Tumor Necrosis Factor-α-Activated Human Adipose Tissue–Derived Mesenchymal Stem Cells Accelerate Cutaneous Wound Healing through Paracrine Mechanisms

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Human adipose tissue–derived mesenchymal stem cells (ASCs) stimulate regeneration of injured tissues by secretion of various cytokines and chemokines. Wound healing is mediated by multiple steps including inflammation, epithelialization, neoangiogenesis, and proliferation. To explore the paracrine functions of ASCs on regeneration of injured tissues, cells were treated with tumor necrosis factor-α (TNF-α), a key inflammatory cytokine, and the effects of TNF-α-conditioned medium (CM) on tissue regeneration were determined using a rat excisional wound model. We demonstrated that TNF-α CM accelerated wound closure, angiogenesis, proliferation, and infiltration of immune cells into the cutaneous wound *in vivo*. To assess the role of proinflammatory cytokines IL-6 and IL-8, which are included in TNF-α CM, IL-6 and IL-8 were depleted from TNF-α CM using immunoprecipitation. Depletion of IL-6 or IL-8 largely attenuated TNF-α CM-stimulated wound closure, angiogenesis, proliferation, and infiltration of immune cells. These results suggest that TNF-α-activated ASCs accelerate cutaneous wound healing through paracrine mechanisms involving IL-6 and IL-8.

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INTRODUCTION

Wound healing is not a series of individual and independent progressive steps, but a complex process involving inflammation, epithelialization, neoangiogenesis, proliferation, and collagen matrix formation (Singer and Clark, 1999). This complex process is carried out and regulated by numerous growth factors, cytokines, and chemokines such as epidermal growth factor, transforming growth factor-β, platelet-derived growth factor, fibroblast growth factor, vascular endothelial growth factor, granulocyte macrophage colony-stimulating factor, IL family, and tumor necrosis factor-α (TNF-α; Steenfos, 1994; Goldman, 2004; Barrientos *et al.*, 2008). Local or topical application of growth factors and cytokines has been reported to promote repair of chronic wounds (Braund *et al.*, 2007).

Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into diverse cell types, including adipocytes, osteoblasts, chondrocytes, and smooth muscle cells (Pittenger *et al.*, 1999; Short *et al.*, 2003; Caplan and Dennis, 2006). They can be isolated from a variety of tissues, such as bone marrow, periosteum, trabecular bone, synovium, skeletal muscle, deciduous teeth, and adipose tissues (da Silva *et al.*, 2006). MSCs accelerated wound healing in several animal models by stimulation of wound closure, re-epithelialization, cellularity, and angiogenesis (Fu *et al.*, 2006; McFarlin *et al.*, 2006; Wu *et al.*, 2007). MSCs have been reported to stimulate tissue regeneration and wound repair by direct differentiation of MSCs to tissue cells or secretion of paracrine factors (Gnecchi *et al.*, 2008; Hocking and Gibran, 2010; Wu *et al.*, 2010). However, an increasing body of evidence suggests that the contribution of MSC differentiation is limited because of poor engraftment and survival of MSCs at the injured site, and paracrine signaling is the primary mechanism accounting for the beneficial effects of MSCs on tissue regeneration (Gnecchi *et al.*, 2008; Herdrich *et al.*, 2008; Hocking and Gibran, 2010). MSCs secreted various cytokines and chemokines, vascular endothelial growth factor, IGF-1, keratinocyte (KC) growth factor, angiopoietin-1, stromal cell-derived factor-1, and macrophage inflammatory

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Abbreviations: ASCs, adipose tissue-derived mesenchymal stem cells; CM, conditioned medium; MSCs, mesenchymal stem cells; TNF-α, tumor necrosis factor-α; vWF, von Willebrand factor

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protein-1 (Kinnaird *et al.*, 2004; Chen *et al.*, 2008; Wu *et al.*, 2010). MSC-conditioned medium significantly promoted endothelial cell tube formation *in vitro* (Wu *et al.*, 2007). In addition, MSC-conditioned medium-stimulated migration of macrophages, KCs, and endothelial cells *in vitro* and infiltration of monocytes/macrophages into wounds (Chen *et al.*, 2008), suggesting a pivotal role of MSC-derived paracrine factors in cutaneous wound healing.

Human adipose tissue-derived MSCs (ASCs) exhibit differentiation potential similar to that of bone marrow-derived MSCs (Zuk *et al.*, 2002). ASCs represent a population of cells surrounding adipocytes in fat tissue (Planat-Benard *et al.*, 2004). ASCs can be easily isolated from subcutaneous adipose tissue of patients (Zuk *et al.*, 2002); therefore, they are regarded as an excellent tool for use in tissue regeneration (Chamberlain *et al.*, 2007; Phinney and Prockop, 2007; Schaffler and Buchler, 2007). Transplantation of ASCs enhances postischemic neovascularization and cutaneous wound healing (Planat-Benard *et al.*, 2004; Ebrahimian *et al.*, 2009). ASCs have been reported to promote tissue regeneration and angiogenesis within injured or inflamed tissues by secretion of various cytokines and chemokines, including vascular endothelial growth factor, stromal cell-derived factor-1, and IL-6 (Phinney and Prockop, 2007; Ebrahimian *et al.*, 2009). Co-transplantation of platelet-rich plasma together with ASCs stimulated wound-healing properties of ASCs in a porcine full-thickness wound model (Blanton *et al.*, 2009), suggesting stimulation of ASC-mediated wound healing by trophic factors produced during the wound-healing process.

Production of TNF-α by a variety of cells including macrophages, monocytes, and mast cells during the inflammatory phase of wound healing has been reported (Locksley *et al.*, 2001; Saika, 2007). TNF-α is the proinflammatory signals controlling wound healing, in a manner that is dependent on concentration and duration of exposure in inflamed or injured tissues (Saika, 2007). TNF-α also works in a paracrine manner, increasing MSCs migration toward chemokines and elevating invasive levels of MSCs by stimulation of expression of matrix metalloproteases (Ponte *et al.*, 2007; Ries *et al.*, 2007). Using shotgun proteomic technology, we have previously identified 118 individual proteins as TNF-α-induced secreted proteins, including IL-6, IL-8, monocyte chemotactic protein-1, CXCL6, cathepsin L, and pentraxin-related protein 3 (Lee *et al.*, 2010). TNF-α-conditioned medium (CM) stimulated chemotactic migration of human monocytes *in vitro* through mechanisms involving IL-6 and IL-8. These results suggest the possibility that TNF-α-stimulated secretion of paracrine factors from ASCs may modulate regeneration of injured tissues *in vivo*.

In the present study, to explore the paracrine functions of TNF-α-stimulated ASCs on tissue regeneration, we examined the effects of TNF-α CM on cutaneous wound healing in a rat excisional cutaneous wound model and characterized the molecular mechanisms by which TNF-α-conditioned medium regulates wound repair *in vivo*.

RESULTS

TNF-α CM accelerates re-epithelialization and wound closure *in vivo*

To determine whether or not TNF-α can regulate the paracrine function of ASCs, the effects of TNF-α CM, which was derived from ASCs, on tissue regeneration were determined using an excisional cutaneous wound-healing model. Compared with PBS- or control CM-treated wounds, topical application of TNF-α CM onto cutaneous wounds accelerated wound closure (Figure 1a). Wound areas were time-dependently diminished under all experimental conditions, whereas TNF-α CM-stimulated decrease of wound area became more evident on day 6 or 9 (Figure 1b). Histological analysis of wounds showed that TNF-α CM accelerated re-epithelialization and wound closure, compared with PBS- or control CM-treated wounds (Figure 1c and Supplementary Figure S1 online).

TNF-α CM stimulates angiogenesis within cutaneous wounds

Angiogenesis is implicated in tissue regeneration and wound healing (Li *et al.*, 2003b). To elucidate the role of TNF-α CM on angiogenesis, we determined the number of blood vessels within cutaneous wounds after treatment with TNF-α CM. As shown in Figure 2a, the numbers of blood vessels containing red blood cells were augmented in TNF-α CM-treated wounds, suggesting enhanced angiogenesis during TNF-α-stimulated wound repair. To further confirm the result that TNF-α CM-stimulated angiogenesis, capillary densities in wounds were quantified by immunostaining of tissue sections for the endothelial cell-specific marker von Willebrand factor (vWF). Compared with PBS- or control CM-treated wounds, the number of blood vessels composed of vWF-positive endothelial cells was increased in TNF-α CM-treated wounds (Figure 2b). Capillary densities containing vWF-positive endothelial cells within cutaneous wounds were time-dependently increased in response to TNF-α CM treatment, with a maximal increase on day 6 (Figure 2c), and decreased to control levels by day 12. These results suggest that TNF-α CM stimulates angiogenesis in an early phase of the cutaneous wound-healing processes.

TNF-α CM increases accumulation of macrophages and cell proliferation during wound healing

Infiltration of monocytes/macrophages normally occurs during an early step of the wound-healing processes and is required for regeneration of injured tissues (Martin and Leibovich, 2005; Adamson, 2009). We have reported that TNF-α CM contains various cytokines and chemokines, and enhances chemotactic migration of human monocytes *in vitro* (Lee *et al.*, 2010). To clarify whether or not TNF-α CM can enhance infiltration of monocytes/macrophages into cutaneous wounds, immunostaining with an antibody against monocyte/macrophage-specific marker CD68 was performed. As shown in Figure 3a, the number of CD68-positive cells was significantly increased in TNF-α CM-treated wounds, compared with PBS- or control CM-treated wounds. Similar to TNF-α CM-stimulated angiogenesis, TNF-α CM-stimulated infiltration of CD68-positive cells peaked on day 6

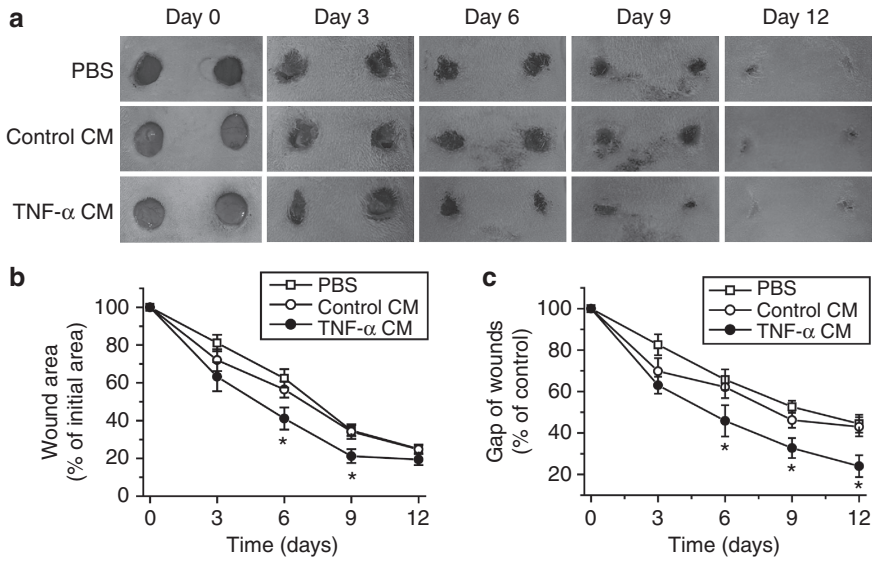

Figure 1. Effects of tumor necrosis factor (TNF)-α-conditioned medium (CM) on cutaneous wound healing and re-epithelialization. (**a**) Excisional wounds were treated daily with phosphate-buffered saline (PBS), control CM, or TNF-α CM and photographed at the time indicated from days 0–12 ($n=8$ for each group and time point). Representative photographs of wounds are shown. (**b**) Measurement of wound sizes at different time points after wound injury. Areas of the wounds were determined by quantitative analysis of wound images using Image J software. Analysis of variance (ANOVA), $^*P<0.05$ versus control CM ($n=16$). (**c**) Histomorphometric analysis of wound gap, which was determined by measurement of the distance between the advancing edges of keratinocytes in the wounded epidermis shown in Supplementary Figure S1 online. ANOVA, $^*P<0.05$ versus control CM ($n=16$).

and decreased to control levels on day 9 (Figure 3b). These results suggest that TNF-α CM stimulates infiltration of monocytes/macrophages into cutaneous wounds.

To assess the effect of TNF-α CM on cell proliferation during wound healing, we determined the number of proliferating cells by immunofluorescence staining of the Ki67 antigen, which is a nuclear protein associated with cell proliferation (Schonk *et al.*, 1989). Immunofluorescence staining of wounds on day 6 showed an increase in number of Ki67-positive cells within the dermal area in response to treatment with TNF-α CM (Figure 3c). Quantification of the immunostaining results showed a significant increase in the number of Ki67-positive proliferating cells on day 6 after topical application of TNF-α CM onto cutaneous wounds, compared with control CM (Figure 3d).

TNF-α CM stimulates cutaneous wound healing through IL-6- and IL-8-dependent mechanisms

We have reported that both IL-6 and IL-8 are largely responsible for TNF-α CM-stimulated chemotaxis of monocytes *in vitro* (Lee *et al.*, 2010). To determine whether or not these cytokines are responsible for TNF-α CM-stimulated cutaneous wound healing, IL-6 and IL-8 were depleted from TNF-α CM by immunoprecipitation with anti-IL-6 and anti-IL-8 antibodies. As shown in Figure 4a, IL-6 and/or IL-8 were specifically depleted from TNF-α CM by immunoprecipitation with a combination of anti-IL-6 and anti-IL-8 antibodies, respectively. Because TNF-α CM-stimulated wound closure peaked on day 6, we next explored the effects of IL-6- or IL-8-depleted TNF-α CM on wound healing on day 6. TNF-α CM-stimulated re-epithelialization and wound closure were completely attenuated by depletion of IL-8 or both IL-8 and IL-6 from TNF-α CM, whereas depletion of IL-6 partially attenuated TNF-α CM-stimulated re-epithelialization and wound closure (Figure 4b and c and Supplementary Figure S2 online). These results suggest that IL-6 and IL-8 are largely responsible for TNF-α CM-stimulated cutaneous wound healing.

IL-6 and IL-8 are involved in TNF-α CM-induced angiogenesis *in vivo*

To explore the question of whether or not IL-6 and IL-8 are involved in TNF-α CM-stimulated angiogenesis, we examined the effects of IL-6- or IL-8-depleted TNF-α CM on angiogenesis during cutaneous wound healing. As shown in Figure 5a, TNF-α CM-stimulated formation of blood vessels containing red blood cells was markedly diminished by depletion of IL-6 or IL-8. Depletion of IL-6 or IL-8 consistently attenuated the TNF-α CM-stimulated increase of vWF-positive blood vessels (Figure 5b and c). In addition, depletion of both IL-6 and IL-8 from TNF-α CM completely abrogated TNF-α CM-stimulated angiogenesis (Figure 5c), suggesting a principal role for IL-6 and IL-8 in TNF-α CM-stimulated angiogenesis.

IL-6 and IL-8 are involved in TNF-α CM-induced infiltration of macrophages and cell proliferation during wound healing

To evaluate the question of whether or not IL-6 and IL-8 are responsible for TNF-α CM-induced infiltration of macrophages into cutaneous wounds, we determined the number of macrophages in dermal wounds that were treated with IL-6- or IL-8-depleted TNF-α CM. Compared with control wounds which were treated with TNF-α CM, the number of CD68-positive cells was markedly reduced in dermal wounds

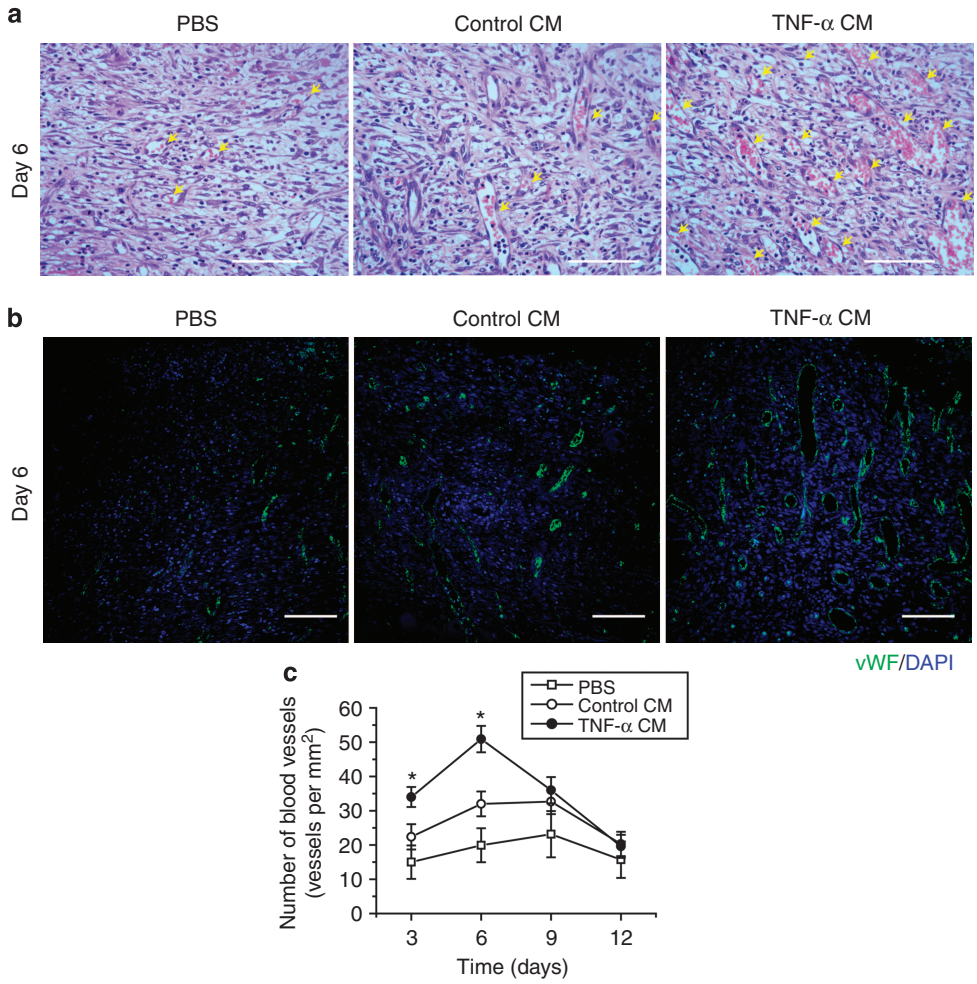

Figure 2. Histological analysis of tumor necrosis factor (TNF)-α-conditioned medium (CM)-induced angiogenesis. (**a**) Cutaneous wounds on day 6 were stained with hematoxylin and eosin (H&E) and photographed with a digital camera (at × 400) mounted on a light microscope. Yellow arrows indicate blood vessels containing red blood cells. Bar = 50 μm. (**b**) For immunostaining of von Willebrand factor (vWF)-positive blood vessels (green), cutaneous wounds on day 6 were stained with anti-vWF antibody and probed with Alexa Fluo488 anti-rabbit secondary antibody, and nuclei (blue) were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Bar = 100 μm. (**c**) The number of vWF-positive blood vessels in each section was counted at the indicated time points. Results are presented as the number of blood vessels per mm^2. Analysis of variance, $*P<0.05$ versus control CM ($n=16$). PBS, phosphate-buffered saline.

treated with IL-6- or IL-8-depleted TNF-α CM (Figure 6a and b). Depletion of IL-8 or both IL-8 and IL-6 completely inhibited infiltration of CD68-positive cells in response to TNF-α CM, whereas depletion of IL-6 partially reduced TNF-α CM-induced infiltration of CD68-positive cells. These results suggest a principal role for IL-8 in TNF-α CM-stimulated infiltration of macrophages into dermal wounds.

We next explored the question of whether or not IL-6 and IL-8 are involved in TNF-α CM-stimulated cell proliferation during wound healing by immunofluorescence staining with anti-Ki67 antibody. Immunofluorescence staining of dermal wounds showed that depletion of IL-6 and/or IL-8 markedly attenuated the TNF-α CM-stimulated increase of Ki67-positive cells within dermal wounds on day 6 (Figure 6c and d). These results suggest that IL-6 and IL-8 are largely responsible for cell proliferation associated with TNF-α CM-stimulated wound healing.

DISCUSSION

We presently showed that TNF-α CM accelerated re-epithelialization, angiogenesis, infiltration of macrophages, and proliferation within cutaneous wounds. Compared with the potent stimulation of cutaneous wound healing by TNF-α CM, control CM from ASCs had no significant impact on cutaneous wound healing. Stimulation of cutaneous wound healing by MSC-conditioned medium, which was prepared by exposure of MSCs to hypoxic condition and further concentrated to 50 times by ultrafiltration, has been demonstrated (Chen *et al.*, 2008). Treatment of MSCs with hypoxic conditions led to increased secretion of various angiogenic cytokines and chemokines. In the present study, we prepared control CM by treatment of ASCs with serum-free medium under normoxic conditions and directly applied control CM to wounds without further concentration of conditioned medium. Therefore, it is likely that low concentration of

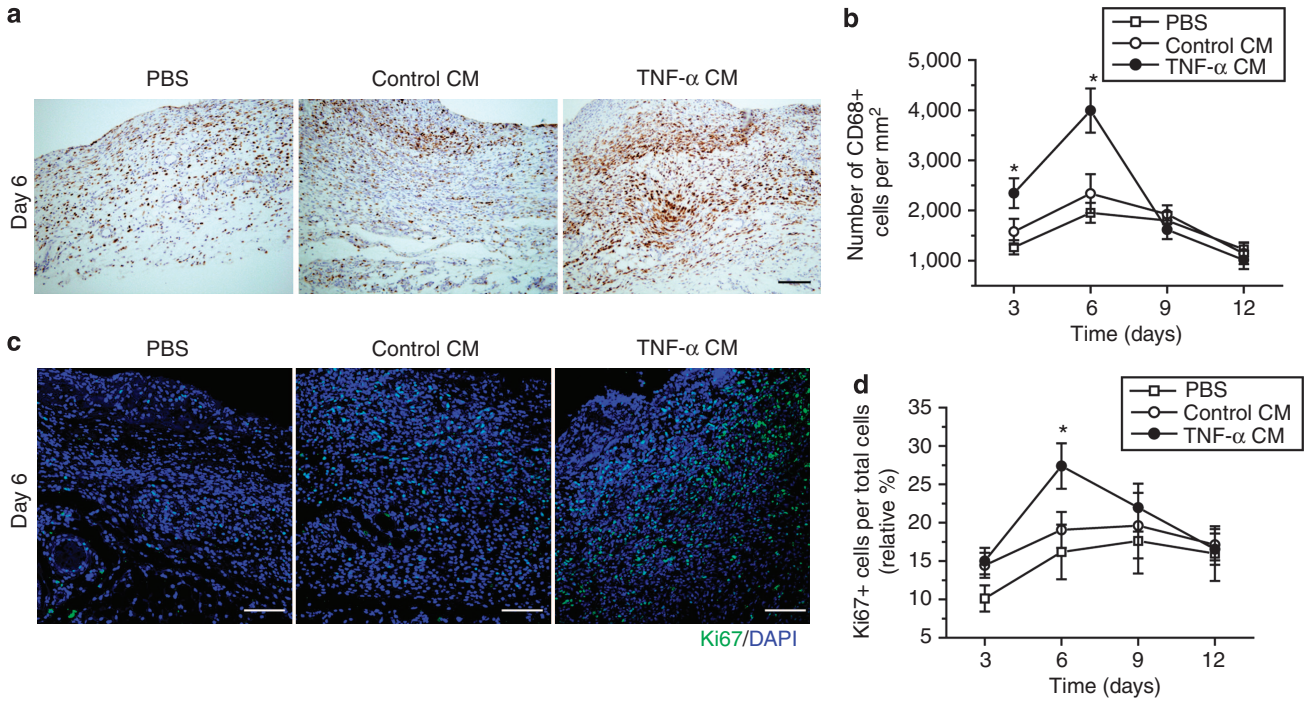

Figure 3. Tumor necrosis factor (TNF)-α-conditioned medium (CM) enhances infiltration of macrophages and cell proliferation in cutaneous wounds. (**a**) Macrophages within cutaneous wounds were detected by immunostaining with anti-CD68 antibody on day 6. Bar = 100 μm. (**b**) CD68-positive cells in each section were counted at the indicated time points. Analysis of variance, *$P<0.05$ versus control CM ($n=16$). (**c**) To determine the effect of TNF-α CM on cell proliferation, wound tissues on day 6 were immunostained with anti-Ki67 antibody (green) and nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (blue). Bar = 100 μm. (**d**) Numbers of Ki67-positive cells and nuclei per field were counted and expressed as the relative percentage of Ki67-positive cells per total cells. Analysis of variance, *$P<0.05$ versus control CM, ($n=16$). PBS, phosphate-buffered saline.

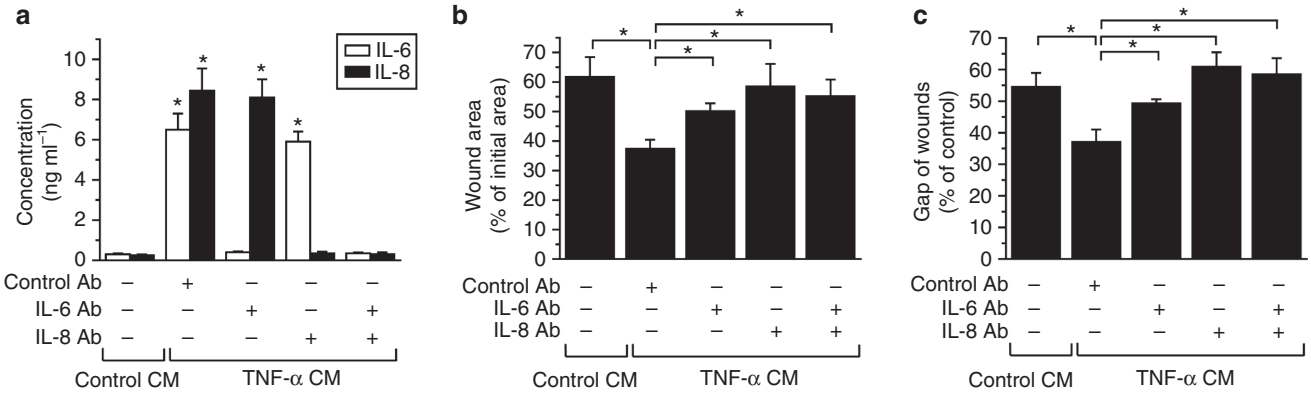

Figure 4. Tumor necrosis factor (TNF)-α-conditioned medium (CM) enhances cutaneous wound healing and re-epithelialization through an IL-6- and IL-8-dependent mechanism. (**a**) TNF-α CM was treated with protein G-agarose beads immobilized with control antibody (normal mouse IgG) or anti-IL-6 and/or anti-IL-8 antibodies for immunoprecipitation of IL-6 and/or IL-8. Concentrations of IL-6 and IL-8 in supernatants were determined by ELISA analysis. (**b**) Wounds were treated daily with control CM, TNF-α CM, or IL-6- and/or IL-8-depleted TNF-α CM. Wound areas were determined by quantitative analysis of the wound images on day 6 after wound injury. (**c**) The gap of wounds was determined by measurement of the distance between the advancing edges of keratinocytes in the wounded epidermis, as shown in Supplementary Figure S2 online. Analysis of variance, *$P<0.05$ versus control CM ($n=16$).

paracrine factors in control CM may be responsible for no effects of control CM on cutaneous wound healing. In contrast, high concentration of paracrine factors in TNF-α CM suggests that TNF-α CM will be highly useful for wound repair and tissue regeneration.

We demonstrated here that IL-6 and IL-8 are largely involved in TNF-α CM-stimulated cutaneous wound healing. Protein levels of IL-6 and IL-8 were hardly detected in control CM and TNF-α treatment drastically upregulated secretion of IL-6 and IL-8 from ASCs (Figure 4a). Using liquid chromatography-coupled with tandem mass spectrometry analysis, we reported that TNF-α CM contained various cytokines and chemokines, including IL-6 and IL-8, CXCL2, CXCL5, CXCL6, CXCL10, monocyte chemotactic protein-1, matrix

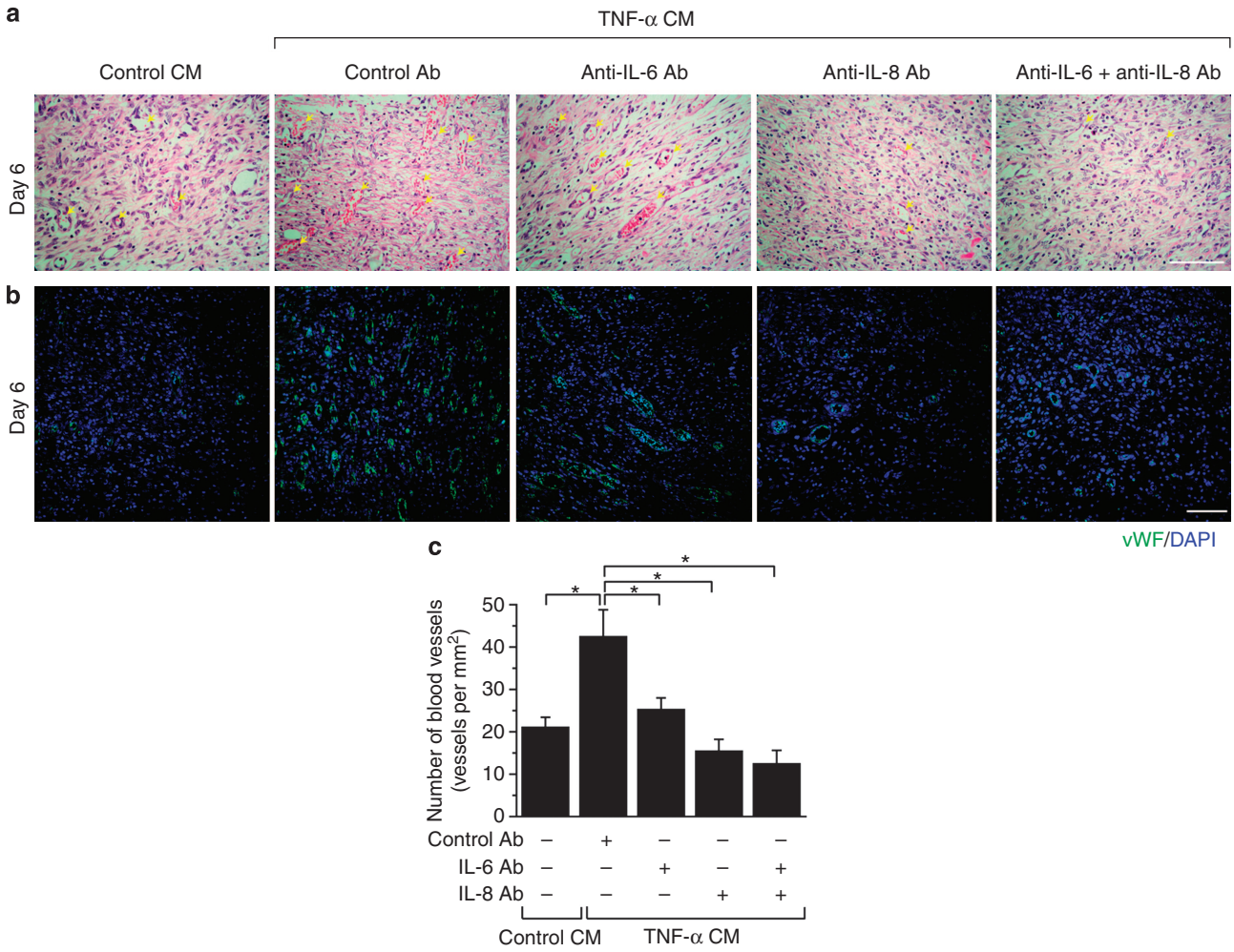

Figure 5. Effects of IL-6- or IL-8-depleted tumor necrosis factor (TNF)-α-conditioned medium (CM) on angiogenesis. (**a**) Cutaneous wounds (Figure 4) were stained with hematoxylin and eosin (H&E) and photographed with a digital camera at ×400 on day 6. Yellow arrows indicate blood vessels containing red blood cells. Bar = 50 μm. (**b**) For immunostaining of von Willebrand factor (vWF)-positive blood vessels (green), cutaneous wounds on day 6 were stained with anti-vWF antibody and probed with Alexa Fluo488 anti-rabbit secondary antibody, and nuclei (blue) were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Overlaid images are shown. Bar = 100 μm. Representative data from eight animals per group are shown. (**c**) The number of vWF-positive blood vessels per mm^2 was quantified on day 6. Analysis of variance, $^*P<0.05$ versus control CM, ($n=16$).

metalloprotease-1, plasminogen activator inhibitor-1, cathepsin L, pentraxin-related protein 3, and complement factors (Lee *et al.*, 2010). Not only cytokines and chemokines, but also matrix metalloproteases and plasminogen activator inhibitors are required for cutaneous wound healing (Lijnen, 2005). Moreover, TNF-α treatment has been shown to stimulate secretion of vascular endothelial growth factor, hepatocyte growth factor, and insulin-like growth factor I from ASCs (Wang *et al.*, 2006). Therefore, it is conceivable that proteases, growth factors, and other paracrine factors may also be key players in TNF-α CM-facilitated wound-healing processes in concert with IL-6 and IL-8, albeit the role of these TNF-α-secreted proteins in wound healing should be further explored. Furthermore, it is possible to hypothesize that exogenously added TNF-α can directly affect wound-healing processes because TNF-α CM contains a significant amount of exogenously added TNF-α (Supplementary Figure S3A online), although TNF-α was not detected in TNF-α CM by proteomic analysis (Lee *et al.*, 2010). However, this possibility can be excluded because immunodepletion of exogenously added TNF-α had no impact on TNF-α CM-stimulated wound-healing processes; wound closure, angiogenesis, and infiltration of immune cells (Supplementary Figure S3 online). Taken together, these results suggest that TNF-α greatly potentiates the paracrine functions of ASCs in cutaneous wound healing through stimulation of secretion of IL-6, IL-8, and other paracrine factors.

Inflammatory cells have a key role in cutaneous wound healing by cleaning up bacteria and tissue debris from the wound (Martin and Leibovich, 2005). Leukocytes (neutrophils, macrophages, mast cells, and lymphocytes) infiltrate wound tissue and regulate healing processes (Singer and Clark, 1999). Conditioned medium from bone marrow-derived MSCs exposed to hypoxic conditions stimulated cutaneous wound healing by recruitment of macrophages and endothelial lineage cells into the wound (Wu *et al.*,

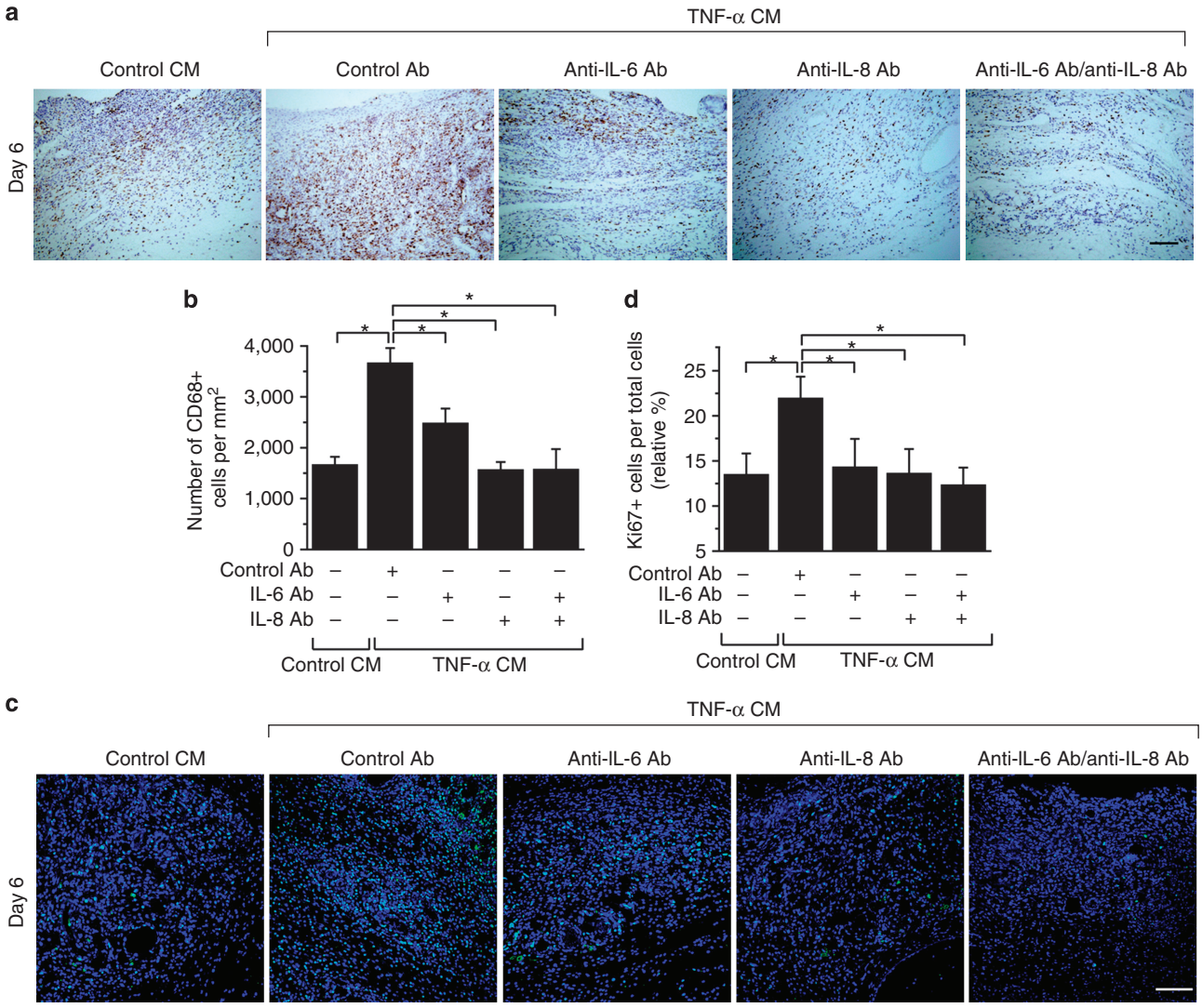

Figure 6. Roles of IL-6 and IL-8 in the tumor necrosis factor (TNF)-α-conditioned medium (CM)-induced infiltration of macrophages and cell proliferation. (**a**) Wounds (Figure 4) were immunostained with anti-CD68 antibody on day 6. Bar = 100 μm. (**b**) The number of CD68-positive cells per mm^2 was counted. Analysis of variance, $^*P<0.05$ versus control CM ($n=16$). (**c**) At day 6 post-wounding, wounds were stained with anti-Ki67 antibody and 4′-6-diamidino-2-phenylindole. The overlaid images show Ki67 (green) and nuclei (blue), respectively. Bar = 100 μm. Representative data from eight animals per group are shown. (**d**) Numbers of Ki67-positive cells and nuclei were determined and results are expressed as the relative percentage of Ki67-positive cells per total cells. Analysis of variance, $^*P<0.05$ versus control CM ($n=16$).

2007; Chen *et al.*, 2008). In the present study, we demonstrated that depletion of IL-6 or IL-8 largely attenuated TNF-α CM-induced infiltration of CD68-positive macrophages into cutaneous wounds. Furthermore, depletion of IL-6 or IL-8 abrogated TNF-α CM-stimulated *in vitro* chemotaxis of human monocytes (Lee *et al.*, 2010), which develop into macrophages within injured tissues. IL-6 functions as a chemoattractant for monocytes (Clahsen and Schaper, 2008). IL-8 was identified as the major bioactive chemoattractant for neutrophils, which arrive first in the wound. IL-8 is increased during TNF-α-induced inflammatory responses and recruits monocytes and neutrophils to the inflamed area (Mukaida, 2000). Therefore, these reports support the notion that IL-6 and IL-8 are key players in TNF-α CM-stimulated infiltration of macrophages *in vivo*.

IL-6 is produced by a variety of cells in wounds, such as epidermal KCs, dermal fibroblasts, and macrophages, which are related to wound healing (Mateo *et al.*, 1994). IL-6 stimulated proliferation of KCs *in vitro* (Grossman *et al.*, 1989) and *in vivo* (Sawamura *et al.*, 1998), which is associated with re-epithelialization of wounds. Use of a full-thickness biopsy wounding model on IL-6 knockout mice provided functional evidence of a role for IL-6 in wound healing (Gallucci *et al.*, 2000; Lin *et al.*, 2003); IL-6 knockout mice exhibited delayed wound healing, which is caused by impaired re-epithelialization, angiogenesis, leukocyte infiltration, and collagen accumulation. In addition, IL-8 has a key role in cutaneous wound healing (Gillitzer and Goebeler, 2001). IL-8 promoted chemotaxis and proliferation of KCs (Tuschil *et al.*, 1992). IL-8 is highly expressed in

human wound fluid and *in vivo* topical application of IL-8 on human skin grafts in a chimeric mouse model induced re-epithelialization by stimulation of KC proliferation (Rennekampff *et al.*, 2000). IL-8 directly enhanced endothelial cell survival, proliferation, and angiogenesis (Li *et al.*, 2003a). These results support the notion that IL-6 and/or IL-8 may be responsible for TNF-α CM-induced wound healing by directly stimulating infiltration of macrophages, re-epithelialization, angiogenesis, and cell proliferation. Because these responses sequentially occurred during wound-healing processes, another possible explanation is that IL-6 and/or IL-8 affect one of wound healing step, which stimulates other steps. For instance, inflammation and accumulation of macrophages are essential for normal wound healing and occur in advance of re-epithelialization, angiogenesis, and proliferation during wound-healing processes (Singer and Clark, 1999). Macrophages have a pivotal role in wound healing by not only cleaning bacteria and damaged tissues, but also secreting angiogenic cytokines and growth factors. Therefore, it is suggested that macrophage activation induced by IL-6 and/or IL-8 indirectly modulates TNF-α CM-stimulated wound healing by acceleration of wound healing-associated sequential steps, including re-epithelialization, angiogenesis, and cell proliferation. The molecular mechanism by which TNF-α CM stimulates cutaneous wound healing should be clarified further.

MATERIALS AND METHODS

Materials

α-Minimum essential medium, trypsin, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN). ELISA kits and neutralizing antibodies for human IL-6 and IL-8 were purchased from BD Biosciences (Bedford, MA). Normal mouse IgG was purchased from Millipore (Billerica, MA). Peroxidase-labeled secondary antibodies were purchased from Amersham Biosciences (Buckinghamshire, UK).

Cell culture and preparation of conditioned medium

ASCs were isolated from human subcutaneous adipose tissue, which was obtained from elective surgeries with patient's consent and TNF-α CM was prepared from ASCs as previously described (Lee *et al.*, 2010). Details are available in Supplementary Text online.

Immunodepletion of IL-6 or IL-8 from TNF-α CM

IL-6 or IL-8 was immunodepleted from TNF-α CM by immunoprecipitation with anti-IL-6 or anti-IL-8 antibodies. Details are available in Supplementary Text online.

Cutaneous wound-healing model

Animal experiments were conducted using protocols approved by the Pusan National University Institutional Animal Use and Care Committee. Male Sprague–Dawley rats weighing 275–300 g were randomly divided into three groups (eight mice per group) and anesthetized using an intraperitoneal injection of ketamine (50 mg kg^{-1}) and xylazine (5 mg kg^{-1}). Details are available in Supplementary Text online.

Immunocytochemistry and histological analysis

Animals were killed and skin samples including the wound and the surrounding skin were excised. Tissue specimens ($n=16$ per group) were fixed in 3% paraformaldehyde for 24 hours and embedded in paraffin. Before sectioning and histological analysis, blocks were cut to expose wounded tissue near the center of each wound. In the present study, three consecutive 6 μm-thick serial sections from the center of the wounds were stained with hematoxylin and eosin for histological analysis. Under light microscopy, the sections were photographed using a model DFC300FX mounted digital camera (Leica, Solms, Germany). Gap of wounds, which was defined as the distance between the advancing edges of KC migration, was measured in three serial sections using Image J software. Capillary density and the numbers of proliferating cells and immune cells were assessed morphometrically by immunostaining with anti-vWF, anti-Ki67, and anti-CD68 antibodies, respectively. The numbers of capillary density and Ki67- or CD68-positive cells in three consecutive serial sections were quantified and averaged. To get a more accurate view of the wound, two 6 μm-thick sections were taken from the specimens at 150 μm intervals and similar results were obtained after hematoxylin and eosin staining and immunostaining of the sections (data not shown). Details are available in Supplementary Text online.

ELISA

Concentrations of IL-6 and IL-8 in conditioned medium from ASCs were determined by ELISA analysis. Details are available in Supplementary Text online.

Statistical analysis

Results of multiple observations are presented as mean ± SD. For multivariate data analysis, group differences were assessed using one-way or two-way analysis of variance, followed by *post hoc* comparisons tested using Scheffe's method. A value of $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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