Chemokine Receptors CCR2 and CX3CR1 Regulate Skin Fibrosis in the Mouse Model of Cytokine-induced Systemic Sclerosis

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Abstract

Background :

Skin fibrotic disorders such as systemic sclerosis (SSc) are characterized by an excessive accumulation of extracellular matrix (ECM), and develop under the influence of certain cytokines. We previously established a mouse model of skin fibrosis induced by exogenous application of cytokines. We have revealed that both the number of macrophages and the levels of macrophage chemoattractant protein-1 (MCP-1) mRNA positively correlate with the extent of skin fibrosis. Macrophages can be divided into two subsets, the first expressing CCR2, and the second expressing CX3CR1.

Objective :

To elucidate the role of skin infiltrating macrophages based on CCR2 and CX3CR1 in this cytokine-induced murine fibrosis model.

Methods :

We examined the amounts of collagen deposited in granulation tissues, the numbers of macrophages and the levels of several mRNA in wild type (WT) mice, $CCR2^{-/-}$ mice, and $CX3CR1^{-/-}$ mice during injections of transforming growth factor-β (TGF-β) followed by injections of connective tissue growth factor (CTGF).

Results :

TGF-β injection increased the expressions of MCP-1, fractalkine, CCR2 and CX3CR1 mRNA in WT mice. The overproduction of collagen induced by TGF-β was significantly reduced by CCR2 deficiency, while collagen contents induced by CTGF were restored to wild-type levels. In contrast, overproduction of collagen in CX3CR1-deficient mice was decreased nearly 50% by both TGF-β and CTGF stimulations.

Conclusion :

The involvement of CCR2/MCP-1 interaction (CCR2-dependent loop) was during the TGF-β phase. In contrast, the fractalkine/CX3CR1 interaction contributes to the initiation of fibrosis by TGF-β and its maintenance by CTGF. Collectively, two subsets of macrophages both cooperatively and independently play important roles in the development of fibrosis.

1. Introduction

SSc is an autoimmune rheumatic disease characterized by fibrosis and vascular injury in the skin and multiple internal organs. The etiology and pathogenesis of SSc remain unclear. Clinically, SSc is typified by excessive collagen deposition, vascular damage, and immunological activation and these clinical features likely interrelated [1] [2] [3] [4]. The central event in the pathogenesis of SSc is an abnormal accumulation of extracellular matrix (ECM) components, predominantly type I and III collagen. The mechanisms that cause excessive fibrosis in SSc remain incompletely understood, however, many studies have suggested that cytokine release from inflammatory cells, endothelial cells, fibroblasts, and other cell types in the involved organs play important roles in the initiation and maintenance of fibrosis [5] [6].

Among these cytokines, transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) are thought to play central roles in the pathogenesis of SSc [7] [8]. To better understand the mechanisms of fibrosis and the contributions of cytokines, we established a unique animal model of skin fibrosis by exogenously administering cytokines [9]. In this model, the *in vivo* effects of cytokines are examined following subcutaneous injection into newborn mice. We found previously that TGF-β induced only transient fibrosis on day 4, despite 7 days of consecutive injections [10]. By contrast, serial injections of CTGF after TGF-β administration caused persistent fibrosis [11]. We also identified a positive correlation between the number of macrophages present, MCP-1 mRNA expression levels, and the extent of skin fibrosis [12]. This finding suggested that macrophages and macrophage related cytokines might indirectly contribute to the development of fibrosis.

Activated macrophages appear to play an important role in fibrosis because they are among the first immune cells recruited during the initial stages of fibrosis. It has long been known that early SSc skin contains an increased number of CD14-positive cells (monocytes/macrophages), compared to normal skin [13]. When activated, macrophages release a number of pro-inflammatory and fibrogenic mediators, such as TGF-β and platelet-derived growth factor (PDGF) [14]. Macrophages are classified into two functional subsets based on their chemokine receptor expression pattern: a short-lived CCR2 subset that is actively recruited to inflamed tissues, and a CX3CR1 subset characterized as resident in non-inflamed tissues [15]. CCR2 is a major receptor for MCP-1, while CX3CR1 is a receptor for fractalkine, a chemokine expressed by endothelial cells.

MCP-1 stimulates collagen production by fibroblasts via endogenous upregulation of TGF-β expression [16]. Previous studies have demonstrated that MCP-1 gene expression is increased during bleomycin-induced pulmonary fibrosis in mice [17] and interstitial kidney fibrosis [18]. In SSc patients, serum levels of MCP-1 are elevated, and spontaneous MCP-1 production by peripheral blood mononuclear cells is increased relative to normal controls [19]. MCP-1 is also strongly expressed in the epidermis, in inflammatory mononuclear cells, and in endothelial cells in the sclerotic skin of SSc patients, but it is not expressed in normal skin. Administration of anti-MCP-1 neutralizing antibody reduced skin sclerosis in bleomycin-treated mice [20]. Furthermore, mice deficient for the MCP-1 receptor CCR2 are protected from fluoresceinisothiocyanate-induced and bleomycin-induced lung fibrosis [21] [22]. Thus, it appears that MCP-1 is critically involved in the pathogenesis of SSc.

Fractalkine is a membrane-bound chemokine that functions not only as a chemoattractant but also as an adhesion molecule, and it is expressed on proinflammatory cytokine activated endothelial cells. The fractalkine receptor, CX3CR1, is expressed on mature monocytes, NK cells, and cytotoxic effector T cells [23]. We previously demonstrated that fractalkine was strongly expressed on endothelial cells in affected skin and lung tissues in SSc patients. Additionally, soluble fractalkine levels were significantly elevated in sera and were associated with the existence of digital ischemia, and severe pulmonary fibrosis. The number of CX3CR1-expressing cells, including monocytes, was increased in the lesional skin and lung tissues from SSc patients with diffuse cutaneous involvement. [24]. Collectively, these data suggest a role for fractalkine as a major mediator of SSc.

In this study, we have investigated whether CCR2/MCP-1 or CX3CR1/fractalkine play a role in the induction and maintenance of skin fibrosis in our animal model, to determine if these molecules might be useful as novel therapeutic targets in fibrotic disorders.

2. Materials and Methods

2.1 Mice

Specific pathogen-free 8-to 10-week-old female BALB/c mice (WT mice) were purchased from Charles River Japan (Yokohama, Japan). CCR2-deficient ($CCR2^{-/-}$) mice and CX3CR1-deficient ($CX3CR1^{-/-}$) mice were generated [25] [26] and backcrossed to a BALB/c background for more than 8 generations in our animal facility. All studies and procedures were approved by the Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science.

2.2 Growth factors

Human recombinant TGF-β3 was purchased from R&D Systems, Inc. (Minneapolis, MN). Human recombinant CTGF was a generous gift from Nosan Corporation. (Yokohama, Japan). Endotoxin levels of TGF and CTGF were <1.0 EU per 1 μg of cytokine as determined by the LAL method respectively.

2.3 In vivo experimental model

TGF-β3 and CTGF were dissolved in phosphate-buffered saline (PBS), to obtain final concentrations of 40 and 20 ng/μl, respectively. Newborn mice were injected with 20 μl of TGF-β3 (800 ng), CTGF (400 ng), or with PBS as a control, into the subcutaneous neck tissue once a day for 3 or 7 consecutive days. Serial injections of the two growth factors (TGF-β3 for days 1-3 days and CTGF for days 4-7) were also conducted (Fig. 1). The amounts of growth factors used in these experiments were previously shown to be optimal in our skin fibrotic animal models using BALB/C mice [10, 11].The mice were euthanized with an overdose of diethyl ether 24 hours after the final injection, and tissue samples were obtained from the site of injection. Parts of the tissue samples were fixed in 10% neutral buffered formalin and processed for routine histological examination and the measurement of collagen content. The remaining tissue was embedded in Tissue-Tek OCT compound (Miles, IN), snap frozen in liquid nitrogen, and stored at –70 ^{O}C prior to use. Ten serial 6-μm sections were cut from each frozen sample embedded in OCT compound. The sections were subjected to RNA extraction.

2.4 Measurement of collagen content in tissue samples

Tissue samples were embedded in paraffin and sections, approximately 15 μm thick, were obtained. Groups of 10 to 20 sections were deparaffinized after incubation with xylol, xylol:ethanol (1:1), ethanol, water:ethanol (1:1), and water. We used only granulated areas of the sections. Individual samples were placed in small test tubes and covered with 0.2 ml of a saturated solution of picric acid in distilled water that contained 0.1% Fast green FCF and 0.1% Sirius red F3BA. The samples were rinsed several times with distilled water until the fluid was colorless. One milliliter of 0.1 N NaOH in absolute methanol (1:1; v:v) was added and the eluted color was read in a spectrophotometer at 540 nm and 605 nm. The method is based on the selective binding of Sirius red F3BA and Fast green FCF to collagens and non-collagenous proteins, respectively [27]. Each experimental sample was run in triplicate.

2.5 RNA isolation and cDNA synthesis

Total RNA was extracted from frozen granulation tissue samples using RNeasy (Qiagen, Valencia, CA) spin columns, according to the manufacturer's protocol, with the addition of a DNase digestion step. RNA concentration and purity was determined spectrophotometrically by measuring fluorescence at 260 nM and 280 nM. Total RNA (100 μg) was reverse transcribed into cDNA in a total volume of 20 μg using a Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions.

2.6 Taqman quantitative real-time PCR analysis

Four microliters of cDNA were used as a template for real-time PCR, which was carried out in an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Foster city, CA). The sequences of the mouse α 2 chain of the type I collagen (COL1A2) primers and probe used are 5'-CAC CCC AGC GAA GAA CTC ATA-3' (forward), 5'-GCC ACC ATT GAT AGT CTC TCC TAA C-3'(reverse) and 5'-CGC CCA GGC CAA CAA GCA TGT C-3' (probe). Intron-spanning primers were used to minimize the possibility of co-amplifying genomic DNA. The primers for MCP-1, fractalkine, CCR2, CX3CR1 and CTGF were purchased from Applied Biosystems. We used the Taqman rodent GAPDH control reagent (PE Applied Biosystems) as an internal control. PCR (1 x (50^{O}C, 2 min, 95^{O}C, 10 min), 40 x (95^{O}C, 15 sec, 60^{O}C, 1 min) was performed in the presence of 0.6 x Taqman Universal PCR master mix (PE Applied Biosystems), forward and reverse primers and a sequence- specific fluorescent probe. Optimal probe and primer concentrations were determined for each assay

to ensure maximum specificity. Relative units (RU) were calculated by the comparative C_T method. First, the C_T for the target amplification (FAM) and the C_T for the endogenous control (VIC; GAPDH) were determined for each sample. The Difference between the C_T for the target and the C_T for the internal control, called ΔC_T, was calculated to normalize for the differences in the amounts of total nucleic acid added to each mixture. The ΔC_T of the calibrator was subtracted from the ΔC_T of each experimental sample to give $\Delta\Delta C_T$. The amount of target normalized to an endogenous control and relative to the calibrator, was then calculated using the equation $2^{(-\Delta\Delta CT)}$. Each experimental sample was run in triplicate.

2.7 Histological examination and immunohistochemistry

The formalin-fixed and paraffin-embedded specimens were cut to a thickness of 4 mm and stained with hematoxylin and eosin. Immunohistochemical analysis using antimouse F4/80 antibody (clone A3-1, ACM, UK), CD11b (clone M1/70, BD Biosciences, San Jose, CA), Gr1(clone RB6-8C5, BD Biosciences) at a dilution 1:100 was performed with biotinylated anti-mouse IgG as secondary antibody. Visualization was done with the standard streptavidin-biotin-coupled immunoperoxidase technique (Histofine kit, Nichirei, Tokyo, Japan). The measurement of macrophages was performed by averaging the number of cells identified by positive for the F4/80 or CD11b staining per visual field in five high power fields (magnification, x 200), respectively. Each experimental sample was run in triplicate.

2.8 Macrophage isolation

Magnetic cell sorting technology (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to purify macrophages according to the manufacture's instructions. CD11b microbeads were used to purify macrophages from spleen cells.

2.9 Cell culture *in vitro*

Primary fibroblast cultures were established from dorsal skin of newborn wild-type mice as described. Fibroblasts were grown at 37°C in a 5% CO_2 atomosphere in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) with 10% fetal calf serum (Nunc, Roskilde, Denmark), 100 U/ml penicillin/streptomycin in 6-well multi-dishes. Passages 3-5 were used for the experiments. After cells reached confluence, the culture medium was discarded and cells were starved using DMEM with 0.1% bovine serum

albumin and nonessential amino acids (Sigma, St. Louis, MO) for 24 hours prior to initiating TGF-β3 or CTGF stimulation. TGF-β3 or CTGF was added to the cell culture medium to a final concentration of 10 ng/mL, 150 ng/mL after 24 hours starvation. The cells were then cultured for 24 hours. For macrophage-fibroblast cocultues, macrophages ($5x10^5$ cells per well) were added in the upper wells (insert) of a 6-well transwell plate (0.4 mm pore size; BD Falcon) simultaneously with TGF-β3. At the end of each experiment, total RNA was isolated from fibroblasts in the lower chamber using RNeasy (Qiagen, Valencia, CA) as above. To examine a contamination by endothelial cells, we added the FACS analyses of skin fibroblasts. $CD31^+$ cells were not detected in isolated skin fibroblasts by FACS analysis (data not shown).

2.10 Flow cytometry

Abs used in this study included PE-conjugated anti-CCR2 mAb (R&D Systems, Minneapolis, MN), APC-conjugated anti-CX3CR1 mAb(R&D Systems). Single-cell suspensions of isolated macrophages were incubated with the Abs for 30 min at 4°C. The cells washed and fixed with 1% paraformaldehyde in PBS. Stained Samples were analyzed on FACSVantage SE (BD Biosciences), analyzing data from 10^5 cells. Data were analayzed using the FlowJo (Tree Star, Ashland,OR) software. Positive and negative populations of cells were determined using unreactive isotypematched mAbs (BD Biosciences) as controls for background staining.

2.11 Statistical Analysis

Values were expressed as the mean ± SEM. Student's *t* test was used to evaluate the statistical differences between the groups.

3. Results

3.1 Semi-quantitative analysis of collagen content in skin fibrosis induced by exogenous injection of TGF-β and CTGF in CCR2-KO and CX3CR1-KO mice

We previously showed that serial injections of CTGF after TGF-β induced persistent fibrotic tissue formation in newborn mice [11], leading to increased amounts of deposited collagen in granulation tissue [12]. We next wanted to analyze the requirements for the chemokine receptors CCR2 and CX3CR1 in this same model system in order to examine potential roles for chemokines in SSc. In this study, we injected 800 ng of TGF-β on days 1-3 followed by 400 ng of CTGF on days 4-7. Since the profibrogenic effects of TGF-β1, -β2, and -β3 were almost identical in previous experiments [10], we used TGF-β3 (henceforth referred to as TGF-β) for all experiments in the present study.

Serial injections of CTGF following TGF-β administration caused a prominent collagen accumulation in WT mice (Fig. 2A, E). By contrast, the collagen content was not increased in $CCR2^{-/-}$ mice until day 4, resulting in a significant reduction of local collagen as compared with WT mice ($p<0.005$) (Fig. 2A, C). However, during days 5-8, when CTGF was injected, enhanced collagen production was observed in $CCR2^{-/-}$ mice, resulting in equivalent collagen content levels in $CCR2^{-/-}$ mice and WT mice at day 8 (Fig. 2A, F). In $CX3CR1^{-/-}$ mice, collagen levels were decreased by approximately 50% on days 4 and 8 as compared with WT mice ($p<0.05$ and $p<0.005$, respectively) (Fig.2A, D, G). These semi-quantitive results were for the most part consistent with the histological findings. The collagen contents injected with PBS as control were not increased in WT, $CCR2^{-/-}$, or $CX3CR1^{-/-}$ mice (data not shown).

3.2 mRNA levels for the α2 chain of type 1 collagen in granulation tissues

We next investigated mRNA levels for the α2 chain of type I collagen (COL1A2) in granulation tissues. The levels of COL1A2 were determined by real-time PCR and normalized against the GAPDH mRNA level in each sample. As shown in Figure 3**,** just TGF-β induced a significant increase in the amount of COL1A2 mRNA in WT mice on day 4 ($p<0.05$). The level of COL1A2 mRNA at day 4 was reduced in $CCR2^{-/-}$ and $CX3CR1^{-/-}$ mice, but were still greater than that observed in WT mice in the absence of TGF-β. These data suggest that the increased collagen content observed after TGF-β administration are due to alterations at the transcriptional level. As shown in Figure 4**,** CTGF following TGF-β induced

an increase in the amount of COL1A2 mRNA in WT mice on day 8.

3.3 Profile of inflammatory cells in this animal model of skin fibrosis

It has been reported that the numbers of macrophages are increased in fibrotic tissues [28]. In our model, TGF-β injection increased the number of lesional macrophages on day 4, and these numbers continued to increase with subsequent CTGF injections on days 4-7 [12]. The numbers of accumulated F4/80-positive cells on day 4 and 8 were significantly decreased in $CCR2^{-/-}$ mice, as compared to WT mice ($p<0.05$ and $p<0.001$) (Fig. 5A, C). $CX3CR1^{-/-}$ mice also exhibited an approximately 30% reduction in macrophage infiltration on day 8 ($p<0.005$, as compared to WT mice) (Fig. 5A, D). The numbers of accumulated CD11b-positive cells on day 4 and 8 were significantly decreased in $CCR2^{-/-}$ mice, as compared to WT mice ($p<0.05$ and $p<0.05$) (Fig. 5E). $CX3CR1^{-/-}$ mice also exhibited a reduction in macrophage infiltration on day 8 ($p<0.05$, as compared to WT mice) (Fig. 5E). The numbers of accumulated Gr1-positive cells on day 4 and 8 were not decreased in $CCR2^{-/-}$ and $CX3CR1^{-/-}$ mice, as compared to WT mice (Fig. 5F). The numbers of macrophages were not increased in WT, $CCR2^{-/-}$, or $CX3CR1^{-/-}$ mice injected with PBS as control (data not shown).

3.4 Chemokine and chemokine receptor mRNA levels in granulation tissues

To examine whether chemokines produced by macrophages and inflammatory cells play a role in skin fibrosis, we performed real-time PCR analysis. TGF-β injection increased the expressions of MCP-1, fractalkine, CCR2 and CX3CR1 mRNA on day 4 in WT mice (Fig. 6). Similarly, we examined the expression of these molecules in $CCR2^{-/-}$ mice and $CX3CR1^{-/-}$ mice. As expected, CCR2 and CX3CR1 mRNA were undetectable in their respective knock-out mice. In $CX3CR1^{-/-}$ mice and $CCR2^{-/-}$ mice, each chemokine level was low, as compared to treated WT controls. In $CX3CR1^{-/-}$ mice and $CCR2^{-/-}$ mice, each chemokine receptor level was low, as compared to treated WT controls. CTGF following TGF-β injection increased the expressions of MCP-1 and fractalkine on day 8 in WT mice (Fig. 7).

3.5 Expression of CCR2 and CX3CR1 on WT Macrophages

Flowcytometric analysis showed that WT $CD11b^{+}$ gated cells expressed CCR2 and CX3CR1 protein (Fig. 8).

3.6 Chemokine and chemokine receptor mRNA levels in fibroblast cultures

In macrophage-fibroblast cocultures, cultured fibroblasts possessed increased levels of not only MCP-1 mRNA, but also CTGF mRNA ($p<0.0005$ and $p<0.05$), compared with fibroblasts cultured alone. Stimulation with TGF-β significantly augmented fractalkine and CTGF mRNA expression in cultured fibroblasts ($p<0.0005$ and $p<0.0005$), and tended to augment COL1A2 mRNA. Fibroblast stimulation with CTGF augmented CTGF mRNA levels ($p<0.05$), providing evidence for an autocrine feedback loop. In contrast, CTGF stimulation of fibroblasts did not alter fractalkine mRNA levels (Fig. 9).

4. Discussion

In the current study using a cutaneous fibrosis model, in which fibrosis is induced by serial TGF-β and CTGF injections, we have shown that macrophage-related chemokines play important roles in the development of this condition. This is consistent with our previous finding of increased number of macrophages at the CTGF injection site following TGF-β administration [12]. The current study suggests that interactions between fibroblasts and immune cells, including macrophages, contribute to the induction and maintenance of fibrosis via increased production of cytokines and chemokines.

MCP-1 is produced by macrophages, fibroblasts, endothelial cells, and other cells. MCP-1 is the predominant chemoattractant and activator of $CCR2^+$ monocytes and T cells. In addition to its chemoattractant activities, this chemokine induces Th2 cell polarization [29]. MCP-1 directly stimulates collagen production by fibroblasts via specific receptors and endogenous upregulation of TGF-β expression [16] Our *in vivo* data also revealed that serial injections of TGF-β increased MCP-1 mRNA and CCR2 mRNA levels. Conversely, the loss of CCR2 attenuated macrophage infiltration as well as skin fibrosis at day 4. Also, in macrophage-fibroblast *in vitro* cocultures, fibroblasts displayed increased levels of both MCP-1 and CTGF mRNAs. The up-regulated collagen levels induced by TGF-β were significantly reduced by CCR2 deficiency, while collagen levels induced by CTGF were unaffected. Thus, MCP-1 is required for TGF-β-mediated fibrosis induction in our mouse model. Collectively, the involvement of MCP-1/CCR2 interaction (CCR2-dependent loop) may be involved in the pathogenesis of skin fibrosis in which TGF-β plays a role.

Our data and previous studies estimate the following hypothesis regarding the role of MCP-1/CCR2 in the development of tissue fibrosis in SSc: First, MCP-1 augments the recruitment of CCR2-expressing macrophages and T cells into tissues, and promotes the interaction of $CCR2^+$ macrophages and fibroblasts. Then, TGF-β and PDGF produced by activated CCR2-expressing macrophages stimulate collagen synthesis by fibroblasts. At the same time, MCP-1 preferentially induces T cell differentiation to Th2 cells rather than Th1 cells, and IL-4 –secreting Th2 cells migrate to the fibroblasts. In turn, activated fibroblasts produce MCP-1, leading to the amplification loop for the development of tissue fibrosis.

Fractalkine is a membrane-bound chemokine that functions not only as a chemoattractant but also as an adhesion molecule. Fractalkine is expressed by endothelial cells that are activated by proinflammatory cytokines. The fractalkine receptor, CX3CR1, is

expressed on mature monocytes, NK cells, and cytotoxic effector T cells. Our data also revealed that serial injections of TGF-β increased fractalkine mRNA and CX3CR1 mRNA expression levels in granulation tissues. Additionally, the blockade of CX3CR1 attenuated macrophage infiltration and skin fibrosis. Furuichi *et al* have reported that CX3CR1 deletion attenuates macrophage infiltration as well as late-phase renal interstitial fibrosis and renal function after ischemic-reperfusion injury in a mouse model. In this model, CX3CR1 was found to be expressed mainly in kidney-infiltrating macrophages [30]. Our data linking CX3CR1 to skin fibrosis are consistent with a report stating that CX3CR1 is important for macrophage accumulation in the wound site and that angiogenic and profibrotic macrophage products are reduced in wounded skin in the absence of CX3CR1 [31]. The number of CX3CR1-expressing cells including monocytes were found to be increased in the lesional skin and lung tissues from SSc patients with diffuse cutaneous involvement [24]. Thus, infiltrating $F4/80^{+}$ macrophages are assumed to be mostly CX3CR1 positive. Fractalkine augments the recruitment of CX3CR1-expressing mononuclear cells into the affected tissue of SSc, leading to inflammation and vascular injury. A short-lived CCR2-expressing macrophage subset is actively recruited to inflamed tissues, while the CX3CR1-expressing subset has been characterized as resident in non-inflamed tissue [15]. In the current study, we found that the number of accumulated macrophages was decreased in $CCR2^{-/-}$ mice at day 4, while $CX3CR1^{-/-}$ mice also exhibited moderately reduced macrophage infiltration at day 8. $CX3CR1^{-/-}$ mice also showed less collagen accumulation after both TGF-β and CTGF stimulations, as compared to WT mice.

In vitro, we observed that stimulation with TGF-β significantly augmented fractalkine expression in cultured fibroblasts. To our knowledge, this is the first study to demonstrate the expression of fractalkine in skin fibroblasts. Ruth *et al* previously reported increased expression of fractalkine and CX3CR1 in synovial tissue fibroblasts in rheumatoid arthritis (RA) patients as well as in an adjuvant-induced arthritis model in rats [32]. Our data revealed that stimulation with TGF-β significantly augments CTGF mRNA expression in cultured fibroblasts, in the absence of macrophages. Thus, the fractalkine/CX3CR1 pathway may play an important role in the induction of fibrosis via both direct effects on fibroblasts, and indirect effects mediated by cytokine release from CX3CR1-macrophages and leukocytes in the lesional tissues. We have demonstrated that fractalkine was strongly expressed on endothelial cells in the affected skin and lung tissues. Soluble fractalkine levels were

significantly elevated in sera and were associated with the existence of digital ischemia, and severity of pulmonary fibrosis [24]. Other studies have shown that Raynaud's phenomenon, a type of ischemia-reperfusion, usually precedes the development of skin sclerosis. Therefore, it is possible that endothelial cell injury caused by recurring ischemia-reperfusion induces inflammatory cell infiltration and cytokine production, leading to the development of tissue fibrosis.

Fractalkine-dependent adhesion of monocytes is greatly enhanced by MCP-1 [33]. Both chemokine pathways may work independently and cooperatively to support the firm adhesion of monocytes *in vivo*. Our data demonstrated that up-regulated transcription of fractalkine and CX3CR1 mRNA in TGF-β-induced fibrosis tended to be reduced by CCR2 deficiency. Also, up-regulated transcription of MCP-1 and CCR2 mRNA in TGF-β-induced fibrosis tended to be reduced by CX3CR1 deficiency.

In summary, fibrosis is a complex process that may involve multiple cytokines and chemokines, which crosstalk with effector cells. MCP-1/CCR2 and fractalkine/CX3CR1 play important roles of the induction and maintenance of fibrosis, likely via differing mechanisms. Therefore, these molecules should be considered as potential therapeutic targets in patients with fibrotic disorders.

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References

[1] Charles C, Clements P, Furst DE: Systemic sclerosis: hypothesis-driven treatment strategies. Lancet 2006; 367: 1683-91.

[2] Denton CP, Black CM: Targeted therapy comes of age in scleroderma. Trends Immunol 2005; 26: 596-602.

[3] Varga J, Abraham D: Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007; 117: 557-67.

[4] Varga JA, Trojanowska M: Fibrosis in systemic sclerosis. Rheum Dis Clin North Am 2008; 34: 115-43; vii.

[5] Jimenez SA, Hitraya E, Varga J: Pathogenesis of scleroderma. Collagen. Rheum Dis Clin North Am 1996; 22: 647-74.

[6] Distler J, Distler O: Novel treatment approaches to fibrosis in scleroderma. Rheum Dis Clin North Am 2008; 34: 145-59; vii.

[7] Leroy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM: A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor beta the answer? Arthritis Rheum 1989; 32: 817-25.

[8] Nakerakanti SS, Bujor AM, Trojanowska M: CCN2 is required for the TGF-beta induced activation of Smad1-Erk1/2 signaling network. PLoS One 2011; 6: e21911.

[9] Takehara K: Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol 2003; 30: 755-9.

[10] Shinozaki M, Kawara S, Hayashi N, Kakinuma T, Igarashi A, Takehara K: Induction of subcutaneous tissue fibrosis in newborn mice by transforming growth factor beta--simultaneous application with basic fibroblast growth factor causes persistent fibrosis. Biochem Biophys Res Commun 1997; 240: 292-7.

[11] Mori T, Kawara S, Shinozaki M, Hayashi N, Kakinuma T, Igarashi A, et al.: Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: A mouse fibrosis model. J Cell Physiol 1999; 181: 153-9.

[12] Chujo S, Shirasaki F, Kawara S, Inagaki Y, Kinbara T, Inaoki M, et al.: Connective tissue growth factor causes persistent proalpha2(I) collagen gene expression induced by transforming growth factor-beta in a mouse fibrosis model. J Cell Physiol 2005; 203: 447-56.

[13] Kraling BM, Maul GG, Jimenez SA: Mononuclear cellular infiltrates in clinically

involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. Pathobiology 1995; 63: 48-56.
[14] Kovacs EJ: Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. Immunol Today 1991; 12: 17-23.
[15] Geissmann F, Jung S, Littman DR: Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003; 19: 71-82.
[16] Gharaee-Kermani M, Denholm EM, Phan SH: Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem 1996; 271: 17779-84.
[17] Zhang K, Gharaee-Kermani M, Jones ML, Warren JS, Phan SH: Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol 1994; 153: 4733-41.
[18] Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TN, Salant DJ, et al.: RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. J Exp Med 1997; 185: 1371-80.
[19] Hasegawa M, Sato S, Takehara K: Augmented production of chemokines (monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1alpha) and MIP-1beta) in patients with systemic sclerosis: MCP-1 and MIP-1alpha may be involved in the development of pulmonary fibrosis. Clin Exp Immunol 1999; 117: 159-65.
[20] Yamamoto T, Nishioka K: Role of monocyte chemoattractant protein-1 and its receptor,CCR-2, in the pathogenesis of bleomycin-induced scleroderma. J Invest Dermatol 2003; 121: 510-6.
[21] Moore BB, Paine R, 3rd, Christensen PJ, Moore TA, Sitterding S, Ngan R, et al.: Protection from pulmonary fibrosis in the absence of CCR2 signaling. J Immunol 2001; 167: 4368-77.
[22] Okuma T, Terasaki Y, Kaikita K, Kobayashi H, Kuziel WA, Kawasuji M, et al.: C-C chemokine receptor 2 (CCR2) deficiency improves bleomycin-induced pulmonary fibrosis by attenuation of both macrophage infiltration and production of macrophage-derived matrix metalloproteinases. J Pathol 2004; 204: 594-604.
[23] Umehara H, Tanaka M, Sawaki T, Jin ZX, Huang CR, Dong L, et al.: Fractalkine in rheumatoid arthritis and allied conditions. Mod Rheumatol 2006; 16: 124-30.

[24] Hasegawa M, Sato S, Echigo T, Hamaguchi Y, Yasui M, Takehara K: Up regulated expression of fractalkine/CX3CL1 and CX3CR1 in patients with systemic sclerosis. Ann Rheum Dis 2005; 64: 21-8.
[25] Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, et al.: Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proc Natl Acad Sci U S A 1997; 94: 12053-8.
[26] Combadiere C, Potteaux S, Gao JL, Esposito B, Casanova S, Lee EJ, et al.: Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. Circulation 2003; 107: 1009-16.
[27] Lopez-De Leon A, Rojkind M: A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. J Histochem Cytochem 1985; 33: 737-43.
[28] Ishikawa O, Ishikawa H: Macrophage infiltration in the skin of patients with systemic sclerosis. J Rheumatol 1992; 19: 1202-6.
[29] Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ: Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature 2000; 404: 407-11.
[30] Furuichi K, Gao JL, Murphy PM: Chemokine receptor CX3CR1 regulates renal interstitial fibrosis after ischemia-reperfusion injury. Am J Pathol 2006; 169: 372-87.
[31] Ishida Y, Gao JL, Murphy PM: Chemokine receptor CX3CR1 mediates skin wound healing by promoting macrophage and fibroblast accumulation and function. J Immunol 2008; 180: 569-79.
[32] Ruth JH, Volin MV, Haines GK, 3rd, Woodruff DC, Katschke KJ, Jr., Woods JM, et al.: Fractalkine, a novel chemokine in rheumatoid arthritis and in rat adjuvant-induced arthritis. Arthritis Rheum 2001; 44: 1568-81.
[33] Green SR, Han KH, Chen Y, Almazan F, Charo IF, Miller YI, et al.: The CC chemokine MCP-1 stimulates surface expression of CX3CR1 and enhances the adhesion of monocytes to fractalkine/CX3CL1 via p38 MAPK. J Immunol 2006; 176: 7412-20.

LEGENDS

Figure 1

Cytokine-induced fibrosis model. Serial injections of the two growth factors (TGF-β3 for days 1-3 days and CTGF for days 4-7) were conducted.

Figure 2

A. The amounts of collagen deposited in granulation tissues in WT mice, $CCR2^{-/-}$ mice, and $CX3CR1^{-/-}$ mice during injections of 800 ng of TGF-β on days 1-3 followed by injections of 400 ng of CTGF on days 4-7. Control WT mice were treated with PBS. Values are the mean ± SEM of 4-7 mice in each group. *$p<0.05$, **$p<0.01$, *** $p<0.005$. B-G. Representative histopathology from WT mice (B), $CCR2^{-/-}$ mice (C), and $CX3CR1^{-/-}$ mice (D) on day 4, and WT mice (E), $CCR2^{-/-}$ mice (F), and $CX3CR1^{-/-}$ mice (G) on day 8.

Figure 3

mRNA levels for the α2 chain of type I collagen in granulation tissues from WT mice, $CCR2^{-/-}$ mice, and $CXCR3^{-/-}$ mice on day 4 after TGF-β injections. The expression levels of COL1A2 mRNA were determined by real-time PCR and normalized against the GAPDH mRNA expression level in each sample. Values are the mean of 4-6 mice in each group. *$p<0.05$.

Figure 4

mRNA levels for the α2 chain of type I collagen in granulation tissues from WT mice, $CCR2^{-/-}$ mice, and $CXCR3^{-/-}$ mice on day 8 after CTGF following TGF-β injections. The expression levels of COL1A2 mRNA were determined by real-time PCR and normalized against the GAPDH mRNA expression level in each sample. Values are the mean of 4-6 mice in each group. *$p<0.05$.

Figure 5

Kinetics of macrophage recruitment assessed by immunohistochemical analysis using anti-mouse F4/80, CD11b and Gr1 antibody. WT , $CCR2^{-/-}$, and $CX3CR1^{-/-}$ mice were

injected with TGF-β and CTGF. Control WT mice were injected with PBS. A. The total numbers of F4/80-positive cells counted in five high power fields at x200 are shown. B-D. Representative photographs from WT mice (B), $CCR2^{-/-}$ mice (C), and $CX3CR1^{-/-}$ mice (D) on day 8. E. The total numbers of CD11b-positive cells counted in five high power fields at x200 are shown. F. The total numbers of Gr1-positive cells counted in five high power fields at x200 are shown. Values are the mean of ± SEM from 4-7 mice in each group. *$p<0.05$, **$p<0.005$, ***$p<0.001$.

Figure 6

mRNA levels of chemokines and chemokine receptors in granulation tissues on day 4. The expression levels of MCP-1, fractalkine, CCR2, and CX3CR1 mRNA in granulation tissues from WT mice, $CCR2^{-/-}$ mice, and $CXCR1^{-/-}$ mice 4 days after TGF-β injections were determined by real-time PCR and normalized against the GAPDH mRNA transcript level in each sample. Values are the mean of 4-6 mice in each group. *$p<0.05$, **$p<0.005$.

Figure 7

mRNA levels of chemokines and chemokine receptors in granulation tissues on day 8. The expression levels of MCP-1 and fractalkine mRNA in granulation tissues from WT mice, $CCR2^{-/-}$ mice, and $CXCR1^{-/-}$ mice 8 days after CTGF following TGF-b injections were determined by real-time PCR and normalized against the GAPDH mRNA transcript level in each sample. Values are the mean of 4-6 mice in each group.

Figure 8

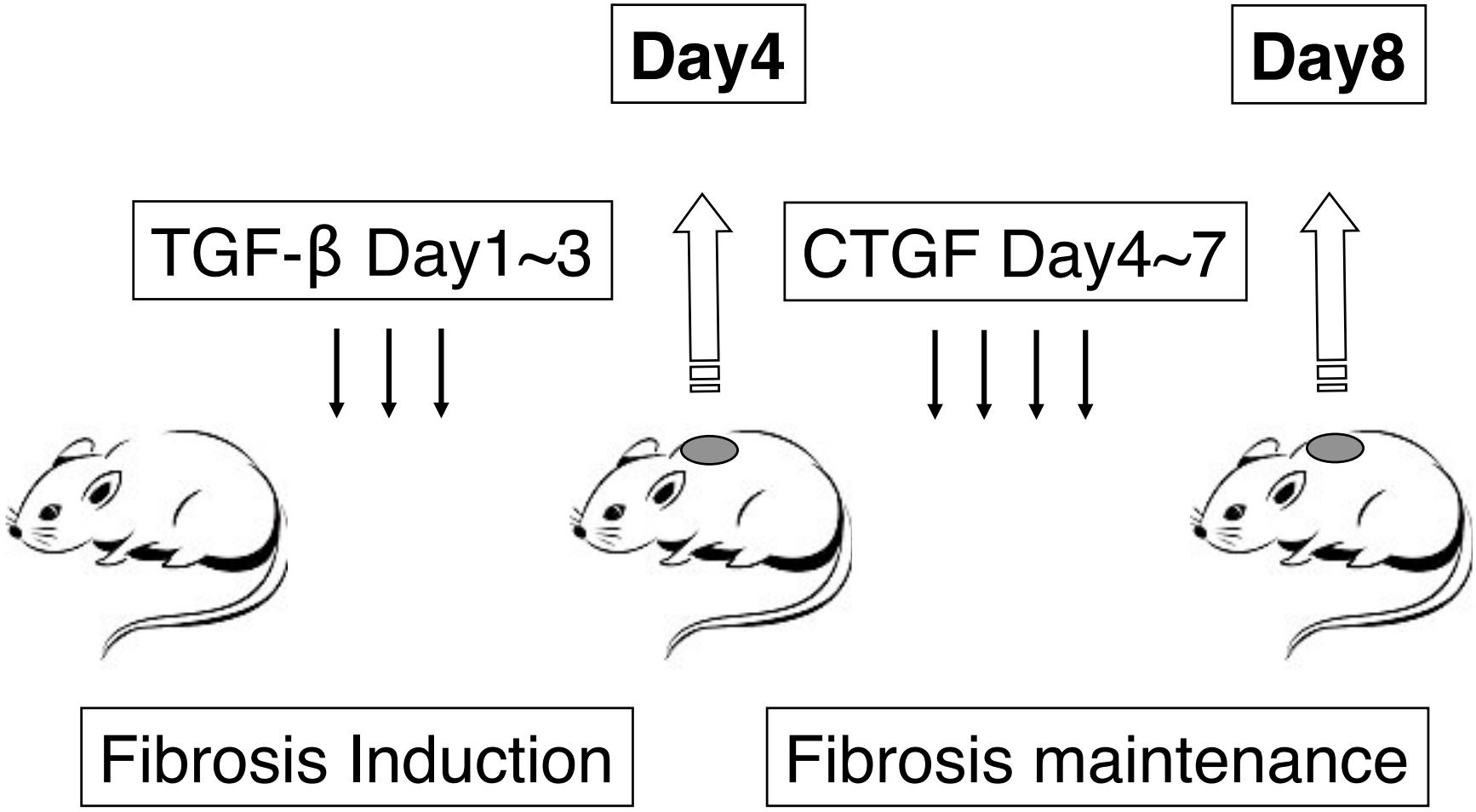
Representative histgrams of CCR2 and CX3CR1 expression on WT Macrophages. Bold lines (open regions) in the histgram represent WT CD11b-gaeted cells, and thin lines (shaded regions) in the hidtogram reperesent isotype control staining.

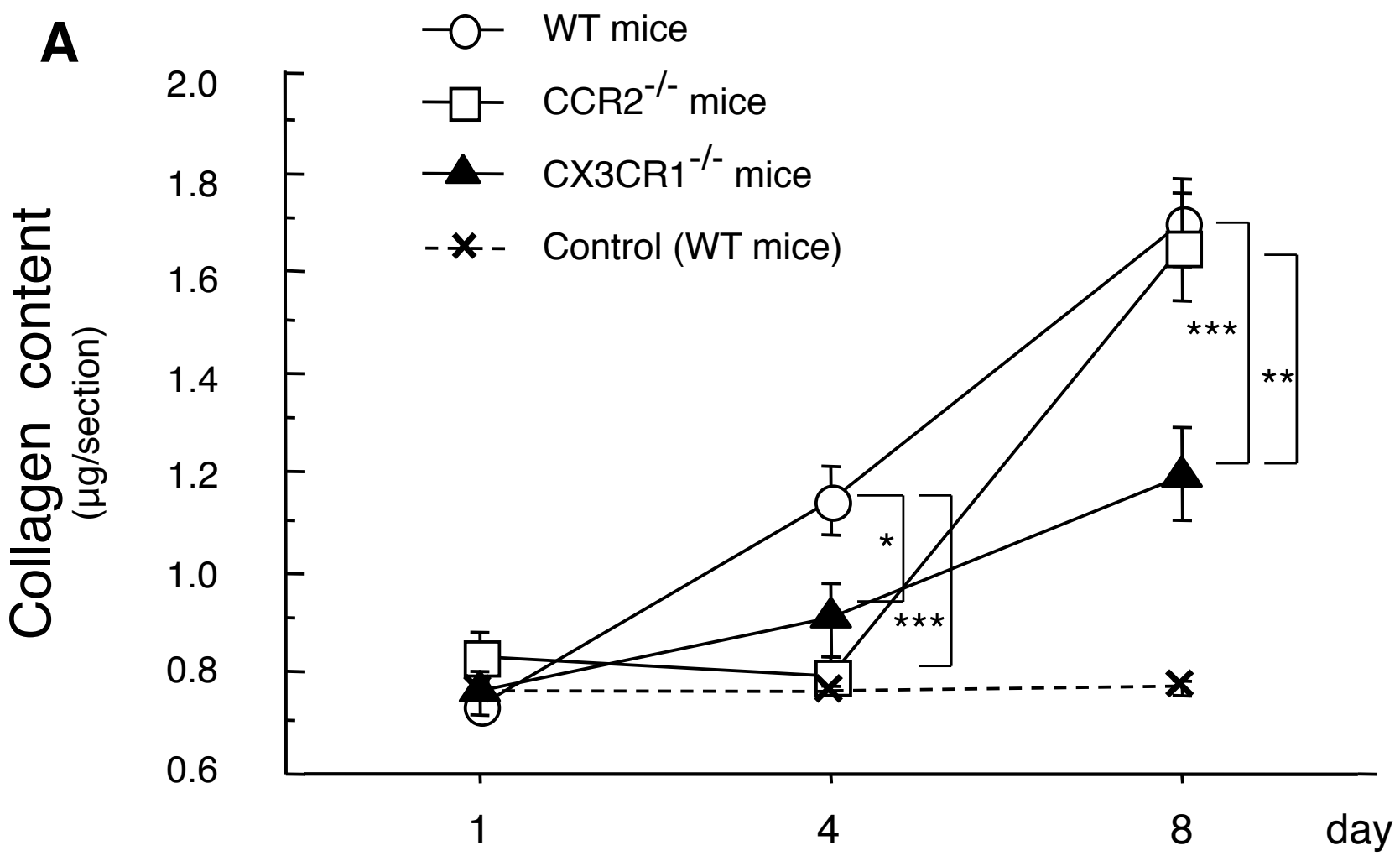
Figure 9

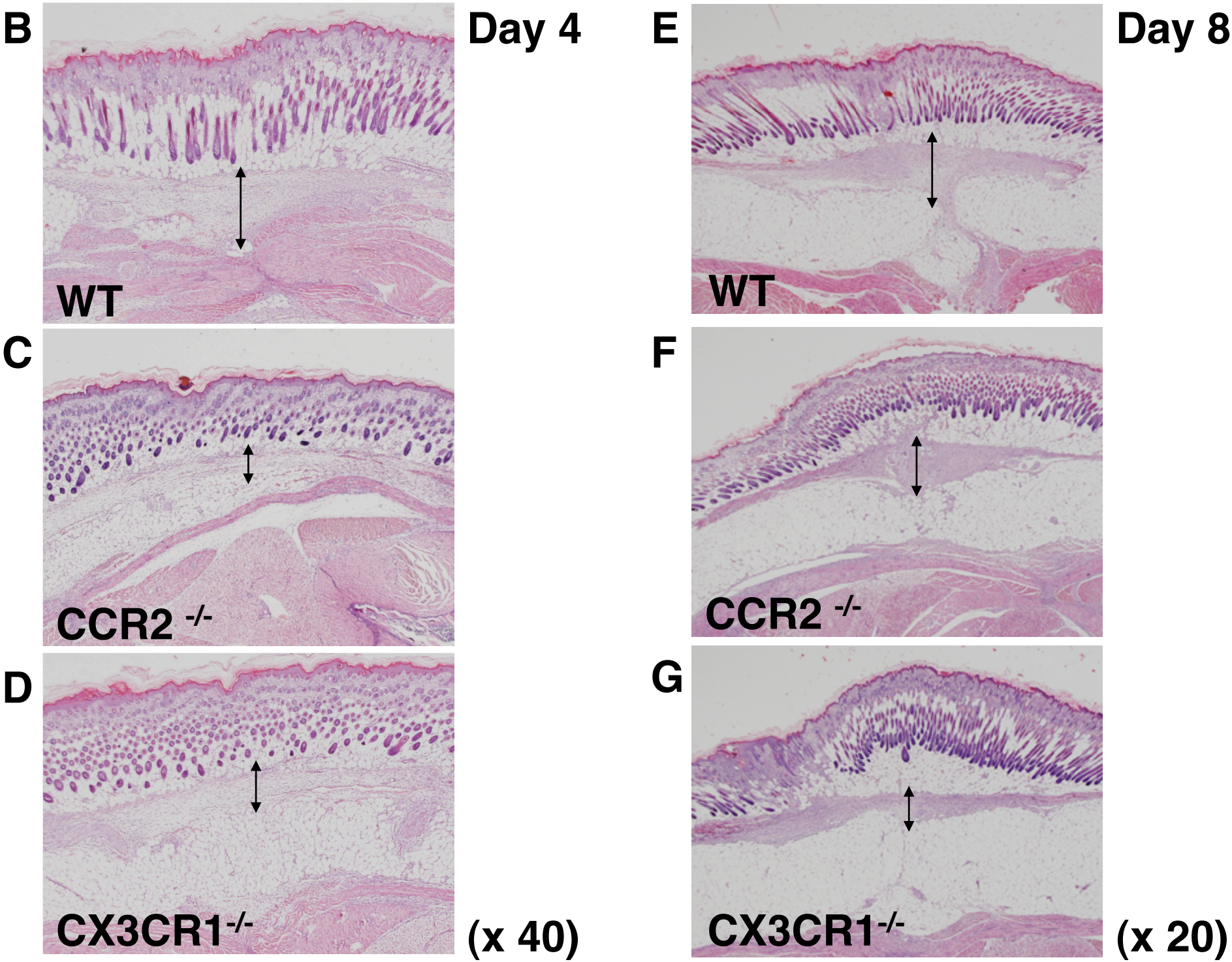
Chemokines and chemokine receptor mRNA levels from in vitro-cultured fibroblasts.

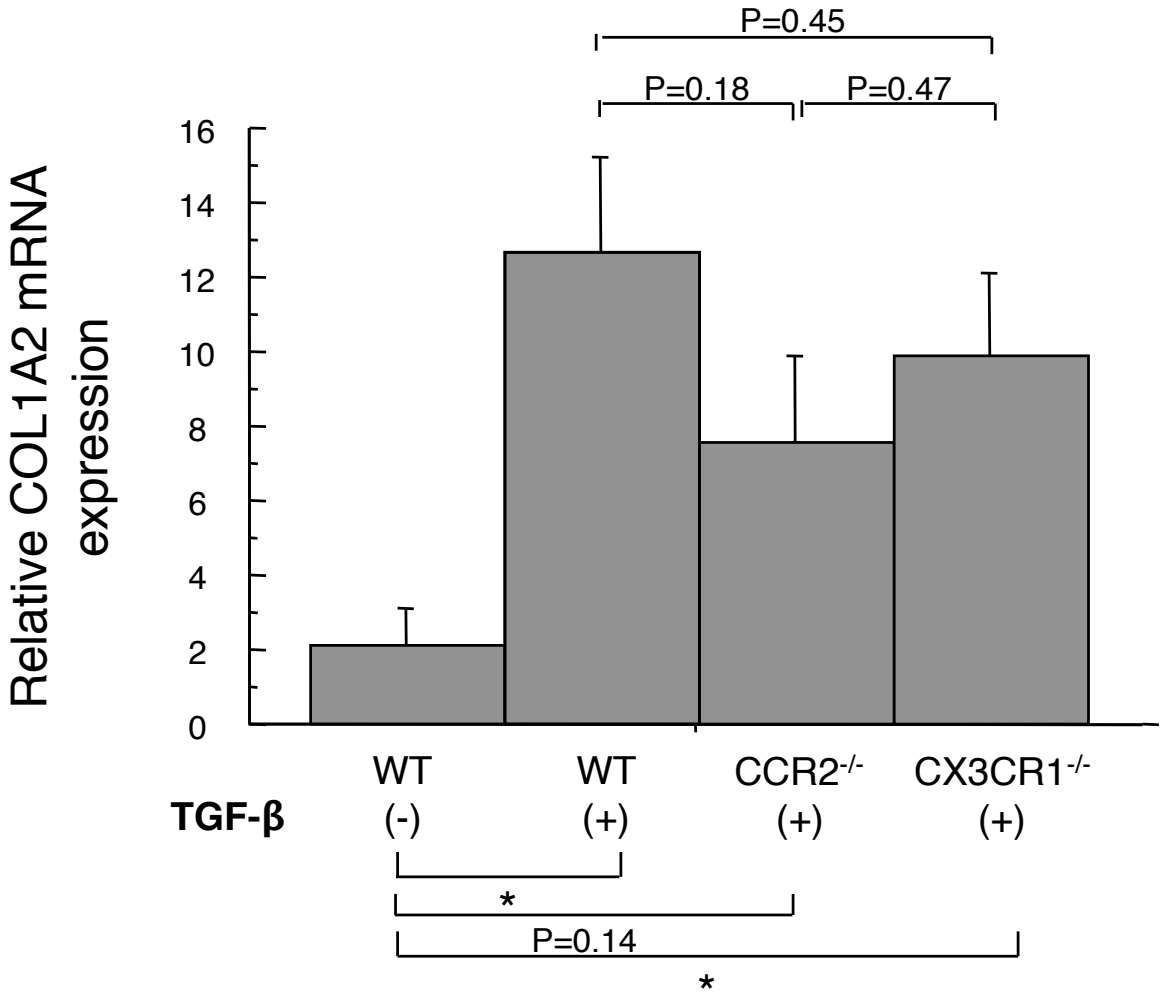
Primary fibroblast cultures were established from dorsal skin of newborn WT mice. TGF-β or CTGF was added to the cell culture medium to a final concentration of 10 ng/ml or 150 ng/ml, respectively. For macrophage-fibroblast cocultures, macrophages ($5x10^5$ cells per well) were

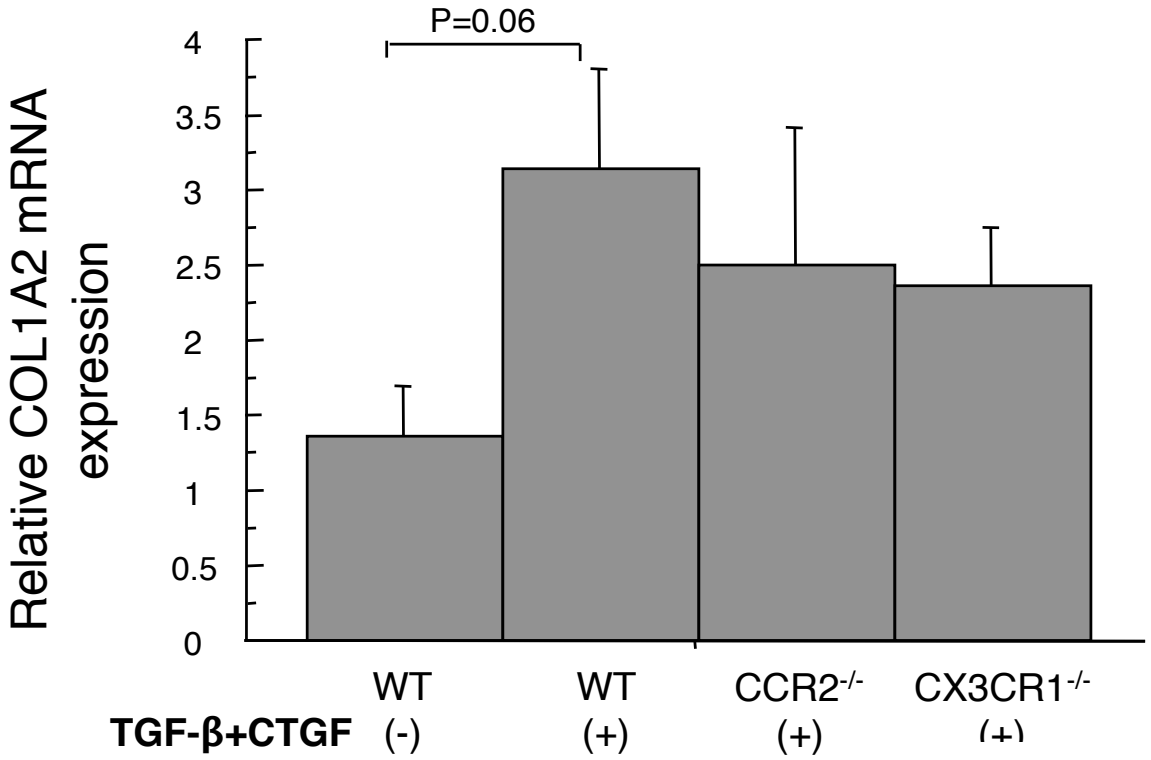
added to the upper wells. Total RNA was isolated from only fibroblasts, and expression levels were determined by real-time PCR and normalized against the GAPDH mRNA transcript level in each sample. Results were obtained from 4 samples in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0005$.

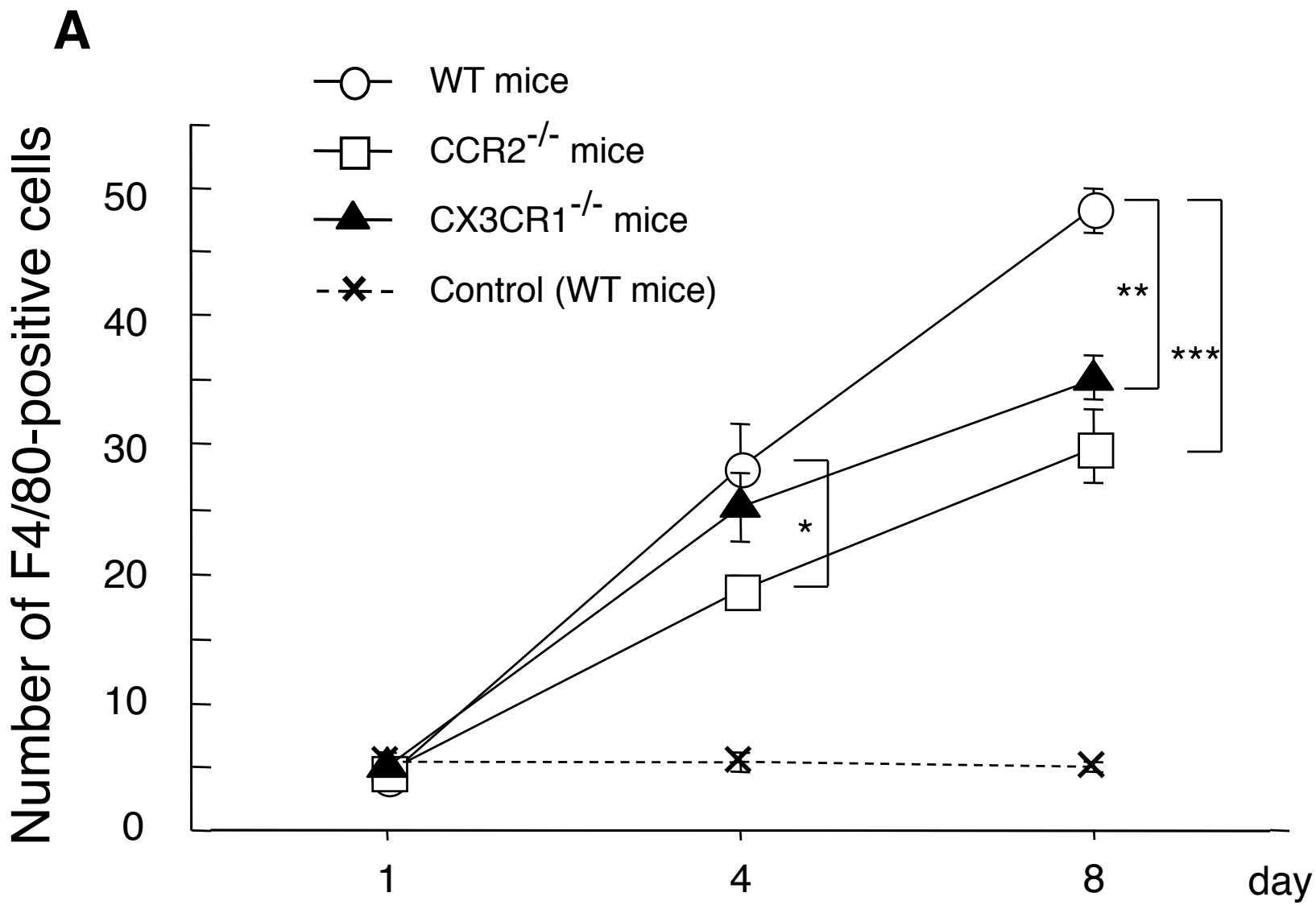

Day4
Day8
TGF-β Day1~3
CTGF Day4~7
Fibrosis Induction
Fibrosis maintenance

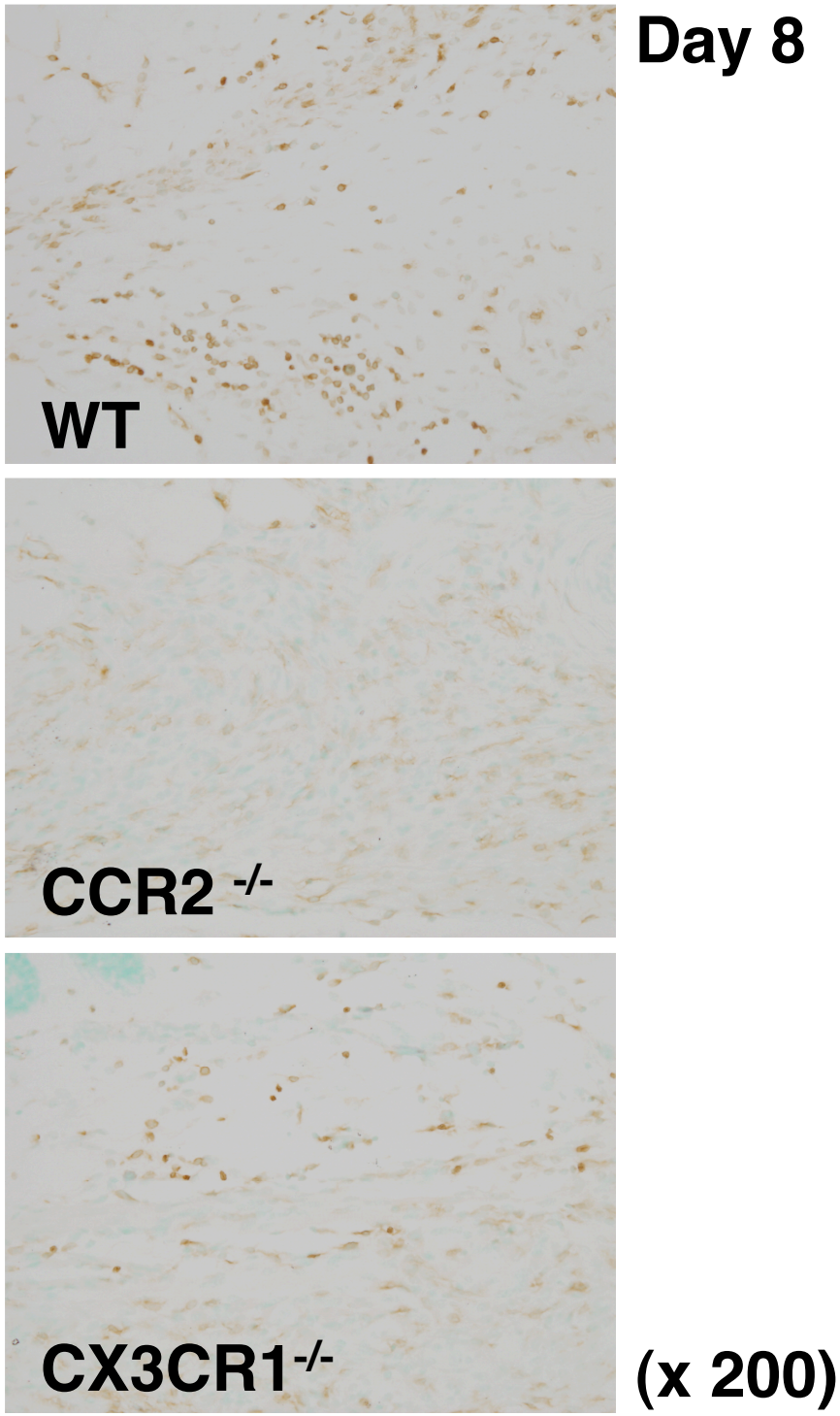

A
WT mice
CCR2-/- mice
CX3CR1-/- mice
Control (WT mice)
Collagen content
(μg/section)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
1
4
8
day
*
***
***
**

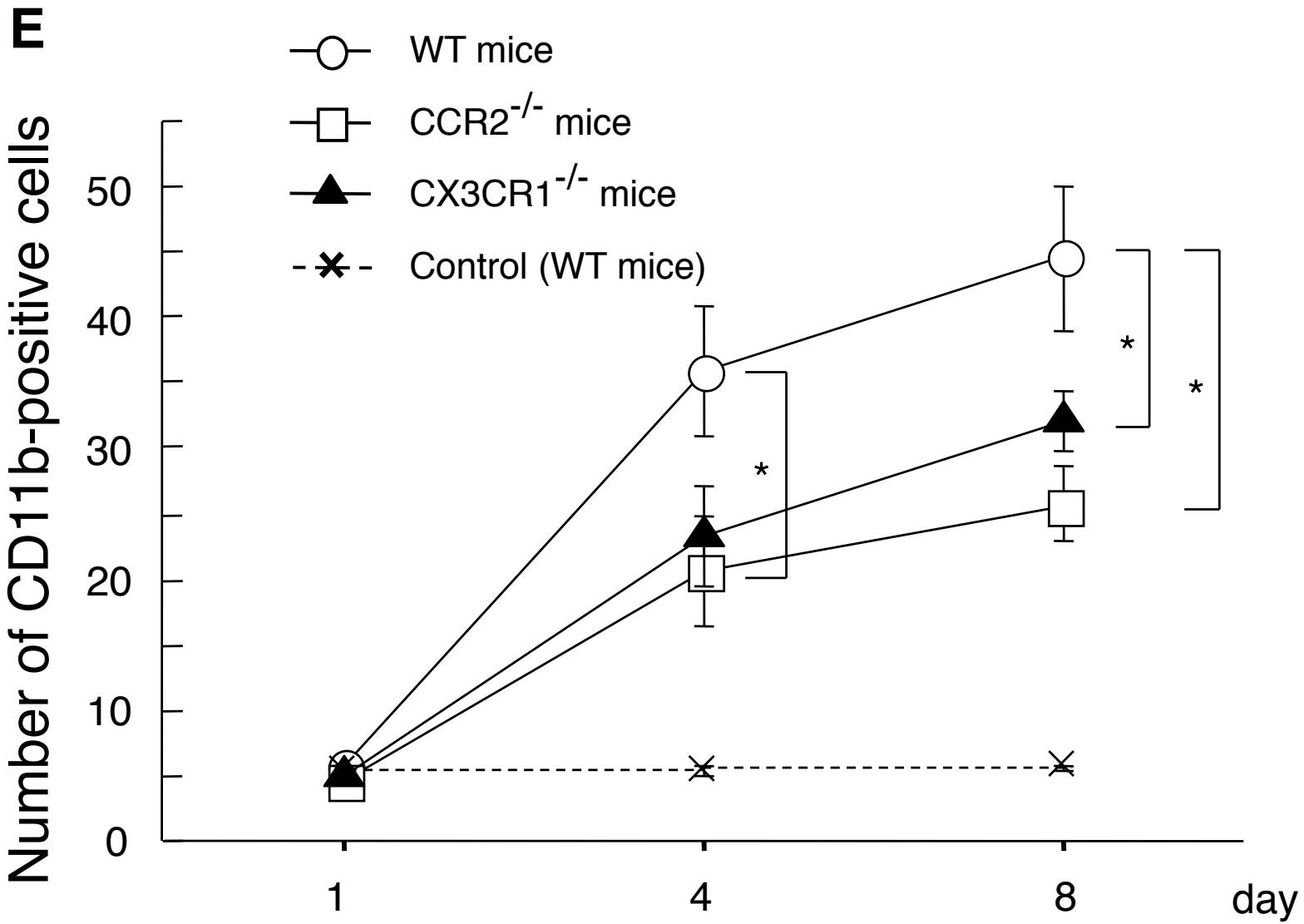

B
Day 4
WT
C
CCR2 -/-
D
CX3CR1-/-
(x 40)
E
Day 8
WT
F
CCR2 -/-
G
CX3CR1-/-
(x 20)


P=0.45
P=0.18
P=0.47
Relative COL1A2 mRNA expression
16
14
12
10
8
6
4
2
0
WT
(-)
WT
(+)
CCR2-/-
(+)
CX3CR1-/-
(+)
TGF-β
*
P=0.14
*

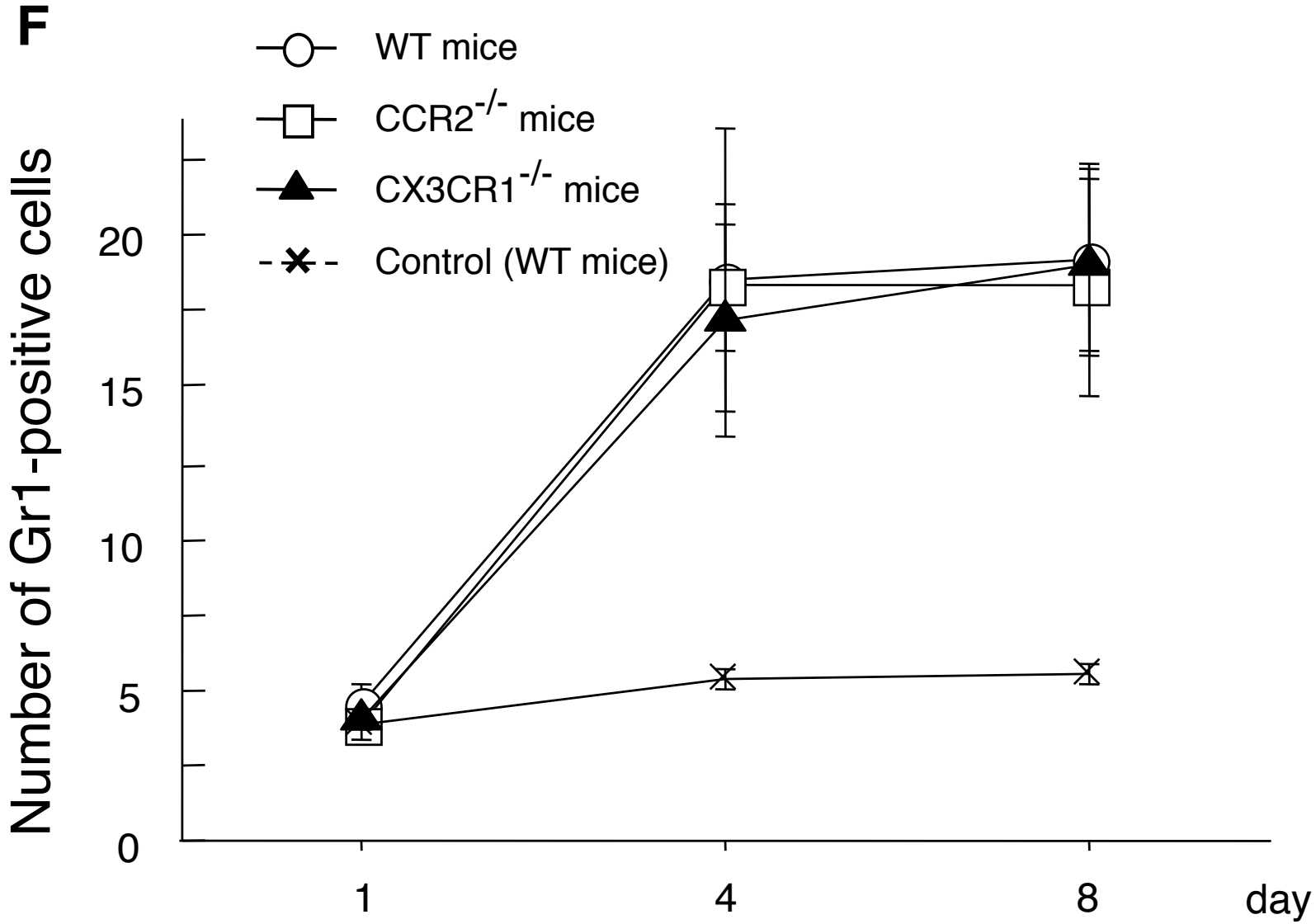

P=0.06
Relative COL1A2 mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
WT
CCR2-/-
CX3CR1-/-
TGF-β+CTGF
(-)
(+)
(+)
(+)


A
WT mice
CCR2-/- mice
CX3CR1-/- mice
Control (WT mice)
Number of F4/80-positive cells
0
10
20
30
40
50
1
4
8
day
*
**
***

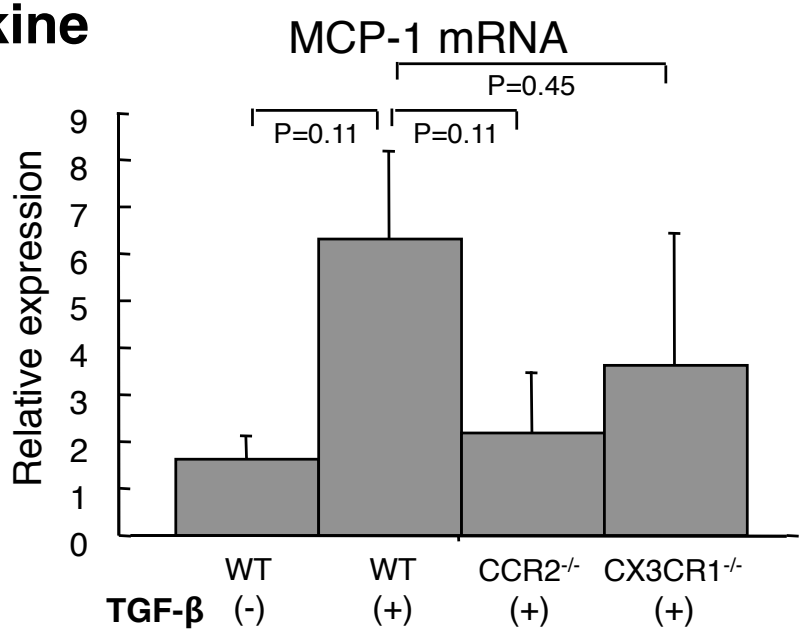

B
Day 8
WT
C
CCR2 -/-
D
CX3CR1-/-
(x 200)

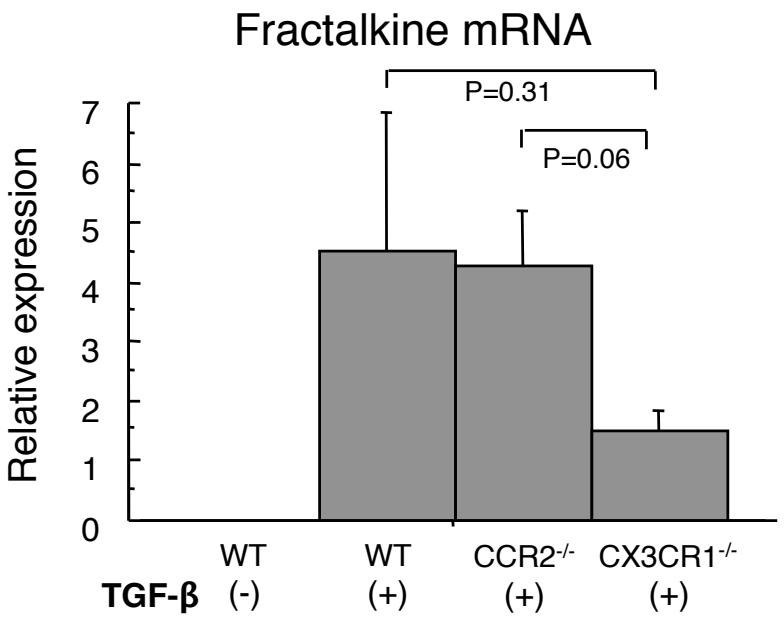

E
WT mice
CCR2-/- mice
CX3CR1-/- mice
Control (WT mice)
Number of CD11b-positive cells
50
40
30
20
10
0
1
4
8
day
*
*
*

F

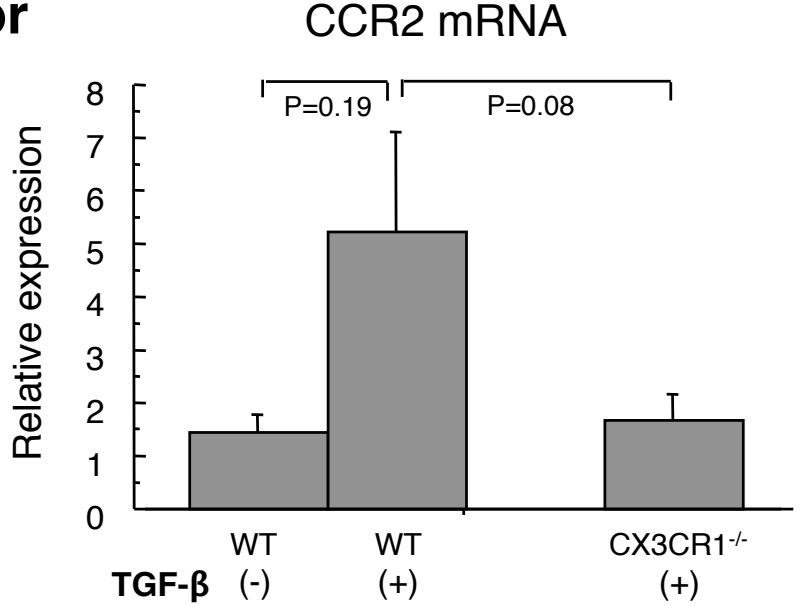

WT mice
CCR2-/- mice
CX3CR1-/- mice
Control (WT mice)
Number of Gr1-positive cells
0
5
10
15
20
1
4
8
day

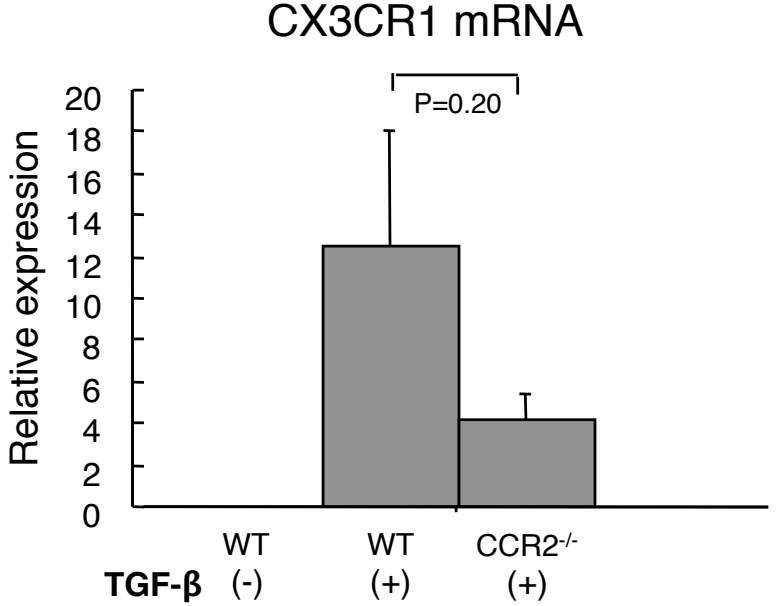
Chemokine



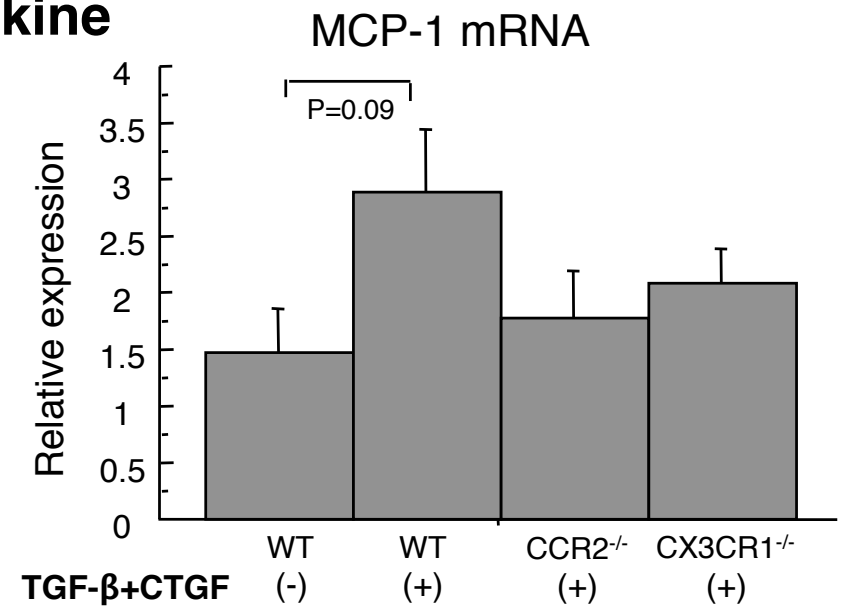


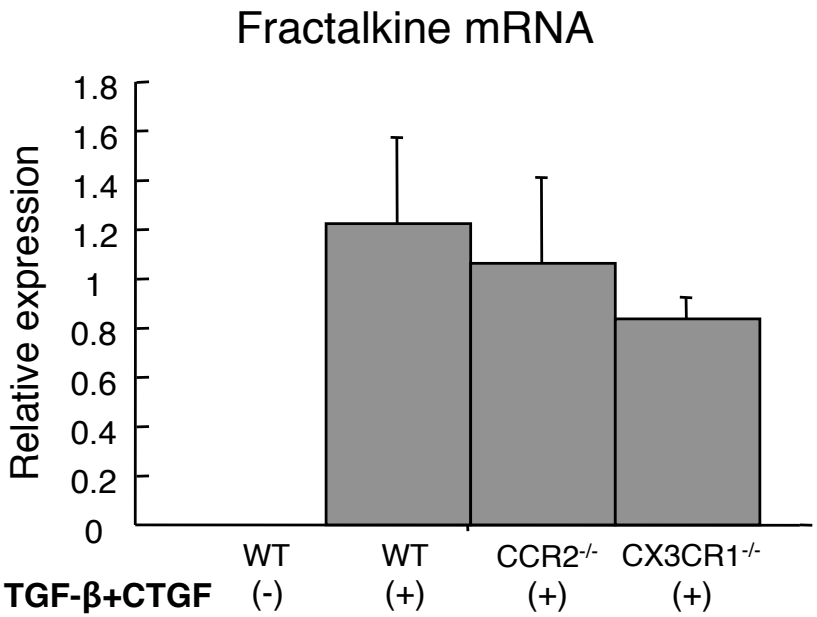
Chemokine receptor

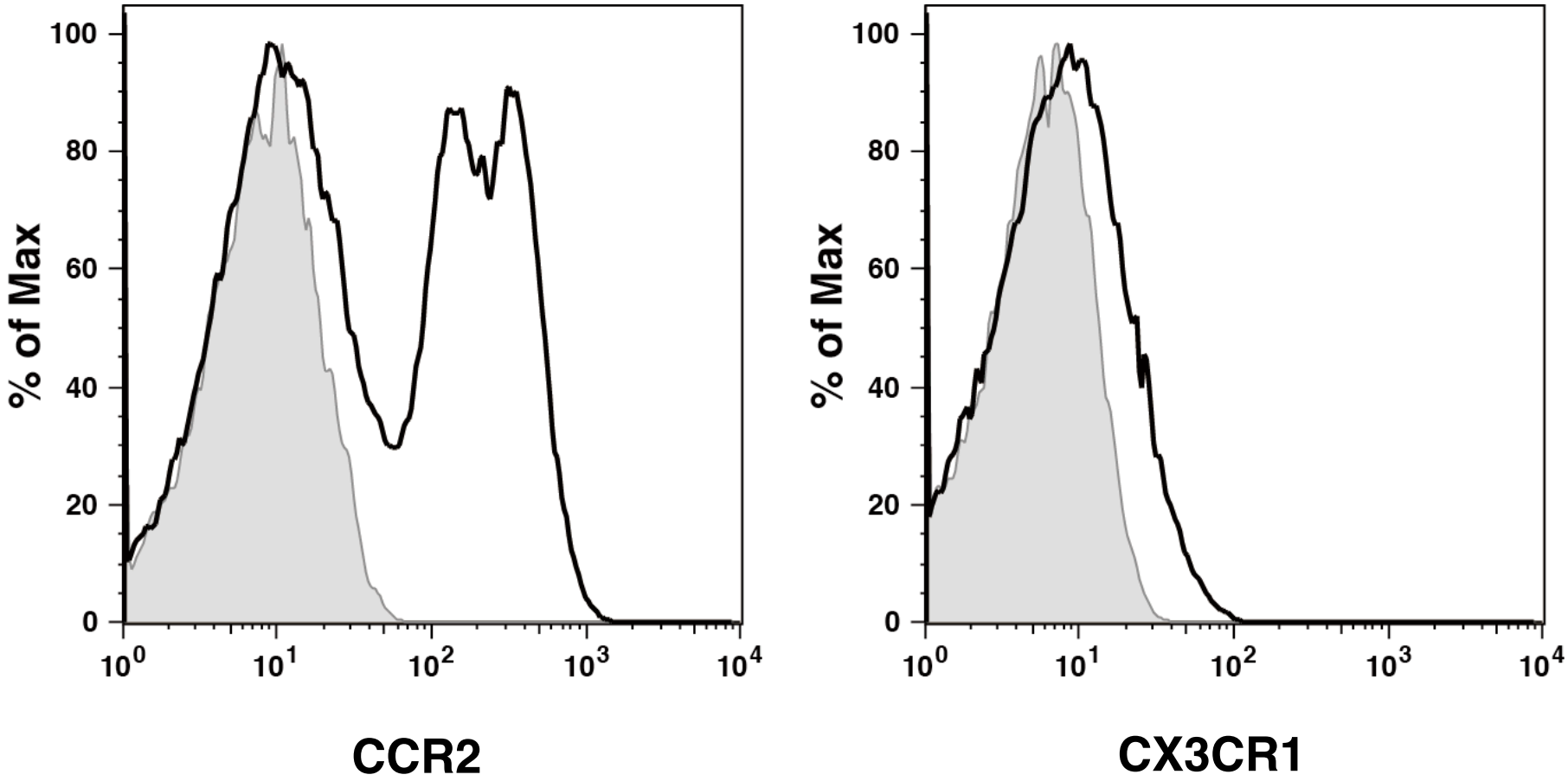




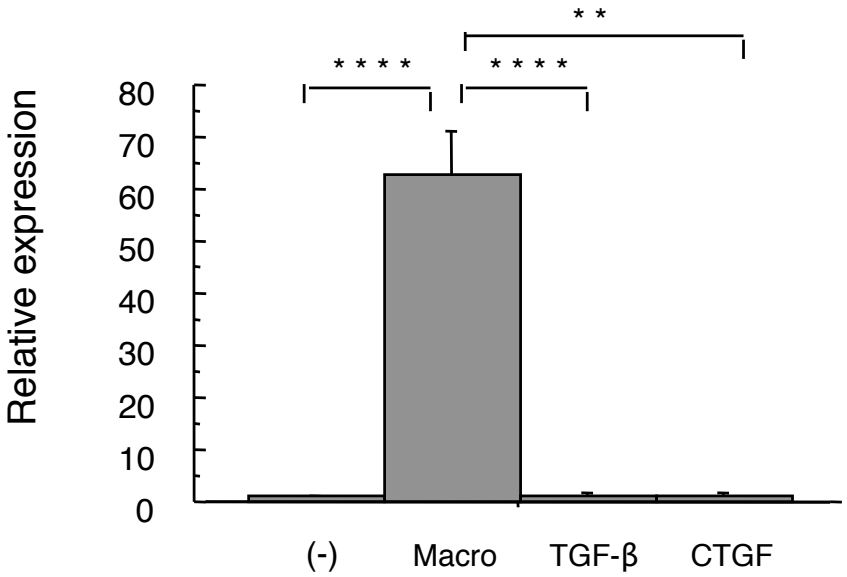
Chemokine


MCP-1 mRNA
Relative expression
4
3.5
3
2.5
2
1.5
1
0.5
0
P=0.09
WT
WT
CCR2-/-
CX3CR1-/-
TGF-β+CTGF
(-)
(+)
(+)
(+)

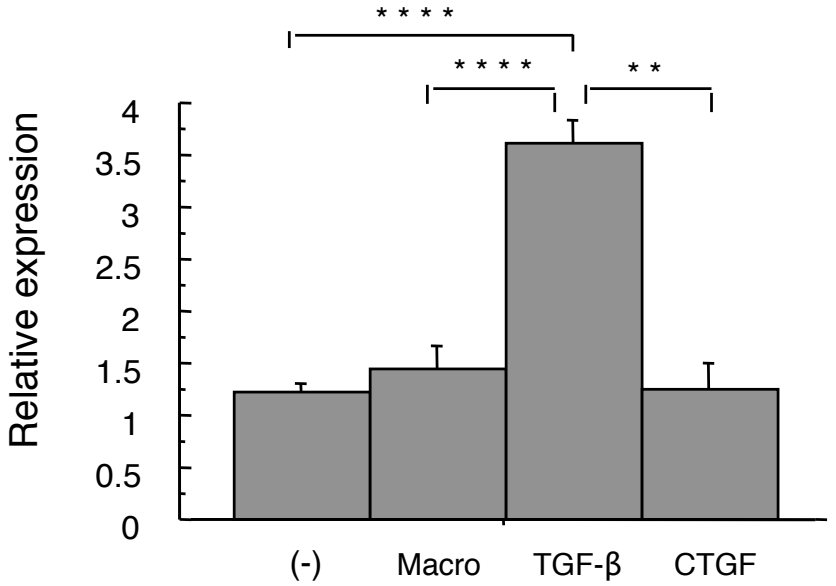

Fractalkine mRNA
Relative expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
WT
CCR2-/-
CX3CR1-/-
TGF-β+CTGF
(-)
(+)
(+)
(+)


% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
CCR2
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
CX3CR1

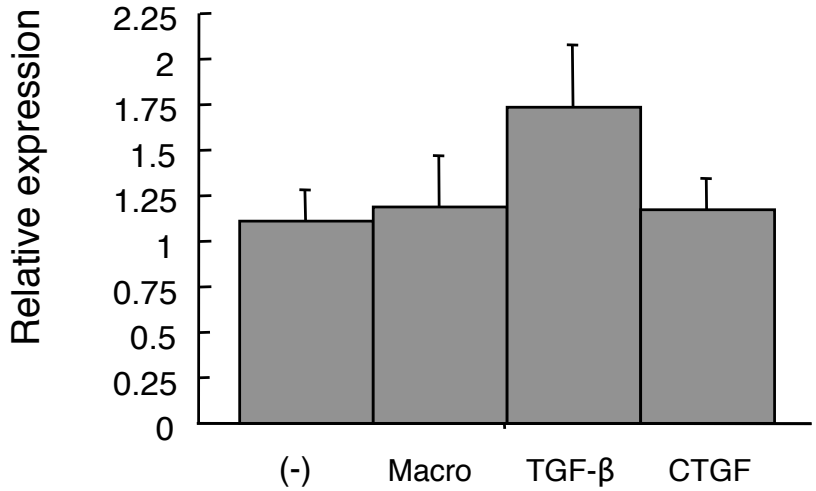
MCP1 mRNA



Fractalkine mRNA



COL1A2 mRNA



CTGF mRNA