Loss of Tumor Necrosis Factor α Potentiates Transforming Growth Factor β -mediated Pathogenic Tissue Response during Wound Healing

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Animal cornea is an avascular transparent tissue that is suitable for research on wound healing-related scarring and neovascularization. Here we show that loss of tumor necrosis factor α (TNF α) potentiates the undesirable, pathogenic response of wound healing in an alkali-burned cornea in mice. Excessive invasion of macrophages and subsequent formation of a vascularized scar tissue were much more marked in TNF α -null knockout (KO) mice than in wild-type mice. Such an unfavorable outcome in KO mice was abolished by *Smad7* gene introduction, indicating the involvement of transforming growth factor β or activin/Smad signaling. Bone marrow transplantation from wild-type mice normalized healing of the KO mice, suggesting the involvement of bone marrow-derived inflammatory cells in this phenomenon. Co-culture experiments showed that loss of TNF α in macrophages, but not in fibroblasts, augmented the fibroblast activation as determined by detection of α -smooth muscle actin, the hallmark of myofibroblast generation, mRNA expression of collagen 1 α 2 and connective tissue growth factor, and detection of collagen protein. TNF α in macrophages may be required to suppress undesirable excessive inflammation and scarring, both of which are promoted by transforming growth factor β , and for restoration of tissue architecture in a healing alkali-burned cornea

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The cornea is an avascular tissue of the eye that must remain transparent to refract light properly. An organized extracellular matrix structure is essential to the maintenance of the transparency. An alkali burn to the cornea is a serious problem that may cause severe and permanent visual impairment by scarring.^{1,2} Influx of inflammatory cells, ie, macrophages, activation of corneal fibroblasts (keratocytes), and subsequent tissue scarring in association with myofibroblast generation and neovascularization are all involved after alkali injury in the cornea.^{3,4} Activation of keratocytes results in the generation of myofibroblasts with an increased tissue contraction and extracellular matrix expression.⁵

Cytokines/growth factors are believed to orchestrate cell behaviors in a healing burned cornea.^{6,7} One of the cytokines up-regulated in an alkali-burned cornea is the pro-inflammatory pleiotropic cytokine tumor necrosis factor α (TNF α). However, the role of TNF α in modulation of cellular responses (inflammation and fibrosis) in an injured tissue has not been completely elucidated. The pro-inflammatory nature of TNF α is supported by observations that administration of anti-TNF α neutralizing antibody has therapeutic effects on an experimental arthritis animal model, as well as in human rheumatoid arthritis.^{8,9} Beneficial effects resulting from the loss of the TNF α receptor have also been reported in other tissues.¹⁰ However, studies using TNF α knockout (KO) mice or TNF α -

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receptor-deficient mice suggest other biological roles. For example, administration of a bacterial antigen induces an intense systemic inflammatory response in TNF α -receptor-deficient mice at the time when this reaction is completely resolved in wild-type (WT) mice.¹¹ More strikingly, lack of TNF α does not reduce the severity of experimental autoimmune arthritis,¹² inconsistent with the clinical efficacy of TNF α -neutralizing antibody in rheumatoid arthritis patients. Additionally, bleomycin-induced pulmonary fibrosis is more severe in KO mice or in TNF α -receptor KO mice than in WT mice.^{13–15}

The present study was conducted to elucidate the roles of TNF α in the process of wound healing by using a model of corneal alkali burn in mice. The results show that loss of TNF α potentiates undesirable actions of transforming growth factor β (TGF β) or activin/Smad in the tissue repair process, resulting in severe and persistent inflammation, fibrosis and neovascularization. Bone marrow transplantation (BMT) from WT mice normalizes healing of the KO mice, suggesting the involvement of BM-derived inflammatory cells in this phenomenon. In vitro co-culture experiments suggest that loss of TNF α in macrophages, but not in ocular fibroblasts, augments the fibrogenic behaviors of fibroblasts with overaction of TGF β /Smad signal as determined by the expression pattern of α -smooth muscle actin (α SMA), the hallmark of myofibroblast generation, expression of connective tissue growth factor (CTGF), and collagen protein. TNF α expressed in macrophages seems to be required for termination of the wound healing response, suppression of excessive cellular reaction, and resultant restoration of tissue architecture.

Materials and Methods

Experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University and conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Alkali Burn in Mouse Eyes

Three microliters of 1 N sodium hydroxide solution was applied to the right eye of 4-week-old KO mice or WT mice to produce an ocular surface alkali burn under both general and topical anesthesia.^{16,17} Ofloxacin ointment was topically administered twice a week in the first 2 weeks and then once a week until week 8 to reduce the risk of bacterial contamination. The eyes with obvious bacterial infection were excluded from the study. Eyes of 40 KO and 40 WT mice were histologically examined at weeks 1, 2, 3, 4, and 8 after alkali burn ($n = 5$ for paraffin sections and $n = 3$ for cryosections in each experimental condition).

Expression of mRNAs of cytokines was assayed by real-time RT-PCR. The burned corneas of 12 KO and 12 WT mice were obtained at weeks 1, 2, and 4 after burn and processed for RNA extraction and real-time RT-PCR

as previously reported.^{16,17} Four uninjured corneas of two KO and two WT mice were included to obtain the basal expression level of each cytokine.

Nine corneas obtained from nine WT or nine KO mice were burned and excised at weeks 1, 2, and 8. Corneas were homogenized in tissue lysis buffer (CellLytic MT; Sigma-Aldrich, St. Louis, MO) containing proteinase inhibitors (Complete protease inhibitor cocktail tablet; Roche Diagnostics, Mannheim, Germany) using an ultrasound homogenizer. Three corneas were used for each experimental condition at each time point. The samples were centrifuged, and protein concentration in each sample was adjusted. The samples were then mixed with 3 $\times$ sample buffer. The protein (10 μ g) was processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fractioned proteins were transferred to polyvinylidene difluoride membrane for incubation with antibodies against phospho-Smad2 (1:1000 in phosphate-buffered saline; Chemicon, Temecula, CA) and actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).¹⁷ Detection of phospho-Smad2 was first performed on the polyvinylidene difluoride membrane, and actin was detected after stripping the antibodies. Immunoreactive bands were visualized on Lumino Analyzer LAS1000 (Fuji Film, Tokyo, Japan) using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

BMT and Ocular Alkali Burn

We examined the roles of TNF α in BM-derived inflammatory cells in the healing process of the burned cornea by using BMT. WT or KO mice were sacrificed, and their BM cells were obtained by flushing the tibia and femur with PBS. A total of 2×10^5 BM cells was transplanted via tail vein to KO mice that had received whole body irradiation of 10 Gy 1 day before BM cell injection (WT-to-KO group or KO-to-KO group, respectively). Similarly, KO BM cells were transplanted to WT mice (KO-to-WT group). The mice were then processed for alkali burn in the right cornea 3 weeks after BMT, and 3 weeks later the corneas were processed for histology or RNA extraction ($n = 6$ in each group). Repopulation of transplanted BM was confirmed by RT-PCR detection of TNF α mRNA in the spleens of transplanted mice. The tissue was processed for histology/immunohistochemistry or extraction of RNA.

Treatment of Corneal Alkali Burn in KO Mice with Smad7 Gene Transfer

Mouse Smad7 cDNA was introduced to KO burned corneas as previously described.¹⁷ The burned cornea of adult KO mice ($n = 12$) received a mixture of recombinant adenoviruses carrying CAG (cytomegalovirus enhancer, chicken β -actin promoter plus a part of the 3'-untranslated region of rabbit β -globin) promoter-driven Cre (Cre-Ad) and mouse Smad7 cDNA under the LNL promoter (Smad7-Ad group) or Cre-Ad (for control) at 2 and 24 hours and at days 5, 10, and 15 after alkali exposure. Cre recombinase expressed via the CAG pro-

Table 1. Sequences Used in This Study

Transcript	Sequence*
mTGF β 1	F: 5'-gca aca tgtgga actcta ccagaa-3' R: 5'-gac gtc aaaagacagcca ctc-3'
mMCP-1	P: 5'-acc ttg gta accggctgc tgaccc-3' F: 5'-tgg ctcagccag atgcagt-3' R: 5'-cca gcc tac tca ttgggatca-3'
MVEGF	P: 5'-ccc cac tca cct gctgctactcattca-3' F: 5'-agcggagaaagc atttgttg-3' R: 5'-caa cgcgag tct gtgttt ttg-3'
mSmad7	P: 5'-cca aga tccgcagacgtgtaaatgttc-3' F: 5'-gac tccaggacg ctgttg gt-3' R: 5'-cca tgg ttg ctg catgaact-3'
mCol 1A2	P: 5'-agt gttccc tgg ttctccatcaaggct-3' F: 5'-aag ggtccc tctggagaacc-3' R: 5'-tct agagccaggagacc ca-3'
mTNF α	P: 5'-cag ggt ctt ctt ggtgctcccggat-3' F: 5'-aca gaaagc atg atccgcga-3' R: 5'-tct gggcca tag aactga tg-3'

*F, forward primer; R, reverse primer; P, probe.

moter deletes the stuffer polyA through the Cre/LoxP system. Preliminary experiments showed that there was no obvious difference in the histology or in healing at the macroscopic level in an alkali-burned mouse eye with Cre-Ad application or without application of adenovirus. At each administration the eye received 3.0×10^7 plaque forming units/3 μ l. The cornea was histologically examined at week 3 after wounding. The efficiency of gene transfer was previously evaluated by co-infection of Cre-Ad and green fluorescent protein under control of the Cre/LoxP system.¹⁷

Immunohistochemistry

Deparaffinized sections (5 μ m) or fixed cryosections (7 μ m) were processed for immunohistochemistry as previously reported.^{18,19} The following antibodies were diluted in PBS: rabbit polyclonal anti-phospho-Smad2 antibody (1:100; Chemicon), rabbit polyclonal anti-keratin 12 antibody²⁰ (1 μ g/ml), mouse monoclonal anti- α SMA antibody (1:100; Neomarker, Fremont, CA), goat polyclonal anti-TNF α antibody (1:100; Santa Cruz Biotechnology), and rat monoclonal anti-CD31 (platelet endothelial cell adhesion molecule [PECAM]) antibody (1:100, Santa Cruz Biotechnology). Immunohistochemistry for TGF β 1 and - β 2 was performed as previously reported.^{21,22} The presence of monocytes/macrophages was examined by using rat monoclonal F4/80 anti-macrophage antigen antibody (Clone A3-1, 1:400; BMA Biomedicals, August, Switzerland). The number of labeled cells in the central cornea (200- μ m length) was determined in four or five corneas for each condition. Negative control staining was performed with omission of each primary antibody and did not yield specific staining (not shown). Data at each time point was statistically analyzed by using an unpaired *t*-test, and *P* < 0.05 was accepted as statistically significant.

Detection of mRNAs of TNF α , TGF β 1, MCP-1, and VEGF in Burned Corneas

Expression of TNF α mRNA in tissue was evaluated by RT-PCR.¹⁶ Real-time RT-PCR for mRNAs of mouse TGF β 1, monocyte/macrophage-chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and collagen I α 2 was performed at weeks 1, 2, and 4 using primers and probes shown in Table 1 as previously reported.^{17,18}

Cell Culture Experiments

Effects of Exogenous TGF β 1 and TNF α on Fibrogenic Gene Expression in WT Ocular Fibroblasts

The eye shells (including cornea and sclera) of WT mice after natal day 1 were minced and explanted in a collagen-coated 60-mm culture dish (Iwaki Glass, Tokyo, Japan) for the outgrowth of ocular fibroblasts. Intraocular structures were carefully removed before being minced. Immunohistochemistry showed that cells expressed α SMA following two or three passages, suggesting they acquired a myofibroblastic phenotype. Thus we used the cells without passage for analysis of α SMA expression, and those at later passages were used for determination of the expression level of collagen and CTGF.

Cells were passaged and grown to confluency in 60-mm culture dishes and then treated with recombinant human TGF β 1 (0.5 or 1.0 ng/ml; R&D Systems, Minneapolis, MN), recombinant human TNF α (5.0 or 10.0 ng/ml; R&D Systems), or vehicle control in the medium supplemented with 3% fetal calf serum for 24 hours. The cells were processed for total RNA extraction and real-time RT-PCR for collagen I α 2 and CTGF. Five dishes were prepared for each culture condition. Data at each time point was statistically analyzed by analysis of variance.

mRNA Expression of TGF β 1 and VEGF by KO Macrophages

Mouse macrophages were obtained from the peritoneal space using a glycogen stimulation method. In brief, 5% sterilized oyster glycogen (Sigma-Aldrich) was injected into the peritoneal space of either a WT or KO mouse. After 4 days the peritoneal cavity was irrigated with culture medium to harvest macrophages. Approximately 90% of the cells obtained by this method were positive for F4/80. The cells were allowed to adhere to 60-mm culture dishes for 6 hours in culture medium, and then nonadherent cells were washed out with PBS. RNA extracted from the adherent cells (macrophages) was analyzed by real-time RT-PCR for mRNA of TGF β 1 or VEGF. Three specimens were prepared for each condition. Data at each time point were statistically analyzed by using the unpaired *t*-test.

mRNA Expression of Collagen I α 2 and CTGF by KO Ocular fibroblasts

Mouse ocular fibroblasts were obtained from KO mice after natal day 1 and cultured as described above. The cells were then treated with recombinant human TGF β 1 (1.0 ng/ml) in the medium supplemented with 3% fetal calf serum for 24 hours. The cells were processed for total RNA extraction and real-time RT-PCR for collagen I α 2 and CTGF. Three specimens were prepared for each condition. Data at each time point were statistically analyzed by using the unpaired *t*-test.

Co-culture of Fibroblasts and Macrophages

To investigate the differential roles of TGF β and TNF α expressed by fibroblasts and macrophages, we performed co-culture experiments using these two cell types obtained from WT and KO mice. A suspension of WT or KO macrophages (2.4×10^6 cells) was added to confluent WT/KO fibroblast cultures in 60-mm dishes in culture medium supplemented with 3% fetal calf serum and further incubated for 24 hours before extraction of total RNA for real-time RT-PCR for mRNA expression of collagen I α 2 and CTGF. Five dishes were prepared for each culture condition.

To confirm the alteration of collagen I α 2 mRNA expression correlated with protein expression, we quantified the collagen protein in culture medium by using a Sircol Collagen Assay Kit (Biocolor, Belfast, Northern Ireland) as previously reported.^{23,24} In brief, as described above, WT/KO ocular fibroblasts and WT/KO macrophages were co-cultured in a medium supplemented with 3% fetal calf serum and 50 mg/ml β -aminopropionitrile fumarate (a lysyl oxidase inhibitor) in 60-mm culture dishes ($n = 4$ in each condition) and incubated for 72 hours. The medium was harvested and was allowed to react with Sirius Red dye. The collagen-dye complex was precipitated by centrifugation. The dye was removed from the precipitated collagen with 0.5 N sodium hydroxide, and the absor-

bance at 540 nm was measured. Collagen in the medium was determined with optical density of serially diluted standards.

To determine the roles of TGF β /Smad signal in fibroblasts in augmentation of expression of collagen I α 2 and CTGF with KO macrophages, we blocked TGF β /Smad signaling in fibroblasts in the co-culture by using adenoviral gene transfer of Smad7 to WT fibroblasts¹⁷ before adding macrophages to the culture. Our previous experiments showed that the adenoviral gene transfer by the Cre/LoxP system works well to introduce Smad7 cDNA to cultured fibroblasts.¹⁷ WT ocular fibroblasts were treated with a mixture of Smad7-adenovirus and Cre-adenovirus (Smad7-Ad group) or with Cre-adenovirus alone as control (Cre-Ad group) at a multiplicity of infection of 100 for 2 hours. The medium containing adenoviral vectors was removed, and the fibroblasts were incubated at 37°C for 48 hours, at which time mouse macrophages (2.4×10^6 cells) were added to the fibroblast culture and incubated for an additional 24 hours before RNA extraction. Five dishes were prepared for each culture condition.

We mimicked the loss of TNF α in macrophages by adding a neutralizing anti-TNF α antibody (10 μ g/ml; R&D Systems, Catalog no. AF-410-NA), while control cultures received nonimmune goat IgG (10 μ g/ml; Sigma-Aldrich 15256, preservative-free). Our previous real-time RT-PCR results showed no, or very minimal, TNF α mRNA expression in fibroblasts. The co-culture was conducted with WT fibroblasts pretreated with either Cre-Ad or Smad7-Ad. Five dishes were prepared for each culture condition. After 24-hour incubation total RNA was extracted. In these co-culture experiments, the extracted total RNA was processed for real-time RT-PCR for collagen I α 2 or CTGF as previously reported. Data at each time point were statistically analyzed by using analysis of variance.

Because the ocular fibroblasts expressed α SMA after two or three passages, we used the outgrowth fibroblasts without any passage for co-culture with macrophages for Western blotting for α SMA. Primary fibroblast outgrowth was co-cultured with WT or KO macrophages and further incubated for 48 hours, and α SMA was detected as previously reported.¹⁷

To further mimic the healing of corneal stroma in vivo, we established a three-dimensional collagen gel co-culture system using WT ocular fibroblasts and WT/KO macrophages. Because the ocular fibroblasts used in the above experiments expressed α SMA after two or three passages, we used fibroblasts without any passage. The ocular fibroblasts obtained from the primary outgrowth (3.5×10^6) were mixed with WT or KO macrophages (4.8×10^6) in 1 ml of collagen gel (collagen Cell Culture System, Chemicon) according to the protocol provided by the manufacturer. The gel with the cells was incubated in wells of 24-well culture plates for 48 hours in a medium supplemented with 3% fetal calf serum. The gel was then fixed with 4% paraformaldehyde and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin (H&E) or processed for immunocytochemistry for F4/80 antigen or α SMA.

Figure 1. Healing of alkali-burned corneas in $\text{TNF}\alpha$ -knockout mice. **A:** Incidence (%) of the eyes with epithelial defects or corneal ulcerations at each time point. At all time points the percentage is higher in $\text{TNF}\alpha$ -null (KO) mice than in wild-type (WT) mice. **B:** The corneas of WT (**a**, **c**, and **e**) and KO mice (**b**, **d**, and **f**) at weeks 2, 3, or 8, respectively. Stromal opacification and neovascularization are more marked in the healing corneas of KO mice as compared with those of WT mice throughout this interval. At week 2 the corneas of KO mice have a central ulceration, which is not seen in WT mice. Stromal neovascularization is still detectable in KO mice, but not WT mice, at week 8. **C:** Histology of burned corneas stained with H&E. No histological differences were seen in the healing corneas of the WT (**c**) and KO (**d**) mice at week 1. However, at week 2 severe inflammation and central ulceration with degraded stroma are observed in the KO (**f**) but not WT corneas (**e**). At weeks 3 and 8 inflammation has decreased in the corneas of WT mice (**g** and **i**), whereas stromal tissue disorganization and significant inflammation are still detected in KO corneas (**h** and **j**). Scale bar = 100 μm .

Results

Loss of $\text{TNF}\alpha$ Worsens the Outcome of Healing of the Cornea after Alkali Burn

To examine the role of $\text{TNF}\alpha$ in modulation of the wound healing response in an alkali-burned cornea, we first histologically evaluated healing of corneas of $\text{TNF}\alpha$ KO or WT littermates following alkali burn. At each time point the incidence and degree of epithelial defect/ulceration (Figure 1A), opacification (Figure 1B), and neovascularization (Figure 1B) in the burned cornea was more severe in KO mice than WT controls. Even at week 8, healing tissue in KO mice still contained inflammatory cells and exhibited a thickened edematous stroma, as compared with WT burned corneas that were nearly healed with minimal inflammation (Figure 1C). This suggests that $\text{TNF}\alpha$ is required for the normal suppression or termination of the wound healing response to avoid excessive inflammation.

Myofibroblast Generation, Macrophage Infiltration, and Stromal Neovascularization in the Injured Cornea Were More Prominent in KO Mice Than in WT Mice

Differentiation of fibroblasts to myofibroblasts, as determined by αSMA expression, is one of the hallmarks of corneal stromal scarring.⁵ Healing burned corneas contain many myofibroblasts in both WT and KO mice at week 1. After week 2, however, the majority of corneal fibroblasts were not labeled with anti- αSMA in WT mice, whereas many cells still stained for αSMA in KO mice (Figure 2, A and B).

We determined the number of F4/80-positive cells in the central cornea to assess macrophage infiltration. At weeks 1 and 2 after injury, there was no difference in the number of macrophages in the central zone of the healing burned cornea between WT mice and KO mice, whereas after week 3 the number of labeled cells was higher in KO corneas than in WT corneas (Figure 2, C and D).

Neovascularization of the corneal stroma also likely contributes to stromal opacification and is associated with inflammation.^{25–27} Immunohistochemistry for CD31 (PECAM) detected marked neovascularization in KO corneas at all time points examined, with neovascularization substantially reduced in eyes of WT mice (Figure 2E).

Cytokine Expression

To locate the source of $\text{TNF}\alpha$ in the healing cornea, we performed dual immunostaining for $\text{TNF}\alpha$ and F4/80 antigen. $\text{TNF}\alpha$ was detected in the healing epithelium and F4/80-labeled macrophages in WT mice (Figure 3A).

Immunohistochemistry showed that protein expression of $\text{TGF}\beta 1$ and $\text{TGF}\beta 2$ in WT corneas was more marked at week 1 but less prominent at week 4, as compared with KO corneas (Figure 3, B and C). Real-time RT-PCR repeated using three independent samples showed mRNA expression of $\text{TGF}\beta 1$, VEGF, and MCP-1 was higher in WT than in KO corneas at week 1 but was higher in KO mice at week 4 (see Supplemental Figure 1 at <http://ajp.amjpathol.org>).

To examine the activation status of $\text{TGF}\beta$ signaling, expression of C-terminally phosphorylated Smad2 was examined by immunohistochemistry and Western blotting. Its expression indicates the activation of the $\text{TGF}\beta$ /

Figure 2. Analysis of stromal healing in burned corneas. **A:** Expression of α SMA in stromal cells at weeks 1 (**a** and **b**), 2 (**c** and **d**), 3 (**e** and **f**), and 8 (**g** and **h**) after alkali burn of either WT (**a**, **c**, **e**, and **g**) or KO (**b**, **d**, **f**, and **h**) mice. Immunofluorescence showed that healing burned corneal stroma contains similar numbers of α SMA-positive myofibroblasts in both WT and KO mice at week 1. At weeks 2 and 3, however, the majority of stromal keratocytes (corneal fibroblasts) were not labeled for α SMA in WT mice, whereas almost all of the keratocytes were positive for α SMA in KO mice. At week 8 almost all of the keratocytes were negative for α SMA in WT mice, but the KO corneal stroma still contained myofibroblasts. Bar, 100 μ m. **B:** Bar chart of the number of myofibroblasts determined as described in Materials and Methods. At and after week 2 the number of myofibroblasts is higher in KO mice than in WT mice. * $P < 0.05$; ** $P < 0.01$; bar, mean $\pm$ SD. **C:** The distribution of F4/80-positive macrophages in representative areas of the burned corneal stroma. At weeks 1 and 2 there was little difference in macrophages in the central zone of the healing burned cornea between WT mice (**a** and **c**) and KO mice (**b** and **d**), whereas after week 3 the number of F4/80-labeled cells was higher in KO mouse corneas than in WT mouse corneas (**e** and **f**). Dotted lines indicate the location of corneal endothelium. Bar, 100 μ m. **D:** Bar chart of the number of macrophages determined as described in Materials and Methods. At and after week 3 the number of myofibroblasts is higher in KO mice than in WT mice. * $P < 0.05$; ** $P < 0.01$; bar, mean $\pm$ SD. **E:** In the healing stroma at weeks 2 (**a** and **b**), 3 (**c** and **d**), or 8 (**e** and **f**), the expression of CD31 (PECAM) is higher in KO corneas as compared to WT corneas. At week 2 very little neovascularization is detectable in WT stroma, whereas the affected stroma is populated with dense blood vessels in a KO mouse. At weeks 3 and 8, almost no CD31(PECAM)-labeled blood vessels are detected in the healing WT stroma, while expression is still high in the stroma of KO mice. Scale bar = 100 μ m.

Smad signaling pathway. Expression of C-terminal phospho-Smad2 was higher in KO tissue than in WT tissue at weeks 2 and 8 (Figure 4, A and B).

Smad7 is up-regulated by many ligands, including TGF β . Smad7 mRNA expression level was higher in KO corneas as compared with WT corneas throughout the intervals examined, especially at 4 weeks after burn (Figure 4C). These findings suggest that the TGF β /Smad signal was more activated in the absence of TNF α .

Repair of the Burned Cornea in Mice following BMT

Macrophages that infiltrate into the healing burned cornea reportedly represent the cell type most involved in the pathogenesis of scarring and neovascularization and are also a source of TNF α .^{22,25} We hypothesized that TNF α -null inflammatory cells might be involved in the phenotype (marked inflammation, neovascularization, myofibroblast generation, and scarring) observed in the KO stroma. To explore this hypothesis, we examined the healing of corneas of KO mice that had received BMT from either WT or KO mice (WT-to-KO or KO-to-KO group, respectively). Using RT-PCR we detected TNF α

mRNA in the spleen of mice of the WT-to-KO group, indicating that WT BM had successfully reconstituted in KO mice (Figure 5A), whereas no TNF α was detected in spleens of KO-to-KO BMT mice. Three weeks after alkali burning, marked neovascularization with ulceration was observed in the cornea of a KO-to-KO group mouse, whereas the cornea of a WT-to-KO group mouse exhibited much less neovascularization without epithelial defect (Figure 5B). RT-PCR of RNA samples extracted from healing corneas revealed expression of TNF α mRNA in the cornea of a WT-to-KO group mouse but not in a KO-to-KO group cornea (Figure 5C). H&E staining showed markedly more inflammation and thickening in corneal stroma of a KO-to-KO mouse as compared with the cornea of a WT-to-KO mouse (Figure 5D, panels a and b). Expression of α SMA and laminin in keratocytes and macrophage invasion was greater in KO-to-KO mice as compared with WT-to-KO mice (Figure 5, D (panels c–h), F, and G). This result indicates that TNF α produced by BM-derived inflammatory cells has an important role in local wound healing in the cornea.

To further examine the role of inflammatory cell-derived TNF α in the healing process, we transplanted KO BM to WT mice and performed alkali burning of the cornea. The

Figure 3. Expression of growth factors in burned corneas. **A:** Double immunostaining at week 2 after burn shows that TNF α (green) is detected in epithelium (epi, green), endothelium (endo, green), and macrophages (yellow dual stain), in healing stroma of a WT cornea (**a**), whereas TNF α is not detected in KO macrophages (red) (**b**). Panels **c** and **d** represent 4',6-diamidino-2-phenylindole nuclear localization in panels **a** and **b**, respectively. Bar, 10 μ m. Immunohistochemical detection of TGF β 1 (**B**) and TGF β 2 (**C**) shows that protein expression of active TGF β 1 and TGF β 2 is higher in WT mice at week 1. At week 4 immunoreactivity for TGF β 1 in the affected stroma is higher in KO mice and that of TGF β 2 is similar between WT and KO. Scale bar = 100 μ m.

results showed that transplantation of KO BM to WT mice did not yield KO-like healing in WT mice (data not shown). The possible mechanisms of this phenomenon are presented in the Discussion.

Adenoviral Gene Transfer of Smad7 Rescues Burned Corneas of KO Mice

Because it appears that TNF α counteracts many biological effects of TGF β , we hypothesized that loss of TNF α might potentiate the actions of TGF β in healing tissue, resulting in more marked inflammation, neovascularization, and scarring as compared with a WT cornea. To explore this hypothesis, we examined the effects of Smad7 cDNA introduction on the healing of a KO burned cornea as previously reported.¹⁷ Smad7 gene transfer rescued the abnormal healing process in KO mice (Figure 6A). Histology showed less inflammation (less macrophage invasion), fewer myofibroblasts, and decreased expression of laminin in stroma of a KO burned cornea treated with Smad7-adenoviral gene transfer compared to a KO cornea infected with control adenovirus (Figure 6, B–D).

Cell Culture Experiments

To examine the roles of TGF β and TNF α in the regulation of gene expression of wound healing-related components, we performed cell culture experiments. Exogenous TGF β 1 up-regulated mRNA expressed collagen I α 2 and CTGF in cultured WT ocular fibroblasts in a dose-dependent manner. TNF α treatment minimally affected the expression of these components, but adding exogenous TNF α to WT cultures treated with TGF β completely abolished its up-regulation of collagen I α 2 expression and reduced CTGF mRNA up-regulation (Figure 7).

Expression of TGF β 1 and VEGF mRNA in cultured KO macrophages was similar to that in WT macrophages (data not shown). Cultured macrophages did not express CTGF. There was also no difference in the degree of up-regulation of collagen I α 2 and CTGF mRNA in re-

Figure 4. Expression of C-terminal phospho-Smad2 protein and Smad7 mRNA. **A:** Activation status of TGF β /Smad is examined by the expression pattern of C-terminal phospho-Smad2. Expression of phospho-Smad2 in a KO cornea (**c**) is similar to that of a WT cornea at week 1. It is then more marked in KO corneas at weeks 2 (**g**) or 4 (not shown) as compared with WT corneas (**e**). At week 8 its expression is similar between WT (**i**) and KO (**k**) corneas as seen by immunostaining. Panels **b**, **d**, **f**, **h**, **j**, and **l** indicate the nuclear 4',6-diamidino-2-phenylindole staining of the sections in panels **a**, **c**, **e**, **g**, **i**, and **k**, respectively. Scale bar, 10 μ m. **B:** Western blotting shows that the expression level of C-terminal phospho-Smad2 in KO tissues is higher than in WT tissues at weeks 2 and 8. **C:** Smad7 mRNA expression level was higher in KO mice than in WT mice throughout the interval examined of 1 to 4 weeks after burn. Three sets of experiments were done, and all of the data are shown in **C**.

Figure 5. Repair of burned corneas of mice that received BMT. **A:** RT-PCR detected TNF α mRNA in spleens of KO mice that received WT BM (WT-to-KO group, lanes 3 and 4) but not in the samples of KO mice that received KO BM (KO-to-KO group, lanes 1 and 2), indicating that WT BM had successfully transplanted to KO mice. **B:** 3 weeks after alkali burning, marked neovascularization with central corneal ulceration is observed in corneas of the KO-to-KO group, whereas corneas of mice in the WT-to-KO group exhibited less neovascularization without epithelial defect. **C:** RT-PCR of RNA samples extracted from healing burned corneas revealed expression of TNF α mRNA in a cornea of WT-to-KO group mice (lanes 1 and 2) but not in KO-to-KO group corneas (lanes 3 and 4). **D:** Histology by H&E staining shows much more inflammation in corneas of KO-to-KO mice (**a**) as compared to corneas of WT-to-KO mice at week 3 (**b**). Macrophage invasion as detected by F4/80 antigen (**c** and **d**) and expression of α SMA (**e** and **f**) and laminin (**g** and **h**) in keratocytes were more marked in KO-to-KO mice (**c**, **e**, and **g**) as compared with WT-to-KO mice (**d**, **f**, and **h**). Bar, 100 μ m. **E** and **F:** The number of myofibroblasts (**E**) and macrophages (**F**) in the stroma as determined by the method described in the text. * $P < 0.05$; bar, mean $\pm$ SD.

sponse to exogenous TGF β 1 between WT and KO fibroblasts in culture (data not shown).

We showed that invading macrophages are one of the cell types expressing TNF α in burned corneas and that TNF α from BM-derived cells has an important role in local wound healing in the cornea. To examine the role of macrophages in the regulation of fibrogenic cytokine expression in fibroblasts, we co-cultured fibroblasts and macrophages. The same number of macrophages was directly added to each fibroblast monolayer, because direct attachment of macrophages to the cells is reportedly required for activation of TGF β secreted by macrophages.^{28,29} The results showed that the co-culture of ocular fibroblasts with

KO macrophages up-regulated mRNA expression of CTGF and collagen α 2 more prominently than that seen with WT macrophages, regardless of the genotype of the fibroblasts (Figures 8A and 9A). We confirmed this up-regulation of collagen α 2 mRNA expression in fibroblasts with co-cultured KO macrophages, which led to increased collagen protein production by Sircol collagen assay (Figure 9B).

Our preliminary experiments showed that up-regulation of collagen α 2 mRNA expression in WT fibroblasts co-cultured with KO macrophages was abolished by further addition of anti-TGF β antibody in the medium (data not shown). We then tested the role of TGF β /Smad signaling in fibroblasts on this phenomenon. Up-regulation

Figure 6. Treatment of alkali-burned corneas of KO mice with adenoviral gene transfer of Smad7 cDNA. **A:** Adenoviral gene transfer of mouse Smad7 cDNA rescued the abnormal healing process in KO mice. Marked neovascularization and shrinkage of the eyeball, presumed to be due to contraction of myofibroblasts, are observed in a Cre-Ad-treated cornea (**a**), whereas these features are much reduced in a cornea with Smad7-Ad (**b**). **B:** H&E histology indicates more marked inflammation in the thickened stroma (presumably due to edema) in a Cre-Ad-treated cornea (**a**), which is much less severe with enhanced tissue healing in a Smad7-Ad-treated cornea (**b**). Immunohistochemical examinations show less macrophage invasion (**c** and **d**), fewer myofibroblasts (**e** and **f**), and decreased expression of laminin (**g** and **h**) in corneal fibroblasts (keratocytes) in KO burned corneas treated with Smad7 gene transfer (**b**, **d**, **f**, and **h**) than in KO corneas with Cre-Ad (**a**, **c**, **e**, and **g**). **C** and **D:** The number of myofibroblasts (**C**) and macrophages (**D**) in the stroma determined as described in the Methods. * $P < 0.05$, ** $P < 0.01$; scale bar = 100 μ m.

Figure 7. Expression of mRNA of collagen Ia2 chain or CTGF in WT ocular fibroblasts treated with TGFβ1, TNFα, or both. TGFβ1 up-regulates mRNA of collagen Ia2 (A) and CTGF (B) in a dose-dependent manner, whereas TNFα does not exhibit any effect on expression. However, addition of TNFα at 10 ng/ml completely abolishes up-regulation of collagen Ia2 mRNA (A) by TGFβ1 and decreases expression of CTGF (B) by TGFβ1. Data represent mean ± SD from five specimens in each condition. **P* < 0.05; ***P* < 0.01; bar = mean ± SD.

of mRNA expression of CTGF and collagen Ia2 by WT/KO ocular fibroblasts in co-culture with KO macrophages was counteracted by pretreatment of fibroblasts with Smad7-Ad, indicating a significant role of TGFβ/Smad signal in fibroblasts for this phenomenon (Figures 8C and 9B). The effect of knocking out TNFα in co-cultured macrophages was reproduced by further addition of anti-TNFα antibody to co-cultures of WT macrophages/WT fibroblasts (Figures 8D and 9C).

Myofibroblast Generation in Vitro

To avoid spontaneous myofibroblastic conversion, we used primary outgrowth of ocular fibroblasts, because passaging these cells two or three times induced a myofibroblastic phenotype in this experimental system. Western blotting showed that primary ocular fibroblast outgrowth expressed αSMA protein more prominently when co-cultured with KO macrophages, regardless of the fibroblast genotype (Figure 10A). To mimic in vivo conditions, we performed three-dimensional collagen gel co-culture of fibroblasts and macrophages. We examined αSMA expression of WT fibroblasts in collagen gel three-dimensional culture with co-cultured WT or KO macrophages (Figure 10B, panels a and b). A few ocular fibroblasts were labeled with anti-αSMA antibody when co-cultured with WT macrophages (Figure 10B, panel c), whereas many αSMA-positive myofibroblasts were observed when cultured with KO macrophages (Figure 10B, panel d), indicating that KO mac-

Figure 8. Expression of collagen Ia2 in WT/KO ocular fibroblasts co-cultured with WT/KO macrophages. **A:** Co-culture experiments show that the presence of KO macrophages with either WT or KO ocular fibroblasts up-regulates mRNA expression of collagen Ia2 in fibroblasts as compared with cultures with WT macrophages. **¹*P* < 0.01 as compared with the level in WT fibroblast/WT macrophage culture; *²*P* < 0.05 as compared with the level in KO fibroblast/WT macrophage culture. There is no statistical difference between data in WT fibroblast/WT macrophage culture and in KO fibroblast/WT macrophage culture. **B:** Sircol collagen assay shows that the collagen protein production by WT/KO fibroblasts coincides with the collagen Ia2 mRNA expression pattern. *¹*P* < 0.05 as compared with the level in WT fibroblast/WT macrophage culture; *²*P* < 0.05 as compared with the level in KO fibroblast/WT macrophage culture. There is no statistical difference between data in WT fibroblast/WT macrophage cultures and in KO fibroblast/WT macrophage cultures. **C:** In co-cultures of WT fibroblasts and KO macrophages, pretreatment of fibroblasts with Smad7 adenoviral gene transfer reduced collagen Ia2 mRNA expression in fibroblasts to the level in Smad7-adenovirus-treated fibroblasts. **¹*P* < 0.01 as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture; *²*P* < 0.01 as compared with the level in Cre-adenovirus-treated WT fibroblast/KO macrophage culture. **D:** In co-cultures of WT fibroblasts and WT macrophages, adding anti-TNFα neutralizing antibody increased expression of collagen Ia2 mRNA in fibroblasts. Enhancement of collagen Ia2 expression by anti-TNFα antibody was counteracted by pretreatment of fibroblasts with Smad7 overexpression. *¹*P* < 0.05 as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture in the presence of control IgG; *²*P* < 0.05 as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture in the presence of anti-TNFα antibody. Bar, mean ± SD. Data represent mean ± SD from five specimens in each condition.

rophages activated the fibroblasts more than the WT fibroblasts even in three-dimensional culture.

Discussion

In the present study we show that loss of TNFα potentiates the pathogenic tissue response in a mouse cornea burned with sodium hydroxide, resulting in marked neovascularization and scarring. Macrophage invasion and myofibroblast generation were enhanced in KO corneas compared to WT corneas in the later phase of healing. Although macrophage invasion in the burned tissue was similar between WT and KO mice at week 1, it was more prominent in KO corneas than in WT corneas at and after week 2. At week 2 the central area of the affected KO cornea was severely ulcerated, whereas WT corneas were already resurfaced. Increased number of invading macrophages is expected to result in an up-regulation of cytokine expression in the healing tissue. Indeed, our repeated real-time RT-PCR suggested that mRNA ex-

Figure 9. Expression of CTGF in WT/KO ocular fibroblasts co-cultured with WT/KO macrophages. **A:** Co-culture experiments show that the presence of KO macrophages with either WT or KO ocular fibroblasts up-regulates mRNA expression of CTGF in fibroblasts as compared with cultures with WT macrophages. $^{***}P < 0.01$ as compared with the level in WT fibroblast/WT macrophage culture; $^{***}P < 0.01$ as compared with the level in KO fibroblast/WT macrophage culture. There is no statistical difference between data in WT fibroblast/WT macrophage culture and in KO fibroblast/WT macrophage culture. **B:** In co-cultures of WT fibroblasts and KO macrophages, pretreatment of fibroblasts with Smad7 adenoviral gene transfer reduced the level of CTGF mRNA expression in WT fibroblasts to that with WT macrophages as compared with the culture with fibroblasts with Cre overexpression. $^{***}P < 0.01$ as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture; $^{***}P < 0.01$ as compared with the level in Cre-adenovirus-treated WT fibroblast/KO macrophage culture. **C:** In co-cultures of WT fibroblasts and WT macrophages, adding anti-TNF α neutralizing antibody increased expression of CTGF mRNA in WT fibroblasts. Enhancement of CTGF expression by anti-TNF α antibody was counteracted by pretreatment of fibroblasts with Smad7 overexpression. $^{***}P < 0.01$ as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture in the presence of control IgG; $^{***}P < 0.01$ as compared with the level in Cre-adenovirus-treated WT fibroblast/WT macrophage culture in the presence of anti-TNF α antibody. Data represent mean $\pm$ SD from five specimens in each condition.

pression of TGF β , MCP-1,³⁰ and VEGF^{25–27} in the healing stroma of alkali-burned mouse corneas increased from week 1 to week 4 (Supplemental Figure 1; <http://ajp.amjpathol.org>). Epithelial recovery was delayed in KO mice as compared with WT mice. The phenomena observed (macrophage invasion, myofibroblast generation, neovascularization, and ulcer formation) are all considered to be TGF β -dependent.^{17,31–34} We detected more matrix metalloproteinase activity in KO corneas during healing as compared with WT corneas by using *in situ* zymography (data not shown), although we have not determined which matrix metalloproteinase family member was involved. We then attempted to uncover the mechanism underlying this phenomenon and determined that loss of TNF α in macrophages, but not in local mesenchymal cells, potentiates TGF β action in healing corneal tissue (Figure 11).

TNF α is believed to promote tissue inflammation, but loss of TNF α did not reduce, and even augmented, inflammation, scarring, and neovascularization in the burned cornea. Other reports support our findings. For example, loss of TNF α has no effect on the degree of joint inflammation in an experimental arthritis model,¹² and loss of TNF α receptor also does not attenuate tissue damage and inflammation upon exposure to a bacterial antigen.¹¹ Pulmonary fibrosis induced by adenoviral overexpression of active TGF β 1 is augmented by loss of the TNF α receptor.¹³ Experimental bleomycin-induced

pulmonary fibrosis was also more severe in TNF α KO mice as compared with WT mice, attributed by the authors to suppression of apoptosis of macrophages and prolonged inflammation.¹⁴ Along with this, overexpression of TNF α attenuated pulmonary fibrosis.¹⁵ Our present finding and these reports indicate that TNF α serves to suppress or terminate inflammation in tissues in the resolution phase of inflammatory diseases or the wound healing process.

TGF β /Smad signaling is a key mediator in fibrosis and inflammation in the healing tissues, including burned cornea.^{31–34} Cross-talk between TNF α signaling and TGF β /Smad signaling has been reported.^{35–38} TNF α signaling inhibits the TGF β /Smad pathway by multiple mechanisms, including induction of Smad7, inhibition of Smad3 by c-Jun N-terminal kinase activation of AP-1, and down-regulation of TGF β receptor expression.^{35–38} As previously reported in dermal fibroblasts,³⁹ the present study showed that TNF α counteracted induction of CTGF by TGF β 1 in cultured ocular fibroblasts, and this might also occur in the healing cornea *in vivo*. Because Smad2 phosphorylation was more marked in KO burned tissues as compared with WT tissue at weeks 2 to 4, and because adenoviral Smad7 overexpression rescued the abnormal healing in a KO mouse cornea, loss of TNF α might allow overactivation of TGF β /Smad signaling, leading to enhanced expression of TGF β -induced cytokines, ie, TGF β 1 and MCP-1.^{40–43}

Figure 10. Expression of α SMA in mouse ocular fibroblasts co-cultured with macrophages in monolayer and three-dimensional collagen gel culture. **A:** The fibroblast outgrowth was used without passage to avoid spontaneous fibroblast-myofibroblast conversion. Primary fibroblast outgrowth expressed α SMA protein more prominently when co-cultured with KO macrophages regardless the fibroblast genotype. **B:** To mimic in vivo conditions, we performed three-dimensional collagen gel co-culture of fibroblasts and macrophages. Fibroblasts were mixed with macrophages and put into the collagen gel at the first passage to avoid spontaneous fibroblast-myofibroblast conversion. In both WT fibroblast/WT macrophage culture (**a**) and WT fibroblast/KO macrophage culture (**b**), WT fibroblasts exhibit an elongated typical fibroblastic shape. A few ocular fibroblasts were labeled with anti- α SMA antibody when co-cultured with WT macrophages (**c**), whereas many α SMA-positive myofibroblasts were observed when cultured with KO macrophages (**d**), indicating that KO macrophages activated the fibroblasts more so than WT fibroblasts. In both collagen gel cultures, macrophages are labeled with F4/80 antibody (**e** and **f**). Scale bar = 10 μ m.

Interactions between fibroblasts and macrophages in an injured tissue are considered to be important in regulation of the healing response. We developed a hypothesis that loss of $\text{TNF}\alpha$ in macrophages, but not in corneal fibroblasts, might augment $\text{TGF}\beta$ signaling in both fibroblasts and macrophages based on our observations that 1) macrophages in the burned cornea express $\text{TNF}\alpha$, 2) exogenous $\text{TNF}\alpha$ counteracts the up-regulation of expression of collagen $\text{I}\alpha 2$ and CTGF mRNAs by $\text{TGF}\beta$ in ocular fibroblasts, and 3) up-regulation of expression of collagen $\text{I}\alpha 2$ and CTGF and collagen protein in ocular fibroblasts by $\text{TGF}\beta$ is similar between WT and KO fibro-

Figure 11. Proposed mechanism of augmented effects of $\text{TGF}\beta$ on healing by KO macrophages in injured corneas. Effects of $\text{TGF}\beta$ secreted by macrophages on fibroblast behavior are counteracted by $\text{TNF}\alpha$ secreted by macrophages through multiple mechanisms, including suppression of $\text{TGF}\beta$ and induction of apoptosis in macrophages themselves. KO macrophages lack $\text{TNF}\alpha$ to suppress $\text{TGF}\beta$ effects, resulting in accelerated up-regulation of $\text{TGF}\beta$ -dependent gene expression.

blasts, indicating that loss of $\text{TNF}\alpha$ in corneal fibroblasts might not have a significant role in excess tissue fibrosis. To explore this hypothesis, we performed BMT and co-culture experiments. Transplantation of WT BM to KO mice rescued the abnormally augmented healing response of a KO cornea, indicating that invasion of BM-derived inflammatory cells into the affected cornea is involved in the KO phenotype of corneal healing. The majority of inflammatory cells that invade the burned cornea are blood cell-derived and thus contained transplanted BM-derived cells. However, transplantation of KO BM to WT mice did not yield KO-like healing in WT mice (data not shown). The healing cornea of these mice demonstrated slightly more neovascularization and scarring than that seen in WT mice, but much less than that seen in KO mice. Dual immunostaining for $\text{TNF}\alpha$ and F4/80 antigen revealed that macrophages in the burned cornea were heterogeneous with both WT and KO macrophages being present, indicating that the mice are chimeric.

Similar chimerism has been reported in other mouse BMT models,^{44,45} although it was not determined whether this chimeric condition resulted from long-lived tissue macrophages that were resistant to irradiation or the survival of a small number of the recipient's bone marrow cells. We think that the presence of $\text{TNF}\alpha$ derived from a small number of surviving WT macrophages in the tissue masked the effects of lack of $\text{TNF}\alpha$ in KO macrophages derived from transplanted BM. We also found the effects of systemic administration of anti- $\text{TNF}\alpha$ neutralizing antibody on the healing process of this corneal alkali burn model in C57/BL6 mice as follows. We administered the antibody (2 μ g/g of body weight), intraperitoneally on alternate days,^{46,47} from 1 day before the animal received alkali burn in an eye until week 2. Control mice received nonimmune IgG. The results of this experiment, however, did not show any obvious change in the healing of corneal burns. Although the reason for the discrepancy

between the results from experiments with a neutralizing antibody and results from those in TNF α -null mice has not been determined, it may be that even with the antibody a small amount of active TNF α in tissues might be enough to mask the effects of reduction of the systemic level of TNF α by neutralization. The phenotype of the TNF α KO mice¹² and the phenotype of ligand neutralization by antibody administration⁸ also do not coincide with each other in an experimental arthritis animal model.

The co-culture experiments showed that ocular fibroblasts, regardless of their genotype, co-cultured with KO macrophages express more collagen α 2, collagen protein, and CTGF as compared with the cells cultured with WT macrophages. Anti-TNF α antibody increased and anti-TGF β antibody (preliminary data) reduced collagen α 2 expression in a co-culture of WT fibroblasts and WT macrophages. Moreover, pretreatment of WT fibroblasts with *Smad7* gene transfer reversed the increase in the expression of collagen α 2 or CTGF by the cells co-cultured with KO macrophages to the level in *Smad7*-adenovirus-treated WT fibroblasts co-cultured with WT macrophages. This finding was further reproduced by the co-culture experiment using anti-TNF α neutralizing antibody to block TNF α activity in the culture. These *in vitro* results strongly support the notion that TNF α derived from macrophages is required for termination or suppression of excessive wound healing/fibrotic reaction in an injured cornea. Although it has been reported that circulating BM-derived stem cells settle at the site of tissue injury and differentiate to mesenchymal cells in experimental atherosclerosis or pulmonary fibrosis in animals,^{48–50} our co-culture study excludes the importance of the genotype of local mesenchymal cells in the KO phenotype of healing. Expression of α SMA is also an important hallmark for tissue fibrosis, but our cultured ocular fibroblasts acquired α SMA expression after passage. Thus, we used the cells without passage for the analysis of α SMA expression when co-cultured with macrophages and showed that KO macrophages induced more α SMA expression fibroblasts regardless of the genotype as compared with WT macrophages. Collagen gel three-dimensional co-culture also showed KO macrophage-accelerated fibroblast-myofibroblast conversion as compared with KO macrophages, further supporting our conclusion.

TNF α receptor p55 appears to accelerate re-epithelialization of cutaneous full-thickness wounds.⁵¹ Healing of this injury in the TNF α receptor p55-null mice exhibits enhanced neovascularization and up-regulation of expression of TGF β 1 and collagen⁵¹ that are similar to findings seen in a burned cornea of mice that lack TNF α . However, the p55 receptor-null mouse showed a reduction of inflammation at the site of cutaneous injury⁴⁸ unlike a ligand-null mouse. The p55-null mice retain the p65 receptor, thus retaining certain responses to TNF α . This mouse is not equivalent to a TNF-null mouse, because it has maintenance of cellular behaviors mediated by the p65 receptor. Further study is needed to determine the reason for this difference between the phenotypes of mice lacking ligand or receptor.

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