Accelerated wound healing in mice by on-site production and delivery of CXCL12 by transformed lactic acid bacteria

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Impaired wound closure is a growing medical problem associated with metabolic diseases and aging. Immune cells play important roles in wound healing by following instructions from the microenvironment. Here, we developed a technology to bioengineer the wound microenvironment and enhance healing abilities of the immune cells. This resulted in strongly accelerated wound healing and was achieved by transforming Lactobacilli with a plasmid encoding CXCL12. CXCL12-delivering bacteria administrated topically to wounds in mice efficiently enhanced wound closure by increasing proliferation of dermal cells and macrophages, and led to increased TGF-β expression in macrophages. Bacteria-produced lactic acid reduced the local pH, which inhibited the peptidase CD26 and consequently enhanced the availability of bioactive CXCL12. Importantly, treatment with CXCL12-delivering Lactobacilli also improved wound closure in mice with hyperglycemia or peripheral ischemia, conditions associated with chronic wounds, and in a human skin wound model. Further, initial safety studies demonstrated that the topically applied transformed bacteria exerted effects restricted to the wound, as neither bacteria nor the chemokine produced could be detected in systemic circulation. Development of drugs accelerating wound healing is limited by the proteolytic nature of wounds. Our technology overcomes this by on-site chemokine production and reduced degradation, which together ensure prolonged chemokine bioavailability that instructed local immune cells and enhanced wound healing.

macrophage | chemokine | blood flow | diabetes | *Lactobacillus reuteri*

An open wound in the skin can cause severe discomfort and provide an entry for invading bacteria. During the inflammation phase of wound healing, immune cells accumulate in response to alarm signals, cytokines, and chemokines released by injured or activated cells (1–5). The chemokine CXCL12 (Stromal cell-Derived Factor 1α) is associated with beneficial effects in models of cutaneous wounds (6, 7) and binds CXCR4 expressed by immune cells and keratinocytes (8). Macrophages and neutrophils represent the major immune cell populations at the wound site, where they are essential for keeping invading microorganisms at bay and also for fueling the healing process by secreting additional chemokines, growth factors, and matrix digesting enzymes. During the course of healing, macrophages shift phenotype toward an anti-inflammatory one and subsequently promote tissue restitution. This shift is induced by macrophage phagocytosis of cell debris and by microenvironmental signals (9) such as CXCL12 (10, 11). Immune suppression therapies (12) and experimental immune cell depletion (13, 14) delay wound healing, revealing a pivotal immune cell involvement.

Chronic wounds are often associated with underlying pathologic processes that increase susceptibility for acquiring wounds (e.g., peripheral neuropathies) and/or reduced healing abilities as seen in persons with arterial or venous insufficiencies or diabetes or who are receiving systemic steroid-treatment (15, 16). Standard care for chronic ulcers comprises surgical or chemical removal of necrotic tissue, repeated dressing changes, and antibiotics to fight infections (17). Several experimental and clinical trials have investigated the effects of local application of growth factors alone or coupled to different biomaterials on different types of chronic wounds, but with modest results so far (18). The poor translational success is at least in part a result of the proteolytic nature of the wound, limiting drug bioavailability, and highlights the importance of addressing clinical feasibility and read-out when designing the delivery system.

This study aimed to accelerate wound healing by targeting the function of immune cells through local bioengineering of the wound microenvironment. To achieve this, a technology optimized to deliver chemokines directly to wounded skin was developed, whereby lactic acid bacteria were used as vectors. *Lactobacillus reuteri* bacteria were transformed with a plasmid encoding the chemokine CXCL12 previously associated with beneficial effects in models of healing (6, 7) and blood-flow restoration (19, 20).

Significance

Chronic wounds comprise a growing clinical problem that represents >3% of the health care budget in industrialized countries. Drug development is hampered by the proteolytic nature of the wounds, which greatly limits drug bioavailability. Here, we present a technology that circumvents this by on-site production and reduced chemokine degradation. Lactobacilli bacteria were transformed into CXCL12-producing vectors to bioengineer the wound microenvironment after topical application. Consequently, the immune cells driving the healing process were reinforced, which greatly accelerated wound closure in healthy mice, in mouse models of hyperglycemia and peripheral ischemia, and in a wound model using human skin disks. Initial safety studies demonstrated that neither bacteria nor the chemokine produced was detected in systemic circulation following application to open wounds.

Author contributions: E.V., F.H., P.P., S.R., and M.P. designed research; E.V., E.Ö., A.M., H.L., P.P., and S.R. performed research; F.H., P.P., and S.R. contributed new reagents/analytic tools; E.V., E.Ö., A.M., H.L., P.P., S.R., and M.P. analyzed data; M.P. supervised the project; and E.V. and M.P. wrote the paper.

Conflict of interest statement: The technology of transformed *Lactobacillus reuteri*-producing chemokines is filed for patent protection (PCT/EP2015/081146, WO2016/102660), and drug candidates using this technology are being developed by a company of which E.V., S.R., and M.P. are shareholders.

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Bacteria-produced lactic acid reduced the pH in the wound and thereby potentiated the effects of the produced CXCL12 by prolonging its bioavailability. The overall result of topical wound treatment with this on-site chemokine delivery system was strongly accelerated wound closure to an extent not reported before.

Results

Specific and Efficient Plasmid Expression in Transformed *L. reuteri*. Bacterial production and secretion of CXCL12 following transformation with pSIP_CXCL12 depended completely on addition of the promoter induction peptide [induced CXCL12-producing Lactobacilli (LB_CXCL12); Fig. S1*B*], and no CXCL12 was detected in the supernatant from noninduced bacteria or bacteria carrying the luciferase plasmids (pSIP_Luc, LB_Luc). Addition of the activation peptide to LB_Luc resulted in immediate luciferase expression that peaked during the first 2 h and lasted for >10 h (Fig. S1*C*). Following topical application to cutaneous wounds in mice, the bacteria were restricted to the wound surface and plasmid expression was high for the first 1 h (Fig. 1 *A* and *B*). Treatment of wounds with CXCL12-producing *L. reuteri* resulted in increased CXCL12 levels in adjacent skin 2 d after wound induction independent of bacterial dose (Fig. 1 *C*–*G*), indicating that bacterial adhesion to the wound is a dose-limiting factor.

CXCL12-Producing L. reuteri Accelerates Wound Healing. Daily administration of CXCL12-producing *L. reuteri* (2×10^7 cfu) to wounds resulted in accelerated wound closure compared with untreated wounds or wounds treated with control *L. reuteri*, in which the effect was most prominent during the first 24 h (Fig. 2 *A* and *B*) and wound area over time (area under the curve) was reduced (Fig. 2*C*). The time to complete or partial wound closure was reduced for wounds treated with CXCL12-producing *L. reuteri* compared with untreated wounds or wounds treated with control *L. reuteri* (Fig. 2*D* and Fig. S2 *A*–*C*). No additional effect on wound healing was demonstrated by increasing the dose of CXCL12-producing *L. reuteri* (Fig. S2 *D* and *E*), which agrees with measured levels of bacteria-delivered CXCL12 (Fig. 1 *D*–*G*). CXCL12 delivered by a different bacterial strain, *Lactococcus lactis*, also accelerated wound healing (Fig. S2 *F* and *G*).

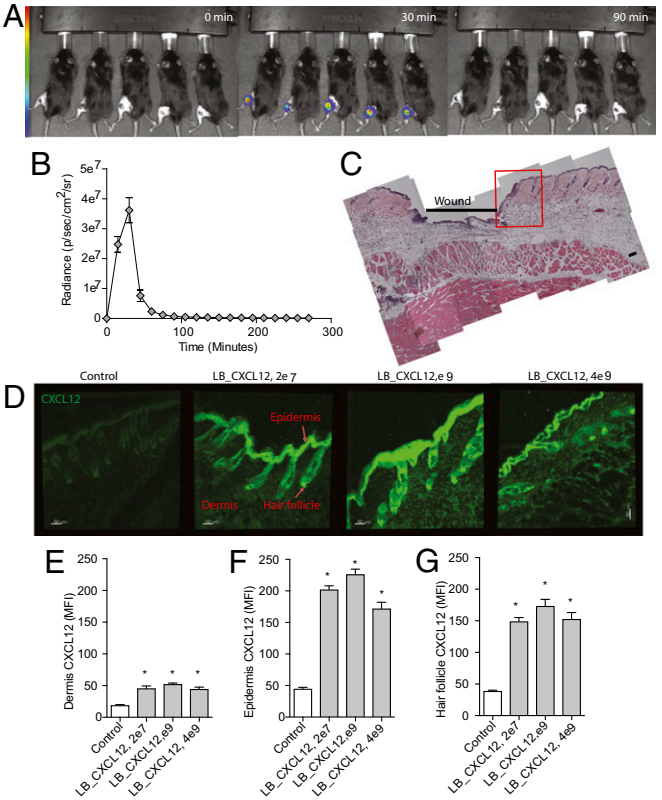

Fig. 1. Local delivery of CXCL12 to wounds by *L. reuteri*. (*A*) Luminescent *L. reuteri* added to hind-limb wounds in anesthetized mice. (*B*) Quantification of plasmid expression ($n = 5$). (*C*) Overview image of a skin wound (red square shows area of analysis in the dermis). (Scale bar, 100 μm.) (*D*) Skin next to the wound stained for CXCL12 (green) in mice receiving no treatment (control) or different doses of CXCL12-producing *L. reuteri* (2×10^7, 1×10^9, and 4×10^9 cfu LB_CXCL12). Levels of CXCL12 were quantified in the dermis (*E*), epidermis (*F*), and hair follicles (*G*) after treatment with different doses of LB_CXCL12 (*$P < 0.05$).

On-Site Production of CXCL12 and Lactic Acid Increases Bioavailability. When fresh supernatants from CXCL12-producing *L. reuteri* or different concentrations of recombinant CXCL12 (rCXCL12) were applied topically to wounds once daily, wound closure was not accelerated (Fig. 3 *A* and *B*). However, rCXCL12 (0.2 μg) given every 10th minute for 1 h once daily to mimic continuous delivery accelerated wound closure (Fig. 3*B*), demonstrating that sustained CXCL12 delivery is needed to promote wound healing.

CXCL12 is processed at the NH_2 terminus to an inactive form [CXCL12(3-68)] by membrane-bound CD26 (dipeptidylpeptidase IV) expressed in wounds (21–23). The activity of CD26 is pH-dependent (24, 25), and enzymatic activity of CD26 was higher at pH 8.3 than pH 7.3, as described before, whereas there was no enzymatic activity at pH 6.3 or pH 5.3 (Fig. S3 *A*–*D*). Consequently, CXCL12 was totally inactivated by CD26 at pH 8.3 within 30 min and inactivated to a partial extent at pH 7.3. In contrast, CXCL12 was not processed at pH 6.3 and pH 5.3 (Fig. 3*C*). Lactic acid produced by *L. lactis* and *L. reuteri* causes a decrease in pH of 0.2–0.5 over the cultured period despite strain-specific differences in growth (Fig. S3 *E*–*H*). To investigate if pH-dependent degradation of CXCL12 within the wound affects healing, rCXCL12 (0.2 μg) was administered in buffers with different pH (pH 7.35, pH 6.35, pH 5.35). Intriguingly, only at pH 6.35 did CXCL12 induce rapid wound closure (Fig. 3 *D* and *E*). These data provide evidence that concomitant lactic acid production by the bacteria enhance bioavailability of the delivered chemokine.

CXCL12 Induces Proliferation and Increases Macrophage-Produced TGF-β and CXCL12. Wound induction increased the number of proliferating cells in the dermis and epidermis at the wound edge at 24 h, in addition to increased density of macrophages and TGF-β–expressing macrophages (Table S1). When wounds were treated with CXCL12-producing *L. reuteri*, the number of proliferating cells increased further (Fig. 4 *A* and *B* and Fig. S4*A*), as did the levels of TGF-β in the dermis closest to the wound and the number of wound-associated macrophages (Fig. 4 *C* and *D* and Fig. S4*B*), of which an increased fraction expressed TGF-β (Fig. 4*E*). Approximately 50% of the TGF-β^+ macrophages within wounds expressed the mannose receptor 1 (MMR), a marker for macrophages involved in tissue remodeling (Fig. 4*F*). The wound-associated macrophages also expressed the CXCL12 receptor CXCR4, and the density but not the fraction of $CXCR4^+$ macrophages were increased by CXCL12-producing *L. reuteri* treatment (Fig. 4 *G* and *H* and Fig. S4*C*). Further, the density and fraction of CXCL12-expressing wound-associated macrophages increased with treatment of CXCL12-producing *L. reuteri* (Fig. 4 *I* and *J* and Fig. S4*C*), demonstrating an autocrine feedback previously described (11).

The effect of macrophages in wound healing was elucidated by depleting mice of macrophages. The protocol used resulted in impaired wound healing at 48 h (Fig. 4 *K* and *L*) and 40% reduction of dermis macrophages at the wound. The effect of CXCL12-expressing *L. reuteri* on accumulation of wound macrophages and acceleration of healing was abolished (Fig. 4 *M* and

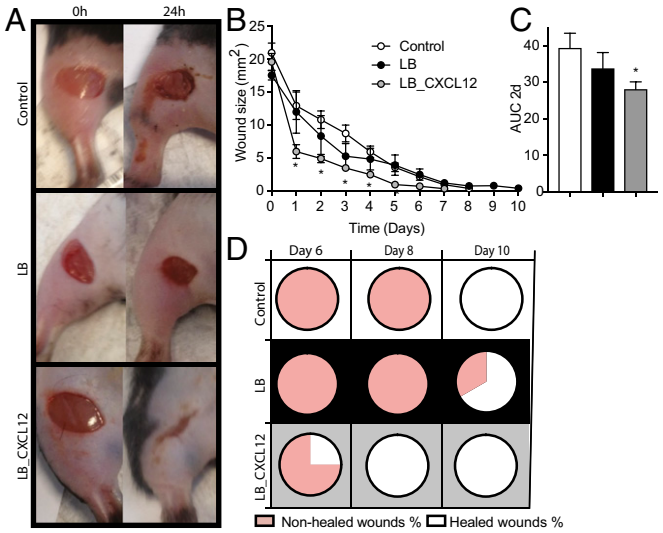

Fig. 2. Accelerated wound healing by treatment with CXCL12-producing *L. reuteri*. (*A*) Wounds receiving no treatment (control), treatment with control *L. reuteri* (LB), and CXCL12-expressing *L. reuteri* (LB_CXCL12) at the time of wound induction (0 h) and at 24 h. Wound sizes (*B*) and accumulated wound surface area at 2 d (*C*). (*D*) Fractions of wounds healed by different treatments at days 6, 8, and 10 (control, $n = 4$; LB, $n = 3$; LB_CXCL12, $n = 8$; $*P < 0.05$).

N), illustrating the causal correlation between wound macrophages and accelerated healing by CXCL12-delivering *L. reuteri*.

Whereas the density of collagen I or fibronectin in the dermis closest to the wound remained unaltered (Fig. S4 *D–F*), CXCL12-expressing *L. reuteri* increased the amount of laminin present at this site (Fig. S4 *D* and *G*).

Improved Reepithelialization of Wounded Human Skin Disks. The effects of CXCL12-producing *L. reuteri* was tested on healthy human skin in an in vitro model of wound reepithelialization, whereby newly formed epidermal sleeves partly cover the exposed dermis in wounded skin disks cultured in 10% FCS medium (positive control) while being barely detectable in disks cultured in 2% FCS medium (negative control; Fig. 5 *A* and *B*). The cells in the epidermal sleeves expressed keratin (Fig. S5*A*), and keratinocytes migrated over the exposed dermis toward the wound center (Fig. S5*B*). The length of the epidermal sleeves (i.e., reepithelialization) was increased after treatment with *L. reuteri* expressing human CXCL12 for 14 d (Fig. 5*B*), and more keratinocytes in the basal layer within 250 μm from the wound edge proliferated (Fig. 5*C*). Few cells were found to proliferate in the dermis beneath the wounds irrespective of conditions (Fig. 5*D* and Fig. S5*C*). Skin morphology was maintained by *L. reuteri* treatment, and epidermis thickening at the wound edge, a hallmark of healing tissues, was macroscopically detected (Fig. 5*A*). Addition of *L. reuteri* to the cultured skin disks lowered the pH of the medium after 24 h (Fig. 5*E*), which did not impact sleeve length or epidermal proliferation (Fig. 5 *B* and *C*). Interestingly, MHC II^+ cells detected in cultured skin disks increased in numbers by hCXCL12-expressing *L. reuteri* (Fig. 5 *F* and *G*), demonstrating macrophage proliferation also in the wound model of human skin.

Improved Wound Healing During Peripheral Ischemia or Hyperglycemia. After induction of hind-limb ischemia (a model of peripheral ischemia), the cutaneous perfusion was reduced by >50% at the day of wound induction (Fig. S6*A*), which resulted in impaired wound healing and prolonged mean time to complete healing (to 10 d; Figs. S2*C* and S6*B*). Treatment with CXCL12-delivering *L. reuteri* again accelerated wound closure compared with untreated or LB-treated wounds, which was most prominent during the first day following wound induction (Fig. 6*A*), resulting in reduced wound area and faster wound healing (Fig. 6 *B* and *C* and Fig. S6 *B–D*).

Mice that were rendered severely hyperglycemic before wound induction had altered wound healing and basal skin blood perfusion (Fig. S6 *E* and *K*), and remained hyperglycemic (>16.7 mmol/L) with maintained body weight during the experiment (Fig. S6 *F* and *G*). In the severely hyperglycemic mice, treatment with *L. reuteri*-delivering CXCL12 accelerated wound closure at 2 d after wound induction (Fig. 6*D*) but not at later time points (Fig. 6 *E* and *F* and Fig. S6 *H–J*). In the moderate hyperglycemic model (>11.1 mmol/L), the wound healing was impaired (Fig. S6*L*) and mice remained moderately hyperglycemic during the experiment (Fig. S6 *M* and *N*). In this model, treatment with *L. reuteri*-delivering CXCL12 accelerated wound closure at day 1 after wound induction (Fig. 6*G*), but not at later time points (Fig. 6 *H* and *I* and Fig. S6 *O–Q*).

Normalized Wound Blood-Flow Response in Hyperglycemic Mice. Blood flow at the site of injury is characterized by rapid vasoconstriction followed by transient hyperemia during the inflammatory phase (26). In mice, the hyperemic phase seen in the skin around the wound (0–300 μm) lasted for 3 d (Fig. S7 *A* and *B*). The hyperemia was absent in mice with hind-limb ischemia and also after treatment with control and CXCL12-expressing *L. reuteri* (Fig. S7 *C* and *D*). In the severely hyperglycemic mice, the basal skin perfusion was reduced already before wound induction (Fig. S6*K*), and the wound-induced hyperemia was absent (Fig. S7*E*). However, treatment with CXCL12-expressing bacteria in hyperglycemic mice normalized the blood-flow increase around the wound (Fig. S7*F*).

Initial Safety and Stability Studies Support Drug Development. No systemic exposure was detected, as circulating CXCL12 did not increase with treatment with CXCL12-producing bacteria (2×10^9 lactobacilli per wound) to open wounds (Fig. 7*A*) despite increased dermal CXCL12 close to the wound (Fig. 1 *D–G*). Further, no translocation of lactobacilli from wound to circulation was detected, even with a 100-fold higher dose (blood cultures).

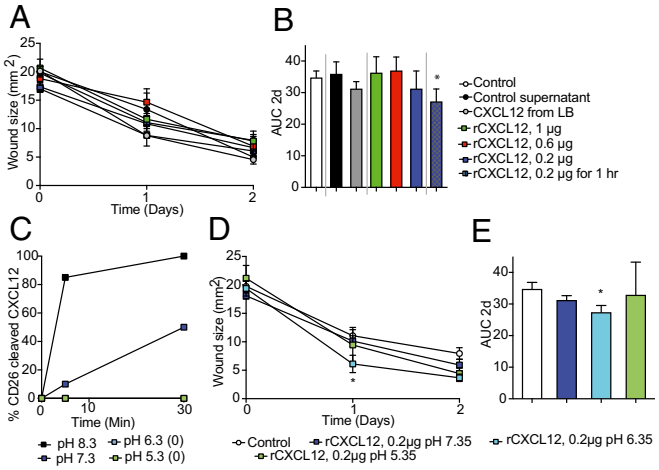

Fig. 3. Accelerated wound healing by prolonged administration of rCXCL12. Wound size (*A*) and accumulated wound surface area (*B*) were quantified for 2 d in mice that were untreated ($n = 15$), treated with supernatants from induced LB (control supernatant, $n = 4$) or LB_CXCL12 (CXCL12 from LB, $n = 5$), or treated with 1.0 μg ($n = 4$), 0.6 μg ($n = 5$) or 0.2 μg ($n = 10$) rCXCL12 at one time point per day or with 0.2 μg rCXCL12 at six time points during 1 h each day ($n = 5$). (*C*) Cleavage of CXCL12 by CD26 at different pH over 30 min in vitro where the curves at pH 6.3 and pH 5.3 overlap. Wound size (*D*) and accumulated wound surface area (*E*) after 0–2 d in mice treated with rCXCL12 in buffers with different pH at one time point daily (control/saline, $n = 12$; rCXCL12 7.35, $n = 10$; rCXCL12 6.35, $n = 4$; rCXCL12 5.35, $n = 4$; $*P < 0.05$).

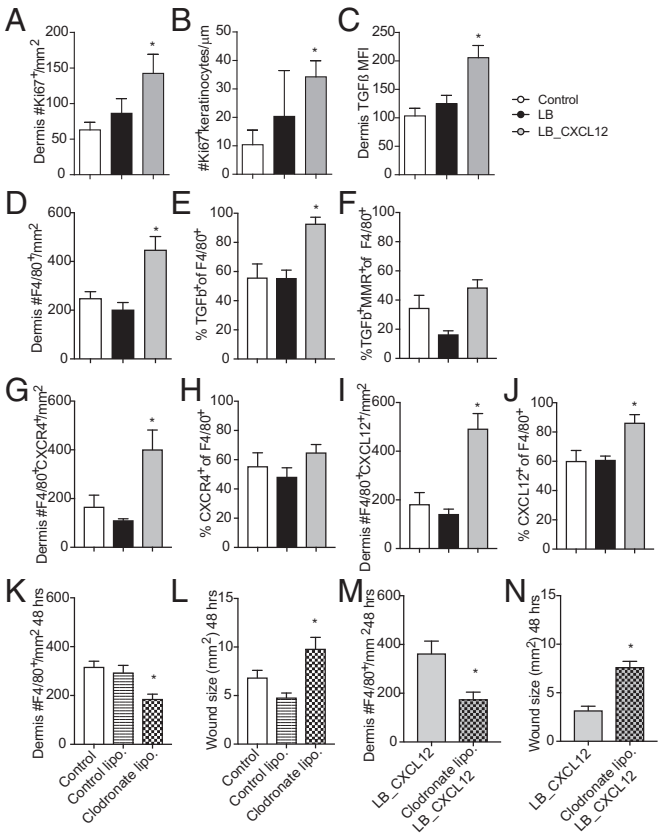

Fig. 4. Early and local effects in the wounds treated with CXCL12-producing *L. reuteri*. (*A* and *B*) Densities of proliferating cells in dermis and epidermis close to the wound edge (0–250 μm) and (*C*) quantifications of dermal TGF-β levels. The density of F4/80$^+$ macrophages is shown (*D*), along with fractions of macrophages expressing TGF-β (*E*) and MMR (*F*) in this area at 24 h after wound induction (*A–F*; $n = 3$ for all groups, $n = 7–16$). The density and fraction of macrophages expressing CXCR4 (*G* and *H*) or CXCL12 (*I* and *J*) in the wound area at 24 h after different treatments are shown: (*K–N*) efficacy of macrophage depletion using clodronate liposomes and the corresponding effect on wound healing in mice in which wounds were untreated (*K* and *L*) or treated with CXCL12-producing *L. reuteri* (*M* and *N*, control, $n = 7$; control and clodronate liposomes, both $n = 5$, $n = 7–13$; LB_CXCL12, $n = 8$; clodronate liposomes LB_CXCL12, $n = 5$, $n = 7–9$; $^*P < 0.05$).

Topical application of *L. reuteri* to wounds did not increase local inflammation-induced hyperemia; in fact, the hyperemic phase was abolished by the treatment (Fig. 7*B*). This indicates that the inflammatory response of wound induction is reduced by *L. reuteri* treatment, and that addition of the bacteria to open wounds does not cause local inflammation.

To facilitate off-the-shelf use, freeze-dried formulations of CXCL12-producing bacteria were developed. Following bacterial resuspension and induction, maintained plasmid expression was detected in vitro (Fig. 7*C*) and in vivo (Fig. 7*D*) in wounds, and maintained biological effect was demonstrated as accelerated wound closure compared with untreated wounds or wounds treated with control *L. reuteri* (Fig. 7 *E* and *F*). Thus, freeze-dried formulations demonstrated maintained stability and efficacy on wound healing and therefore represent an attractive approach for future pharmaceutical development.

Discussion

The present study demonstrates efficient means to accelerate cutaneous wound healing through bioengineering of the wound microenvironment by local overexpression of CXCL12. To achieve this, a system that generates sustained chemokine delivery and increases chemokine bioavailability within the wound was developed by using genetically modified *Lactobacillus* bacteria as vectors (described in Fig. S8). Transformed *Lactobacillus* expressed and released high levels of CXCL12 for 1 h following induction and topical administration to cutaneous wounds. In parallel, these bacteria enhanced chemokine bioavailability by producing lactic acid, which reduced local pH and thereby prevented CXCL12 degradation. This approach resulted in greatly accelerated wound healing in a macrophage-dependent manner in the skin of healthy mice, as well as in mice with reduced skin perfusion or hyperglycemia. Accelerated wound healing was also confirmed in a human skin wound model.

Wound healing is a complex biological process, and therapeutic enhancement has proven difficult. Patients with large and chronic wounds rely on removal of necrotic tissue, changes of wound dressings, and frequent antibiotic therapies to avoid systemic spread of infectious bacteria that colonize the wound (17). Boosting the signals that stimulate healing within the local microenvironment has been explored by using topical administration of certain growth factors and chemokines alone or coupled to different biomaterials (18, 27–30). In preclinical models, local overexpression or administration of chemokine-loaded hydrogels (CXCL12 or CCL2) have been reported to improve wound healing (18, 28, 30, 31), and CXCR2$^{-/-}$ mice were shown to exhibit impaired healing (32). Despite promising experimental effects, clinical translation is hampered at least in part by high levels of proteolytic enzymes within the wound (33), which limits protein bioavailability. Inhibition of these proteolytic enzymes (DPP4/CD26) has yielded interesting results, and improved wound healing was observed in CD26$^{-/-}$ mice (30). DPP4/CD26 inhibition is already in clinical use to lower blood glucose levels, and a recent review reports positive effects of this treatment on healing of diabetic foot ulcers (34). Another way to override local protein

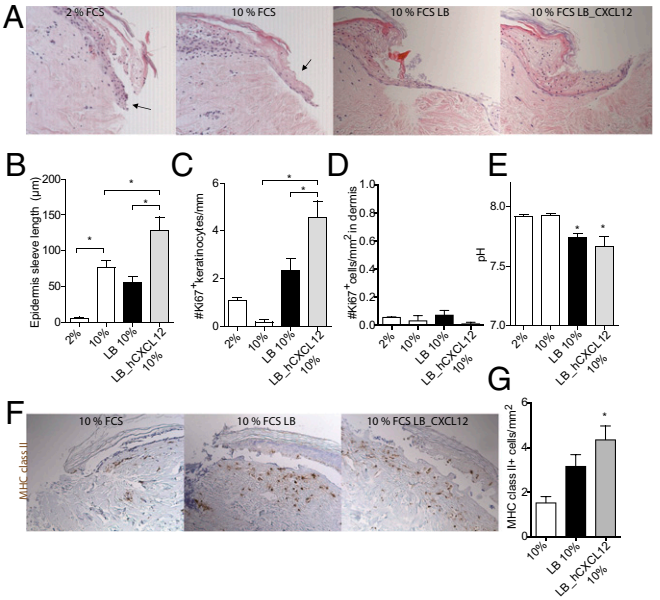

Fig. 5. Reepithelialization of wounds inflicted in cultured disks of human skin after treatment with *L. reuteri* (LB) or LB expressing human CXCL12 (LB_hCXCL12). (*A*) Epidermis and dermis at the wound edge in cultured skin disks after 14 d (arrows indicate newly formed epidermal sleeves). Length of epidermal sleeves were measured from the wound edge (*B*), and the number of basal Ki67$^+$ keratinocytes within 250 μm of the wound edge was measured (*C*) and quantified (*D*, $n = 6$, $n = 6$). (*E*) pH of medium after 24 h of culture. (*F*) Epidermis and dermis at the wound edge in skin disks after 14 d in culture. MHC class II$^+$ cells appear in brown and are quantified in *G* ($n = 6$, $n = 6$); $^*P < 0.05$ as indicated by braces or vs. 10% group.

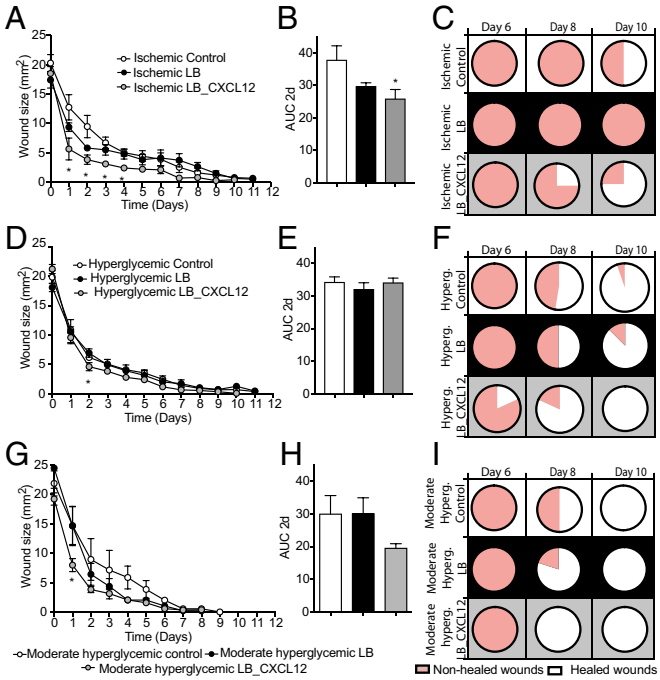

Fig. 6. Wound healing in mice with reduced skin perfusion or hyperglycemia. Wound size over time in mice with skin ischemia (*A*) and accumulated wound surface area quantified for 2 d (*B*). (*C*) Fractions of the wounds healed by the different treatments at days 6, 8, and 10 ($n = 4$ for all groups). (*D*) Wound size over time in severely hyperglycemic mice (blood glucose >16.4 mmol/L) and accumulated wound surface area at 2 d (*E*). (*F*) Fractions of wounds healed by different treatments at days 6, 8, and 10 (hyperglycemic control, $n = 19$; hyperglycemic LB, $n = 8$; hyperglycemic LB_CXCL12, $n = 11$). (*G*) Wound size over time in moderately hyperglycemic mice (blood glucose >11.1 mmol/L) and accumulated wound surface area at 2 d (*H*). (*I*) Fractions of the wounds healed by the different treatments at days 6, 8, and 10 ($n = 4$ for all groups; $^*P < 0.05$).

degradation is to deliver protein-producing cells to the wound. Genetically modified mesenchymal stem cells expressing the chemokine CXCL12 have been reported to accelerate cutaneous wound healing following injection in the wound edge (27). This approach is excellent for experimental delivery of CXCL12, but faces significant difficulties when clinical feasibility and regulatory aspects are taken into account.

The present study uses the probiotic (i.e., health-promoting, nonpathogenic) bacteria *L. reuteri* as local protein-producing bioreactors, which normally are sparsely present on the skin (35). The use of lactobacilli as vectors to deliver protein has been reported in models of colitis, in which IL-10 delivered by *Lactobacillus casei* was shown to ameliorate inflammation (36). Further, *L. lactis* producing active IL-22 is a promising system as a living vaccine adjuvant (37). Lactobacilli produce lactic acid and thereby reduce local pH when applied to wounds. Local pH regulates biological actions of chemokines by affecting their combination and conformation, but also their degradation by extracellular enzymes (25, 38). The superior effect on wound healing by our genetically modified *Lactobacillus* compared with rCXCL12 depended on the local pH reduction by bacterial lactic acid. The reduced pH increased chemokine bioavailability by preventing peptidase CD26 from cleaving and inactivating CXCL12, as previously shown (11).

Macrophages are key players in wound healing. They dynamically change function throughout the healing process, as they phagocytose cellular debris, apoptotic cells, and invading organisms, as well as instruct epithelial and endothelial cells, fibroblasts, stem cells, and other immune cells by releasing multiple paracrine factors (14, 39–41). Consequently, macrophage depletion at early stages of wound healing attenuated epithelialization, granulation tissue formation, and wound contraction (14). TGF-β is a central signaling molecule in tissue restoration, as it stimulates chemotaxis, wound contraction, angiogenesis, and deposition of ECM (42). Antiinflammatory macrophages fuel tissue restoration by being the main source of TGF-β (9, 14). In the present study, the most prominent effect on wound healing by the CXCL12-delivering *L. reuteri* was during the first 24 h following wound induction and treatment, which coincided with increased TGF-β levels in the dermis and higher numbers of TGF-β^+ macrophages. Thus, the observed accelerated wound closure by bacteria-delivered CXCL12 is at least partly the result of an amplified repair program by the increased population of TGF-β–producing macrophages, which further instruct other cells involved in the wound healing process (14).

Wound healing is a metabolically active process, and sufficient blood supply is a prerequisite. Basal blood flow and vasomotion are reduced in limbs in persons with peripheral ischemic diseases or diabetes, and these conditions are highly associated with development of chronic wounds. In the present study, the effect of the CXCL12-delivering *L. reuteri* on wound healing was investigated in models of peripheral ischemic disease and diabetes. The skin perfusion was demonstrated to be reduced in both of these models, and treatment with CXCL12-delivering *L. reuteri* again accelerated wound closure. Further, the initial hyperemic response to wound induction was absent in mice with induced peripheral ischemia or hyperglycemia, but was restored in the hyperglycemic mice treated with CXCL12-delivering *L. reuteri*.

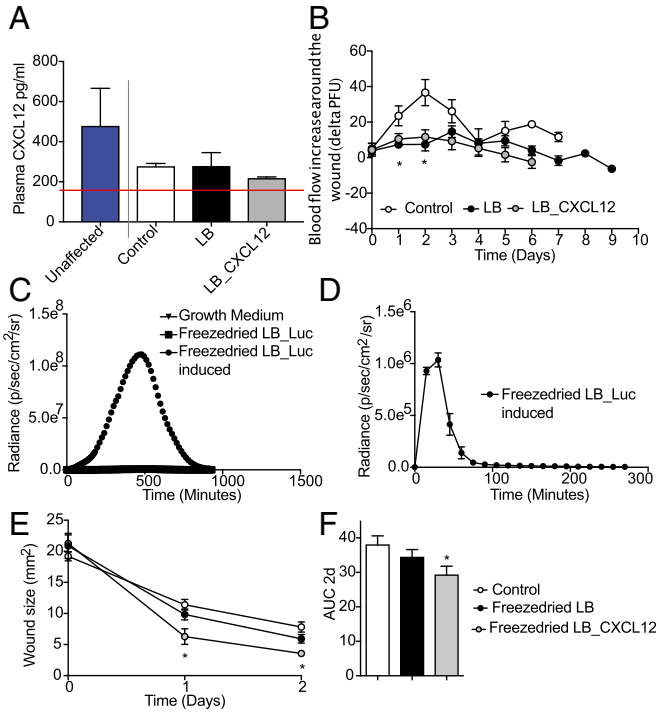

Fig. 7. Safety and pharmacokinetics of LB_CXCL12 for development of clinical wound treatment. (*A*) Plasma levels of CXCL12 in unaffected mice and in mice in which wounds were induced and blood collected after 3 h of treatment ($n = 3$ for all groups). Red line indicates detection limit. (*B*) Difference in perfusion in the skin surrounding the wound (0–300 μm) and an unaffected area of the skin used as reference, that is, delta perfusion (control and LB, $n = 4$; LB_CXCL12, $n = 5$). In vitro expression (*C*, $n = 4$–8) and in vivo expression (*D*, $n = 5$) of LB_Luc immediately resuspended from a freeze-dried formulation applied to the wound surface of 1-d-old cutaneous wounds. Wound size (*E*) and accumulated wound surface (*F*) over time in healthy mice in which wounds were treated with immediately revived control LB or LB_CXCL12 ($n = 5$ for all groups; $^*P < 0.05$).

In summary, we have developed a technology platform of local CXCL12 delivery and prolonged bioavailability in wounds. With the use of this approach, bioengineering of the wound microenvironment resulted in reprogramming of wound macrophages into the restitution phenotype and thereby a unique efficacy on acceleration of cutaneous wound healing in three different mouse models and human skin biopsy specimens. The delivery system was confirmed to be clinically and commercially feasible in preclinical models.

Methods

All experiments were approved by the Uppsala Regional Laboratory Animal Ethical Committee or the Uppsala Ethical Committee.

Experimental Models.

Wound healing in mice. Full-thickness (i.e., epidermis, dermis, and subcutis) wounds were induced on the hind limb of anesthetized C57BL/6 mice by using a sterile punch biopsy needle (5-mm diameter). Wounds were treated daily with 10 μL saline solution, control *L. reuteri*, or CXCL12-expressing *L. reuteri* or *L. lactis*. Bacteria were cultured overnight, reinoculated, and grown to OD 0.5, or resuspended from a freeze-dried formulation and activated 5 min before application with induction peptide SppIP (10 ng/mL) to wounds. rCXCL12-1a (R&D Systems) 0.2 μg (pH 7.35, pH 6.35, or pH 5.35), 0.6 μg, or 1 μg was administered to wounds once daily.

Reepithelialization assay in human skin biopsy specimens. Wounds were induced in the center of each 6-mm-diameter skin disk by removing the epidermis and superficial parts of the dermis by using a 3-mm skin biopsy punch and sterile scissors, and skin disks were cultured in media at 37 °C, 5% carbon dioxide, and 95% humidity for as long as 14 d. Control or hCXCL12-expressing *L. reuteri*, 10^6 bacteria in 10 μL MRS (De Man, Rogosa, and Sharpe agar), were administered centrally in the wounds. At time points 7 d and 14 d, disks were sampled for further analysis.

Reduction of skin perfusion. Hind-limb ischemia was induced in anesthetized mice by ligation and excision of the femoral artery above the superficial epigastric artery branch.

Induction of hyperglycemia. Severe hyperglycemia (blood glucose level >16.7 mmol/L) was induced by a single tail-vein injection of alloxan monohydrate (8 mg/mL, 1 μL/g body weight) dissolved in sterile saline solution. Intermediate hyperglycemia (blood glucose level >11.1 mmol/L for two consecutive days) was induced by i.p. injections of streptozotocin (40 mg/kg for five consecutive days), whereas control groups were injected with saline solution. Blood glucose and body weight was monitored throughout the experiment. The mice reached these blood glucose levels between days 12 and 30.

Statistical Analysis. Data are presented as mean ± SEM. Two-way ANOVA with Bonferroni post hoc test was used to analyze the healing process over time. For analysis of one time point, one-way ANOVA with Bonferroni post hoc test (between more than two groups) or Student's two-tailed unpaired *t* test was used, and $P < 0.05$ was considered statistically significant.

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1. Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. *Nature* 453:314–321.
2. Bianchi ME (2007) DAMPs, PAMPs and alarmins: All we need to know about danger. *J Leukoc Biol* 81:1–5.
3. Orlova VV, et al. (2007) A novel pathway of HMGB1-mediated inflammatory cell recruitment that requires Mac-1-integrin. *EMBO J* 26:1129–1139.
4. Sozzani S, et al. (1995) Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* 155:3292–3295.
5. Phillipson M, Kubes P (2011) The neutrophil in vascular inflammation. *Nat Med* 17:1381–1390.
6. Badillo AT, Chung S, Zhang L, Zoltick P, Liechty KW (2007) Lentiviral gene transfer of SDF-1alpha to wounds improves diabetic wound healing. *J Surg Res* 143:35–42.
7. Feng G, Hao D, Chai J (2014) Processing of CXCL12 impedes the recruitment of endothelial progenitor cells in diabetic wound healing. *FEBS J* 281:5054–5062.
8. Bollag WB, Hill WD (2013) CXCR4 in epidermal keratinocytes: Crosstalk within the skin. *J Invest Dermatol* 133:2505–2508.
9. Boniakowski AE, Kimball AS, Jacobs BN, Kunkel SL, Gallagher KA (2017) Macrophage-mediated inflammation in normal and diabetic wound healing. *J Immunol* 199:17–24.
10. Beider K, et al. (2014) Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype. *Oncotarget* 5:11283–11296.
11. Sánchez-Martín L, et al. (2011) The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression. *Blood* 117:88–97.
12. Bootun R (2013) Effects of immunosuppressive therapy on wound healing. *Int Wound J* 10:98–104.
13. Goren I, et al. (2009) A transgenic mouse model of inducible macrophage depletion: Effects of diphtheria toxin-driven lysozyme m-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. *Am J Pathol* 175:132–147.
14. Lucas T, et al. (2010) Differential roles of macrophages in diverse phases of skin repair. *J Immunol* 184:3964–3977.
15. Demidova-Rice TN, Hamblin MR, Herman IM (2012) Acute and impaired wound healing: Pathophysiology and current methods for drug delivery, part 1: Normal and chronic wounds: Biology, causes, and approaches to care. *Adv Skin Wound Care* 25:304–314.
16. Poetker DM, Reh DD (2010) A comprehensive review of the adverse effects of systemic corticosteroids. *Otolaryngol Clin North Am* 43:753–768.
17. Lipsky BA, et al.; Infectious Diseases Society of America (2012) 2012 Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. *Clin Infect Dis* 54:e132–e173.
18. Demidova-Rice TN, Hamblin MR, Herman IM (2012) Acute and impaired wound healing: Pathophysiology and current methods for drug delivery, part 2: Role of growth factors in normal and pathological wound healing: Therapeutic potential and methods of delivery. *Adv Skin Wound Care* 25:349–370.
19. Kuliszewski MA, Kobulnik J, Lindner JR, Stewart DJ, Leong-Poi H (2011) Vascular gene transfer of SDF-1 promotes endothelial progenitor cell engraftment and enhances angiogenesis in ischemic muscle. *Mol Ther* 19:895–902.
20. Yamaguchi J, et al. (2003) Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 107:1322–1328.
21. Mortier A, Gouwy M, Van Damme J, Proost P, Struyf S (2016) CD26/dipeptidylpeptidase IV-chemokine interactions: Double-edged regulation of inflammation and tumor biology. *J Leukoc Biol* 99:955–969.
22. Baticic Pucar L, Pernjak Pugel E, Detel D, Varljen J (2017) Involvement of DPP IV/CD26 in cutaneous wound healing process in mice. *Wound Repair Regen* 25:25–40.
23. Saboo A, Rathnayake A, Vangaveti VN, Malabu UH (2016) Wound healing effects of dipeptidyl peptidase-4 inhibitors: An emerging concept in management of diabetic foot ulcer-A review. *Diabetes Metab Syndr* 10:113–119.
24. Ohtsuki T, et al. (1998) Negative regulation of the anti-human immunodeficiency virus and chemotactic activity of human stromal cell-derived factor 1alpha by CD26/dipeptidyl peptidase IV. *FEBS Lett* 431:236–240.
25. Proost P, et al. (1998) Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. *FEBS Lett* 432:73–76.
26. Velnar T, Bailey T, Smrkolj V (2009) The wound healing process: An overview of the cellular and molecular mechanisms. *J Int Med Res* 37:1528–1542.
27. Nakamura Y, Ishikawa H, Kawai K, Tabata Y, Suzuki S (2013) Enhanced wound healing by topical administration of mesenchymal stem cells transfected with stromal cell-derived factor-1. *Biomaterials* 34:9393–9400.
28. Zhu Y, et al. (2016) Sustained release of stromal cell derived factor-1 from an antioxidant thermoresponsive hydrogel enhances dermal wound healing in diabetes. *J Control Release* 238:114–122.
29. Wood S, et al. (2014) Pro-inflammatory chemokine CCL2 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response. *PLoS One* 9:e91574.
30. Segers VFM, et al. (2011) Protease-resistant stromal cell-derived factor-1 for the treatment of experimental peripheral artery disease. *Circulation* 123:1306–1315.
31. Hu MS, et al. (2017) Delivery of monocyte lineage cells in a biomimetic scaffold enhances tissue repair. *JCI Insight* 2:96260.
32. Devalaraja RM, et al. (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol* 115:234–244.
33. Yager DR, Nwomeh BC (1999) The proteolytic environment of chronic wounds. *Wound Repair Regen* 7:433–441.
34. Marfella R, et al. (2012) Dipeptidyl peptidase 4 inhibition may facilitate healing of chronic foot ulcers in patients with type 2 diabetes. *Exp Diabetes Res* 2012:892706.
35. Gao Z, Tseng CH, Pei Z, Blaser MJ (2007) Molecular analysis of human forearm superficial skin bacterial biota. *Proc Natl Acad Sci USA* 104:2927–2932.
36. Qiu ZB, et al. (2013) Effect of recombinant Lactobacillus casei expressing interleukin-10 in dextran sulfate sodium-induced colitis mice. *J Dig Dis* 14:76–83.
37. Loera-Arias MJ, et al. (2014) Secretion of biologically active human interleukin 22 (IL-22) by Lactococcus lactis. *Biotechnol Lett* 36:2489–2494.
38. Lambeir AM, et al. (2001) Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. *J Biol Chem* 276:29839–29845.
39. Eming SA, Wynn TA, Martin P (2017) Inflammation and metabolism in tissue repair and regeneration. *Science* 356:1026–1030.
40. Wynn TA, Vannella KM (2016) Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* 44:450–462.
41. Lawrence T, Natoli G (2011) Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nat Rev Immunol* 11:750–761.
42. Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RHJ (2011) Macrophages in skin injury and repair. *Immunobiology* 216:753–762.