Inactivation of the Cannabinoid Receptor CB1 Prevents Leukocyte Infiltration and Experimental Fibrosis

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Objective. Cannabinoids are derivates of the marijuana component Δ^9-tetrahydrocannabinol that exert their effects on mesenchymal cells and immune cells via CB1 and CB2 receptors. The aim of the present study was to evaluate the role of CB1 in systemic sclerosis.

Methods. CB1-deficient ($CB1^{-/-}$) mice and wild-type littermates ($CB1^{+/+}$ mice) were injected with bleomycin. CB1 signaling was activated in vivo with the selective agonist *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA). Bone marrow transplantation experiments were performed to investigate whether the phenotype of $CB1^{-/-}$ mice was mediated by leukocytes or mesenchymal cells. The role of CB1 was also investigated in the TSK-1 mouse model.

Results. $CB1^{-/-}$ mice were protected from bleomycin-induced dermal fibrosis, with reduced dermal thickening, hydroxyproline content, and myofibroblast counts. Inactivation of CB1 decreased the number of infiltrating T cells and macrophages in lesional skin. In contrast, activation of CB1 with ACEA increased leukocyte infiltration and enhanced the fibrotic response to bleomycin. The phenotype of $CB1^{-/-}$ mice was mimicked by transplantation of $CB1^{-/-}$ mouse bone marrow into $CB1^{+/+}$ mice, demonstrating that CB1 exerts its profibrotic effects indirectly by regulating leukocyte infiltration. Consistently, knockdown of CB1 did not prevent fibrosis in the inflammation-independent TSK-1 mouse model.

Conclusion. We demonstrate that the cannabinoid receptor CB1 is crucial for leukocyte infiltration and secondary fibroblast activation and that inactivation of CB1 exerts potent antifibrotic effects in inflammation-driven models of fibrosis.

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology that affects the skin and a variety of internal organs such as the lungs, the heart, and the gastrointestinal tract. Early stages of SSc are characterized by vascular changes and inflammatory infiltrates in involved organs (1). The inflammatory infiltrates are dominated by macrophages and activated T cells. Later stages of SSc are characterized by an excessive accumulation of extracellular matrix components. The resulting fibrosis disrupts the physiologic tissue structure and frequently leads to dysfunction of the affected organs. The accumulation of extracellular matrix results from an increased release of collagen and other components of the extracellular matrix by pathologically activated SSc fibroblasts (2). Activated fibroblasts that have differentiated into myofibroblasts and release excessive amounts of collagen are mostly localized adjacent to inflammatory infiltrates, suggesting that leukocytes may trigger the initiation of fibrosis in early stages of SSc (3). However, the molecular pathways that regulate leukocyte infiltration and subsequent fibroblast activation are incompletely understood (1).

Cannabinoids are derivates of the marijuana component Δ^9-tetrahydrocannabinol. They can be classified into 3 different groups according to their origin. The family of cannabinoids includes endogenous cannabinoids (endocannabinoids) that are synthesized within the human body, plant-derived cannabinoids like the lead compound Δ^9-tetrahydrocannabinol, and synthetic cannabinoids synthesized for therapeutic interventions (4). Unlike early hypotheses, the effects of cannabinoids are not restricted to neurons and to the central nervous system, but cannabinoids affect many cell types (4,5). Cannabinoids are currently being evaluated for the treatment of different tumors, since they inhibit tumor cell proliferation and induce cell cycle arrest in transformed cells (6). Cannabinoids are also crucial for bone homeostasis, since they control the differentiation and proliferation of osteoclast precursor cells and osteoblasts (7). Moreover, cannabinoids have been implicated in the regulation of immune responses by controlling leukocyte activation, cytokine release, and chemotaxis (8).

These pleiotropic effects are mediated by 2 different cannabinoid receptors, CB1 and CB2 (9,10). Despite similarities in the encoding nucleotide sequence, the expression patterns of CB1 and CB2 are distinct and they mediate different or even opposing effects. Striking examples of the distinct roles of CB1 and CB2 are their effects on bone mass. Inactivation of CB1 increases the bone mass, whereas inactivation of CB2 accelerates age-related trabecular bone loss (11,12). The different effects of CB1 and CB2 have stimulated the development of highly selective agonists and antagonists for both receptors, and synthetic and selective CB1 antagonists are available that have been proven to potently inhibit CB1 signaling in humans (13,14).

We demonstrated recently that CB2 exerts antifibrotic effects in the mouse model of bleomycin-induced fibrosis (15). Inactivation of CB2 resulted in increased accumulation of collagen and more pronounced dermal thickening, whereas activation of CB2 effectively prevented bleomycin-induced fibrosis. Intrigued by the opposing roles of CB1 and CB2 in bone homeostasis, we aimed to investigate the role of CB1 in fibroblast activation and fibrosis. We demonstrate that, in contrast to CB2, activation of CB1 results in exacerbation of fibrosis, whereas inactivation of CB1 exerts potent antifibrotic effects. Inactivation of CB1 reduced leukocyte infiltration into lesional skin and prevented subsequent fibroblast activation and collagen accumulation upon challenge with bleomycin.

MATERIALS AND METHODS

Bleomycin-induced dermal fibrosis in CB1-deficient mice. Mice deficient for CB1 (CB1$^{-/-}$ mice) (16) were backcrossed onto a C57BL/6 background for at least 6 generations. Wild-type (WT) C57BL/6 littermates expressing CB1 (CB1$^{+/+}$ mice) were used as controls. Skin fibrosis was induced in 6-week-old male mice by local injections of bleomycin for 4 weeks as described (17). Briefly, 100 μl of bleomycin dissolved in 0.9% NaCl at a concentration of 0.5 mg/ml was administered every other day by subcutaneous injections in defined areas of 1 cm^2 at the upper back. Subcutaneous injections of 100 μl 0.9% NaCl were used as control treatment. Four different groups were analyzed, consisting of 2 groups with CB1$^{-/-}$ mice and 2 groups with CB1$^{+/+}$ mice. One group of CB1$^{-/-}$ mice and 1 group of CB1$^{+/+}$ mice were challenged with bleomycin, while the remaining 2 groups were injected with NaCl. After 4 weeks, mice were killed by cervical dislocation. The 4 groups consisted of 20 mice in total. All animal experiments were approved by the local ethics committee.

Activation of CB1 in experimental fibrosis. To maximally activate CB1 signaling in experimental fibrosis, C57BL/6 mice challenged with bleomycin were additionally treated with N-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA). ACEA is a highly selective CB1 receptor agonist with a K_i value of 1.4 nM and selectivity for CB1 receptors >1,400-fold that for CB2 receptors (18,19). ACEA was purchased from Biozol and dissolved in anhydrous ethanol at a concentration of 10 mg/ml. The working solutions were prepared fresh on the day of the experiments by diluting the stock solutions in NaCl. ACEA was administered by intraperitoneal injections twice a day at a concentration of 7.5 mg/kg for 4 weeks. Treatment with ACEA started in parallel to bleomycin challenge. Mice injected with NaCl and with bleomycin only were used as controls. Fifteen mice were analyzed in these experiments.

Bone marrow transplantation. To analyze the role of bone marrow–derived cells and fibroblasts in the phenotype of CB1$^{-/-}$ mice, bone marrow transplantation experiments were performed (15). Female CB1$^{-/-}$ and CB1$^{+/+}$ mice served as donors of bone marrow. Tibias and femurs were prepared under sterile conditions. Bone marrow cells were flushed from the bone marrow cavities with phosphate buffered saline (PBS) and subsequently filtered through 70-μm nylon meshes (BD Biosciences). Erythrocytes were hemolyzed, and the remaining bone marrow cells were kept on ice until the time of transplantation. For transplantation, 2.0×10^6 bone marrow cells of donor mice were resuspended in 0.1 ml PBS and injected via the tail veins. Male CB1$^{-/-}$ or CB1$^{+/+}$ mice received bone marrow transplants at age 4 weeks. Recipient CB1$^{-/-}$ or CB1$^{+/+}$ mice underwent whole body irradiation at a dose of 4 Gray twice before transplantation to irradiate their bone marrow. Two weeks after bone marrow transplantation, when stable engraftment had occurred, mice were challenged with bleomycin for 4 weeks as described above. Twenty-six mice were analyzed in these experiments.

Inactivation of CB1 in TSK-1 mice. To investigate the role of CB1 in a noninflammatory model of SSc, CB1$^{-/-}$ mice were crossed with TSK-1 mice to generate TSK-1 mice deficient for CB1 (CB1$^{-/-}$ TSK-1 mice). The TSK-1 phenotype is caused by a dominant mutation in the fibrillin 1 gene (20). TSK-1 mice are characterized by accumulation of collagen
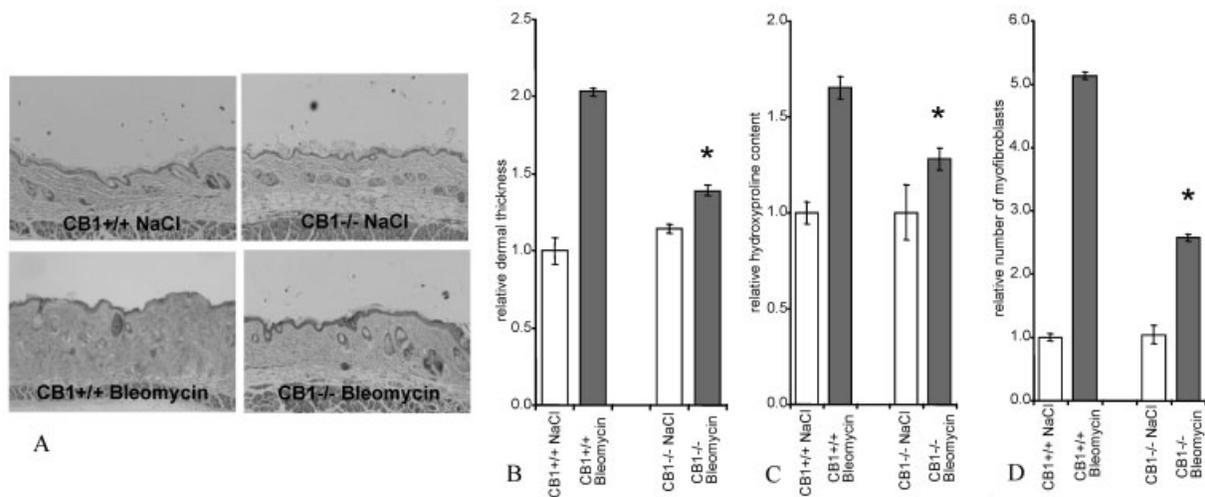
Figure 1. CB1-deficient (CB1$^{-/-}$) mice are protected from dermal fibrosis. **A,** Reduced accumulation of collagen and dermal fibrosis in CB1$^{-/-}$ mice upon challenge with bleomycin. Representative sections are shown (hematoxylin and eosin stained; original magnification × 100). **B,** Decreased dermal thickening in CB1$^{-/-}$ mice compared with their wild-type CB1$^{+/+}$ littermates. **C,** Reduced hydroxyproline content in lesional skin of CB1$^{-/-}$ mice. **D,** Lower myofibroblast counts in CB1$^{-/-}$ mice. CB1$^{+/+}$ control mice were injected with NaCl, and the values for these mice were set at 1.0; the other results were normalized to this value. Values are the mean ± SEM. $*$ = $P \leq 0.05$ versus CB1$^{+/+}$ mice challenged with bleomycin.

fibers in the hypodermis, resulting in progressive hypodermal thickening. In contrast to bleomycin-induced fibrosis, inflammatory infiltrates are absent, and the aberrant activation of fibroblasts is not caused by the release of inflammatory mediators from leukocytes. Similar to SSc fibroblasts, fibroblasts from TSK-1 mice are endogenously activated, with an increased release of collagen that persists for several passages in vitro. Thus, TSK-1 mice are a model for later, inflammation-independent stages of SSc, whereas bleomycin-induced fibrosis represents early, inflammation-dependent stages of SSc.

Genotyping of TSK-1 mice was performed using polymerase chain reaction with the following primers: mutated fibrillin 1/TSK-1, 5′-GTTGGCAACTATACCTGCAT-3′ (forward) and 5′-CCTTTCCTGGTAACATAGGA-3′ (reverse). Four groups with a total of 23 mice were analyzed. One group consisted of TSK-1 mice expressing CB1 (CB1$^{+/+}$). A second group consisted of TSK-1 mice deficient for CB1 (CB1$^{-/-}$). The last 2 groups consisted of pa/pa (control) mice, one group expressing CB1 and the other deficient for CB1. Mice were killed by cervical dislocation at age 10 weeks to analyze the hypodermal thickness, the hydroxyproline content, and the number of myofibroblasts in lesional skin.

Histologic analysis. Lesional skin areas were excised, fixed in 4% formalin, and embedded in paraffin. Five micrometer–thick sections were stained with hematoxylin and eosin. The dermal thickness was analyzed at 100× magnification by measuring the distance between the epidermal–dermal junction and the dermal–subcutaneous fat junction at 4 sites from the lesional skin of each mouse (21). Infiltrating leukocytes in lesional skin of CB1$^{-/-}$ mice, CB1$^{+/+}$ mice, CB1$^{+/+}$ mice treated with ACEA, and bone marrow–transplanted mice were quantified on hematoxylin and eosin–stained sections. Eight different high-power fields from different tissue sites

from each mouse were evaluated for polymorphonuclear cells at 400× magnification by an experienced examiner (JHWD) who was blinded to the treatment. Images were captured with an Eclipse 80i microscope (Nikon) equipped with a DSP 3CCD camera (Sony).

Hydroxyproline assay. The collagen content in lesional skin samples was explored by hydroxyproline assay (22). After digestion of punch biopsy samples (3 mm in diameter) in 6M HCl for 3 hours at 120°C, the pH of the samples was adjusted to 7 with 6M NaOH. Afterward, samples were mixed with 0.06M chloramine T and incubated for 20 minutes at room temperature. Next, 3.15M perchloric acid and 20% p-dimethylaminobenzaldehyde were added, and samples were incubated for an additional 20 minutes at 60°C. The absorbance was determined at 557 nm with a SpectraMax 190 microplate spectrophotometer (Molecular Devices).

Immunohistochemistry for α-smooth muscle actin (α-SMA), CD3, and F4/80. The expression of α-SMA, T cells, and macrophages was quantified in paraffin-embedded sections of lesional skin from CB1$^{-/-}$ mice, CB1$^{+/+}$ mice, CB1$^{+/+}$ mice treated with ACEA, and bone marrow–transplanted mice. Myofibroblasts were identified by staining for α-SMA as described (23,24). After deparaffinization and blocking with 5% horse serum and 3% H$_2$O$_2$, skin sections were incubated with anti–α-SMA antibodies (clone 1A4; Sigma-Aldrich). Polyclonal rabbit anti-mouse antibodies labeled with horseradish peroxidase (HRP) (Dako) were used as secondary antibodies. To quantify the numbers of infiltrating T cells, skin sections were stained for CD3. After deparaffinization, antigen retrieval with Tris–EDTA–Tween, and blocking with 10% goat serum and 0.3% H$_2$O$_2$, sections were incubated with rabbit polyclonal anti-CD3 antibodies (Abcam). Polyclonal HRP-labeled goat anti-rabbit Ig (Dako) were used as secondary
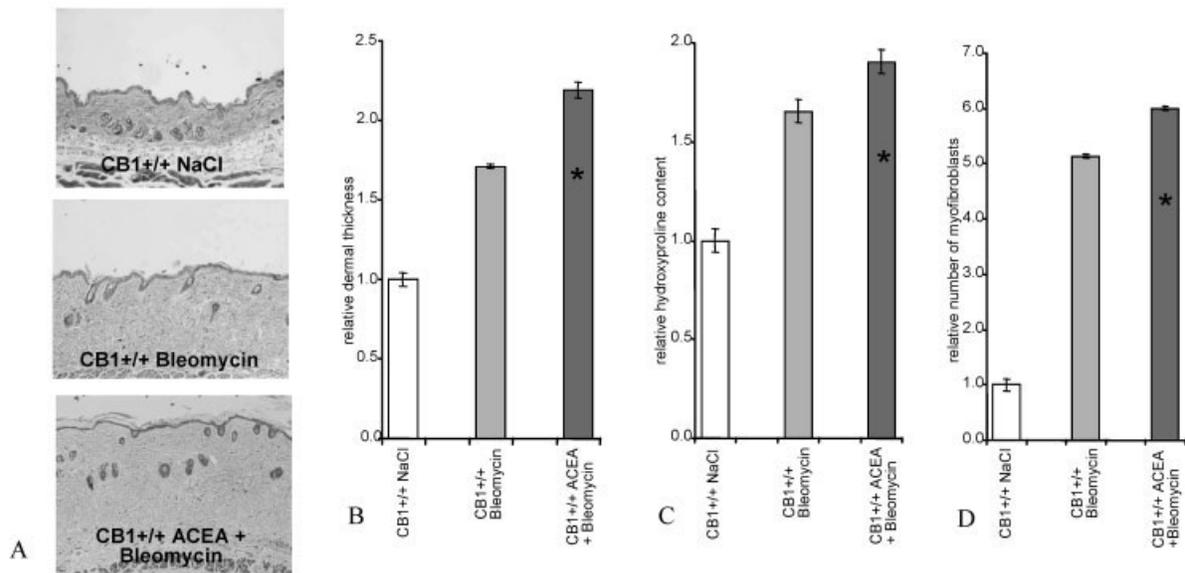
Figure 2. Treatment with the CB1 agonist *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) increases sensitivity to bleomycin-induced fibrosis. **A,** Exacerbation of dermal fibrosis in bleomycin-injected $CB1^{+/+}$ mice upon treatment with ACEA compared with mice injected only with bleomycin. Representative sections are shown (hematoxylin and eosin stained; original magnification × 100). **B–D,** Bleomycin-induced fibrosis in $CB1^{+/+}$ mice aggravated by more pronounced dermal thickening (**B**), increased hydroxyproline content (**C**), and higher myofibroblast counts (**D**) in lesional skin of ACEA-treated mice. $CB1^{+/+}$ control mice were injected with NaCl, and the values for these mice were set at 1.0; the other results were normalized to this value. Values are the mean ± SEM. ∗ = $P \leq 0.05$ versus $CB1^{+/+}$ mice challenged with bleomycin alone.

antibodies. Macrophages were detected by staining for F4/80. After antigen retrieval with proteinase K and blocking, sections were incubated with rat anti-mouse F4/80 antibodies (AbD Serotec). Alkaline phosphatase–labeled polyclonal goat anti-rat antibodies (Abcam) served as secondary antibodies.

Irrelevant isotype antibodies were used for controls. Staining was visualized with 3,3′-diaminobenzidine peroxidase substrate solution (Sigma-Aldrich) (for α-SMA and CD3) or with the use of a BCIP/nitroblue tetrazolium Alkaline Phosphatase Substrate Kit IV (Vector) (for F4/80). Sections stained for α-SMA and F4/80 were counterstained with hematoxylin. The number of myofibroblasts was determined at 200× magnification in 4 different sections from each mouse. T cells and macrophages were counted in 8 different sections of lesional skin of each mouse at 400× magnification. Counting was performed in a blinded manner by an experienced examiner (JHWD).

Statistical analysis. Data are expressed as the mean ± SEM. The Mann-Whitney U test was used for statistical analyses. *P* values less than or equal to 0.05 were considered significant.

RESULTS

$CB1^{-/-}$ mice are protected from bleomycin-induced dermal fibrosis. To evaluate the role of CB1 in fibrosis, $CB1^{-/-}$ mice and their $CB1^{+/+}$ littermates were challenged with bleomycin. No differences in skin archi-

tecture were observed between $CB1^{-/-}$ mice and $CB1^{+/+}$ mice injected with NaCl (Figure 1A). However, $CB1^{-/-}$ mice were protected from bleomycin-induced fibrosis. Dermal thickening upon bleomycin challenge was reduced by (mean ± SEM) 62 ± 3% in $CB1^{-/-}$ mice compared with $CB1^{+/+}$ mice ($P = 0.01$) (Figure 1B). Consistently, the hydroxyproline content in lesional skin of $CB1^{-/-}$ mice was significantly lower than that in lesional skin of $CB1^{+/+}$ mice, with a decrease of 57 ± 6% ($P = 0.05$) (Figure 1C). Myofibroblast counts were also reduced by 62 ± 6% in $CB1^{-/-}$ mice upon bleomycin challenge ($P = 0.01$) (Figure 1D). Together, these data demonstrate that inactivation of CB1 protects against fibrosis.

Activation of CB1 signaling exacerbates experimental fibrosis. The effects of an increased activation of CB1 on experimental fibrosis were evaluated using the highly selective CB1 agonist ACEA (19). Treatment with ACEA during bleomycin challenge resulted in exacerbation of dermal fibrosis (Figure 2A). Dermal thickening was 40 ± 5% more pronounced in ACEA-treated mice than in mice injected with bleomycin alone ($P = 0.01$) (Figure 2B). Consistently, the hydroxyproline content and the number of myofibroblasts were also
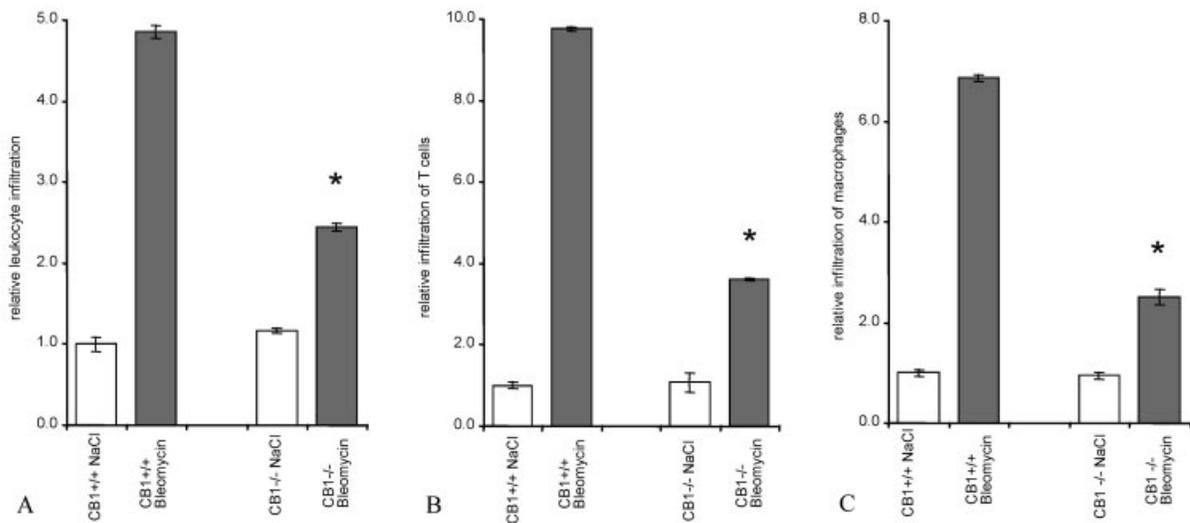
Figure 3. CB1 regulates leukocyte infiltration into lesional skin. Inflammation is reduced in CB1-deficient (CB1$^{-/-}$) mice challenged with bleomycin, as shown by decreased leukocyte counts (**A**) with significantly lower numbers of infiltrating T cells (**B**) and macrophages (**C**) in lesional skin of CB1$^{-/-}$ mice than in that of CB1$^{+/+}$ mice. CB1$^{+/+}$ control mice were injected with NaCl, and the values for these mice were set at 1.0; the other results were normalized to this value. Values are the mean ± SEM. ∗ = $P \leq 0.05$ versus CB1$^{+/+}$ mice challenged with bleomycin.

significantly increased (by 29 ± 6% and 18 ± 3%, respectively) in ACEA-treated mice ($P = 0.05$ and $P = 0.02$, respectively) (Figures 2C and D). Thus, activation of CB1 increases the sensitivity to bleomycin-induced dermal fibrosis.

CB1 regulates leukocyte infiltration. Inflammatory infiltrates consisting mainly of T cells and macrophages are characteristic features of early stages of SSc that are mimicked in the mouse model of bleomycin-induced fibrosis. Infiltrating leukocytes stimulate fibroblast activation and collagen synthesis via the release of profibrotic factors (1). Inflammatory infiltrates were significantly reduced in CB1$^{-/-}$ mice compared with CB1$^{+/+}$ mice challenged with bleomycin, and the number of infiltrating leukocytes was reduced by 63 ± 7% ($P = 0.01$) (Figure 3A). Further subanalyses revealed that T cell and macrophage counts were significantly lower in CB1$^{-/-}$ mice, with decreases of 70 ± 5% and 75 ± 6%, respectively ($P = 0.01$ for each comparison) (Figures 3B and C).

In contrast, treatment with the CB1 agonist ACEA exacerbated the inflammatory response to bleomycin. The total number of infiltrating leukocytes as well as T cell and macrophage counts increased significantly upon treatment with ACEA compared with bleomycin treatment alone (23 ± 9% for the total number of leukocytes [$P = 0.05$], 39 ± 4% for T cells [$P = 0.01$],

and 50 ± 3% for macrophages [$P = 0.01$]) (Figures 4A–C). Together, these data suggest that CB1 stimulates leukocyte activation and tissue infiltration in experimental fibrosis.

CB1 on leukocytes is essential for the profibrotic effects of CB1. The altered number of leukocytes in lesional skin indicates that CB1 might regulate tissue fibrosis by controlling leukocyte activation. To follow up this hypothesis, bone marrow transplantation experiments were performed, and the response to bleomycin was analyzed in the resulting chimeric mice. The number of infiltrating leukocytes was not reduced in CB1$^{-/-}$ mice reconstituted with CB1$^{+/+}$ mouse bone marrow, and these mice were not protected from bleomycin-induced dermal fibrosis (further information is available upon request from the corresponding author). Dermal thickness, hydroxyproline content, and myofibroblast counts in CB1$^{-/-}$ mice with CB1$^{+/+}$ mouse bone marrow were comparable with those observed in CB1$^{+/+}$ mice with CB1$^{+/+}$ mouse bone marrow (Figures 5A–D).

In contrast, CB1$^{+/+}$ mice transplanted with bone marrow cells from CB1$^{-/-}$ mice were protected from bleomycin-induced fibrosis. The numbers of T cells and macrophages as well as the total number of leukocytes in lesional skin of CB1$^{+/+}$ mice with CB1$^{-/-}$ mouse bone marrow were reduced to a degree similar to that observed in CB1$^{-/-}$ mice with CB1$^{-/-}$ mouse bone mar-
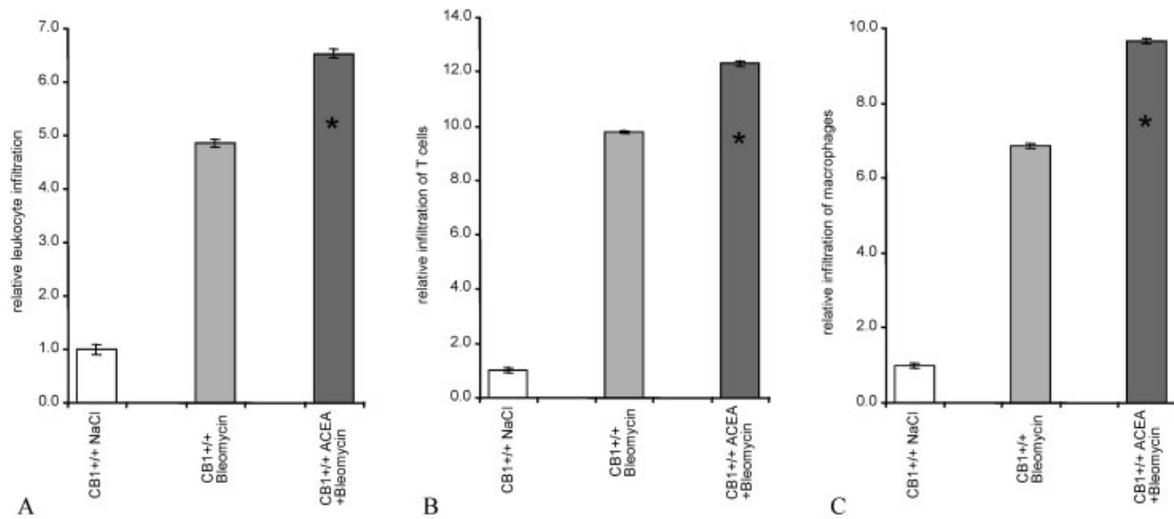
Figure 4. Leukocyte infiltration into lesional skin is regulated by CB1. Shown are increased leukocyte infiltration (**A**) with higher numbers of T cells (**B**) and macrophages (**C**) in mice treated with the CB1 agonist *N*-(2-chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (ACEA). CB1$^{+/+}$ control mice were injected with NaCl, and the values for these mice were set at 1.0; the other results were normalized to this value. Values are the mean ± SEM. * = $P \leq 0.05$ versus CB1$^{+/+}$ mice challenged with bleomycin alone.

row (further information is available upon request from the corresponding author). Moreover, dermal thickening in CB1$^{+/+}$ mice with CB1$^{-/-}$ mouse bone marrow did not differ from that observed in CB1$^{-/-}$ mice with

CB1$^{-/-}$ mouse bone marrow (increases of 46 ± 4% and 39 ± 4%, respectively; $P = 0.34$), but was significantly lower than that in CB1$^{+/+}$ mice with CB1$^{+/+}$ mouse bone marrow (increase of 103 ± 3%) ($P = 0.01$)
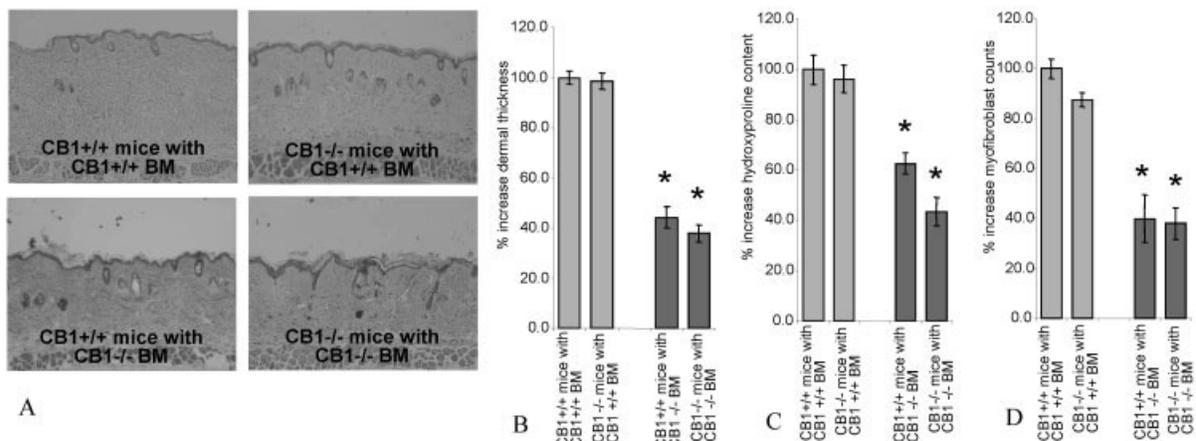

Figure 5. Inactivation of CB1 on leukocytes completely mimics the antifibrotic effects observed in CB1-deficient (CB1$^{-/-}$) mice. **A,** Bone marrow (BM) transplantation experiments revealed that CB1$^{+/+}$ mice transplanted with CB1$^{-/-}$ mouse bone marrow were protected from bleomycin-induced dermal fibrosis to a degree similar to that of CB1$^{-/-}$ mice with CB1$^{-/-}$ mouse bone marrow, whereas no antifibrotic effects were observed in CB1$^{-/-}$ mice with CB1$^{+/+}$ mouse bone marrow. Representative skin sections are shown (hematoxylin and eosin stained; original magnification × 100). **B–D,** Shown are comparable reductions of dermal thickening (**B**), hydroxyproline content (**C**), and myofibroblast counts (**D**) between CB1$^{+/+}$ mice transplanted with bone marrow from CB1$^{-/-}$ mice and CB1$^{-/-}$ mice with CB1$^{-/-}$ mouse bone marrow upon challenge with bleomycin. The values for CB1$^{+/+}$ mice with CB1$^{+/+}$ mouse bone marrow are set at 100%; other results are shown as changes relative to these mice. Values are the mean ± SEM. * = $P \leq 0.05$ versus bleomycin-challenged CB1$^{+/+}$ mice with CB1$^{+/+}$ mouse bone marrow.
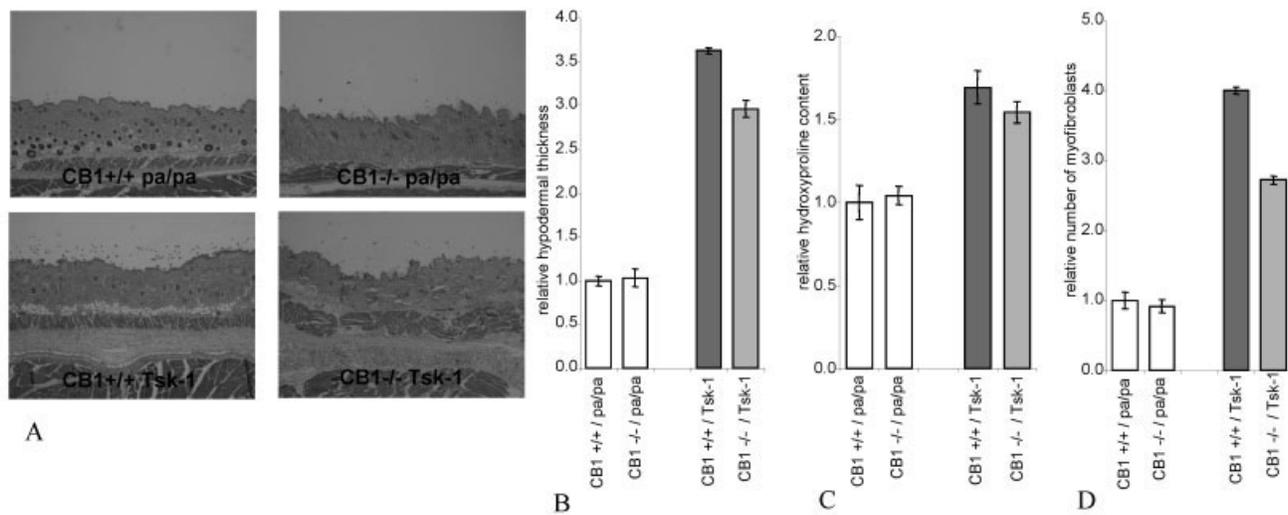
Figure 6. Inactivation of CB1 does not reduce fibrosis in TSK-1 mice. TSK-1 mice are a model of inflammation-independent fibrosis without inflammatory infiltrates and with endogenous activation of fibroblasts. Consistent with the hypothesis that inactivation of CB1 exerts its antifibrotic effects by inhibiting leukocyte infiltration, but not by direct effects on fibroblasts, CB1-deficient ($CB1^{-/-}$) TSK-1 mice were not protected from fibrosis (original magnification $\times$ 100) (**A**). Hypodermal thickness (**B**), hydroxyproline content (**C**), and myofibroblast counts (**D**) did not differ between $CB1^{-/-}$ TSK-1 mice and their $CB1^{+/+}$ TSK-1 littermates. Values for $CB1^{+/+}$ pa/pa (control) mice were set at 1.0; all other results were normalized to this value. Values are the mean $\pm$ SEM.

(Figures 5A and B). Consistently, hydroxyproline content and myofibroblast counts in $CB1^{+/+}$ mice reconstituted with $CB1^{-/-}$ mouse bone marrow were similar to those in $CB1^{-/-}$ mice with $CB1^{-/-}$ mouse bone marrow, but were significantly reduced compared with those in $CB1^{+/+}$ mice with $CB1^{+/+}$ mouse bone marrow (Figures 5C and D). These data demonstrate that CB1 exerts its profibrotic effects indirectly by regulating leukocyte infiltration.

Inhibition of CB1 does not prevent inflammation-independent fibrosis. To confirm that CB1 controls fibroblast activation indirectly by regulating leukocyte infiltration, we analyzed the role of CB1 in the TSK-1 mouse model of SSc. Inflammatory infiltrates are absent in TSK-1 mice, and the increased release of collagen by TSK-1 mouse fibroblasts is caused by endogenous activation and not by the release of profibrotic cytokines from infiltrating leukocytes as in bleomycin-induced fibrosis (20,25). Consistent with the results from bone marrow transplantation and with the hypothesis that inactivation of CB1 exerts its antifibrotic effects by inhibiting leukocyte infiltration and not by direct inhibitory effects on fibroblasts, $CB1^{-/-}$ TSK-1 mice were not protected from fibrosis. No significant differences in hypodermal thickness, hydroxyproline content, and myofibroblast counts were observed between

$CB1^{-/-}$ TSK-1 mice and their $CB1^{+/+}$ TSK-1 littermates (Figures 6A–D).

DISCUSSION

Fibrosis is a major reason for the high morbidity of SSc patients and is also a leading cause of their increased mortality (26). Treatment of fibrosis is a major challenge for physicians, because efficient antifibrotic therapies that target specifically the aberrant activation of SSc fibroblasts are currently not available for clinical use (27). Inactivation of CB1 has previously been shown in an elegant study to decrease the wound healing response to acute liver injury and to inhibit progression of liver cirrhosis (28). Our data constitute the first evidence that the effects of CB1 are not restricted to the liver but are also operative in other fibrotic diseases with significant differences in the histologic changes and in the underlying pathogenesis.

The pathogenesis of skin fibrosis differs from that of liver cirrhosis in several ways, including but not limited to 1) the lack of stellate cells in the skin that are thought to play a key role in the pathogenesis of liver cirrhosis; 2) no role of epidermal-to-mesenchymal transition in the skin, in contrast to the prominent role of this transition in liver cirrhosis; and 3) the unclear role of

bone marrow–derived fibrocytes in the pathogenesis of SSc. These differences are also reflected in differences in genes up-regulated during fibrogenesis and in their expression pattern. These differences in pathogenesis are also reflected by different mechanisms of action of CB1 in SSc and liver cirrhosis. In liver cirrhosis, CB1 seems to exert its profibrotic effects by inhibiting proliferation of hepatic myofibroblasts. In contrast, in experimental SSc we demonstrate that the profibrotic effects are exclusively mediated by the effects of CB1 on leukocytes, and we demonstrate that inhibition of CB1 prevents leukocyte infiltration into lesional skin.

Although additional studies on more complex murine models of SSc, such as in Fra-2–transgenic mice or in caveolin 1–knockout mice (23,29,30), are needed for final conclusions, our findings might have clinical implications, since specific inhibitors of CB1, such as SR141716A, have been developed and have been proven to potently inhibit CB1 signaling in humans (13,14). However, the incidence of depression and fear was increased in patients receiving SR141716A, and suicidal behavior might occur in predisposed patients. It is currently unknown whether these adverse effects were a class effect or were specific for SR141716A. However, these reports warrant a careful monitoring of patients receiving CB1 antagonists.

The 2 cannabinoid receptors, CB1 and CB2, have opposing roles in fibrosis. Inhibition of CB2 exacerbates fibrosis, whereas agonists of CB2 exert antifibrotic effects in different models (15,31,32). In contrast, we demonstrate in the present study that CB1 is profibrotic and that activation of CB1 exerts profibrotic effects, whereas inhibition of CB1 prevents bleomycin-induced dermal fibrosis. These results warrant the use of highly selective drugs that either inhibit CB1 or activate CB2 but that do not affect the other cannabinoid receptor. Nonspecific drugs such as the CB1/CB2 agonist dronabinol, which is approved for the treatment of cachexia and of nausea resulting from chemotherapy, act on both cannabinoid receptors, and their effects on fibrosis are unpredictable. The antifibrotic effects caused by the activation of CB2 might be counterbalanced or even outweighed by the profibrotic effects of the activation of CB1. It will also be interesting to determine the role of endocannabinoids in fibrosis. These endogenous agonists often bind CB1 and CB2, but with different affinities (33). Will the profibrotic effects of the activation of CB1 dominate, or will endocannabinoids preferentially activate CB2 receptors and exert antifibrotic effects?

Early stages of SSc are characterized by an infiltration of affected skin by inflammatory cells, in particular T cells and macrophages (1,2). The infiltrating leukocytes release profibrotic cytokines, such as monocyte chemoattractant protein 1, interleukin-4 (IL-4), and IL-13, that stimulate the collagen synthesis in resident fibroblasts (2,34,35). CB1 might play a crucial role in these early, inflammatory stages of SSc. Our results demonstrate that CB1 stimulates the infiltration of leukocytes into lesional skin in inflammation-driven models of fibrosis. Knockdown of CB1 significantly reduced the numbers of leukocytes in bleomycin-challenged mice, whereas activation of CB1 resulted in increased leukocyte infiltration. These findings indicate that inhibition of CB1 exerts its antifibrotic effects indirectly by orchestrating the infiltration of leukocytes into lesional skin rather than by direct effects on the collagen synthesis of fibroblasts. Consistent with this hypothesis, the release of collagen was not altered in fibroblasts isolated from $CB1^{-/-}$ mice (data not shown). Moreover, the antifibrotic effects caused by general inactivation of CB1 fully resembled those caused by transplantation of $CB1^{-/-}$ mouse bone marrow cells into WT mice. In contrast, no protective effect was observed in $CB1^{-/-}$ mice reconstituted with bone marrow cells from WT mice. Finally, inactivation of CB1 did not exert antifibrotic effects in the TSK-1 mouse model, which serves as an inflammation-independent model with endogenous fibroblast activation.

WIN55,212-2, a nonselective, synthetic cannabinoid receptor agonist, was recently reported to decrease the release of collagen in cultured fibroblasts (36). However, the concentrations of WIN55,212-2 used in that study were very high and induced apoptosis in fibroblasts. Thus, the reduced synthesis of collagen upon incubation with WIN55,212-2 might have been due to toxic effects rather than to specific antifibrotic effects. Together, these data suggest that CB1 positively regulates the infiltration of leukocytes and enhances their migration into lesional skin in SSc. Inactivation of CB1 reduces leukocyte infiltration and prevents the release of profibrotic mediators, which results in decreased activation of resident fibroblasts and protection from fibrosis. CB1 might regulate leukocyte activation on different levels. Besides the effects on migration of leukocytes, blockade of CB1 might shift the balance from a Th2-type to a Th1-type immune response. Activation of CB1 increased the release of the Th2 cytokine IL-4 and suppressed the levels of the Th1 cytokines interferon-γ and IL-12 (37). Pharmacologic inhibition of CB1 shifted the immune response from a Th2-type to a Th1-type response. Since Th2 cytokines such as IL-4 potently stimulate fibroblasts to release extracellular matrix pro-

teins (38,39), prevention of Th2 differentiation might play a major role in the antifibrotic effects observed upon inactivation of CB1.

In summary, we demonstrate that CB1 indirectly regulates the activation of fibroblasts by orchestrating the influx of leukocytes into lesional skin. Activation of CB1 enhanced leukocyte infiltration and inflammation-driven fibrosis, whereas ablation of CB1 exerted potent antifibrotic effects. Thus, CB1 might be a potential molecular target for the treatment of inflammatory stages of SSc.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. J. H. W. Distler had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Marquart, Zwerina, O. Distler, Schett, J. H. W. Distler.
Acquisition of data. Marquart, Zerr, Palumbo, Reich, Tomcik, Horn, Dees, Engel, J. H. W. Distler.
Analysis and interpretation of data. Marquart, Akhmetshina, J. H. W. Distler.

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