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Decreased Macrophage Number and Activation Lead to Reduced Lymphatic Vessel Formation and Contribute to Impaired Diabetic Wound Healing

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Impaired wound healing is a common complication of diabetes. Although it is well known that both macrophages and blood vessels are critical to wound repair, the role of wound-associated lymphatic vessels has not been well investigated. We report that both the presence of activated macrophages and the formation of lymphatic vessels are rate-limiting to the healing of diabetic wounds. We have previously shown that macrophages contribute to the lymphatic vessels that form during the acute phase of corneal wound healing. We now demonstrate that this is a general phenomenon; cells that co-stain for the macrophage marker F4/80 and the lymphatic markers LYVE-1 (lymphatic vascular endothelium hyaluronate receptor) and podoplanin contribute to lymphatic vessels in full-thickness wounds. LYVE-1-positive lymphatic vessels and CD31-positive blood vessels were significantly reduced in corneal wound healing in diabetic mice (*db/db*) ($P < 0.02$) compared with control (*db/+*) mice. Glucose treatment of control macrophages led to the down-regulation of the lymphatic-specific receptor *VEGFR3* and its ligands, vascular endothelial growth factor-C and -D (*VEGF-C*, *-D*). Interleukin-1 β stimulation rescued diabetic macrophage function; application of interleukin-1 β -treated *db/db*-derived macrophages to wounds in *db/db* mice induced lymphatic vessel formation and accelerated wound healing. These observations suggest a poten-

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Wound healing involves a complex interplay among cells, growth factors, and cytokines. The cascade of events that leads to wound healing begins with clotting and the recruitment of inflammatory cells, such as neutrophils and macrophages. The role of macrophages in wound healing-associated inflammation is particularly well documented. Within the first few days after injury, circulating monocytes are recruited into the wound site where they differentiate into macrophages,^{1,2} become activated, phagocytose debris, and produce factors such as vascular endothelial growth factor (VEGF)-A and -C that regulate tissue repair and induce the formation of lymphatic and blood vessels.^{3,4} The critical role of macrophages is demonstrated by the fact that their depletion in wounds leads to a delay in wound healing.^{5,6}

Lymphatic vessels in wounds function to maintain normal tissue pressure by draining the protein-rich lymph from the interstitial space, as well as by facilitating the delivery of cells that mediate the immune response.^{7,8} Delayed wound healing, such as that seen in infections, seems to be due, at least in part, to reduced lymphatic development that leads to persistent edema and delayed removal of debris and inflammatory cells.⁹ However, the origin of the cells involved in the formation of lymphatic vessels in wounds has not been investigated.

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Figure 1. Contribution of macrophages to lymphatic vessel formation in normal wound healing. Sections of granulation tissue 5, 10, and 14 days after wounding stained for F4/80 (green), a macrophage marker; LYVE-1 (red), a marker of lymphatic endothelium; and TOPRO3 (blue), a nuclear marker. Results reveal vessel-like structures composed of cells that are double positive for markers of macrophages and lymphatic cells. Scale bar = 40 μ m. The small window in D14/OVERLAY image shows a higher magnification of a F4/80 and LYVE-1 double-positive structure (white arrowhead). Scale bar = 16 μ m.

We and others have previously shown that monocyte/macrophages contribute to the formation of lymphatic vessels during inflammation.^{10–12} In addition, we demonstrated that macrophages express VEGFR3 (Flt4) and secrete VEGF-C, a ligand for VEGFR3,¹³ which induces lymphatic vessel formation¹³ and has been shown to be essential for reducing tissue edema in wound healing.⁹

Diabetic patients frequently have serious problems with wound repair, and the etiology of this impaired healing process is poorly understood. We have used a genetic model of murine diabetes (*db/db*) to investigate diabetes-associated wound healing. *db/db* mice have an inactivating mutation of the gene encoding the leptin receptor ObR^{14,15} and develop obesity, insulin resistance, and severe diabetes with marked hyperglycemia, resembling adult-onset diabetes mellitus.¹⁴ Similar to human diabetics, wound healing in these animals is markedly delayed.^{5,16,17}

In this study we show in wild-type mice that, as in corneal inflammation,¹⁰ lymphatic vessels that form during the acute phases of excisional wounds are comprised largely of cells that co-stain for the macrophage marker F4/80 and the lymphatic markers LYVE-1 and podoplanin. We document that macrophages contribute to lymphatic vessels in wild-type or heterozygous (+/*db*) spontaneously diabetic mice but not in homozygous *db/db* mice. Exogenous activation of *db/db* macrophages restores their functions and rescues their ability to contribute to wound healing.

Materials and Methods

Animals

Male BKS.Cg-m Leprdb/+ +/J, BKS.Cg-m Leprdb/+ –/J, and wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME; 8 to 10 weeks) were used. All animal protocols were approved by Schepens Animal Care and Use Committee, consistent with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and St. Elizabeth's Institutional Animal Care and Use Committee, consistent with the Guide for the Care and Use of Laboratory Animals.

Corneal Inflammation Model

Corneal inflammation was created by suture placement. Before suture placement on the cornea, each animal was deeply anesthetized with an intraperitoneal injection of ketamine (3 to 4 mg/mouse) and xylazine (0.007 mg/mouse). Using stromal incisions that encompassed more than 120° of the corneal circumference, three 11-0 nylon (MANI, Tochigi, Japan) sutures were placed intrastromally. To obtain standardized angiogenic and lymphangiogenic responses, the outer edge of the suture was placed halfway between the limbus and the line outlined by a 2-mm trephine; the inner edge was equidistant from the 2-mm trephine (Figure 1).

a

c

Red; LYVE-1

Green; CD31

b

Whole-Mount Corneal Staining of Blood and Lymphatic Vessels

Mice were euthanized 7 days after suture placement, and the corneas were excised, rinsed three times in phosphate-buffered saline (PBS), and fixed in acetone for 1 hour. They were then rinsed in PBS, blocked with 2% bovine serum albumin (BSA)-PBS with 0.03% Triton X-100, and incubated overnight at 4°C with rabbit anti-mouse LYVE-1 antibody (1:500; generously provided by Dr. D.G. Jackson, University of Oxford, Oxford, UK).^{18,19} The tissues were then washed, blocked, and stained overnight with rat anti-mouse fluorescein isothiocyanate (FITC)-conjugated CD31 (PE-CAM-1) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), then washed, blocked, and stained with anti-rabbit Cy3-conjugated secondary antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA). Double-stained, whole-mount corneas were visualized under a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY), a Leica TSC-SP2 inverted (Leica, Wetzlar, Germany), and an upright confocal laser-scanning microscope. Digital pictures of the flat mounts were taken using a spot image analysis system (Spot Advanced; Diagnostic Instruments, Inc., Sterling Heights, MI), and the area covered by blood and lymphatic vessels positive for CD31 or LYVE-1,^{18,19} respectively, was quantified using NIH Image software (Image J, <http://rsb.info.nih.gov/ij/>). The total corneal area was calculated using the innermost vessel of the limbal arcade as the border; the areas of blood and lymphatic vessels within the cornea were calculated and normalized to the total corneal area (expressed as a percentage of the cornea covered by vessels). Five mice were used in each experiment, which was conducted three times with similar results. A representative experiment is shown.

Collection and Culture of Peritoneal Macrophages

Thioglycollate-induced macrophages were collected from the peritoneal cavity (peritoneal exudate cells; PECs) of normal 8-week-old male *db/db* and *db/+* mice using previously described methods.²⁰ PECs were washed, resuspended, and cultured for 24 hours at 37°C in a 5% CO₂ (10⁶ cells/35-mm plate) in RPMI 1640 medium (Cambrex, Baltimore, MD) containing 10% BSA (Sigma Chemical Co., St. Louis, MO), 1 × 10⁻⁵ mol/L 2-mercaptoethanol (Sigma-Aldrich Corp.), 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1 mmol/L nonessential amino acid, 1 mmol/L sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Twenty-four hours later, adherent cells were harvested. For incubation in high glucose, macrophages were cultured for 3 days in the same medium with the addition of 30 mmol/L glucose. For stimulation with interleukin (IL)-1β, macrophages were cultured for 24

hours in the same medium with the addition of 20 ng/ml IL-1β following culture in high glucose (BD PharMingen, San Diego, CA). Five mice were used in each experiment, which were each conducted three times with similar results. A representative experiment is shown.

Collection of F4/80- and CD11b-Positive Macrophages from Bone Marrow

Macrophages were collected from bone marrow as previously described²¹ and were cultured for 7 days at 37°C in a 5% CO₂ in RPMI 1640 containing 10% horse serum, 10% controlled processed serum replacement factor 1(CSPR-1), 10% L929 cell-conditioned medium (L929 cells provided by Dr. Joan Stein-Streilein, Harvard Medical School, Boston, MA), 1% minimal essential medium vitamins, 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin and streptomycin. Nonadherent cells were then removed, and adherent cells were harvested from the culture dish with a cell scraper, rinsed in PBS, blocked by incubation for 10 minutes on ice with CD16/32 Fc-block (BD PharMingen) in 1% BSA-PBS, and labeled for 30 minutes on ice with rat anti-mouse phycoerythrin (PE)-conjugated CD11b, rat anti-mouse FITC-conjugated F4/80, or an isotype control antisera (FITC and PE-conjugated nonspecific rat IgG2b*, 1:100; BD PharMingen). Labeled cells were analyzed on an EPIC XL flow cytometer (Beckman Coulter, New York, NY). For stimulation with IL-1β, macrophages were cultured for 24 hours in the same medium with the addition of 20 ng/ml IL-1β. For labeling with DiIAcLDL (Biomedical Technologies, Inc., Stoughton, MA), macrophages were cultured for 2 hours in DiIAcLDL according to the manufacturer's instructions (Invitrogen).

Skin Wound Model

Wounds were created in mice 8 to 10 weeks of age as described previously.¹⁷ In brief, after induction of deep anesthesia by intraperitoneal injection of sodium pentobarbital (160 mg/kg), full-thickness and excisional skin wounds (one per mouse) were created on the backs of the mice using 6-mm skin biopsy punches and were covered with the semipermeable polyurethane dressing OpSite (Smith & Nephew, Massillon, OH). Macrophages (1 × 10⁶ cells/0.2 μl) or saline was injected with a 27-gauge needle into the wound bed around the wound at four sites (total 4 × 10⁶ cells) through the OpSite (*n* = 5). Wound closure was documented with a digital camera (Nikon Coolpix 995; Nikon, Tokyo, Japan) on days 0, 7, and 14. Images were analyzed using the NIH Image J analyzer by tracing the wound margin with a fine resolution computer mouse and calculating pixel area. The areas of the wounds were compared using Fisher's *t*-test.

Figure 2. Angiogenesis and lymphangiogenesis in the corneal suture model assay in *db/db* mice. **a:** A schematic illustrating the method of the suture placement on cornea. **b:** Quantification of lymphangiogenesis and hemangiogenesis in the corneal suture model assay in wild-type C57BL/6 mice. **c:** Hemangiogenesis (CD31; green) and lymphangiogenesis (LYVE-1; orange) in the *db/db* and *db/+* (control) mouse corneal 7 days after suture placement. Graphs display quantification of lymphatic and blood vessel area.

This experiment was conducted twice with similar results. A representative experiment is shown.

Histological Analysis

Wound tissues were harvested at 7 and 14 days after macrophage application. The tissues were fixed in 4% paraformaldehyde for 24 hours and were incubated overnight in 15% and then 30% sucrose before being placed in 100% acetone for 3 hours and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin and eosin and then observed by masked observers who assigned histological scores. In brief, each specimen was given a score of 1 to 12 as previously described¹⁷: 1 to 3, none to minimal cell accumulation and granulation tissue or epithelial migration; 4 to 6, thin, immature granulation tissue dominated by inflammatory cells but with few fibroblasts, capillaries, or collagen deposition and minimal epithelial migration; 7 to 9, moderately thick granulation tissue, dominated by inflammatory cells and more fibroblasts and collagen deposition; and 10 to 12, thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition.

Evaluation of Wound Lymphatic Vessel-Like Structures

Sections of wounds were stained with rabbit anti-LYVE-1 antibody (1:500; Angiobio, Del Mar, CA), hamster anti-mouse podoplanin antibody (Angiobio), rat anti-mouse F4/80, and rat anti-mouse CD31 (1:250; BD PharMingen). Labeling with F4/80 and CD31 was visualized with FITC streptavidin (Vector Laboratories, Burlingame, CA) and biotinylated anti-rat antibody (Vector Laboratories). Lymphatic vessel-like structures were defined as three or more cells associated to form a cord-like structure (see below). Lymphatic vessels were normalized per 1 mm² using percentage of fluorescence area, as described previously.²²

Reverse Transcriptase (RT)-PCR and Real-Time PCR Analysis

For RT-PCR analysis, RNA was extracted using RNA-Stat (Tel-Test Inc., Friendswood, TX), according to the manufacturer's instructions. Total RNA was reverse-transcribed using TaqMan Mutiscribe RT Kit (Applied Biosystems, Foster City, CA), and amplification was performed on the ABI 7300 Real Time PCR System (Applied Biosystems). The following primers and probes were designed using PrimerExpress software (Applied Biosystems): *VEGF-A* (all isoforms): forward, 5'-CATCTTCAAGCCGT-CCTGTGT-3'; reverse, 5'-CAGGGCTTCATCGTTACAG-CA-3'; and 5-carboxyfluorescein (FAM)-CCGCTGATGC-GCTGTGCAGG-BHQ. *VEGFR3*: forward, 5'-TGCTGAAA-GAGGGCGCTACT-3'; reverse, 5'-TGCCGATGTGAATT-AGGATCTTG-3'; and FAM-AGCACCGTGCCCTG-ATGTCGGA-TAMRA.

The relative expression levels of the target gene mRNAs were calculated by the comparative C_T method. All target sequences were normalized to 18S mRNA (VIC-labeled TaqMan reagents; Applied Biosystems) in multiplexed reactions performed in duplicate. Differences in C_T values were calculated for each target mRNA after subtracting the mean value of 18S rRNA (relative expression = 2^{- Δ CT}). Results were analyzed using analysis of variance.

Tube Formation Assay

Adherent cells were used in the tube formation assay as previously described.¹⁰ An aliquot (100 μ l) of growth factor-depleted Matrigel (Becton Dickinson, Bedford, MA) with 100 μ l of EBM-2 (endothelial cell culture medium) (Cambrex Corporation, East Rutherford, NJ) was added to four-chamber slides (Lab-Tek; Nunc Inc., Naperville, IL) and allowed to gel for 30 minutes at 37°C. PECs were preincubated with high glucose (2 $\times$ 10⁶ cells/ml) in 500 μ l of EBM-2 containing 3% fetal bovine serum (normal glucose). Tube formation was monitored over 5 days. Digital pictures were taken using a Spot image analysis system. The areas covered by cell aggregates and tube-like structures were measured at 48 hours using NIH Image software. The total area covered by the Matrigel was outlined (18 mm²); the area of the aggregates and the tube-like structures was calculated and expressed as a percentage of the Matrigel area.

Results

Macrophages Contribute to Lymphatics in Normal Wound Healing

To determine whether the involvement of macrophages in lymphatic vessel formation that we observed during corneal inflammation¹⁰ is common to other forms of wound healing, we investigated the healing of excisional skin wounds. Examination of granulation tissue in wild-type C57BL/6 mice 5 days after wounding revealed lymphatic structures consisting of cells that were double-positive for F4/80 and LYVE-1 (Figure 1; day 5). Cells expressing both F4/80 and LYVE-1 were not detectable in the pre-existing lymphatics in the nonwounded tissue at the wound edge. Double positive cells persisted 10 to 14 days after wounding, though in lower numbers (Figure 1; white arrowhead), and lymphatic structures that remained in the granulation tissue stained for LYVE-1 only. We could not definitively determine whether these LYVE-1-only-positive structures had sprouted from local existing lymphatic vessels or if they were formed *in situ* by F4/80-positive cells that had down-regulated F4/80 expression. However, the fact that lymphatic vessels in the wound at day 14 expressed low levels of F4/80 suggests that new lymphatic vessels were formed from F4/80-positive cells. These observations indicate that, as for corneal inflammation, the lymphatic vessels that assemble transiently during the acute phase (days 5 to 7) of wound

Figure 3. Characterization of PECs from *db/db* and *db/+* mice. **a:** Thioglycollate-induced macrophages were collected from the peritoneal cavity of normal 8-week-old male *db/db* and *db/+* mice and cell numbers compared ($n = 4$). **b:** Real-time RT-PCR analysis of *VEGFR3*, *VEGF-C*, and *VEGF-A* relative expression ($RE = 2^{DCT}$); by PECs from *db/db* and *db/+* mice. **c:** RT-PCR analysis of *VEGFR3* and *VEGF-C* after IL-1 β (20 ng/ml) stimulation on PECs from *db/db* mice.

healing are derived largely from F4/80 macrophages and suggest an important role for macrophages in the formation of lymphatic vessels during the early stages of wound healing.

Lymphangiogenesis in *db/db* Mouse Cornea

Although reduced wound healing-associated angiogenesis has been documented in diabetic patients and in animal models,^{14,23} the status of lymphangiogenesis in diabetic wounds has not been well investigated. We used the corneal suture model of inflammation¹³ to assess lymphangiogenesis in the *db/db* mouse. Lymphangiogenesis and hemangiogenesis in *db/+* mouse cornea were equivalent to that in wild-type mice (Figure 2b).

Thus, *db/+* littermate mice were used as controls in subsequent studies. Quantification of LYVE-1-positive lymphatic vessels and CD31-positive blood vessels in the cornea 7 days after suture placement revealed that both lymphangiogenesis and hemangiogenesis were significantly suppressed in the diabetic mice ($P < 0.02$) as compared with control (*db/+*) mice (Figure 2, b and c).

Macrophage Function in *db/db* Mice

Using the corneal suture model of inflammation and wound healing, we have previously shown that macrophages play a critical role in the formation of lymphatic vessels.¹⁰ As described above, macrophages contrib-

Figure 5. Effect of IL-1 β on tube formation by macrophages. **a:** Schematic of the tube formation assay. **b:** Flow cytometric analysis of cultured macrophages isolated from bone marrow and cultured in L929 cell condition medium. **c:** Quantification of cluster and tube-like structure formation in Matrigel (18 mm²) of db^{+/+} peritoneal macrophages (1 $\times$ 10⁶ cell/ml) plated into Matrigel and grown for 24 hours in high glucose alone or in high glucose with IL-1 β . **d:** Photomicrograph of Matrigel assay in **c**. Statistical analysis was performed using analysis of variance post hoc analysis (Fisher's PLSD).

ute to lymphatics in normal skin wound healing. Based on these observations, we hypothesized that the reduced lymphatic vessels observed in diabetic mice might be due, at least in part, to an altered ability of

Figure 4. Effect of glucose and IL-1 β on control macrophages. Real-time RT-PCR analysis of *VEGFR3*, *VEGF-C*, and *VEGF-A* of db^{+/+} mouse PECs cultured in high glucose (30 mmol/L) for 72 hours ($n = 6$) relative expression (RE) = 2^{DCT} and in the presence or absence of IL-1 β . The statistical analysis was assessed by analysis of variance post hoc analysis (Fisher's PLSD).

Figure 6. Wound healing in *db/db* mice. **a:** Photomicrographs of wounds in *db/db* mice 7 and 14 days after administration of the indicated macrophages (1×10^6 cells), including saline (no macrophages), *db/db* macrophages, *db/db* macrophages treated with IL-1 β , or untreated *db/+* macrophages. **b:** Quantification of newly formed granulation tissue over wounds at days 7 and 14. The area of the wounds at day 7 that received the IL-1 β -treated macrophages was significantly smaller than those that received nontreated *db/db* macrophages or *db/+* macrophages. At day 14, there was a significant difference between the area of the wounds that received IL-1 β -treated *db/db* macrophages and untreated *db/db* macrophages but no difference between IL-1 β -treated *db/db* macrophages or *db/+* macrophages. Statistical analysis was performed by analysis of variance post hoc analysis (Fisher's PLSD).

macrophages to participate in lymphatic vessel formation. To test this possibility, peritoneal macrophages, collected as previously described²⁰ from *db/db* mice and *db/+* controls, were quantified and assessed for their ability to form lymphatic vessels *in vitro*. Significantly fewer macrophages were recruited by thioglycolate stimulation in *db/db* mice than in control (*db/+*) mice ($P = 0.02$) (Figure 3a). Moreover, mRNA levels for *VEGFR3* and its ligands *VEGF-C* and *VEGF-A* were significantly reduced in *db/db*-derived macrophages compared with macrophages from control (*db/+*) mice

($P < 0.05$) (Figure 3b). Stimulation of *db/db*-derived macrophages with an inflammatory cytokine, such as IL-1 β (20 ng/ml), led to a marked increase in *VEGFR3* mRNA and a modest increase in *VEGF-C* expression (Figure 3c).

Effect of High Glucose on Macrophage Function

We next investigated whether the reduced gene expression observed in *db/db*-derived macrophages

Figure 7. Histological assessment of wounds in *db/db* mice treated with macrophages. **a1** through **a8**: Photomicrograph of H&E-stained paraffin sections. **a-5**: Wounds that received IL-1-treated macrophages display many inflammatory cells in their granulation. **a-6**: Arrows indicate cord-like structures in granulation tissue that received IL-1 β -treated-macrophages. **b**: Histological scores at days 7 and 14 after receiving the indicated macrophages. There was more extensive granulation tissue at day 7 in wounds that had received IL-1 β -treated macrophages than in wounds that had received the untreated *db/db* macrophages or the *db/+* macrophages. At day 14, there was a significant difference between the wounds that had received IL-1 β -treated *db/db* macrophages versus untreated *db/db* macrophages but no difference between IL-1 β -treated *db/db* macrophages and *db/+* macrophages. The statistical analysis was performed using analysis of variance post hoc analysis (Fisher's PLSD).

might be due to the effects of elevated glucose that characterizes the diabetic state. Incubation of control (*db/+*) macrophages under glucose conditions that mimic those seen in diabetes (30 mmol/L glucose) led to the suppression of *VEGFR3* and *VEGF-C* mRNA to a

level similar to those measured in *db/db*-derived macrophages (Figure 4). Treatment of the glucose-incubated *db/+* macrophages with IL-1 β (20 ng/ml) attenuated the inhibition of *VEGFR3*, *VEGF-C*, and *VEGF-A* expression.

Figure 8. Effect of IL-1 β treatment on formation of cord-like structures in the granulation tissue. **a:** Immunofluorescence images at day 7 after treatment with untreated *db/db* macrophages (LYVE-1; orange) and IL-1 β -stimulated *db/db* macrophages (LYVE-1; orange). **b:** Quantification of cord-like structures (three or more cells) (1 mm²). Cells were counted under the fluorescence microscope following DAPI staining (data not shown).

We have previously shown that macrophages cultured in Matrigel can form vessel-like structures that express lymphatic markers.¹⁰ Whereas culture of control (*db/+*) macrophages led to the formation of tube-like structures, control macrophages cultured for 72 hours in high glucose (30 mmol/L glucose) did not form tubes (Figure 5c). The glucose-treated macrophages associated with one another but did not assemble into tubes. However, IL-1 β treatment of the glucose-incubated macrophages restored their ability to assemble into vessel-like structures (Figure 5, c and d).

Effect of Macrophage Administration on Wound Healing

We next compared the effect of *db/db*-derived and control macrophages on wound healing. Because the reduced number of macrophages recruited by thioglycolate in *db/db* mice (Figure 3a) makes it technically difficult to collect sufficient macrophages to conduct these studies, macrophages for wound healing studies were harvested from bone marrow. Bone marrow was collected from *db/db* mice and cultured in L929 medium for 7 days.^{21,24,25} More than 95% of the macrophages collected from wild-type C57BL/6 bone marrow using this method express CD11b and F4/80 (Figure 5b).

Macrophages were harvested from *db/db* mouse bone marrow and cultured for an additional 24 hours in normal glucose in the absence or presence of IL-1 β (20 ng/ml). Skin wounds were created in *db/db* mice and 1×10^6 *db/db*-derived macrophages (activated or nonactivated) were injected into the wounds. Wounds that received IL-1 β -activated macrophages exhibited significantly more wound healing at day 7 ($P < 0.0001$), as evidenced by the amount of granulation tissue, compared with wounds that received nonactivated macrophages or saline (Figure 6, a and b). This difference in wound healing was no longer apparent 14 days after treatment (Figure 6b), suggesting that activated macrophages are rate-limiting only during the initial phase of wound healing.

Wound Histology

There were gross differences in wound healing between *db/db* mice that received IL-1 β -treated *db/db*

macrophages and those that received untreated *db/db* macrophages. Histological analysis revealed significant granulation tissue in wounds receiving IL-1 β -treated macrophages (Figure 7); numerous infiltrating cells were evident, some of which appeared to be assembling into cord-like structures (Figure 7, a and b), representing lymphatic vessel-like structures. In contrast, wounds in *db/db* mice treated by saline injection contained little granulation tissue. Tissue sections from each group were scored for the extent of granulation tissue and the number of inflammatory cells by previously described methods¹⁷ at 7 and 14 days after treatment (Figure 7b). Wounds receiving IL-1 β -treated macrophages had thicker granulation tissue and more infiltrating cells at both 7 and 14 days than control wounds that received untreated macrophages. These observations are consistent with the well-documented role of the activated macrophage in wound healing and suggest that insufficient macrophage number and incomplete activation may account, at least in part, for the impaired wound healing observed in diabetes.

Lymphatic-Like Vessels in Wounds Treated with IL-1 β Stimulated *db/db* Macrophages

Seven days after macrophages were applied to wounds, the mice were sacrificed and the wound areas were sectioned and stained with antisera against LYVE-1, CD31, and F4/80. There were more LYVE-1-positive cord-like structures in the granulation tissue of wounds that had received IL-1 β treated *db/db*-derived macrophages than in wounds that had received non-treated *db/db* macrophages (Figure 8). A majority of F4/80-positive cells in the granulation tissue co-stained with LYVE-1 (data not shown). Within the wound itself, the newly formed small lymphatic vessel structures were nearly all double-labeled with LYVE-1 and F4/80 (Figure 9a). However, outside of granulation tissue, there were only a few F4/80-positive cells in the lymphatic vessels (Figure 9b). Most of the cells that made up the lymphatic vessels of the granulation tissue double-stained with LYVE-1 and CD31, whereas none of the cells in blood vessels expressed LYVE-1 (data not

Figure 9. Effect of IL-1 β -treated macrophages on formation of lymphatic structures. Confocal microscopic images of wounds treated with IL-1 β -stimulated *db/db* macrophages. **a:** Small vessel-like structures stained with LYVE-1 (orange) and F4/80 (green) at the edge of the granulation tissue (blue is nuclear staining with TOPRO-3). The **arrow** indicates the lumen that was constituted by cells that stained for both F4/80 (green) and LYVE-1 (red). Scale bar = 20 μ m. **b:** Area outside of the granulation tissue stained with LYVE-1 (orange) and F4/80 (green) (blue is nuclear staining with TOPRO-3). V, vessel; L, lymphatic vessel. Note that some lymphatic vessels did not stain with F4/80 (**arrowhead**), whereas others stained for both F4/80 and LYVE-1 (**arrow**). Scale bar = 40 μ m. **c:** A vessel-like structure in the granulation tissue stained with podoplanin (orange) and F4/80 (green). Scale bar = 40 μ m.

shown). The small lymphatic vessel-like structures stained with antisera against both podoplanin and F4/80 (Figure 9c).

Prelabeled macrophages that had been injected also contributed to LYVE-1 and podoplanin-positive lymphatic-like structures in the wound (Figure 10). Four

Figure 10. The contribution of exogenously administered macrophages to lymphatic vessels in wound in *db/db* mice. Macrophages were isolated from wild-type C57BL/6 mice, prelabeled with DiLDL, and injected into excisional wounds created in diabetic mice. Granulation tissue was examined 7 days later. Top: DiI-prelabeled macrophages stained with LYVE-1 located in granulation tissue 7 days after transplantation. Bottom: DiI-prelabeled macrophages stained with antisera against podoplanin in granulation tissue 7 days after transplantation.

weeks after macrophage injection, when wound-healing inflammation had resolved, there were no lymphatic vessel-like structures that co-labeled with LYVE-1 and F4/80; only LYVE-1-positive lymphatic vessels were evident (data not shown).

Discussion

We have shown that, as we observed during corneal inflammation, macrophages contribute to the formation of lymphatics in healing of excisional wounds. Furthermore, whereas the addition of macrophages isolated from *db/db* mouse bone marrow to wounds in *db/db* mice did not significantly affect wound healing, IL-1 β activation of the *db/db* macrophages restored their ability to contribute to wound healing. Observations of the effects of glucose treatment on macrophages from nondiabetic mice lead us to speculate that high glucose suppression of macrophage function, and the resulting reduced inflammatory response may account, at least in part, for the impaired wound repair that is frequently observed in association with diabetes. Instead of the acute inflammation observed during normal healing, wound healing in the diabetic is characterized by chronic moderate inflammation that is apparently insufficient to move the healing process forward. The role of macrophages as a source of cytokines and growth factors and as a phagocyte during wound healing has been well documented.²⁶ The func-

tion of macrophages has been well investigated in diabetic mice²⁷ but not in humans. Macrophages isolated from diabetic mice have been shown to secrete reduced levels of inflammatory cytokines such as IL-1 β and tumor necrosis factor- α .²⁷ In addition, as macrophages have been shown to play a critical role in keratinocyte deposition of extracellular matrix in wound area,²⁸ reduced macrophages may also have a variety of indirect effects.

Our findings of reduced macrophage number in both the corneal suture model and in peritoneal exudates in *db/db* mice suggest that inadequate macrophage number may be one factor contributing to impaired wound repair. We have previously shown that peritoneal monocytes/macrophages express markers of lymphatic endothelium, including LYVE-1, podoplanin, and Prox-1 and that macrophages contribute to lymphatic vessels during acute inflammation in the cornea.^{10–12} Furthermore, we observed that peritoneal macrophages could form lymphatic vessel-like tubes *in vitro* in a density-dependent manner.¹⁰ Thus, the reduced macrophage number in *db/db* mice may be the cause of the significant reduction in lymphatic structures in the granulation tissue of wounds in *db/db* mice compared with controls, which, in turn, may result in impaired wound healing.

Our observation of VEGF-C expression by macrophages within the inflamed corneal stroma and by bone marrow-derived macrophages¹³ indicates that macrophages are a likely source of VEGF-C in tumors and in

inflammation where they may function to induce lymphatic vessel formation.²⁹ Therefore, it is relevant that the *db/db*-derived macrophages were qualitatively different from the *db/+* macrophages; *db/db* macrophages exhibited significantly reduced *VEGFR3*, *VEGF-C*, and *VEGF-A* mRNA expression relative to *db/+* macrophages. The fact that incubation of macrophages from control mice in high glucose suppressed the expression of *VEGFR3* and *VEGF-C* suggest that this suppression may be secondary to hyperglycemia. IL-1 β stimulation of *db/db*-derived macrophages led to increased expression of lymphatic endothelial markers, including *VEGFR3* and its ligand *VEGF-C*. Consistent with these observations, unstimulated macrophages from *db/+* mice incubated in high glucose formed fewer clusters and tube-like structures in a three-dimensional assay than cells stimulated with IL-1 β . Thus, in addition to the lower number of macrophages, reduced expression of *VEGFR3*, *VEGF-C*, and *VEGF-A* may contribute to impaired wound healing by leading to reduced lymphatic vessel formation.

Our results indicate that activated macrophages with normal function are important for wound repair and participate in the formation of transient lymphatic vessels that seem to regress when inflammation is resolved. It has been previously suggested that lymphangiogenesis is not essential for closure of excisional skin wounds.⁴ We found that the administration of macrophages that could contribute to the formation of lymphatic vessels accelerated and enhanced the formation of the granulation tissue. The early formation of lymphatic vessel-like structures in granulation tissue that strongly expressed LYVE-1 and podoplanin seems to be important for normal wound healing. The *db/db* mice share a number of similarities with human adult-onset diabetes mellitus, including impaired wound healing. Thus, our observations of wound healing rescue by exogenously activated macrophages may hold promise for human therapies.

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