experiments, neutrophils, which can also express CD11b, were gated out by light scatter. However, neutrophils were rarely seen in skin routine histology (Fig. 1) and were therefore unlikely to be a prominent cell population by flow cytometry.

**CD11b<sup>+</sup> cells infiltrating skin express macrophage scavenger receptors**

To confirm that the predominant CD11b-expressing cells in skin on days 14 and 21 were mature macrophages and not NK cells or neutrophils, we performed flow cytometric analysis for macrophage scavenger receptor using Abs that recognize scavenger receptor A (Sc-R-A) types I and II (mAb 2F8) (22) and MARCO (Ab ED31) (23). The percentage of total skin cells expressing 2F8 and MARCO was elevated by day 14 post-BMT, with marked elevation by day 21 post-BMT (Fig. 3B plot). Furthermore, most of the CD11b-positive cells in Scl GVHD skin on day 21 also expressed Sc-R-A when double staining was performed (2F8 or MARCO; Fig. 4), and the proportion of the CD11b<sup>+</sup> infiltrating cells expressing 2F8 increased further by day 21 post-BMT. Sc-R-A type I and II (2F8) expression was greater than that of MARCO. Therefore, Sc-R-A-expressing macrophages are the predominant cells infiltrating the skin of animals with Scl GVHD at early time points, and their influx accompanies skin thickening.

**Activated macrophages infiltrate skin in murine Scl GVHD**

Because macrophage scavenger receptors (MARCO and Sc-R-A types I and II), VLA-4, and CD11b are up-regulated in activated macrophages, we examined the level of expression of these surface markers per cell using flow cytometric analysis, comparing the mean fluorescence intensity of positive cells from animals with Scl GVHD vs that of cells from syngeneic BMT control animals (summarized in Table II, depicted in Fig. 3B overlays). Our results suggest that there may be active Ag presentation in skin at early time points in Scl GVHD. On day 14 post-BMT, VLA-4, MARCO, and Sc-R-A types I and II were up-regulated on macrophages by 34–76% (Table III). The mean fluorescence intensity of cells expressing MARCO and 2F8 did not increase further on day 21. The values were similar for MARCO, VLA-4, and CD11b on day 21, perhaps reflecting an already up-regulated state. We also evaluated histocompatibility class II molecules using an Ab specific to I-A<sup>d</sup> that recognizes immune cells of both donor and recipient animals. We found elevated levels on CD45-positive cells (Table III). In contrast to the up-regulation of scavenger receptor molecules, I-A expression on total CD45<sup>+</sup> and CD11b<sup>+</sup> cells was increased on day 14 post-BMT and remained increased on day 21 post-BMT.

**Chemokine MCP-1, MIP-1α, and RANTES mRNA expression is elevated during early murine Scl GVHD**

In experiments reported previously (1) and here we determined that CD11b<sup>+</sup> monocyte/macrophages are the predominant cells infiltrating skin at early time points in Scl GVHD. To test whether chemokines might play a role in attracting these monocyte/macrophages to skin, we performed semiquantitative RT-PCR analysis of total skin RNA to examine the expression of cutaneous MCP-1, MIP-1α, and RANTES mRNA in Scl GVHD. Messages for all these C-C chemokines were increased in Scl GVHD skin before skin thickening and before infiltration of CD45<sup>+</sup> cells was evident (Fig. 5). On day 7 post-BMT, MCP-1, MIP-1α, and RANTES mRNAs were elevated by approximately 1.5-, 2.1, and 3.7-fold, respectively, in experimental animals compared with controls. These data are plotted in Fig. 5B and compared with influx of CD45<sup>+</sup> cells and skin thickness by image analysis (summary plot of flow cytometric analysis and image analysis of immunostaining and skin thickness; original data not shown). On day 14, MCP-1, MIP-1α, and RANTES mRNA levels remained elevated, and skin thickening was detectable in Scl GVHD, but not controls. By day

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### Table II. Percent increase of activation marker expression on cutaneous cells isolated from skin of mice with Scl GVHD<sup>a</sup>

<table>
<thead>
<tr>
<th>Days Post-BMT</th>
<th>VLA-4</th>
<th>MARCO</th>
<th>2F8</th>
<th>CD11b</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>34.2</td>
<td>55.1</td>
<td>76.3</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>7.2</td>
<td>0</td>
<td>0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Immune cells in skin of mice with Scl GVHD are activated compared to controls by day 14 post-BMT. Percent increase was calculated using the mean fluorescence intensity of cells expressing the specific activation markers VLA-4, MARCO, 2F8, and CD11b [100% × (mean fluorescence for Scl GVHD − mean fluorescence for control)/mean fluorescence for control]. Numbers are an average of data obtained from each group of mice (n = 3).

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### Table III. Percent increase of I-A<sup>d</sup> expression on cutaneous cells isolated from skin of mice with Scl GVHD<sup>a</sup>

<table>
<thead>
<tr>
<th>Days Post-BMT</th>
<th>CD45&lt;sup&gt;+&lt;/sup&gt; cells</th>
<th>CD11b&lt;sup&gt;+&lt;/sup&gt; cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>154.3</td>
<td>44.1</td>
</tr>
<tr>
<td>21</td>
<td>116.8</td>
<td>70.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> I-A<sup>d</sup> expression is up-regulated on cutaneous macrophages of mice with Scl GVHD by day 14 post-BMT [100% × (mean fluorescence for Scl GVHD − mean fluorescence for control)/mean fluorescence for control]. Flow cytometric gates were set on CD45, and CD11b positive cells before determining the I-A expression. Percent increase was calculated by comparing the mean fluorescence intensity of I-A expression in the CD45<sup>+</sup> and CD11b<sup>+</sup> cells in mice with Scl GVHD vs syngeneic BMT controls. Numbers are an average of data obtained from each group of mice (n = 3).
21 post-BMT, when skin was markedly thickened (>40%), both MCP-1 and MIP-1α mRNA were elevated 2.5-fold, while RANTES mRNA was increased 1.9-fold (Fig. 5A). On day 21 we performed flow cytometric analysis of skin cell suspensions double-stained with CD11b and MCP-1. Neutrophils can also express CD11b, but they are not present in routine histology and were gated out by light scatter in the flow experiments. We found that the number of CD11b+MCP-1+ cells is elevated in animals with Scl GVHD (10.9% of total dermal cells compared with 4% in controls). MCP-1+ cells were predominantly CD11b+ cells, because CD11b+ cells were 16–30% of the total cells in skin (Fig. 5C). Although other cells (endothelial cells, keratinocytes) may also secrete MCP-1, their numbers in skin were very low compared with the predominant CD11b+ population in Scl GVHD mice. Therefore, activated donor monocyte/macrophages may produce their own chemoattractant in an autocrine loop at this time point.

Cutaneous TGF-β1, but not TGF-β2 or -β3, mRNA is increased during early murine Scl GVHD

TGF-β1 is a potent fibrogenic cytokine that is known to induce collagen synthesis by fibroblasts in vitro and in vivo. It has been implicated in the pathophysiology of scleroderma by several different methods (24), but the other TGF-β isoforms (TGF-β2 and TGF-β3) may also be involved. We previously reported that at an early time point (day 7 post-BMT), TGF-β1 mRNA levels are elevated approximately 3- to 5-fold by RT-PCR analysis of the skin of experimental animals with Scl GVHD vs syngeneic BMT control animals (1). To characterize the contribution of other TGF-β isoforms to fibrosis in the Scl GVHD model, semiquantitative RT-PCR analysis of total skin RNA was performed. We repeated the TGF-β1 PCR experiment and tested for TGF-β2 and -β3. TGF-β1 mRNA levels in this set of experiments were approximately 4- to 5-fold higher on day 7 and 2-fold higher in experimental animals than in controls on day 21 post-BMT (Fig. 6A). At early time points (days 7 and 14), no changes in cutaneous TGF-β2 and -β3 mRNAs were seen in experimental animals (Fig. 6A). Only very slight increases in TGF-β2 and -β3 mRNAs were observed at later time points (day 21 post-BMT) in experimental animals (statistically insignificant).

Therefore, TGF-β1 is probably the critical isoform of TGF-β driving the cutaneous fibrosis in Scl GVHD and in early fibrosis of scleroderma.

Both cutaneous monocytes/macrophages and T cells make TGF-β1 in murine Scl GVHD

We determined that both monocyte/macrophages and T cells in skin make TGF-β1 mRNA (Fig. 6B) by RT-PCR analysis of total...
RNA from magnetic bead-separated skin cells on days 14 and 21 post-BMT. Residual, negatively selected cells (keratinocytes, fibroblasts, endothelial cells, and other resident skin cells) did not make significant TGF-β1 mRNA. The amount of TGF-β1 mRNA per μg RNA expressed by monocyte/macrophages was approximately 1.9- and 1.4-fold more than that expressed by T cells on days 14 and day 21 post-BMT. This suggests that monocyte/macrophages are the main source of TGF-β1 in Scl GVHD skin, since CD11b<sup>+</sup> cells were approximately 2- to 3-fold more numerous than T cells. These data also suggest that fibroblasts and endothelial cells present in the residual cell preparation do not make significant amounts of TGF-β1 mRNA.

We chose mouse Thy-1.2 microbeads for the positive selection of mouse T cells, a standard method (25). The purity was >90% by flow analysis when we analyzed the Thy-1.2 bead-selected cells stained with anti-CD3 Ab. Although Thy-1.2 may be expressed on other cell populations, such as endothelial cells, the numbers of non-T cells expressing Thy-1.2 in skin would be very small compared with the number of cutaneous infiltrating T cells.

**Anti-TGF-β Ab treatment reduces CD11b<sup>+</sup>, 2F8<sup>+</sup> monocyte/macrophage influx into skin in murine Scl GVHD**

We hypothesize that monocyte activation by host-reactive donor T cells is an initiating event in Scl GVHD and sclerodermatosis that may lead to increased cutaneous TGF-β production and skin fibrosis. We previously demonstrated that starting on day 7 and by day 14 post-BMT, the percentages of CD11b<sup>+</sup> monocyte/macrophages infiltrating skin are markedly increased, and by day 21, monocyte/macrophages are increased approximately 5- to 6-fold in Scl GVHD compared with control animals. This early infiltration of monocyte/macrophages is accompanied by up-regulation of TGF-β1 mRNA synthesis and prominent skin thickening (1).

![Figure 6](http://www.jimmunol.org/)  
**Figure 6.** Cutaneous TGF-β1 is the critical TGF-β isoform, and monocyte/macrophages and T cells are the main cutaneous cell populations making TGF-β1 in early Scl GVHD. A, TGF-β1, but not TGF-β2 or -β3, mRNA is up-regulated in skin of animals with early Scl GVHD. The plotted data show fold up-regulation of mRNA for TGF-β1, -β2, and -β3 by RT-PCR analysis of total skin RNA (E/C). B, Single-cell suspensions from back skin were separated by magnetic beads for monocyte/macrophages (CD11b) and T cells (Thy-1.2). Total RNA was prepared from separated cells and residual cells, and subjected to RT-PCR analysis. The purity of monocytes and T cells was defined by flow cytometry (>90%) for each population by staining with anti-CD11b and anti-CD3 Ab. Both CD11b<sup>+</sup> and Thy-1.2<sup>+</sup>, but not residual cells, synthesize TGF-β1 mRNA. **Upper panel,** Scanned agarose gels showing ethidium bromide-stained PCR products. Data from a representative animal per group are shown (n = 3–5). **Lower panel,** Plotted values showing the ratio of TGF-β1 mRNA to β-actin RT-PCR products (p = 0.005, using unpaired t test to compare the ratio of TGF-β1 mRNA to β-actin of Thy-1.2<sup>+</sup> cells population to that of CD11b<sup>+</sup> cell population on day 14; p = 0.002 on day 21).

![Figure 7](http://www.jimmunol.org/)  
**Figure 7.** A–E. Anti-TGF-β Abs administered on days 1 and 6 post-BMT effectively block immune cells infiltrating skin and prevent collagen I synthesis in Scl GVHD. A–E. Immunostaining results on acetone-fixed frozen skin sections from syngeneic BMT control, Scl GVHD, and anti-TGF-β Ab-treated Scl GVHD mice on day 21 post-BMT (scale bar = 50 μm). A, Stained with anti-CD11b Ab; B, anti-2F8 Ab, a class A scavenger receptor; C, anti-CD3 Ab; D, anti-I-A<sup>d</sup> Ab; E, type I collagen antiserum. Staining with isotype control Abs (on the same slide with specific Ab) is always negative (not shown). Micrographs show immunostaining results from a representative animal per group (n = 3–5).
gave similar results, which confirmed CD11b staining data (Fig. 7B, plotted in Fig. 8).

Anti-TGF-β Ab treatment reduces T cell infiltration into skin in murine Scl GVHD

In classic GVHD, donor T cells initiate disease by recognizing recipient Ag as foreign and providing signals to cytotoxic T cells and NK cells, causing epithelial injury. In Scl GVHD, donor T cells may activate monocyte/macrophages, thereby initiating fibrosis disease via a different subset of effector cells (note the absence of significant epithelial injury in Fig. 1). Therefore, we analyzed cutaneous T cells as well as monocyte/macrophages in Scl GVHD. By day 14 post-BMT, there was a >4-fold increase in the number of cutaneous CD3+ T cells (5–8% of total skin cells) by flow cytometric analysis of Scl GVHD animals compared with syngeneic BMT animals (Fig. 3A; flow histogram not shown). By day 21 post-BMT, the number of T cells was increased further (17.3 ± 5.9% of total skin cells) in experimental animals. In contrast, in anti-TGF-β Ab-treated experimental animals T cell infiltration of skin was markedly reduced and comparable to that in controls, as shown in the immunostaining data (Fig. 7C, plotted in Fig. 8). Therefore, anti-TGF-β Abs significantly block cutaneous influx by CD3+ T cells as well as monocyte/macrophages.

Anti-TGF-β Ab treatment reduces I-A- cells in skin in murine Scl GVHD

Anti-TGF-β Ab treatment also reduced the numbers of I-A- cells in skin of Scl GVHD mice (Fig. 7D, plotted in Fig. 8).

Anti-TGF-β Ab decreases skin type I collagen synthesis in murine Scl GVHD

By image analysis, skin and lung fibrosis were abrogated by the administration of blocking Abs to TGF-β in murine Scl GVHD (1). Immunostaining and RT-PCR analysis were performed to detect type I collagen synthesis. By day 21 post-BMT, type I collagen protein (Fig. 7E, plotted in Fig. 8) in skin of experimental animals receiving anti-TGF-β Abs was comparable to that in controls. Pro(α1)I collagen mRNA expressed in skin of anti-TGF-β Ab-treated mice with Scl GVHD was actually less than that in controls (Fig. 9) as determined by semiquantitative RT-PCR analysis of total RNA.

Anti-TGF-β treatment blocks the elevation of cutaneous TGF-β-1 mRNA in murine Scl GVHD

Because we hypothesized that skin fibrosis is TGF-β1 driven, RT-PCR was performed on RNA from skin of anti-TGF-β Ab-treated experimental animals to determine whether anti-TGF-β Ab has any effect on TGF-β1 mRNA expression. By day 21 post-BMT, cutaneous TGF-β1 mRNA expressed by experimental animals receiving anti-TGF-β Abs was comparable to the baseline in controls (Fig. 9), consistent with the marked reduction in cutaneous mononuclear cell infiltrates and abrogation of skin thickening in treated animals.

Discussion

Murine Scl GVHD may best model a subset of scleroderma patients with explosive, rapidly progressive, fibrosing disease that evolves over a period of months rather than years. This group of patients is potentially ideal for early therapy, before irreversible organ damage occurs. We have shown here and in previous studies that during murine Scl GVHD, a succession of early events (infiltration of skin by donor monocyte/macrophages and T cells, and up-regulation of TGF-β1 and MCP-1, MIP-1α, and RANTES chemokine mRNA) is temporally related to increased collagen mRNA synthesis, skin thickening, and lung fibrosis (summarized in Table IV), a composite of multiple experiments. We have also shown that macrophages expressing markers of activation and Ag presentation (CD11b, I-A, SR-A, and VLA-4) are the predominant cells infiltrating skin during early time points in murine Scl GVHD, when skin thickening is first apparent. To our knowledge this is the first description of macrophage SR-A induction in scleroderma or Scl GVHD. Abs to TGF-β prevent not only skin thickening and lung fibrosis, but also the infiltration and possible activation of mononuclear cells into skin. If immune cell migration is prevented, skin thickening does not occur. Our data provide a foundation for interventions in early scleroderma at several different points in the immune cascade of Scl GVHD: chemokine production, T cell and monocyte/macrophage activation and homing to skin, T cell and monocyte function in skin, and direct inhibition.
of fibrogenic TGF-β1 itself. We also show that presumably functional donor immune cells infiltrate skin early in disease.

Cells infiltrating skin in early Scl GVHD are of donor origin

Scleroderma is a disease of unknown etiology that occurs most commonly in women after childbearing years. Recent reports describe persistent HLA-compatible fetal cells in the skin and blood of women with scleroderma that occur at a much higher incidence than in healthy women (26, 27). These data suggest that a state of microchimerism could lead to a chronic graft-vs-host (or host-vs-graft) type of reaction in these women with scleroderma (28). In our studies numerous mononuclear cells infiltrating skin at early time points in Scl GVHD are of donor origin, since we detected Y-chromosome-specific sequences from donor male animals in the skin of female experimental animals with Scl GVHD, but not in controls (Fig. 2). The presence of donor cells in the skin by day 14 post-BMT also correlates well with these data and with our previously published results showing increased levels of TGF-β1 by day 7, T cell and monocyte/macrophage infiltration into skin by day 14 by routine histology and flow cytometric analysis, detectable skin thickening by day 14, and subsequent collagen mRNA up-regulation by day 21 (1). We are examining the Ag-presenting capability of cells infiltrating skin in this model in separate experiments. If donor mononuclear cells are involved in initiating Scl GVHD, can disease be effectively treated by the administration of specific Abs or antagonists directly or indirectly against the infiltrating effector cells and their functions? Possible antagonists include Abs to activation/homing markers of monocytes (VLA-4, CD11b), integrins on endothelial cells, and blocking peptides or molecules to abrogate immune cell signaling. Is fibrosing disease reversible? Can disease be transmitted and accelerated by adoptive transfer of cells?

### Macrophage scavenger receptors and autoimmunity

Macrophage scavenger receptors are a diverse family of proteins that bind a wide variety of ligands (reviewed in Ref. 29). ScR-A are pattern-recognition receptors that have an intrinsic ability to recognize specific elements unique and essential to self-vs-nonself discrimination (29, 30). ScR-A have been implicated in the recognition and phagocytosis of apoptotic thymocytes and have been strongly implicated in contributing to the development of atherosclerotic plaques in heart disease (31, 32). ScR-A type I and II (detected with mAb 2F8) expression has been identified in marginal zone macrophages of spleen, alveolar macrophages, and macrophages of heart, gut, and cortical and medullary regions of thymus (29). MARCO expression is far more restricted and has been localized primarily to macrophages in splenic marginal zone area where active Ag presentation occurs. MARCO expression can be induced on tissue macrophages in response to inflammatory stimuli (33, 34). MARCO-expressing cells are highly phagocytic macrophages that have been implicated in Ag processing and the induction of anti-self immune responses due to their ability to present modified self-Ags. Ligation of macrophage ScR-A does not cause costimulatory molecule up-regulation, which could explain the minimal CD11b up-regulation in cutaneous macrophages in Scl GVHD (Table II) (33).

We have not yet determined whether ScR-A up-regulation during Scl GVHD is due to cutaneous influx of already activated immune cells or maturation of newly infiltrating monocytes to macrophages in skin, with ScR-A up-regulation on cutaneous cells during Ag presentation. Activation could occur very early after BMT, before our first time point at day 7 in these experiments. The up-regulation of ScR-A, however, in conjunction with an increase in I-A molecules (an MHC class II Ag) suggests that there may be active Ag presentation at early time points in cutaneous Scl GVHD. The involvement of scavenger receptors in this putative Ag presentation may explain the development of GVHD due to presentation of modified self-Ags by activated macrophages. The concomitant early events of macrophage ScR-A activation and extensive T cell and macrophage infiltration into skin is a critical area of investigation to identify steps that trigger autoimmunity in Scl GVHD and scleroderma. Both protein (including 2F8 Ab) and nonpeptide ScR-A inhibitors have been described that will be interesting to test in our murine Scl GVHD model (35, 36).
TGF-β2, and -β3 may also be involved in various fibrosing diseases (38, 39). However, our data demonstrate that TGF-β1 appears to be the critical isoform driving cutaneous fibrosis in Scl GVHD because TGF-β1 mRNA is elevated in animals with Scl GVHD compared with controls at the earliest time point (day 7 post-BMT), while the other isoforms are approximately equivalent. We have not looked at time points earlier than 7 days post-BMT, while the other isoforms are approximately equivalent. However, there could be a few potent TGF-β-producing cells at the early time points. Our experiments do not evaluate that possibility. Secondly, TGF-β1 itself is a very strong chemotactant for mononuclear cells, particularly monocyte/macrophages (40, 41) and fibroblasts, and may recruit monocyte/macrophages via chemotaxis and/or increased monocyte/matrix adhesion. In sites of inflammation, several types of cells, including monocyte/macrophages, T cells, and even fibroblasts, are able to produce TGF-β (42). Our data demonstrate that both cutaneous macrophages and T cells isolated by magnetic bead separation produce TGF-β1 mRNA in animals with Scl GVHD. We have not directly examined TGF-β mRNA production by fibroblasts and endothelial cells, but they are represented in the residual cells, which have no detectable TGF-β1 mRNA production by RT-PCR analysis (Fig. 6B). In early Scl GVHD, TGF-β1 produced by activated T cells and/or monocyte/macrophages activated by donor T cells may coordinate with MCP-1 and RANTES in attracting more monocyte/macrophages and T cells to skin. By blocking TGF-β with Abs, we may have also affected the chemotactant function of TGF-β. Those infiltrating cells responding to TGF-β1 are also capable of producing TGF-β1, as demonstrated by the increased mRNA expression by RT-PCR (Fig. 6B). Cutaneous fibroblasts are stimulated to synthesize collagens, thereby causing increased collagen deposition and skin fibrosis.

C-C chemokines may be involved in the pathogenesis of early Scl GVHD

Chemokines may be a potential new target for the treatment of fibrosing disease. Chemokines and recruitment of immune cells. It is well established that chemokines are produced locally in tissue and can selectively recruit different subsets of leukocytes to inflammatory sites (43). MCP-1, MIP-1α, and RANTES belong to the C-C chemokine family and attract mainly monocytes and T cells, respectively. In our studies (Fig. 5), C-C chemokines MCP-1, MIP-1α, and RANTES mRNA are elevated in experimental animals with Scl GVHD at a very early time point (day 7 post-BMT) before significant numbers of CD45+ immune cells, including monocytes, infiltrate skin. At later time points (days 14 and 21 post-BMT), MCP-1 and MIP-1α mRNA remain elevated and show further increases, paralleled by a significant increase in the numbers of monocyte/macrophages infiltrating skin and subsequent skin thickening. Our data suggest the biologic relevance of these chemokines in fibrosis. In contrast, the early up-regulation of RANTES mRNA is followed by a decrease on day 21 post-BMT, in almost a mirror image pattern compared with monocyte chemokines. This is an intriguing observation, suggesting interplay between early T cell activation of monocytes, then possible dampening of the early T cell effect and replacement by a monocyte effect as the disease progresses. We are exploring this hypothesis in separate experiments.

Chemokine effects on matrix deposition. Chemokines may also affect the homeostasis of extracellular matrix itself. MCP-1 and RANTES may be involved in the fibrotic pathway by modulating collagen turnover or type I and IV collagen deposition directly (by sending signals to fibroblasts via macrophages) (7, 11) or indirectly through the stimulation of TGF-β (44) (Fig. 1).

Chemokine interactions with TGF-β. Our data show that mRNA levels of TGF-β1, MCP-1, and RANTES are increased at early time points in Scl GVHD (Fig. 5). TGF-β1 can induce the up-regulation of MCP-1 and RANTES expression at both mRNA and protein levels in vitro and in vivo. MCP-1 and MIP-1α can increase the secretion of TGF-β from macrophages, which, in turn, increases the expression of collagen types I and III (45–47), suggesting that complex interactions between these C-C chemokines and TGF-β1 may occur in our model, as in other inflammatory conditions.

In summary, TGF-β1 is one of the most important cytokines in stimulating collagen synthesis and matrix deposition; however, fibrosis is a complex process that may involve multiple cytokines and chemokines, for which the interactions are incompletely understood.

Effects of anti-TGF-β Ab in Scl GVHD

Polyclonal anti-TGF-β Abs that block all TGF-β isoforms appear to have complex and multiple effects in Scl GVHD when administered early in disease. We have shown that when mononuclear cell (mainly monocytes and T cells) migration into skin is blocked, up-regulation of macrophage activation markers (ScR-A and I-A) is decreased, TGF-β1 mRNA levels are not elevated, type I collagen mRNA and protein synthesis are reduced, and skin thickening does not occur. This inhibition of fibrosis may occur via chemokine-TGF-β interactions, monocyte/macrophage activation, and/or monocyte homing or at multiple levels. The Scl GVHD model provides a unique opportunity to further investigate these pathways in vivo, to better understand monocyte/macrophage function, and to design novel interventions for fibrosing diseases, including scleroderma and Scl GVHD.

Other potential inhibitors of fibrosis and treatments for scleroderma

In addition to TGF-β, C-C chemokines, particularly MCP-1 and RANTES, may be actively involved in Scl GVHD by attracting monocyte/macrophages and T cells into skin and possibly interacting with TGF-β1, thereby affecting collagen deposition and contributing to the progression of fibrosis. Thus, blocking Abs or peptide antagonists to MCP-1, RANTES, or C-C chemokines and chemokine receptor inhibitors may be potential new therapies for mononuclear cell-driven progressive fibrosing diseases such as Scl GVHD and scleroderma. A better understanding of the roles of these effector cells (T cells and monocytes) may be useful in predicting the course of the disease as well.

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