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Immunopathology and Infectious Diseases

B-Lymphocyte Depletion Reduces Skin Fibrosis and Autoimmunity in the Tight-Skin Mouse Model for Systemic Sclerosis

Minoru Hasegawa,* Yasuhito Hamaguchi,† Koichi Yanaba,† Jean-David Bouaziz,† Junji Uchida,† Manabu Fujimoto,* Takashi Matsushita,* Yukiyo Matsushita,* Mayuka Horikawa,* Kazuhiro Komura,* Kazuhiko Takehara,* Shinichi Sato,‡ and Thomas F. Tedder†

From the Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; the Department of Dermatology,‡ Nagasaki University Graduate School of Biomedical Science, Nagasaki, Japan; and the Department of Immunology,† Duke University Medical Center, Durham, North Carolina*

Systemic sclerosis (scleroderma) is an autoimmune disease characterized by excessive extracellular matrix deposition in the skin. A direct role for B lymphocytes in disease development or progression has remained controversial, although autoantibody production is a feature of this disease. To address this issue, skin sclerosis and autoimmunity were assessed in tight-skin mice, a genetic model of human systemic sclerosis, after circulating and tissue B-cell depletion using an anti-mouse CD20 monoclonal antibody before (day 3 after birth) and after disease development (day 56). CD20 monoclonal antibody treatment (10 to 20 μg) depleted the majority (85 to 99%) of circulating and tissue B cells in newborn and adult tight-skin mice by days 56 and 112, respectively. B-cell depletion in newborn tight-skin mice significantly suppressed (~43%) the development of skin fibrosis, autoantibody production, and hypergammaglobulinemia. B-cell depletion also restored a more normal balance between Th1 and Th2 cytokine mRNA expression in the skin. By contrast, B-cell depletion did not affect skin fibrosis, hypergammaglobulinemia, and autoantibody levels in adult mice with established disease. Thereby, B-cell depletion during disease onset suppressed skin fibrosis, indicating that B cells contribute to the initiation of systemic sclerosis pathogenesis in tight-skin mice but are not required for disease maintenance. *(Am J Pathol 2006, 169:954–966; DOI: 10.2353/ajpath.2006.060205)*

Systemic sclerosis (SSc, scleroderma) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs.[1,2] Although the molecular basis for SSc pathogenesis is unknown, hypergammaglobulinemia, polyclonal and memory B-cell hyperactivity, and altered B-cell homeostasis are found in SSc patients,[3–5] with B-cell-associated transcripts up-regulated in lesional skin.[6] Disease-specific autoantibodies reactive with DNA topoisomerase I, RNA polymerases, and fibrillin-1 further suggests that activated B cells contribute to disease pathogenesis.[7–10] Moreover, B cells from SSc patients overexpress CD19,[11] a signal transduction molecule that regulates B-cell responses to self and foreign antigens. A *CD19* gene polymorphism also correlates with SSc susceptibility.[12] Despite these findings, a role for B cells in SSc pathogenesis has remained controversial.[13]

The tight-skin ($Tsk^{/+}$) mouse serves as a model for SSc,[14] with increased synthesis and accumulation of collagen and other extracellular matrix proteins in the skin.[15] Homozygous mice die *in utero*, whereas heterozygous $Tsk^{/+}$ mice survive but develop skin fibrosis.[16] A tandem duplication within the *fibrillin-1* gene causes tissue hyperplasia and the SSc-like phenotype.[17–21] However, there is also an immunological component to disease pathogenesis because $CD4^+$ T

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S.S. and T.F.T. share senior authorship.

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Address reprint requests to Thomas F. Tedder, Box 3010, Department of Immunology, Room 353 Jones Building, Research Dr., Duke University Medical Center, Durham, NC 27710. E-mail: thomas.tedder@duke.edu.

cells, mast cells, and other immunocompetent cells contribute to skin fibrosis in Tsk$^{/+}$ mice.[22–25] For example, the infusion of bone marrow and spleen cells from tight-skin mice into normal mice induces a Tsk-like cutaneous phenotype and autoantibodies.[22,26] Importantly, the adoptive transfer of enriched B or T cells alone into normal syngeneic mice does not cause skin fibrosis, whereas B cells alone increased autoantibody production.[22] T helper 2 (Th2) cells and T-cell-secreted profibrogenic cytokines contribute specifically to the fibrotic processes in scleroderma.[23,27,28] By contrast, CpG oligodeoxynucleotide or interleukin (IL)-12 inhibit skin sclerosis in Tsk$^{/+}$ mice by stimulating a Th1 immune response.[29,30] Transforming growth factor (TGF)-β and IL-4 may contribute directly to skin fibrosis because they induce hyperresponsive collagen production in Tsk$^{/+}$ fibroblasts.[28,31] Reciprocally, skin fibrosis is prevented in Tsk$^{/+}$ mice bearing IL-4, IL-4 receptor, STAT6, or TGF-β gene mutations or by the administration of anti-IL-4 monoclonal antibody (mAb) to newborn Tsk$^{/+}$ mice.[27,28,32–34] Disrupting IL-4 rescues mice homozygous for the tight-skin mutation from embryonic death and also diminishes TGF-β production by fibroblasts.[33] The phenotypic characteristics of SSc patients and Tsk$^{/+}$ mice are similar, except Tsk$^{/+}$ mice have pulmonary emphysema and cardiac hypertrophy[14] that are not inhibited by anti-IL-4 mAb, the absence of CD4$^+$ T cells, or IL-4, IL-4 receptor, TGF-β, or Stat6 deficiencies.[23,27,32,34] Tsk$^{/+}$ B cells also display a hyperresponsive phenotype, with enhanced CD19-induced $[Ca^{2+}]_i$ responses, higher levels of CD19 tyrosine phosphorylation,[13,35] impaired CD22 regulation of signal transduction,[36] and the production of autoantibodies against SSc-specific target autoantigens, such as topoisomerase I, RNA polymerase I, and fibrillin-1.[37,38] There is also a correlation between the concentration of serum anti-topoisomerase I autoantibodies in Tsk$^{/+}$ mice and histological and biochemical alterations in the skin.[39] Likewise, human autoantibodies to fibrillin-1 activate normal human fibroblasts in culture through the TGF pathway to recapitulate the scleroderma phenotype.[10] Thereby, CD19 deficiency in Tsk$^{/+}$ mice down-regulates B-cell function, improves skin sclerosis (~36%), and inhibits autoimmunity.[35] However, whether B cells initiate, contribute to disease progression, or play a role in the maintenance of established disease remains unresolved.

B-cell depletion using CD20 mAb-based immunotherapy is an effective treatment for rheumatoid arthritis and other autoimmune diseases,[40] but it has not been examined in SSc patients in which current therapies do not improve or suppress the development of skin sclerosis. Likewise, therapeutic strategies that improve skin sclerosis in adult Tsk$^{/+}$ mice with disease have not been identified. Therefore, a role for B cells in SSc pathogenesis was assessed in Tsk$^{/+}$ mice using mouse anti-mouse CD20 mAbs that provide a preclinical test for B-cell depletion immunotherapy that is amenable to mechanistic studies and genetic manipulation.[41–44]

Materials and Methods

Immunotherapy

Tsk$^{/+}$ and wild-type littermates were generated by crossing Tsk$^{/+}$ males (C57BL/6; Jackson Laboratory, Bar Harbor, ME) with wild-type females. CD20$^{-/-}$ mice were as described.[41] Female mice were given 5 to 250 μg of sterile IgG2a (MB20-11) anti-mouse CD20 mAb[41] or isotype-matched control mAb in PBS. In most cases, mice were injected with mAb every 2 weeks, for a total of four treatments. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science or the Duke University Animal Care and Use Committee.

Flow Cytometry Analysis

Antibodies used included phycoerythrin (PE)-conjugated CD19 (MB19-1)[45] mAb; fluorescein isothiocyanate-conjugated B220, CD3, CD4, CD8, and NK1.1 mAbs; and Cy5-conjugated mAb (all from BD Pharmingen, San Diego, CA). Thy1.2 mAb (Caltag, Burlingame, CA) was used for T-cell enumeration. For mouse CD20 expression, biotin-conjugated mouse anti-mouse CD20 (MB20-18)[41] mAb was used, with biotin-conjugated anti-human CD20 (B1) mAb (Beckman-Coulter, Hialeah, FL) used as a negative control and streptavidin PE used as a developing reagent (Southern Biotechnology, Birmingham, AL). For two- or three-color immunofluorescence analysis, single-cell lymphocyte suspensions (0.5 to 1 $\times$ 10^6) were stained at 4°C using predetermined optimal concentrations of mAb for 20 minutes as described.[46] Cells with the forward and side light scatter properties of lymphocytes were analyzed on a FACScan flow cytometer (BD Immunocytometry Systems, San Diego, CA).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Anti-nuclear antibodies were assessed using HEp-2 substrate cells (Medical and Biological Laboratories, Nagoya, Japan) as described.[11] Antibody and autoantibody levels were determined by ELISA as described.[11,47] Topoisomerase I antibody levels were quantified using Medical and Biological Laboratories ELISA kits. Serum interferon (IFN)-γ and IL-4 levels were quantified using R&D Systems ELISA kits (Minneapolis, MN). Recombinant peptide representing the proline-rich C-terminal region (amino acids 395 to 446) of fibrillin-1 was generated as described[9,38] and used as the substrate for fibrillin-1-specific ELISAs as described.[35]

Skin Fibrosis and Tissue Pathology

Skin was taken from the para-midline, lower back region as full thickness sections extending down to the body

wall musculature. Tissues were fixed in 10% formaldehyde solution for 24 hours, embedded in paraffin, cut into sections, stained with hematoxylin and eosin (H&E), and independently examined by three investigators in a blinded manner. Hypodermal thickness, defined by depth of the subcutaneous loose connective tissue layer (ie, the hypodermis or superficial fascia) beneath the panniculus carnosus, was measured for multiple transverse perpendicular sections using an ocular micrometer. Dermal thickness was defined as the thickness of skin from the top of the granular layer to the junction between the dermis and subcutaneous fat. Ten random measurements were taken per tissue section. To quantify hydroxyproline (collagen) content, 6-mm punch biopsies from shaved dorsal skin were treated with chloroform/methanol (2:1, v/v) to remove fat, dried by centrifugation under vacuum, weighed, acid-hydrolyzed for 24 hours at 110°C, dried, redissolved in 200 μl of water, and filtered through Millipore filters. Samples (20-μl aliquots) were diluted 10-fold and analyzed for composition in an amino acid analyzer (Hewlett-Packard, Palo Alto, CA). Lungs inflated after harvesting and hearts were fixed in 10% formaldehyde solution, embedded in paraffin, and stained with H&E.

Cytokine Expression

B-cell-enriched splenocyte preparations (≥93% B220$^+$) were generated by depleting Thy1.2 mAb-coated cells using magnetic beads (Dynal Biotech, Oslo, Norway). Total RNA from full-thickness skin sections, splenocyte suspensions, or B16 F10 mouse melanoma cells (American Type Culture Collection, Manassas, VA) was converted into cDNA using a reverse transcription system (Promega, Madison, WI). Transcript levels were quantified by real-time polymerase chain reaction (PCR) using sequence-specific primers and probes designed by predeveloped TaqMan assay reagents (one cycle of 50°C for 2 minutes, 95°C for 10 minutes; 40 cycles of 92°C for 15 seconds, 60°C for 60 seconds) and an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). The relative expression of target transcripts and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript PCR products was determined using the ΔΔCt method[48] using cytokine mRNA levels in wild-type mice as calibrators in triplicate assays with the mean Ct used for calculations.

Statistical Analysis

The Mann-Whitney *U*-test was used for verifying significant differences between sample means and Bonferroni's test was used for multiple comparisons.

Results

B-Cell Depletion

Mature CD20$^+$ B cells are significantly reduced in wild-type mice by day 2 after intravenous treatment with CD20 mAb (MB20-11, 250 μg/mouse).[42,43] We therefore determined whether subcutaneous, intravenous, and intraperitoneal treatment depleted B cells to the same extent throughout a range of mAb concentrations in 2-month-old wild-type mice. Single mAb doses as low as 5 μg given intravenously, intraperitoneally, or subcutaneously depleted most mature bone marrow, blood, spleen, and lymph node B cells after 7 days as determined by immunofluorescence staining with flow cytometry analysis (Figure 1A; data not shown). Small numbers of CD19^{low} B220$^+$ B cells persisted within the blood, spleen, and lymph nodes after CD20 mAb treatment (Figure 1B). Such cells predominantly represented cells with a pre-B/immature B-cell phenotype and were likely to be recent emigrants from the bone marrow as described.[43] However, subcutaneous, intravenous, or intraperitoneal CD20 mAb treatments did not significantly alter the frequency or apparent composition of these residual B220$^+$ B-cell populations. After CD20 mAb (5 μg) treatment, the pharmacokinetics of CD20 mAb treatments varied slightly during the first 15 to 60 minutes after mAb administration depending on the route of administration, but mAb clearance and B-cell reconstitution were similar between subcutaneous and intravenous treatments, with increased numbers of circulating and tissue B220$^+$ cells first observed at 2 weeks (not shown; Figure 1C).

The effectiveness of subcutaneous CD20 mAb treatment was examined using newborn mice, in which intravenous treatment is not possible. Skin sclerosis in Tsk$^{/+}$ mice may start immediately after birth, but skin tightness is recognizable by 7 days after birth and develops fully by ~2 months of age.[14,49] When 3-day-old wild-type and Tsk$^{/+}$ mice were treated with MB20-11 mAb (subcutaneously, 10 μg/mouse), mature CD19$^+$B220$^+$ spleen B-cell numbers were reduced by 85 to 90% 7 days later. Treating 8-week-old wild-type and Tsk$^{/+}$ littermates with MB20-11 mAb (20 μg s.c.) reduced CD19$^+$ B220$^+$ B-cell numbers by 94 to 98% in the bone marrow, blood, spleen, and lymph nodes 7 days later, while circulating and tissue T-cell numbers were unchanged. Isotype-control mAb treatments did not affect circulating or tissue B-cell numbers. Therefore, B-cell depletion was effective in Tsk$^{/+}$ littermates when CD20 mAb was given subcutaneously at low doses.

The effectiveness of long-term B-cell depletion in newborn Tsk$^{/+}$ mice was assessed by MB20-11 mAb (10 or 20 μg) treatment on day 3 after birth, with repeated treatment on days 17, 31, and 45. At 8 weeks of age, the majority of circulating, spleen, and lymph node CD19$^+$ B220$^+$ B cells were depleted to similar extents in both Tsk$^{/+}$ and wild-type littermates (85 to 99%; Figure 1B and Table 1), without affecting T-cell numbers or the relative ratios of circulating or spleen CD4$^+$/CD8$^+$ T cells (data not shown). Small numbers of B220$^+$ B cells persisted in Tsk$^{/+}$ and wild-type littermates even with 20-μg mAb doses, 1 to 3% for blood and 5 to 6% for spleen and lymph nodes (Table 1). The residual B cells present in both Tsk$^{/+}$ and wild-type littermates predominantly represented B220$^+$ cells with a CD19^{low} pre-B/immature B-cell phenotype (Figure 1B) as described.[43] Thus, B-cell depletion was effective for the entire treatment pe-

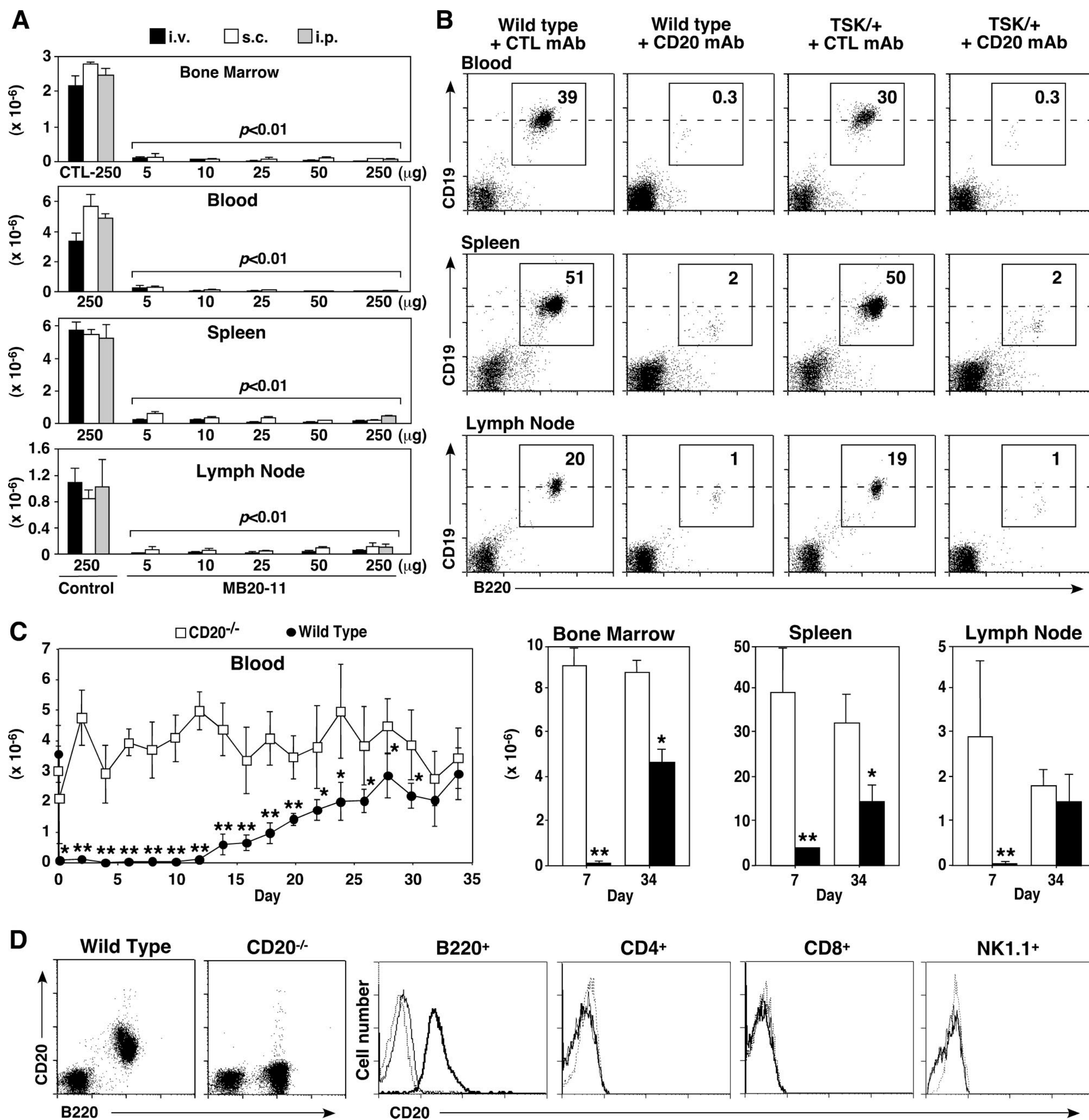

Figure 1. Equivalent B-cell depletion after intravenous, subcutaneous, or intraperitoneal CD20 mAb treatments. **A:** Two-month-old wild-type mice (two to three mice per dose) were given CD20 mAb at the dose indicated or were treated with an isotype-matched control (CTL, 250 μg) mAb. Seven days after mAb administration, tissue B-cell ($B220^+$) numbers were quantified after immunofluorescence staining with flow cytometry analysis. Bone marrow mature B-cell (IgM^+B220^{hi}) numbers were from bilateral femurs. Blood B cells were $B220^+$ cells/ml. Lymph node B cells were from bilateral inguinal and axillary node pairs. **B:** Equivalent B-cell depletion in $Tsk^{/+}$ and wild-type littermates after CD20 mAb treatment. Representative blood, spleen, and peripheral lymph node B-cell frequencies after CD20 or isotype-matched control (CTL) mAb treatment as determined by immunofluorescence staining with flow cytometry analysis. Mice were treated subcutaneously with mAb (20 μg) on day 3 after birth, with repeated treatment on days 17, 31, and 45. Numbers indicate the percentage of gated $B220^+CD19^+$ B cells present in 56-day-old mice. **C:** Duration of B-cell depletion in 2-month-old wild-type (**closed circles** and **bars**) and $CD20^{-/-}$ (**open circles** and **bars**) littermates (five mice per group) given 5 μg of CD20 mAb intravenously on day 0. Tissue B-cell ($B220^+$) numbers were quantified on the days indicated as in **A**. Significant differences between sample means are indicated: $^*P < 0.05$; $^{**}P < 0.01$. **D:** Representative CD20 expression by blood B, T, and NK cells from 2-month-old wild-type and CD20-deficient ($CD20^{-/-}$) mice (≥3 mice). $B220^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, and NK ($NK1.1^+CD3^-$) cells were assessed by two- or three-color immunofluorescence staining using the MB20-18 anti-mouse CD20 mAb (thick line) with flow cytometry analysis. Data are shown as representative dot plots ($B220^+$ cells) and histograms (B, T, and NK cells). Isotype-matched control mAb staining (dashed line) or results from leukocytes of $CD20^{-/-}$ mice stained with MB20-18 mAb (thin line) are shown as negative controls.

riod, with no indication of differences in CD20 mAb effectiveness between $Tsk^{/+}$ and wild-type littermates. Furthermore, B-cell depletion had no obvious effects on the normal development, maturation, or connective tissues of juvenile mice because no morphological changes or alterations in movement or development were detected in mice as determined by observation and microscopic examination of tissue sections.

Table 1. Tissue B-Cell Depletion after CD20 mAb Treatment

		$CD19^+B220^+$ B cell numbers ($\times 10^{-6}$)*	
Mouse genotype	mAb, dose	56-Day-old mice† (% depletion)	
		Blood	Spleen
Wild type	CTL, 20 μg	3.9 ± 0.3	42.5 ± 4.1
Wild type	CD20, 10 μg	0.39 ± 0.02 (90%)	5.4 ± 0.4 (87%)
Wild type	CD20, 20 μg	0.1 ± 0.03 (97%)	2.5 ± 0.3 (94%)
Tsk/+	CTL, 20 μg	4.1 ± 0.4	44.1 ± 3.7
Tsk/+	CD20, 10 μg	0.45 ± 0.03 (89%)	6.5 ± 0.3 (85%)
Tsk/+	CD20, 20 μg	0.04 ± 0.01 (99%)	2.4 ± 0.2 (95%)

*Values (±SEM) indicate cell numbers present in tissues of ≥5 mice for each group: blood shown as cells/ml, and lymph node as pooled bilateral inguinal and axial lymph nodes.
†Control (CTL) or CD20 mAb given subcutaneously on days 3, 17, 31, and 45, with tissues harvested on day 56.
‡Control (CTL) or CD20 mAb given subcutaneously on days 56, 70, 84, and 98, with tissues harvested on day 112.

(*table continues*)

T and NK Cell Expression of Mouse CD20

Although mouse CD20 expression by B-cell subsets has been examined extensively, its expression by other leukocyte subsets has only been partially assessed.[41] Moreover, small subpopulations of human T cells and NK cells have been reported to express CD20 at low levels as determined by immunofluorescence staining with flow cytometry analysis.[50–53] Therefore, CD20 expression by mouse T cells and NK cells was assessed. $B220^+$ cells represented the predominant, if not exclusive, source of CD20 expression among blood leukocytes from wild-type mice, whereas there was no mAb reactivity with leukocytes from CD20-deficient mice (Figure 1D). $CD4^+$ T cells, $CD8^+$ T cells, and $NK1.1^+$ cells did not express CD20 at detectable levels in wild-type mice. In fact, CD20 mAb staining was equivalent for T and NK cells from wild-type and CD20-deficient mice. Thus, CD20 expression appears to be B-cell restricted, consistent with the selective depletion of B cells after mAb treatment.

B-Cell Depletion Suppresses Skin Fibrosis in Tsk/+ Mice

The benefit of B-cell depletion in newborn Tsk/+ mice was assessed by MB20-11 or control mAb (10 or 20 μg) treatment on day 3 after birth, with repeated treatments on days 17, 31, and 45. Skin fibrosis in B-cell-depleted Tsk/+ mice was then assessed by histopathology of skin sections from 56-day-old mice. In Tsk/+ mice, hypodermal thickness was increased eightfold because of hyperplasia of the subcutaneous loose connective tissue layer (Figures 2 and 3A) as reported.[35] Dermal thickness from the top of the granular layer to the junction between the dermis and subcutaneous fat was similar between control mAb-treated Tsk/+ (50 ± 4 μm) and wild-type (52 ± 5 μm) littermates. Subcutaneous fat and panniculus carnosus thickness was also similar between Tsk/+ and wild-type littermates. However, CD20 mAb treatment reduced mean dermal and hypodermal thickness in Tsk/+ mice by 31% (10 μg, $P < 0.05$) and 43% (20 μg, $P < 0.01$), respectively, compared with control mAb treatment, although these tissues were significantly thicker than those of control mAb-treated wild-type littermates ($P < 0.01$, Figure 3A). B-cell depletion in wild-type mice did not affect dermal or hypodermal thickness.

Skin fibrosis was also assessed by quantifying the hydroxyproline content of skin biopsies. Hydroxyproline content was increased by 42% in 56-day-old Tsk/+ mice relative to wild-type littermates ($P < 0.01$, Figure 3A). However, CD20 mAb treatment (20 μg) reduced hydroxyproline content by half in Tsk/+ mice ($P < 0.01$), although skin hydroxyproline content remained significantly higher than in wild-type littermates ($P < 0.05$). Hydroxyproline content was also significantly ($P < 0.05$) reduced in Tsk/+ mice treated with 10 μg of CD20 mAb. Despite this, CD20 mAb treatment (10 or 20 μg) did not significantly affect the onset or severity of pulmonary emphysema in Tsk/+ mice because their alveoli remained three to four times larger than in control mice, as described.[32] Thus, early B-cell depletion suppressed skin fibrosis in Tsk/+ mice.

B-Cell Depletion Does Not Reverse Skin Fibrosis in Tsk/+ Mice

Whether untreated or treated with control mAb, Tsk/+ mice developed significant skin fibrosis and maximal skin thickness by ~56 days of age (Figures 2 and 3A; data not shown). To identify time points in disease progression when B-cell depletion could inhibit or reverse disease, 14-, 28-, and 56-day-old Tsk/+ mice were treated with CD20 mAb (20 μg) bi-weekly until days 70, 84, or 112. Thickness and hydroxyproline content of skin from Tsk/+ mice was significantly reduced when they were treated with CD20 mAb from day 14 until day 70 (Figure 3B, $P < 0.05$). However, there was no significant difference in skin thickness for mice treated during days 28 to 84 (Figure 3C). Thus, B-cell depletion remained effective for reducing skin fibrosis between days 14 and 28. To assess whether B-cell depletion could reverse disease, 56-day-old Tsk/+ mice were treated with CD20 mAb on days 56, 70, 84, and 98. By day 112, B-cell depletion was equally effective in wild-type and Tsk/+ littermates, with small numbers of $CD19^{low}$ $B220^+$ B cells persisting in

Table 1. Continued

CD19$^+$B220$^+$ B cell numbers ($\times 10^{-6}$)*			
	112-Day-old mice‡ (% depletion)		
Lymph node	Blood	Spleen	Lymph node
1.1 ± 0.1	4.4 ± 0.6	42.6 ± 3.9	1.2 ± 0.2
1.0.15 ± 0.01 (86%)	ND	ND	ND
0.07 ± 0.01 (94%)	0.10 ± 0.03 (98%)	1.5 ± 0.2 (96%)	0.07 ± 0.01 (94%)
1.3 ± 0.2	4.8 ± 4.0	49.2 ± 4.1	1.3 ± 0.2
1.0.19 ± 0.02 (85%)	ND	ND	ND
0.07 ± 0.01 (95%)	0.10 ± 0.02 (98%)	1.7 ± 0.1 (97%)	0.06 ± 0.01 (95%)

Tsk$^{/+}$ and wild-type littermates, 2% for blood, 3 to 4% for spleen, and 5 to 6% for lymph nodes (Table 1). Because the extent of B-cell depletion was similar, if not identical, between Tsk$^{/+}$ and wild-type littermates (Table 1) there was no indication of differences in CD20 mAb effectiveness between mice with and with-

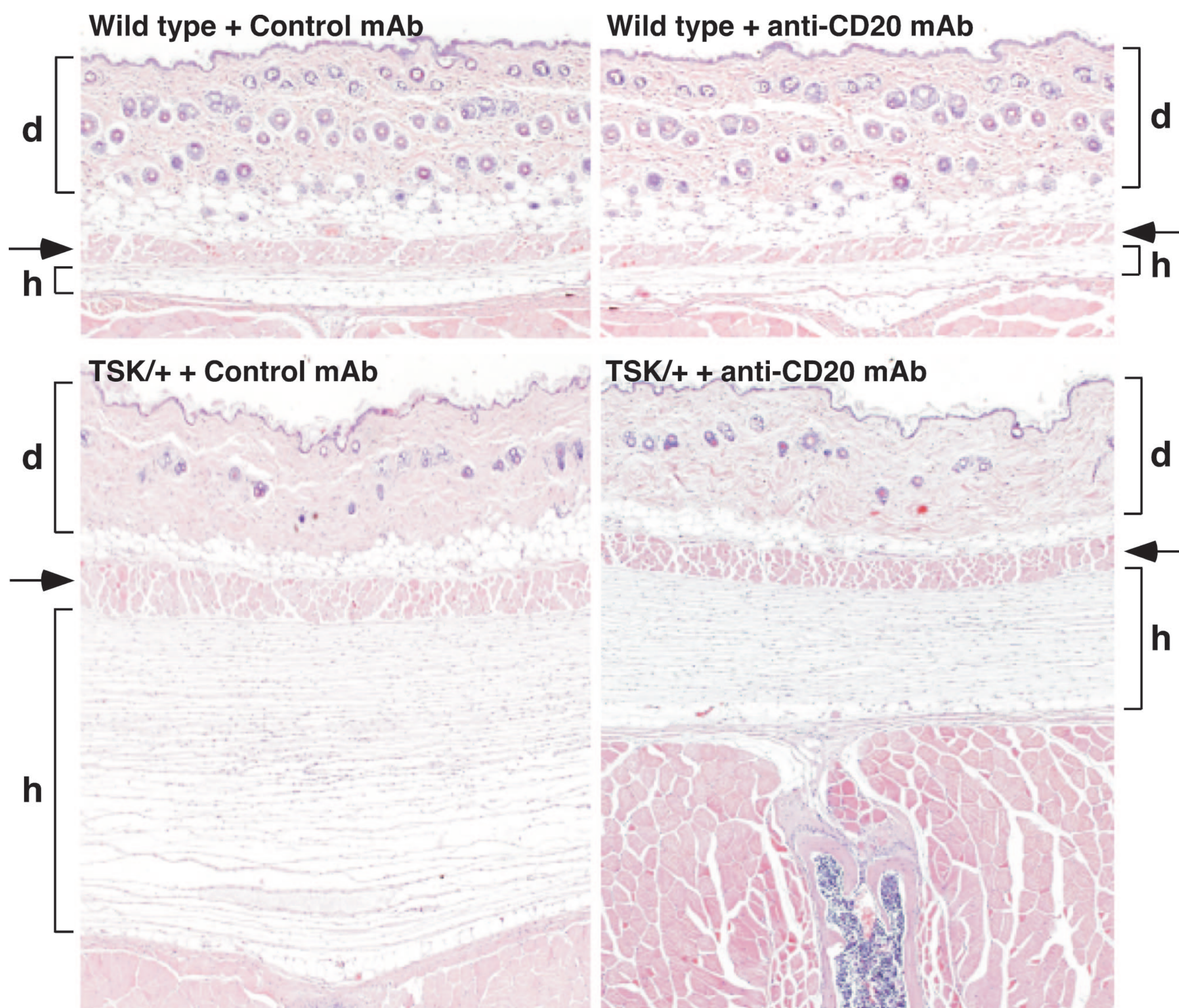

Figure 2. Fibrosis of dorsal skin from 56-day-old Tsk$^{/+}$ and wild-type littermates treated with CD20 or isotype-matched control (CTL) mAb. Mice were given mAb (20 μg) subcutaneously on day 3 after birth, with repeated treatment on days 17, 31, and 45. Representative H&E-stained histological sections. Dermis is indicated by (d), with the loose connective tissue layer (ie, the hypodermis or superficial fascia) beneath the panniculus carnosus (**arrow**) indicated by (h). Results represent those obtained with ≥5 mice of each group. Original magnifications, ×40.

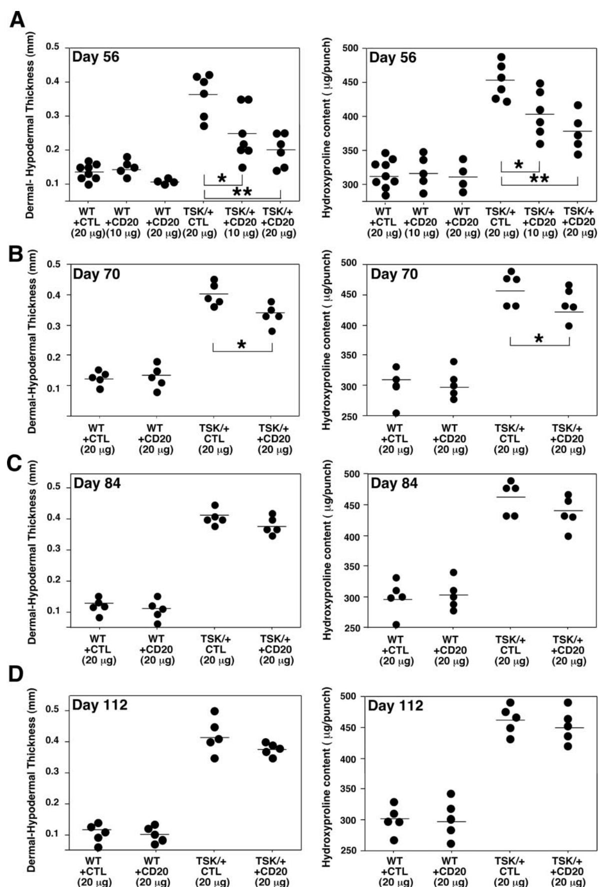

Figure 3. Fibrosis of dorsal skin from Tsk/+ and wild-type littermates treated with CD20 or isotype-matched control (CTL) mAb. **A:** Dermal and hypodermal thickness and hydroxyproline content of 6-mm skin punch biopsies from mice given mAb subcutaneously on day 3 after birth, with repeated treatment on days 17, 31, and 45 with tissues harvested on day 56 as described in Figure 2. **B:** Littermates were given mAb subcutaneously on days 14, 28, 42, and 56 after birth, with 6-mm skin punch biopsies harvested on day 70. **C:** Littermates were given mAb subcutaneously on days 28, 42, 56, and 70 after birth, with 6-mm skin punch biopsies harvested on day 84. **D:** Littermates were given mAb subcutaneously on days 56, 70, 84, and 98 after birth, with tissues harvested on day 112. All values represent results from individual mice with horizontal bars representing means. Significant differences between sample means are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

out disease. CD20 mAb treatment did not affect T-cell numbers (data not shown). Regardless, CD20 mAb treatment did not significantly affect fibrosis or the hydroxyproline content of skin biopsies from 112-day-old Tsk/+ mice (Figure 3D). Likewise, B-cell depletion did not significantly affect emphysema severity in Tsk/+ mice (not shown). Myocardial hypertrophy in ~4-month-old Tsk/+ and wild-type mice was indistinguishable from that of mice with comparable heart weights (129 ± 15 mg versus 118 ± 14 mg, respectively; n = 5). CD20 mAb treatment did not affect heart size or total weight in wild-type or Tsk/+ mice (119 ± 12 mg versus 125 ± 13 mg, respectively; n = 5). Thus, B-cell depletion can suppress the development of skin sclerosis early after disease development but does not alter established sclerosis or emphysema in Tsk/+ mice.

B-Cell Depletion Inhibits Autoantibody Generation in Tsk/+ Mice

Although a pathogenic role for autoantibodies in SSc development remains uncertain,[10] autoantibody and serum immunoglobulin levels were assessed in Tsk/+ mice as markers for effective B-cell depletion and hyperactivity. B-cell depletion starting on day 3 significantly inhibited both IgM and IgG anti-fibrillin 1 autoantibody production in 56-day-old mice treated with 20 µg of CD20 mAb (Figure 4A). However, IgM or IgG anti-fibrillin 1 autoantibody levels were not significantly altered when B-cell depletion was initiated in 56-day-old mice (Figure 4B). Serum anti-nuclear antibody generation was also assessed in 56-day-old Tsk/+ and wild-type littermates treated repeatedly with CD20 mAb since day 3 after birth. Anti-nuclear antibodies with a homogenous chromosomal staining pattern were detected in 33% (5 of 15) of Tsk/+ mice treated with control mAb. However, anti-nuclear antibodies were rarely detected in B-cell-depleted Tsk/+ mice (20 µg of CD20 mAb, 1 of 15; 10 µg, 1 of 15), or wild-type mice treated with CD20 (20 µg, 0 of 15) or control (20 µg, 1 of 15) mAb. Similarly, 56-day-old Tsk/+ mice treated with control mAb produced significantly higher levels of rheumatoid factor and topoisomerase I- or single-stranded DNA-specific autoantibodies when compared with control mAb-treated wild-type littermates ($P < 0.05$, Figure 4C). However, rheumatoid factor and autoantibody levels were decreased significantly in B-cell-depleted Tsk/+ mice (10 or 20 µg of CD20 mAb) relative to control mAb-treated Tsk/+ littermates when assessed by ELISA (Figure 4C) or immunoprecipitation assays (data not shown). By contrast, initiating B-cell depletion in 56-day-old Tsk/+ mice did not significantly affect rheumatoid factor or anti-topoisomerase I and anti-DNA autoantibody levels by day 112 (Figure 4D). Thus, B-cell depletion dramatically inhibited autoantibody production in Tsk/+ mice when started early, but not after disease development.

B-cell depletion significantly reduced serum antibody levels in both Tsk/+ and wild-type littermates when first given 3 days after birth (Figure 4E). Tsk/+ mice treated with control mAb had significantly elevated IgM, IgG1, IgG2a, and IgG2b levels compared with wild-type littermates ($P < 0.05$). However, Tsk/+ mice treated with CD20 mAb (10 or 20 µg) had significantly decreased IgM, IgG1, IgG2a, IgG2b, and IgG3 levels compared with control mAb-treated Tsk/+ littermates. In fact, IgG2a ($P < 0.05$), IgG2b ($P < 0.01$), and IgG3 ($P < 0.001$) levels in CD20 mAb-treated Tsk/+ mice were lower than those in control mAb-treated wild-type littermates. By contrast, B-cell depletion did not affect serum antibody levels in Tsk/+ or wild-type mice when CD20 mAb treatment was started 56 days after birth (Figure 4F). Thus, B-cell depletion inhibited the development of hypergammaglobulinemia in young Tsk/+ mice but did not reverse established hypergammaglobulinemia.

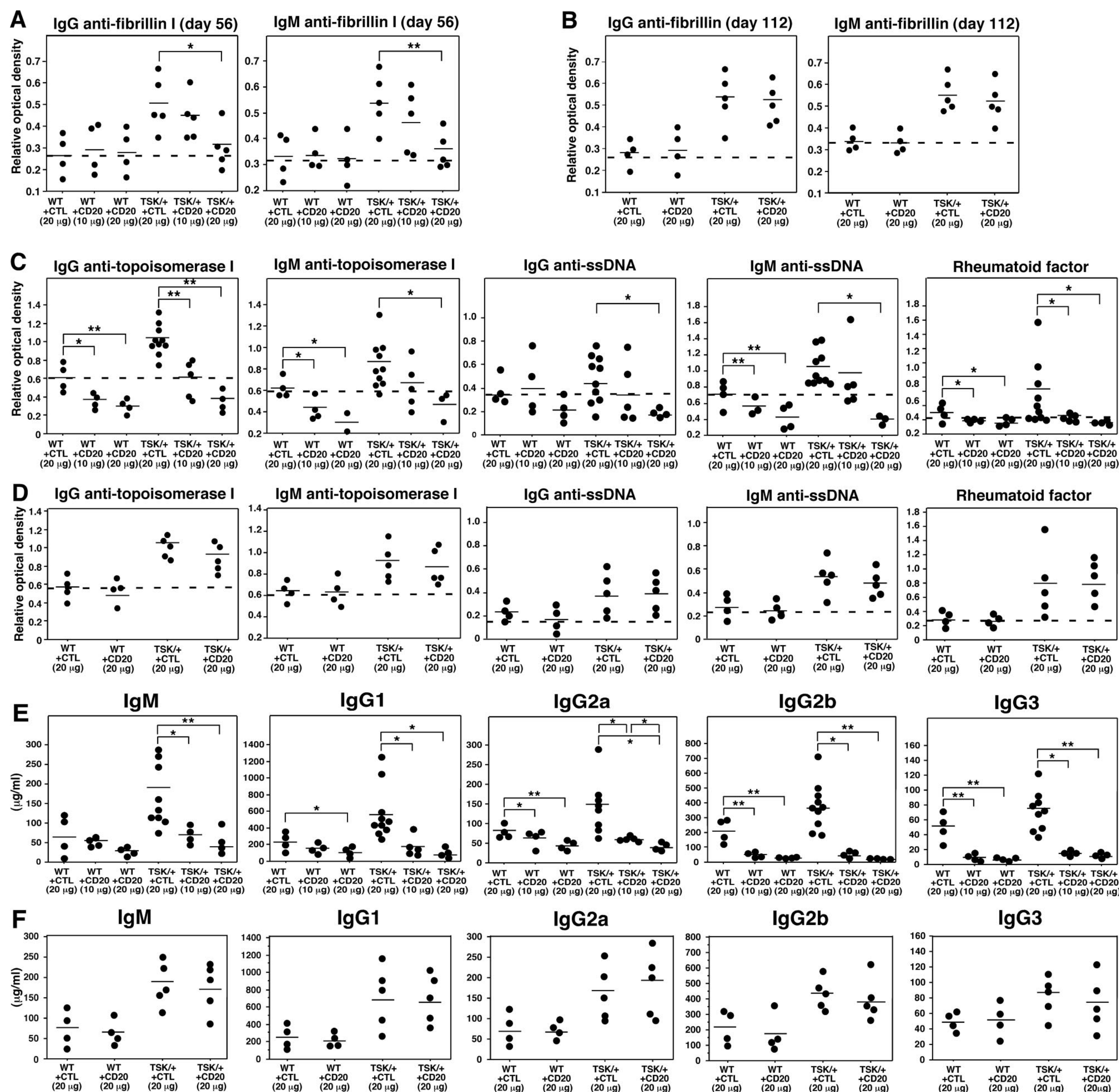

Figure 4. B-cell depletion inhibits autoantibody production and hypergammaglobulinemia in Tsk$^{/+}$ mice. Mice were treated with CD20 mAb (20 μg) bi-weekly (**A**, **C**, **E**) since day 3 after birth (days 3, 17, 31, and 45) and serum was harvested on day 56, or (**B**, **D**, **F**) since day 56 after birth (days 56, 70, 84, and 98) with serum harvested on day 112. Relative serum antibody, fibrillin-, topoisomerase I-, and single-stranded DNA-specific antibody and rheumatoid factor levels were determined by ELISA, with assay background results (+2 SDs) indicated by a horizontal dashed line. Horizontal bars represent mean autoantibody and antibody levels for each group. Significant differences between sample means are indicated: *$P < 0.05$; **$P < 0.01$.

Skin B-Cell Depletion in Tsk$^{/+}$ Mice

B-cell-associated transcripts are up-regulated in lesional skin from SSc patients.[6] It was therefore assessed whether CD20 mAb-mediated skin B-cell depletion might influence skin fibrosis in young Tsk$^{/+}$ mice. Tsk$^{/+}$ and wild-type littermates were treated with CD20 or control mAb (20 μg) at 3 and 17 days, with B-cell-associated transcripts quantified in skin from 3-week-old mice, a time in which blood and spleen CD19^{+} B220^{+} B cells were depleted by $> 93\%$. Transcripts for CD19, CD20, and light chains was compared with CD138 (syndecan-1) and T-cell CD3 transcript levels. CD138 is expressed by plasma cells, epithelial cells, vascular smooth muscle cells, endothelium, and neural cells. However, B- and T-cell transcripts were not detectable in the skin of Tsk$^{/+}$ and wild-type littermates by real-time PCR analysis compared with water-only control reactions or RNA from melanoma cells (Table 2, not shown). By contrast, CD138 transcripts were readily detected in the skin,

Table 2. Absence of B- and T-Cell Transcripts in the Skin of $Tsk^{/+}$ Mice

Sample	mAb	Relative mRNA expression					
		CD3	CD19	CD20	κ	λ	CD138
WT skin	CTL	4 ± 1	<1	4 ± 1	21 ± 3	2 ± 1	164 ± 9
WT skin	CD20	5 ± 1	<1	4 ± 1	20 ± 4	1 ± 1	164 ± 20
$Tsk^{/+}$ skin	CTL	5 ± 1	<1	3 ± 1	22 ± 2	2 ± 1	135 ± 14
$Tsk^{/+}$ skin	CD20	4 ± 1	<1	2 ± 1	13 ± 5	2 ± 1	162 ± 14
WT B cells*	None	25 ± 5	10,789 ± 355	80,903 ± 776	86,588 ± 585	5898 ± 63	4 ± 1
WT splenocytes	None	552 ± 22	3237 ± 87	27,623 ± 581	20,936 ± 342	2555 ± 50	2 ± 1

Values (±SEM) indicate relative mRNA expression levels as determined by real-time PCR in the dorsal skin and spleens of 21-day-old $Tsk^{/+}$ or wild-type (WT) littermates (six mice per group) after CD20 or control (CTL) mAb treatment (20 μg) on days 3 and 17 of age.

*Splenocytes from wild-type mice were enriched for B cells by depleting $Thy1.2^{+}$ T cells.

while spleens and B-cell-enriched splenocytes expressed high levels of CD19, CD20, κ, λ, and CD3 transcripts. Thus, B cells or plasma cells were not found in the skin of young $Tsk^{/+}$ mice, even in the absence of CD20 mAb treatment.

B-Cell Depletion Modulates Cytokine Expression

Whether B-cell depletion within lymphoid tissues influenced proinflammatory cytokines critical for the development of skin sclerosis in $Tsk^{/+}$ mice[27,30,34] was assessed by real-time PCR analysis. $Tsk^{/+}$ and wild-type littermates were treated with either CD20 or control mAb (20 μg) at 3 and 17 days with mRNA expression quantified in skin from 3-week-old mice. In the skin, TGF-β mRNA levels were elevated in $Tsk^{/+}$ mice (183 ± 15%) relative to wild-type littermates (100 ± 10, $P < 0.01$, $n = 5$; Figure 5A). However, B-cell depletion significantly reduced TGF-β transcript levels in $Tsk^{/+}$ mice ($P < 0.05$) to 134 ± 13% of wild-type littermate levels, but did not reduce TGF-β levels in wild-type mice (92 ± 9%). IL-4, IL-6, and IL-10 mRNA levels were increased by 70, 84, and 56%, respectively, in control mAb-treated $Tsk^{/+}$ mice compared with wild-type littermates ($P < 0.01$, Figure 5A). By contrast, TNF-α (44%), IL-2 (31%), and IFN-γ (29%) mRNA levels were significantly decreased in $Tsk^{/+}$ mice compared with wild-type littermates ($P < 0.01$). However, B-cell depletion significantly reduced IL-4, IL-6, and IL-10 mRNA levels in $Tsk^{/+}$ ($P < 0.01$) and wild-type ($P < 0.01$) littermates. B-cell depletion also significantly increased TNF-α, IL-2, and IFN-γ mRNA levels in $Tsk^{/+}$ ($P < 0.05$), but not wild-type littermates. Thus, B-cell depletion significantly altered skin cytokine profiles in young $Tsk^{/+}$ mice.

Spleen IL-4, IL-2, and IFN-γ mRNA levels were decreased by 48, 66, and 45%, respectively, in control mAb-treated $Tsk^{/+}$ mice compared with wild-type littermates ($P < 0.01$, Figure 5B). By contrast, IL-6 (76%), IL-10 (68%), and TNF-α (29%) mRNA levels were significantly increased in $Tsk^{/+}$ mice ($P < 0.05$), compared with wild-type littermates. CD20 mAb-induced B-cell depletion significantly increased IL-4 (84%), IL-2 (59%), and IFN-γ (79%) mRNA levels in $Tsk^{/+}$ mice ($P < 0.05$) but also resulted in increased IL-4, IL-6, IL-10, TNF-α, IL-2, and IFN-γ mRNA levels in wild-type ($P < 0.05$) littermates. By contrast, TGF-β mRNA expression was not significantly elevated in Tsk mice (126 ± 12%) relative to wild-type littermates (100 ± 15, $n = 5$). Furthermore, B-cell depletion did not significantly affect TGF-β transcript levels in either wild-type or $Tsk^{/+}$ littermates. Although increased cytokine transcript levels in $Tsk^{/+}$ and wild-type littermates may

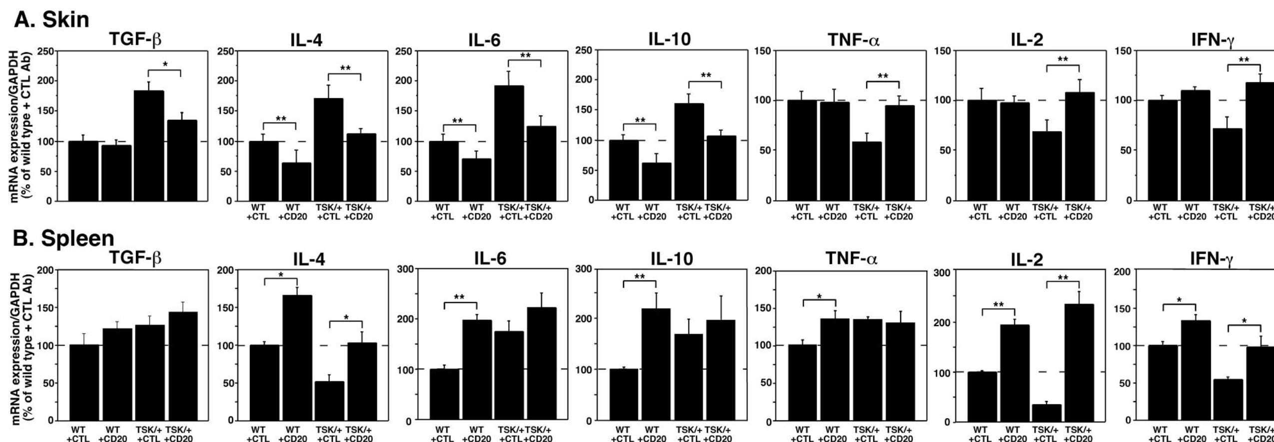

Figure 5. B-cell depletion alters cytokine production in the skin and spleens of $Tsk^{/+}$ mice. Cytokine mRNA levels in the dorsal skin (**A**) or among spleen lymphocytes (**B**) of 21-day-old $Tsk^{/+}$ or wild-type littermates after CD20 or control mAb treatment (20 μg) on days 3 and 17 of age. Transcript levels were quantified by real-time reverse transcriptase-PCR analysis and were normalized relative to endogenous GAPDH levels. Values represent mean percentages (±SEM) relative to transcript levels of wild-type littermates treated with CTL mAb (100%, broken horizontal lines), with ≥10 mice (**A**) or four mice (**B**) in each group. Values significantly different from controls are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$.

result from the increased ratio of T cells to B cells, these results document that B-cell depletion nonetheless significantly altered the cytokine profiles in spleens of young $Tsk^{/+}$ mice. To determine whether B-cell depletion altered the systemic balance of Th1/Th2 cytokines, serum levels of IFN-γ (representative Th1 cytokine) and IL-4 (representative Th2 cytokine) were measured. However, neither cytokine was detectable (n = 10 mice for each group), consistent with earlier findings of others that it is not possible to detect IL-4 in $Tsk^{/+}$ mouse serum.[34] Thus, B-cell depletion differentially affected skin and spleen cytokine levels, which may have therapeutic benefits.

Discussion

The current study verifies that B cells play an important contributory role during cutaneous fibrosis in $Tsk^{/+}$ mice. B-cell depletion by CD20 mAb treatment not only inhibited the development of autoimmunity in $Tsk^{/+}$ mice, but inhibited skin fibrosis by 31 to 43% when initiated early in the course of disease development (Figures 2 and 3). B-cell depletion also rebalanced skin cytokine transcript levels in $Tsk^{/+}$ littermates with progressing sclerosis and significantly altered spleen cytokine transcript levels (Figure 5). Although the extent of B-cell depletion was similar in newborn and adult $Tsk^{/+}$ mice (Table 1), sclerosis, autoantibody levels, and hypergammaglobulinemia in mice with established disease was not altered by CD20 mAb-mediated B-cell depletion (Figures 2 and 4). Thus, B cells significantly influenced cutaneous fibrosis during disease development but were not necessary for the maintenance of disease in $Tsk^{/+}$ mice.

Demonstrating that B-cell depletion reduces skin fibrosis in $Tsk^{/+}$ mice provides a basis for identifying the mechanisms through which B cells may influence autoimmune disease. B cells can produce autoantibodies, influence cytokine expression and promote Th2 cell development, function as antigen-presenting cells, and contribute to local inflammation. Although theoretical and not observed in the current studies, B-cell depletion in young wild-type and $Tsk^{/+}$ mice might also affect skin development, which is not yet complete at 3 days after birth. Early B-cell depletion in $Tsk^{/+}$ mice also prevented autoantibody production and hypergammaglobulinemia, whereas B-cell depletion in adult $Tsk^{/+}$ mice with disease did not reduce autoantibody levels or hypergammaglobulinemia (Figure 4). Preventing the generation of autoreactive B cells and the production of anti-fibrillin-1 antibodies plus other autoantibodies may be important because correlations exist between the concentration of serum anti-topoisomerase I autoantibodies and histological and biochemical alterations in the skin of $Tsk^{/+}$ mice.[39] Autoantibodies to fibrillin-1 also activate cultured human fibroblasts to recapitulate the fibrotic SSc phenotype *in vitro*.[10] Although autoantibodies correlate with B-cell hyperactivity and disease in $Tsk^{/+}$ mice, further studies are needed to determine whether autoantibodies contribute directly to disease *in vivo*.

The influence of B-cell depletion on skin and spleen cytokine levels provides the most likely mechanistic explanation for B-cell depletion affecting skin fibrosis. B-cell depletion reduced skin TGF-β transcript levels and also reduced IL-4, IL-6, and IL-10 mRNA expression in young $Tsk^{/+}$ mice, while significantly increasing TNF-α, IL-2, and IFN-γ production (Figure 5A). This may explain reduced skin fibrosis because Th2 cytokines such as IL-4 and IL-6 stimulate the synthesis of extracellular matrix proteins in dermal fibroblasts,[54,55] whereas Th1 cytokines such as IFN-γ and TNF-α suppress fibroblast collagen production *in vitro*.[56] A relative shift to Th2 rather than Th1 cytokines can also induce tissue fibrosis.[56] Likewise, blocking IL-4 function inhibits skin fibrosis in newborn $Tsk^{/+}$ mice,[27,34] whereas IL-12 production can prevent sclerosis and autoimmunity in $Tsk^{/+}$ mice.[30] Normalized cytokine expression after CD20 mAb treatment did not appear to result from the depletion of skin B cells, because it was difficult to detect an increase in either B or T cells in the skin of $Tsk^{/+}$ mice by immunohistochemistry (not shown) or by real-time PCR amplification of B- and T-cell transcripts (Table 2). Thus, it is likely that B-cell depletion inhibited skin fibrosis by altering cytokine production by T cells circulating through the skin or indirectly affecting serum cytokines. For example, IL-10 produced by activated B cells inhibits IL-12 production by dendritic cells, which promotes Th2 T-cell differentiation.[57] In support of this, CD20 mAb treatment significantly altered cytokine transcript levels within the spleens of treated mice (Figure 5B), and may thereby alter the central and peripheral balance of Th1/Th2 cells. Consistent with this, numerous studies have documented a role for IL-4 produced postnatally in skin hyperplasia and indicated that $CD4^+$ T cells of the Th2 phenotype influence the skin manifestations of the scleroderma-like syndrome in $Tsk^{/-}$mice.[28] Moreover, normalizing tissue IL-4 levels may reduce tissue fibrosis induced by TGF-β, because IL-4 induces TGF-β gene expression and IL-4 and TGF-β interact in regulating collagen gene expression *in vitro*.[32,33] At least two mechanisms for B-cell depletion affecting T-cell cytokine production are possible. First, B-cell depletion may affect T cell-B cell interactions that would normally promote Th2 cell generation and/or cytokine production. Alternatively, the initial depletion of most peripheral B cells followed by the continued removal of maturing B cells as they emerge from the bone marrow may induce activation of monocytes/macrophages within CD20 mAb-treated mice and thereby alter their maturation, regulatory functions, and/or cytokine production. Thus, B-cell depletion in $Tsk^{/+}$ mice may have pleiotrophic effects on the lymphocyte networks that influence T cell function and cytokine production within lymphoid tissues and influence skin-infiltrating inflammatory cells.

The current results are consistent with studies showing that B cells do not cause skin fibrosis in $Tsk^{/+}$ mice[18,49,58] but do contribute to disease pathogenesis.[22,26] Although some studies have concluded that B cells play no role in skin sclerosis, this interpretation could have resulted from

several factors, including small sample sizes,[18,58] mixed genetic backgrounds of mice,[18,49,58] and reliance on stretchable skin length[58] or dermal thickness[18] as disease parameters. Dermal thickness was similar in Tsk$^{/+}$ and wild-type littermates in the current (Figure 2) and previous studies,[14,23,24,49] while hypodermal thickness, including a subcutaneous connective tissue layer, is increased eightfold in Tsk$^{/+}$ C57BL/6 mice (Figure 2A), as reported.[24] Hypodermal thickness in Tsk$^{/+}$ BALB/c mice is only two- or threefold increased, potentially complicating some studies.[49] Baxter and colleagues[59] have recently confirmed that dermal thickness and dermal collagen content of Tsk$^{/+}$ and wild-type mouse skin are comparable. Based on this, Baxter and colleagues[59] concluded that Tsk$^{/+}$ mice are not useful for studying anti-fibrotic therapeutics, despite their greater skin-fold thickness. However, the current study and a number of previous studies have already demonstrated normal dermal thickness in Tsk$^{/+}$ mice, but increased hypodermal thickness,[14,23,24,39,49] increased collagen production,[25,32,39,60] and augmented TGF-β mRNA expression[22] in the skin. That the sites of fibrosis are different between human SSc (dermis) and Tsk$^{/+}$ mice (hypodermis) may represent species-specific differences in skin architecture. Thus, although results from Tsk$^{/+}$ mice cannot be simply translated into human therapies, the skin sclerosis and autoimmunity of Tsk mice make this a very useful animal model for skin fibrosis and scleroderma. Because the current model system is not complicated by genetic background differences and does not require adoptive lymphocyte transfers, this strategy suggests the utility of B-cell-targeted therapies in SSc patients with early disease and provides a pathway for mechanistic studies of SSc pathogenesis.

This study indicates that B-cell-directed therapies may benefit SSc patients when given during early stages of disease because skin fibrosis was diminished in Tsk$^{/+}$ mice after B-cell depletion between days 3 and 14, and before 28 days after birth (Figure 3, A and B). Likewise, anti-IL-4 mAb treatments reduce Tsk$^{/+}$ mouse skin thickening when initiated during the first week after birth.[34] Halofuginone, an inhibitor of type-1 collagen synthesis and skin sclerosis, is effective in newborn and 4-week-old Tsk$^{/+}$ mice.[61] Similarly, the development of pulmonary emphysema in Tsk$^{/+}$ mice was not affected by B-cell depletion or by other immunomodulatory treatments.[23,27,32,34] Thus, B cells and other components of the immune system contribute to early disease initiation or progression while established skin sclerosis and pulmonary emphysema are likely to involve additional factors. Because T cells, mast cells, and other immune cells also contribute to skin fibrosis in Tsk$^{/-}$mice, it remains possible that inhibiting T-cell and mast cell function in addition to B-cell depletion may have additive effects. Small subpopulations of human blood and bone marrow CD4$^+$ and CD8$^+$ T cells, and NK cells have been reported to express CD20 at low levels as determined by immunofluorescence staining with flow cytometry analysis.[50–53] Although human T and NK cells have never been shown to produce CD20 transcripts or the CD20 protein directly, these findings have lead to the suggestion that depletion of these cells contributes to the therapeutic benefit of CD20 mAb treatment in humans.[53] In mice, circulating CD4$^+$ and CD8$^+$ T cells, or NK cells did not express detectable CD20 cell surface protein (Figure 1D). Similarly, mouse bone marrow, blood, spleen, or lymph node leukocytes do not express detectable CD20 as determined by immunofluorescence staining of T cells, NK cells, dendritic cells, or monocytes/macrophages (K. Yanaba, J.-D. Bouaziz, and T.F. Tedder, manuscript in preparation). These results in mice are in agreement with the vast majority of studies assessing CD20 expression by human leukocytes, where CD20 expression has been found to be B-cell-restricted.[62] Thus, it remains likely that B-cell depletion may have secondary effects on T cells that influence skin sclerosis.

Although the positive results of this study may not translate to currently available CD20 mAb therapies in humans, the finding that intravenous, subcutaneous, and intraperitoneal CD20 mAb treatments depleted tissue B cells equally in mice (Figure 1) suggest that low CD20 mAb doses may effectively treat some autoimmune diseases. The 10-μg doses of CD20 mAb ($\sim$1.5 mg/m^2) given to 2-month-old mice (Figure 1) were 250-fold lower than the 375-mg/m^2 doses of rituximab normally given to oncology patients four times during the course of a month. Moreover, SSc patients may benefit from repeated low-dose subcutaneous CD20 mAb treatments given during early disease onset compared with intermittent high-dose mAb infusions. Consistent with this, B-cell depletion was beneficial after early disease onset in Tsk mice (Figure 3B). A failure of CD20 mAb treatment to reduce disease or autoantibody levels in adult Tsk$^{/+}$ mice also mimics findings in numerous human autoimmune diseases after CD20 mAb treatment,[40] presumably because of the presence of long-lived CD20-negative plasma cells or incomplete B-cell depletion. It is likely that early B-cell depletion in Tsk$^{/+}$ mice prevented hypergammaglobulinemia and diminished autoantibody production by eliminating mature B cells before plasma cell differentiation. Alternatively, autoreactive B cells generated later during the course of disease progression may persist after CD20 mAb treatment. Nonetheless, although autoantibodies correlate with B-cell hyperactivity and disease in Tsk$^{/+}$ mice, further studies are needed to determine whether autoantibodies contribute to disease and to identify the role for B cells in directing T-cell function *in vivo*. Because no therapy has been proven effective in suppressing or improving skin sclerosis in controlled studies of SSc patients or in Tsk$^{/+}$ mice with disease manifestations, and CD20 mAb treatment has few side effects,[40] therapies that effectively eliminate B cells or simply reduce B-cell hyperactivity during early disease onset may treat SSc or contribute to the therapeutic benefit of treatments yet to be identified.

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