Systemic Anti-TNFα Treatment Restores Diabetes-Impaired Skin Repair in *ob/ob* Mice by Inactivation of Macrophages

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To date, diabetes-associated skin ulcerations represent a therapeutic problem of clinical importance. The insulin-resistant type II diabetic phenotype is functionally connected to obesity in rodent models of metabolic syndrome through the release of inflammatory mediators from adipose tissue. Here, we used the impaired wound-healing process in *obese/obese* (*ob/ob*) mice to investigate the impact of obesity-mediated systemic inflammation on cutaneous wound-healing processes. Systemic administration of neutralizing monoclonal antibodies against tumor necrosis factor (TNF)α (V1q) or monocyte/macrophage-expressed EGF-like module-containing mucin-like hormone receptor-like (Emr)-1 (F4/80) into wounded *ob/ob* mice at the end of acute wound inflammation initiated a rapid and complete neo-epidermal coverage of impaired wound tissue in the presence of a persisting diabetic phenotype. Wound closure in antibody-treated mice was paralleled by a marked attenuation of wound inflammation. Remarkably, anti-TNFα- and anti-F4/80-treated mice exhibited a strong reduction in circulating monocytic cells and reduced numbers of viable macrophages at the wound site. Our data provide strong evidence that anti-TNFα therapy, widely used in chronic inflammatory diseases in humans, might also exert effects by targeting "activated" TNFα-expressing macrophage subsets, and that inactivation or depletion of misbehaving macrophages from impaired wounds might be a novel therapeutic clue to improve healing of skin ulcers.

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INTRODUCTION

The last two decades have seen an explosive global increase in people diagnosed with diabetes mellitus (Zimmet *et al.*, 2001). The diabetic skin ulceration represents an unmet medical problem in many diabetic individuals. Diabetic ulcers still have a poor prognosis (Apelqvist *et al.*, 1995) and the 3-year survival rates after limb amputations are between 50 and 59%, as assessed for Italy and Sweden, respectively (Apelqvist *et al.*, 1993; Faglia *et al.*, 2001). By contrast, the efforts to identify novel pharmacological approaches to improve significantly severe diabetes-impaired healing conditions have failed and only recombinant platelet-derived growth factor is now available for treatment of foot ulcers (Wiemann *et al.*, 1998).

In the past decade, rodent models of obesity and diabetes have been extensively used to unravel the functional connection between an expanding adipose tissue and an increasing insulin resistance. Initial experiments evidenced the pro-inflammatory tumor necrosis factor (TNF)α as an adipose tissue-derived mediator that might directly contribute to insulin resistance in obese rodents (Hotamisligil *et al.*, 1993). However, recent studies now implicate bone marrow-recruited macrophages located within adipose tissue rather than resident adipocytes to represent the cellular source of pro-inflammatory mediators in obesity (Weisberg *et al.*, 2003). Interestingly, plasma TNFα levels were also dependent on adipose tissue mass in humans (Kern *et al.*, 1995; Mantzoros *et al.*, 1997), and clinical studies confirmed that the presence of inflammatory mediators predicts the development of type II diabetes mellitus (Schmidt *et al.*, 1999; Pradhan *et al.*, 2001).

Here, we used the *obese/obese* (*ob/ob*) mouse as a model system of impaired wound healing. These mice are characterized by severe diabetes and obesity syndromes (Coleman, 1978). The diseased phenotype is mediated by the functional loss of the *ob* gene product: the 16 kDa cytokine leptin (Zhang *et al.*, 1994). Leptin is now well established as an adipocytokine, which is secreted from adipocytes and functions in the regulation of neuroendocrine circuits, energy

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homeostasis, and as an important mediator of immune-mediated diseases and inflammatory processes (Tilg and Moschen, 2006). Systemic leptin administration into wounded *ob/ob* mice led to the resolution of the diabetic phenotype and improved the severely disturbed healing conditions by acting as a mitogen for wound keratinocytes, attenuating the inflammatory wound response and restoring insulin signaling in wound tissue (Frank *et al.*, 2000; Goren *et al.*, 2003, 2006).

Interestingly, also systemic application of an anti-TNFα antibody markedly increased insulin receptor expression in wound tissue (Goren *et al.*, 2006). These recent findings strongly suggest a possible functional connection between adipose tissue-derived TNFα and wound-healing disorders in *ob/ob* mice, although the mechanism still remains unknown. TNFα is a major product of classically activated macrophages (Gordon, 2002; Mosser, 2003); however, it must not necessarily be secreted. TNFα is synthesized as a membrane protein, which can be cleaved by TNFα-converting enzyme to allow the subsequent release of the cytokine from activated macrophages (Solomon *et al.*, 1999).

In this study, we provide strong evidence that improved skin repair in diabetic and obese mice after systemic administration of a TNFα-neutralizing antibody was functionally connected to markedly reduced monocyte/macrophage numbers in *ob/ob* mice. Thus, our data strongly suggest that impaired wound-healing conditions in *ob/ob* mice were driven by TNFα-expressing macrophages, which occurred as a consequence of metabolic disorders in the animals. Moreover, our findings prove a beneficial effect of a TNFα neutralization strategy in impaired wound healing, which was mediated by a yet unknown induction of cell death of activated macrophages in disturbed wound tissue, and thus extend its therapeutic potential beyond the treatment of chronic inflammatory diseases.

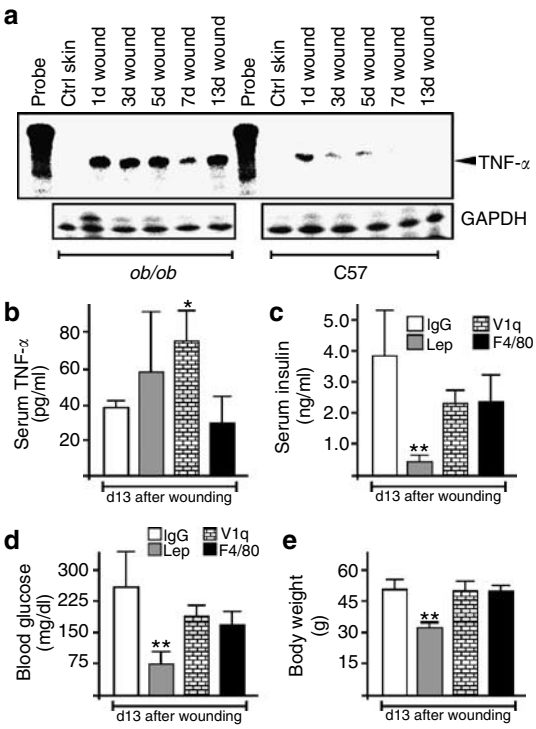

Figure 1. Anti-TNFα (V1q) and anti-macrophage (F4/80) treatments did not improve the metabolic syndrome in *ob/ob* mice. (**a**) RNase protection assay demonstrating TNFα mRNA expression in non-wounded back skin (ctrl skin) and wound tissue isolated from C57Bl/6 and *ob/ob* mice. The time after injury is indicated for each lane. Levels of TNFα (**b**) and insulin (**c**) in serum, blood glucose (**d**), and body weight (**e**) in *ob/ob* mice at day 13 after wounding. Mice had been treated with leptin (starting at 2 days before wounding, one application per day), V1q- and F4/80-specific rat monoclonal antibodies or a nonspecific rat IgG (injections at days 7, 9, and 11 post-wounding for the antibody treatment). $^{**}P<0.01$; $^{*}P<0.05$ vs IgG-treated animals. Bars indicate the mean ± SD obtained from four individual animals ($n=4$).

RESULTS

Short-term anti-TNFα (V1q) and anti-macrophage (F4/80) treatments did not improve metabolic syndrome in wounded *ob/ob* mice

Adipose tissue-derived TNFα is considered to be functionally involved in obesity-associated insulin resistance (Hotamisligil *et al.*, 1993; Wellen and Hotamisligil, 2005). To elucidate the potential mechanism of obesity-related TNFα interference with skin repair in *ob/ob* mice (Goren *et al.*, 2006), we first analyzed circulating levels of TNFα in serum of the animals. To this end, we injected a monoclonal antibody against murine TNFα (V1q) (Echtenacher *et al.*, 1990) into wounded *ob/ob* mice. However, we had to avoid any interference with physiologic functions of normally expressed TNFα during the acute phase of skin repair (Figure 1a), as we intended to restrictively interfere only with a potential pathophysiological role of TNFα during diabetes-impaired wound-healing conditions. To reach this goal, we administered the rat monoclonal V1q antibody at days 7, 9, and 11 upon injury by intraperitoneal injection. Specific antibody treatment was controlled by intraperitoneal administration of a nonspecific rat IgG at days 7, 9, and 11 after wounding. Consistent with published data (Hotamisligil *et al.*, 1993), we found low levels of TNFα in the sera of *ob/ob* mice and V1q treatment did not reduce circulating TNFα (Figure 1b). Even a 16-day systemic leptin treatment of the mice, which served as control for correction of the metabolically disturbed phenotype (Figure 1c–e) and impaired healing in *ob/ob* mice (Pelley-mounter *et al.*, 1995; Frank *et al.*, 2000; Ring *et al.*, 2000; Goren *et al.*, 2003, 2006), did not reduce serum TNFα levels (Figure 1b). In clear contrast to unaltered TNFα levels upon antibody or leptin regimens, we could observe a dramatic improvement of wound morphology in leptin- and TNFα-treated animals compared to the IgG-administered control group (Figure 2). One possibility to explain the discrepancy between unchanged circulating TNFα levels and improved skin repair might arise from binding of V1q to membrane-bound TNFα in monocytes/macrophages (Solomon *et al.*, 1999; Lügering *et al.*, 2001) with a subsequent depletion of the cells by induction of apoptosis and/or complement activation (Nauta *et al.*, 2002). To experimentally mimic this condition in the mice, we included the monocyte/macrophage-specific F4/80 rat monoclonal antibody (Austyn and Gordon, 1981; Hirsch *et al.*, 1981) into our experimental

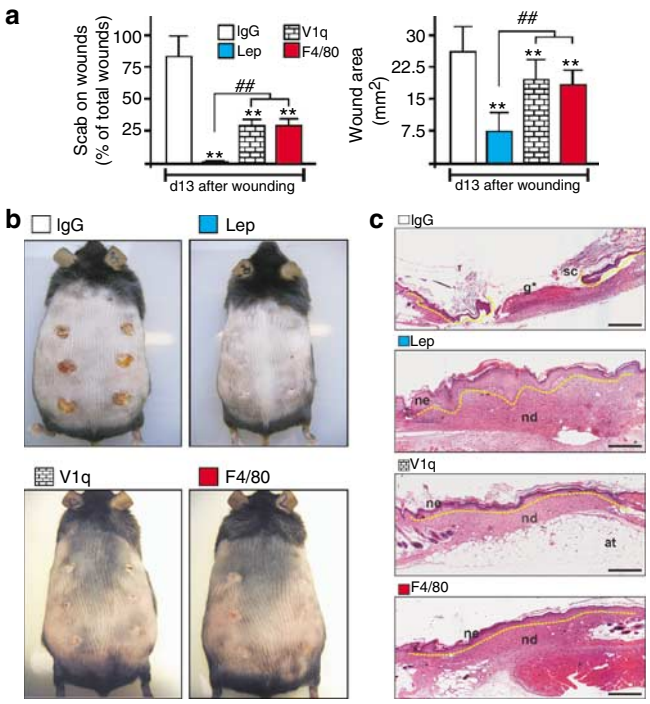

Figure 2. Analysis of wound morphology. (**a**) Presence of scab-covered wounds and wound area of 13 day wounds in *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice. $^{**}P<0.01$ *versus* IgG-treated animals. $^{\#\#}P<0.01$ as indicated by the brackets. Bars indicate the mean ± SD from 24 wounds ($n=24$) from four individual animals ($n=4$). (**b**) photographs of 13-day back skin wounds in *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice. (**c**) Representative histological analyses of 13 day wound tissue isolated from individual *IgG-*, *leptin-*, *V1q-*, and F4/80-treated *ob/ob* mice as indicated. Formalin-fixed, paraffin-embedded 6 μm sections were counterstained using eosin/hematoxylin. Abbreviations: *at*, adipose tissue; *gt*, granulation tissue; *gt**, atrophied gt; *nd*, neo-dermis; *ne*, neo-epidermis, *sc*, scab. Bar = 1 mm.

setup by systemic application at days 7, 9, and 11 upon wounding. Both the V1q and F4/80 antibodies did not significantly improve the disturbed metabolic phenotype of *ob/ob* mice, as the animals remained severely hyperinsulinemic (Figure 1c), hyperglycemic (Figure 1d), and obese (Figure 1e).

Short-term systemic anti-TNFα (V1q) and anti-macrophage (F4/80) treatments strongly improved healing of chronic wounds in *ob/ob* mice with persisting metabolic syndrome

Most remarkably, we observed a profound improvement of impaired wound tissue in response to the administration of the V1q or F4/80 antibodies. Here, it is important to note that changes in chronic wounds toward a properly healing wound area occurred within 6 days, as we applied the V1q, F4/80, or the nonspecific control antibodies only three times from day 7 after wounding and subsequently analyzed wound morphology as early as day 13 after wounding. As shown in Figure 2, anti-TNFα (V1q) or anti-monocyte/-macrophage (F4/80) treatments resulted in a general resolution of chronic wound conditions in the animals. We found a significant reduction of persisting scabs on the backs of antibody-treated animals (Figure 2a, left panel). This parameter served as an initial readout for wound epithelialization, as scabs are lost after formation of a robust neo-epidermal coverage of the wound site. In parallel, wound areas were also significantly reduced by antibody treatment (Figure 2a, right panel). Leptin administration of animals, starting at day 2 before wounding, is well established to mediate a robust improvement of impaired wounds in *ob/ob* mice (Frank *et al.*, 2000; Ring *et al.*, 2000; Goren *et al.*, 2003, 2006). The above-mentioned improved wound-healing conditions upon leptin, V1q, and F4/80 applications (Figure 2a) are documented by photographs of representative animals shown in Figure 2b. As a next step, we performed a histological analysis of wound tissue from the different experimental settings. First, we observed a poor tissue regeneration in IgG-treated control mice and wound areas were characterized by atrophied wound margin epithelia. Additionally, the granulation tissue appeared to be reduced (Figure 2c, IgG panel). By contrast, systemic leptin administration, which exerts its actions in functional connection to the resolution of the diabetic phenotype (Figure 1c–e), mediated an improvement of wound morphology as assessed by the formation of both a robust multilayered neo-epithelium and a dense neo-dermis (Figure 2c, lep panel). Although effects on wound morphology appeared to be not as pronounced compared to leptin treatment, both V1q and F4/80 antibody administrations mediated the formation of an epithelial coverage of the wound area, which was paralleled by the formation of a dense neo-dermis. Thus, treatment strategies using the antibodies were able to drive a rapid formation of an epithelial coverage onto chronic wounds in the presence of persisting hyperglycemia and, thus, a diabetic phenotype in the mice (Figure 1c–e).

Short-term anti-TNFα (V1q) or anti-macrophage (F4/80) treatments markedly attenuated wound inflammatory conditions

Leptin-driven skin repair in *ob/ob* mice was associated with a marked decline in wound inflammation following the acute phase of repair, whereas chronic healing conditions exhibited a sustained inflammatory response in *ob/ob* as well as *db/db* mice (Wetzler *et al.*, 2000; Goren *et al.*, 2003). Thus, it was interesting to investigate the inflammatory status of V1q- or F4/80-improved wound tissue in *ob/ob* mice. We observed a strong attenuation of the inflammatory response at the wound site upon targeting both the TNFα or macrophage-specific antigens in the mice (Figure 3). IL-β, TNFα, or cyclooxygenase (COX)-2 mRNA levels were significantly reduced upon systemic antibody application. However, chemokine (C-C motif) ligand 2 (CCL2) mRNA expression at the wound site was much less influenced by the treatments (Figure 3a). Nevertheless, we found a clear reduction of IL-1β, TNFα, and CCL2 proteins in healing-improved wound tissue isolated from leptin-, V1q-, and F4/80-treated mice (Figure 3b). These data provide strong evidence that the beneficial effects of both antibodies in disturbed healing are functionally linked to the reduction of exacerbated wound inflammatory conditions in obese and diabetic mice.

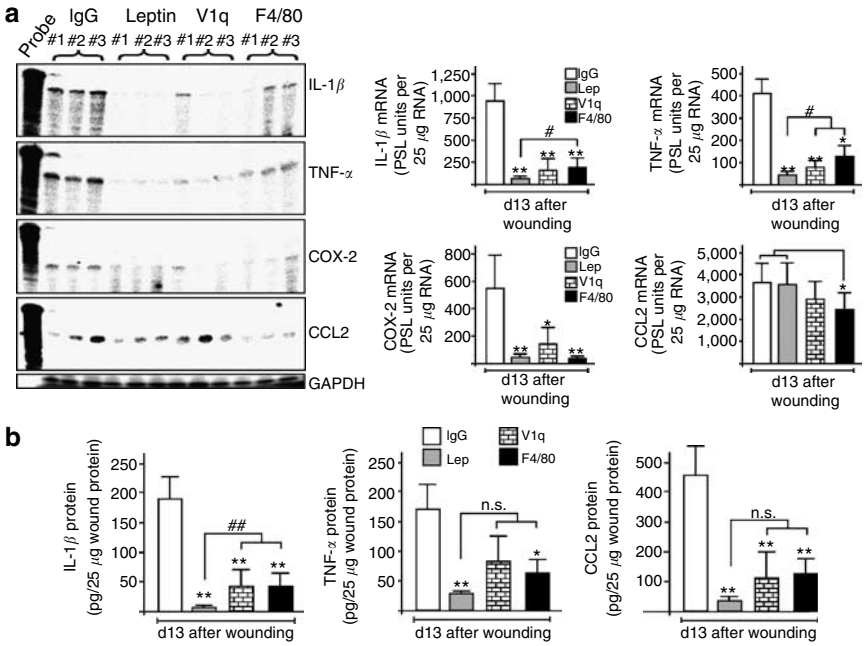

Figure 3. V1q and F4/80 attenuate wound inflammation in impaired wounds of *ob/ob* mice. Mice had been treated with a nonspecific IgG or leptin (starting at 2 days before wounding, one application per day), or V1q and F4/80 (injections at days 7, 9, and 11 after wounding). (**a**) IL-1β, TNFα, COX-2, and CCL2 mRNA expression in 13-day wound tissue isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated. #1–3 represent individual animals. For the RNase protection assay (left panels), every experimental time point depicts three wounds ($n=3$) isolated from a single individual mouse. A glyceraldehyde-3-phosphate dehydrogenase hybridization is shown as a loading control. 1,000 cpm of the hybridization probe were used as a size marker. Statistical analysis of IL-1β, TNFα, COX-2 and CCL2 mRNA expression is shown in the right panels. $^{**}P<0.01$; $^{*}P<0.05$ *vs* IgG-treated animals. $^{\#}P<0.05$ as indicated by the brackets. Bars indicate the mean$\pm$SD from 12 wounds ($n=12$) from four individual animals ($n=4$). (**b**) IL-1β-, TNFα-, and CCL2-specific ELISA analyses from 13-day wound lysates isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated. $^{**}P<0.01$; $^{*}P<0.05$ *versus* IgG-treated animals. $^{\#\#}P<0.01$; NS, not significant as indicated by the brackets. Bars indicate the mean$\pm$SD from eight wounds ($n=8$) from four individual animals ($n=4$).

Short-term anti-TNFα (V1q) or anti-macrophage (F4/80) treatments reduced circulating monocytes and resident wound macrophage numbers

Here, we hypothesized a similar mode of action for V1q and F4/80 in *ob/ob* mice *in vivo* via a potential depletion of macrophages by complement activation. Indeed, both antibodies mediated an accelerated skin repair and an attenuated wound inflammation in *ob/ob* mice in the presence of a severe diabetic phenotype. To strengthen our hypothesis, we focused our investigations on the monocyte/macrophage cell type as the potential target cell to explain the effects of both applied antibody strategies. First, we determined the impact of systemic application of leptin, V1q, and F4/80 on circulating monocyte numbers. FACS analysis using a PE-coupled F4/80 antibody determined circulating monocytes to represent a 13.4$\pm$3.1% fraction as compared to the total leukocyte count (Figure 4). Interestingly, leptin did not significantly change numbers of circulating monocytes. By contrast, FACS analysis revealed a dramatic loss (about 80%) of monocytic cell numbers in the circulation of *ob/ob* mice upon treatment with either V1q or F4/80 antibodies (Figure 4). Loss of monocytic cells was profound and resulted in remaining monocytic fractions of 3.7$\pm$1.0% for V1q or 2.5$\pm$0.3% for F4/80 treatment, respectively. These data provided strong experimental evidence that both antibodies,

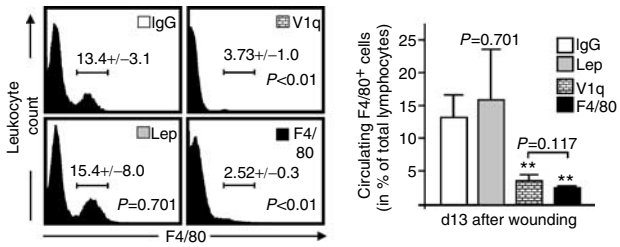

Figure 4. Determination of circulating F4/80-positive monocytes in *ob/ob* mice. Mice had been treated with a nonspecific IgG or leptin (starting at 2 days before wounding, one application per day), or V1q and F4/80 (injections at days 7, 9, and 11 after wounding). At day 13 after wounding, fresh blood from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice was analyzed for F4/80-positive monocytes by FACS analysis as indicated. $^{**}P<0.01$ *versus* IgG-treated animals. Bars indicate the mean$\pm$SD from four individual animals ($n=4$).

which were not functionally related with respect to their antigenic target molecule, exhibited their therapeutic potential by targeting the identical cell type. Moreover, subsequent analysis of wound macrophage numbers translated our antibody-mediated effects from the circulation into the poorly regenerating peripheral wound tissue. As shown in Figure 5a, improved wound tissue isolated from V1q- and F4/80-treated *ob/ob* mice exhibited a reduction in wound macrophage

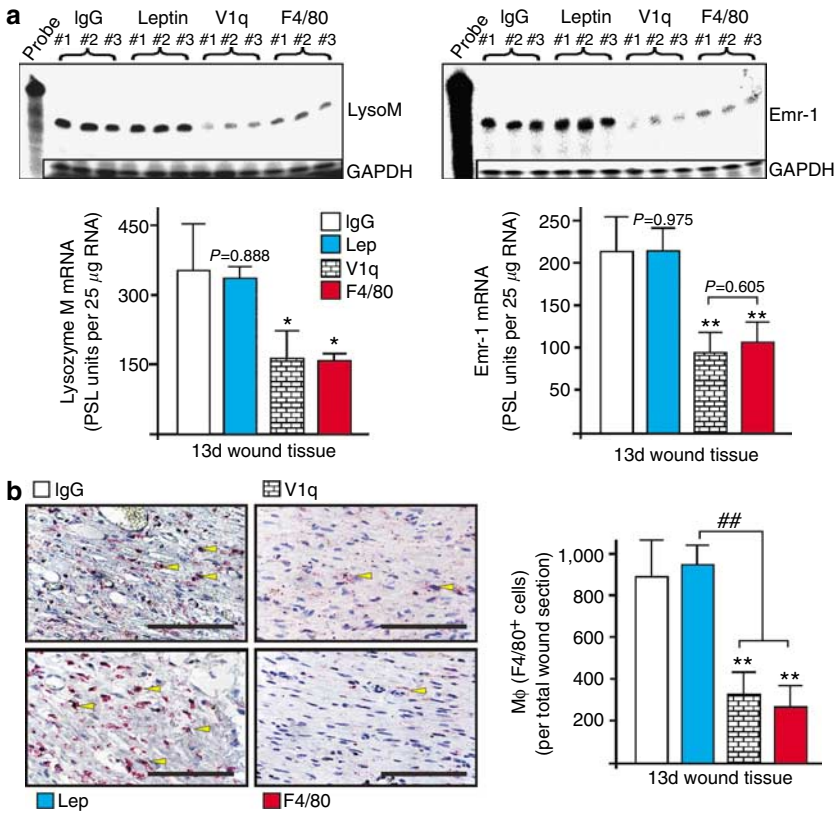

Figure 5. V1q and F4/80 reduce of macrophage numbers in impaired wounds of *ob/ob* mice. Mice had been treated with a nonspecific IgG or leptin (starting at 2 days before wounding, one application per day), or V1q and F4/80 (injections at days 7, 9, and 11 after wounding). (**a**) Lysozyme M and Emr-1 mRNA expression in 13-day wound tissue isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated. #1–3 represent individual animals. For the RNase protection assay (upper panels), every experimental time point depicts three wounds ($n=3$) isolated from a single individual mouse. A glyceraldehyde-3-phosphate dehydrogenase hybridization is shown as a loading control. 1,000 cpm of the hybridization probe were used as a size marker. A statistical analysis of lysozyme M and Emr-1 mRNA expression is shown in the lower panels. $^{**}P<0.01$; $^{*}P<0.05$ *versus* IgG-treated animals. Bars indicate the mean ± SD from 12 wounds ($n=12$) from four individual animals ($n=4$). (**b**) Immunohistological determination of wound macrophage numbers *in situ*. Sections (6 μm) were stained for macrophage-specific antigen F4/80. Representative higher magnification sections isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice are shown in the left panels. Bar = 50 μm. Absolute numbers of F4/80-positive wound macrophages from total wound sections are given in the right panel. $^{**}P<0.01$ *versus* IgG-treated animals. $^{\#\#}P<0.01$ as indicated by the brackets. Bars indicate the mean ± SD from four wounds from four individual animals ($n=4$).

numbers. A quantitative analysis of constitutively expressed macrophage-specific lysozyme M and Emr-1 mRNA species in wound tissue (Figure 5a) and the immunohistological staining for F4/80-positive cells (Figure 5b) both demonstrated an approximately 50–60% reduction of wound macrophages in V1q- and F4/80-improved healing conditions. In contrast to both antibody treatments, these data suggested a different mode of action for leptin to improve repair in *ob/ob* mice, as the remaining high numbers of wound macrophages did not drive an exacerbated wound inflammation, but appeared to be silenced as a direct consequence of leptin action (Figure 3).

Short-term anti-TNFα (V1q) or anti-macrophage (F4/80) treatments triggered apoptosis of wound macrophages

As we had observed a strong depletion of monocytes from the circulation after V1q and F4/80 treatments, which were related to reduced numbers of wound macrophages, we were finally interested in infiltrating macrophage conditions in the context of the applied diverse treatment regimens and tissue environments. To this end, we performed a terminal deoxyribonucleotidyl transferase-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay in a double-labeling experiment, which allowed us a simultaneous detection of F4/80- and TUNEL-positive apoptotic cells within different wound tissues. Interestingly, we observed the lowest percentage of TUNEL-positive and thus apoptotic macrophages in wounds isolated from phosphate-buffered saline, as well as leptin-treated *ob/ob* mice (Figure 6a–c). Impaired wounds in IgG-treated animals were thus characterized by high numbers of viable macrophages (Figure 6a–c), which most probably triggered an augmented and sustained inflammatory response at the wound site (Figure 3) that obviously interfered with repair (Figure 2). By contrast, high numbers of viable wound macrophages upon leptin administration here indeed suggested that leptin did not exert its beneficial effects on skin repair by interfering with monocyte viability (Figures 4 and 6) but through downregulation of wound macrophage inflammatory responses (Figure 3). More importantly, wound tissue from V1q- and F4/80-treated *ob/ob* mice was characterized

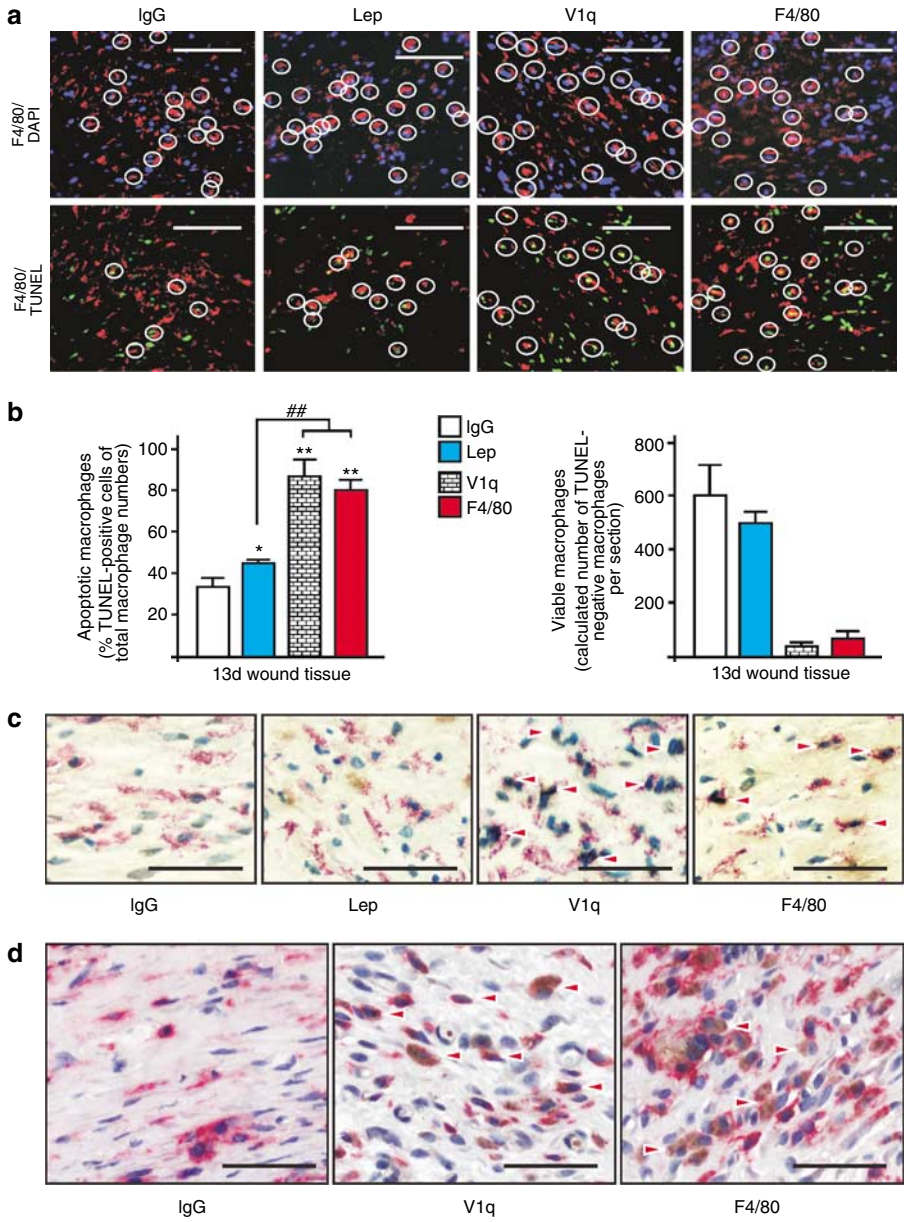

Figure 6. Wound macrophages in V1q- and F4/80-treated *ob/ob* mice are in an apoptotic state. (**a**) Paraffin-fixed sections (6 μm) of 13-day wound granulation tissue isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated were simultaneously stained for macrophage-specific F4/80 and apoptotic cells by TUNEL using a fluorescence-based technique. Nuclei were visualized by 4,6-diamidino-2-phenylindole staining. 4,6-Diamidino-2-phenylindole/F4/80 double-positive cells have been indicated by circles in the upper panels. As TUNEL labels only nuclear structures, we restricted our counting of macrophage/TUNEL double-positive cell numbers to 4,6-diamidino-2-phenylindole/F4/80 double-positive cells, which have also been indicated by circles in the lower panels. Note that we have chosen areas with higher numbers of F4/80-stained macrophages within the granulation tissues for the presented figure to visualize our quantitative analysis. The comparable numbers of F4/80-stained macrophages for all four treatment groups presented here does not reflect total numbers throughout the wound. Bar = 50 μm. A statistical analysis is given in panel **b** (left panel). $^{**}P<0.01$; $^{*}P<0.05$ *versus* IgG-treated animals. $^{\#\#}P<0.01$ as indicated by the brackets. Bars indicate the mean ± SD from three wounds from three individual animals ($n=3$). The right panel combines data from (**b**) and Figure 5b, showing the calculated number of viable macrophages in 13-day wound sections from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated. (**c**) Paraffin-fixed sections (6 μm) of 13-day wound granulation tissue isolated from *IgG-*, *leptin-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated were simultaneously stained for macrophage-specific F4/80 (red) and apoptotic cells by TUNEL (brown) using a peroxidase-based technique. TUNEL-positive macrophages are indicated. Bar = 40 μm. (**d**) Granulation tissue (13-day wounds) from *IgG-*, *V1q-*, and *F4/80*-treated *ob/ob* mice as indicated was analyzed for macrophage-specific F4/80 (red) and processed active caspase-3 (brown). F4/80 and active caspase-3 double-positive macrophages are indicated. Bar = 30 μm.

by highest numbers of apoptotic and thus non-viable macrophages (Figure 6a–c). Interestingly, immunohistological staining for processed and thus active caspase-3 strongly suggested that systemic antibody treatments exerted its actions via induction of a controlled macrophage cell death. These data show that nearly all (more than 80%) infiltrating macrophages that were reduced in number upon V1q and F4/80 applications additionally appeared to be in an

apoptotic state, and thus were no longer able to interfere with skin repair even under severe diabetic conditions in the animals.

DISCUSSION

A series of wound-healing experiments performed by Leibovich and Ross (1975) in the 1970s established a prime role of activated macrophages in skin repair. These ''classical'' studies using a guinea pig wound model demonstrated that depletion of macrophages under sterile conditions resulted in the failure of animals to clear wound tissue of matrix, dead and damaged cells, fibrin, and debris (Leibovich and Ross, 1975). In a recent review, Leibovich questioned his early statement that ''it has largely been considered taboo to tinker too heavily with the inflammatory response at the wound site for fear of aborting this process'' (Martin and Leibovich, 2005). Indeed, observations from chronic wound-healing conditions in humans, as well as mice, strongly support the wound macrophage to interfere with the normal repair process under conditions of impaired healing. It is known that macrophage numbers are markedly elevated in human chronic leg ulcers (Rosner *et al.*, 1995; Loots *et al.*, 1998) and in chronic wounds of genetically diabetic and obese *db/db* and *ob/ob* mouse models (Wetzler *et al.*, 2000; Goren *et al.*, 2003). Nevertheless, although the presence of elevated macrophage numbers at chronic wound sites strongly suggested a key role of this cell type in the control and orchestration of pathobiology in impaired wound tissue, most of the experimental work in the field restrictively focused on the role of soluble mediators. Not unexpected, an aberrant expression of diverse protein-type mediators has been reported in disturbed healing conditions in a series of animal studies (for a review see Werner and Grose, 2003). Unfortunately, however, therapeutic approaches to improve disturbed wound healing in humans and mice by exogenous administration of missing or dysregulated factors by application of single recombinant protein mediators have failed (Jeffcoate and Harding, 2003).

Obviously, resident cells in diabetic ulcers are phenotypically altered with respect to their sensitivity toward exogenous signals (Falanga, 2005; Goren *et al.*, 2006). Thus, it is reasonable to argue that a yet poorly understood mechanism drives a complex dysregulation of inter- and intracellular signaling machineries that normally enable the proper and accurate communication of resident cells embedded into healing wound tissue and thus causes the failure to pharmacologically correct wound-healing disorders through application of recombinant mediators. Interestingly, one such underlying mechanism might emerge from studies using obese and diabetic rodents. TNFα is now discussed as a pivotal mediator connecting obesity and insulin resistance (Hotamisligil *et al.*, 1993), but low levels of free-circulating serum TNFα even in obese mice suggested a different mode of action for the therapeutically applied anti-TNFα antibody. Recent studies implicated macrophages rather than adipocytes as the cellular source of obesity-associated TNFα that is functionally connected to conditions of insulin resistance. Adipose tissue macrophages were bone marrow-derived, recruited via chemokine (C-C motif) receptor 2 activation, and most likely responsible for adipose tissue-derived TNFα production in obese mice (Weisberg *et al.*, 2003, 2006).

Macrophages must be in an activated state to produce TNFα (Gordon, 2002; Mosser, 2003). As TNFα did not appear in high levels in the circulation of obese mice (Hotamisligil *et al.*, 1993; this study), it is tempting to argue that TNFα might not be released from activated macrophages during developing obesity to mediate conditions of increasing insulin resistance. This notion is strongly supported by observations that inflammatory stimuli induced a membrane-bound form of TNFα in monocytes or macrophages, which might be subsequently released by TNFα-converting enzyme cleavage under inflammatory conditions (Solomon *et al.*, 1999). Moreover, data from our study provide evidence that obesity-related disturbances might be mediated by a central pool of circulating TNFα-positive monocytes from which mononuclear cells enter diverse organ systems. Obviously, anti-TNFα treatment of *ob/ob* mice depleted these activated monocytes from the circulation and caused a marked reduction of macrophage numbers in disturbed wound sites in the animals. More importantly, depletion of circulating and wound monocytic cells in *ob/ob* mice resulted in a rapid and profound transformation of chronic wounds into healing skin tissue.

Additionally, our data demonstrate that the obesity-changed macrophage cell type itself and not the action of an obesity-induced TNFα molecule is in the center of disturbed skin repair in diabetes. Thus, TNFα neutralization using systemically applied antibodies has to be regarded as an epiphenomenon with respect to the observed beneficial effects of the anti-TNFα therapy on impaired wound healing, as we obtained a comparable effect with respect to circulating monocyte numbers and improval of skin repair using an antibody directed against the monocyte/macrophage-specific membrane protein F4/80 (Austyn and Gordon, 1981; Hirsch *et al.*, 1981). Therefore, the mode of action of infliximab, a TNFα-neutralizing antibody used for therapy of chronic inflammatory diseases in humans, might be more diverse with respect to its TNFα-binding properties than initially expected. This hypothesis is strongly supported by the work of Lügering *et al.* (2001), who reported a marked induction of apoptosis in circulating monocytes by infliximab in patients with Crohn's disease. In accordance, our V1q and F4/80 treatments of wounded diabetic mice also targeted obesity-activated monocytes in the circulation by reducing their total numbers. Moreover, the remaining monocytes that had actually escaped this process and reached the chronified wound tissue were obviously in an apoptotic state and could no longer interfere with the healing process. These findings are strongly supported by the potency of the anti-TNFα antibodies infliximab and adalumimab, but not the soluble receptor etanercept, to selectively mediate caspase-dependent apoptosis in cultured human monocytes, a process that is paralleled by a marked decrease in cytokine release from the cells (Shen *et al.*, 2005, 2006). In summary, our observations indicate a pivotal role for activated macrophages in the development of diabetes-impaired wound

healing and strongly suggest a pharmacologic targeting of those activated macrophages as a promising therapeutic approach to improve impaired wound-healing conditions in general.

MATERIALS AND METHODS

Animals

Female C57Bl/6 and C57BL/6J-*ob/ob* mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained under a 12 h light/12 h dark cycle at 22°C until they were 8 weeks of age. At this time, they were caged individually, monitored for body weight and wounded as described below.

Treatment of mice

Murine recombinant leptin (Calbiochem, Bad Soden, Germany) was injected intraperitoneally (2 μg/g body weight, one injection per day, from 2 days before wounding until day 13 after wounding) and purified rat monoclonal antibodies directed against TNFα (V1q) (Echtenacher *et al.*, 1990) (Abcam Ltd, Cambridge, UK), F4/80 (Austyn and Gordon, 1981; Hirsch *et al.*, 1981) (SeroTec, Düsseldorf, Germany), or a nonspecific rat IgG (Santa Cruz, Heidelberg, Germany) were injected intraperitoneally (1 μg/g body weight) in 0.5 ml phosphate-buffered saline at days 7, 9, and 11 after injury.

Wounding of mice

Wounding of mice was performed as described previously (Frank *et al.*, 1999; Stallmeyer *et al.*, 1999). Briefly, mice were anesthetized and six full-thickness wounds (5 mm in diameter, 3–4 mm apart) were made on the back of each mouse. An area of 7–8 mm in diameter was excised at the indicated time points for analysis. As a control, a similar amount of skin was taken from the backs of non-wounded mice. For each experimental time point, tissue from four wounds each from four animals ($n=16$ wounds, RNA analysis) and from two wounds each from four animals ($n=8$ wounds, protein analysis) were combined and used for RNA and protein preparation. All animal experiments were carried out according to the guidelines and were approved by the local Ethics Animal Review Board.

RNA isolation and RNase protection analysis

RNA isolation and RNase protection assays were carried out as described previously (Chomczynski and Sacchi, 1987; Frank *et al.*, 1999). The cDNA probes were cloned using RT-PCR. The probes corresponded to nt 481–739 (for IL-1β, NM008361), nt 541–814 (for TNFα, NM013693), nt 796–1063 (for COX-2, M64291), nt 623–323 (for CCL2, NM011333), nt 23–296 (for lysozyme M, BC002069), nt 2561–2845 (for Emr-1, X93328), and nt 163–317 (for glyceraldehyde-3-phosphate dehydrogenase, NM002046) of the published sequences.

Immunohistochemistry

Mice were wounded as described above. Animals were killed at day 13 after injury. Complete wounds were isolated from the back and fixed in formalin. Bisected wounds were embedded in paraffin. Immunohistochemistry from 6 μm deparaffinized sections was performed as described (Stallmeyer *et al.*, 1999). The monoclonal antibody directed against macrophage-specific F4/80 antigen was from Serotec, Düsseldorf, Germany. The anti-active caspase-3 antibody was obtained from Innovative Diagnostik Systeme (Hamburg, Germany).

Determination of apoptotic macrophages in wound tissue by TUNEL staining

Isolated 13 day wound tissue from *ob/ob* mice was fixed according to the HOPE (Hepes glutamic acid buffer mediated organic solvent protection effect) method and subsequently embedded in low-melting paraffin (Innovative Diagnostik Systeme, Hamburg, Germany). Sections (6 μm) of HOPE-fixed wound tissue was stained for apoptotic cells using the fluorescence-based dead end calorimetric TUNEL system (Promega, Mannheim, Germany) or the peroxidase-based ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon, Millipore, Schwalbach, Germany) according to the instructions of the manufacturer. Sections were subsequently incubated with macrophage-specific rhodamine-coupled F4/80 antibody (1:200 dilution, Serotec). Nuclei were counterstained using 4,6-diamidino-2-phenylindole staining (Sigma, Deisenhofen, Germany).

Flow cytometry

Two hundred microliter whole blood of ob/ob mice was mixed with 5 ml RBC lysis buffer (0.74% NH_4Cl) and incubated for 30 minutes to lyse red blood cells. The remaining leukocytes were washed, plated at 10^5 cells/well and incubated with 50 μl phycoerythrin-conjugated anti-F4/80 antibody (BD Biosciences, Heidelberg, Germany) (1:100 diluted in phosphate-buffered saline containing 1% fetal calf serum) for 30 minutes at 4°C. Cells were washed and analyzed for surface F4/80 expression using a FACSCalibur flow cytometer (BD Biosciences).

ELISA

Total wound lysate (25 μg), from 13 day wounds, was analyzed for the presence of immunoreactive IL-1β, TNFα, or CCL2 by ELISA using the respective Quantikine murine ELISA kits (R&D systems, Wiesbaden, Germany).

Determination of blood glucose and blood insulin levels

Blood glucose levels were determined using the Accutrend sensor (Roche Biochemicals, Mannheim, Germany) and serum insulin was analyzed by ELISA (Crystal Chemicals, Chicago) as described by the manufacturer.

Statistical analysis

Data are shown as means $\pm$ SD. Data analysis was carried out using the unpaired Student's *t*-test with raw data.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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