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Wound healing is a well-coordinated process that involves inflammatory mediators and cellular responses; however, if any disturbances are present during this process, tissue repair is impaired. Chronic wounds are one of the serious long-term complications associated with diabetes mellitus. The chemokine receptor CCR4 and its respective ligands, CCL17 and CCL22, are involved in regulatory T cell recruitment and activation in inflamed skin; however, the role of regulatory T cells in wounds is still not clear. Our aim was to investigate the role of CCR4 and regulatory T cells in cutaneous wound healing in diabetic mice. Alloxan-induced diabetic wild- type mice (diabetic) developed wounds that were difficult to heal, differently from $CCR4^{-/-}$ diabetic mice ($CCR4^{-/-}$ diabetic), and also from anti-CCL17/22 or anti-CD25–injected diabetic mice that presented with accelerated wound healing and fewer regulatory T cells in the wound bed. Consequently, $CCR4^{-/-}$ diabetic mice also presented with alteration on T cells population in the wound and draining lymph nodes; on day 14, these mice also displayed an increase of collagen fiber deposition. Still, cytokine levels were decreased in the wounds of $CCR4^{-/-}$ diabetic mice on day 2. Our data suggest that the receptor CCR4 and regulatory T cells negatively affect wound healing in diabetic mice.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that results in high levels of glucose in the blood and, if untreated, can lead to severe complications. More than 415 million people worldwide are affected, and if this trend continues, 642 million people will have diabetes by 2040. This disease caused the death of 5 million people in 2015, according to the International Diabetes Federation (2015) (Rocha et al., 2016; Trikkalinou et al., 2017). People with diabetes have a higher risk of developing serious health problems, such as blindness, cardiovascular accidents, nephropathy, neuropathy, foot ulcerations, impaired wound healing, and amputations (Cavanagh et al., 2005; Falanga, 2005; Ghosh et al., 2015; Jeffcoate et al., 2004). Approximately 15% of individuals with DM will develop a foot ulcer during their lifetime, and it is estimated that 8 out of every 10 non-traumatic amputations are due to DM, of which 85% are the consequence of foot ulcer progression (Brem and Tomic-Canic, 2007; Singh et al., 2005). Foot ulcers are a major type of chronic wound, which lead patients to lose their jobs, suffer prejudices, and have a reduced quality of life. Chronic wounds affect 1–2% of the population in developed countries, and it is associated with comorbidities, such as ischemia, venous stasis disease, and DM (Gottrup, 2004). Moreover, wound care imposes a substantial financial burden on the health care system (Bjarnsholt et al., 2008), but currently, there are no effective treatments for chronic wounds.

Wound healing is a complex event consisting of three overlapping but distinct phases: inflammation, cell proliferation, and remodeling (Acosta et al., 2008; Gurtner et al., 2008). The inflammatory phase is characterized by platelet aggregation and fibrin clot formation, which favors the migration of leukocytes, such as neutrophils and macrophages, which in turn clear the wound area (DiPietro, 1995;

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Abbreviations: dLN, draining lymph node; DM, diabetes mellitus; RT-PCR, reverse transcriptase PCR; Treg, regulatory T cell; WT, wild type

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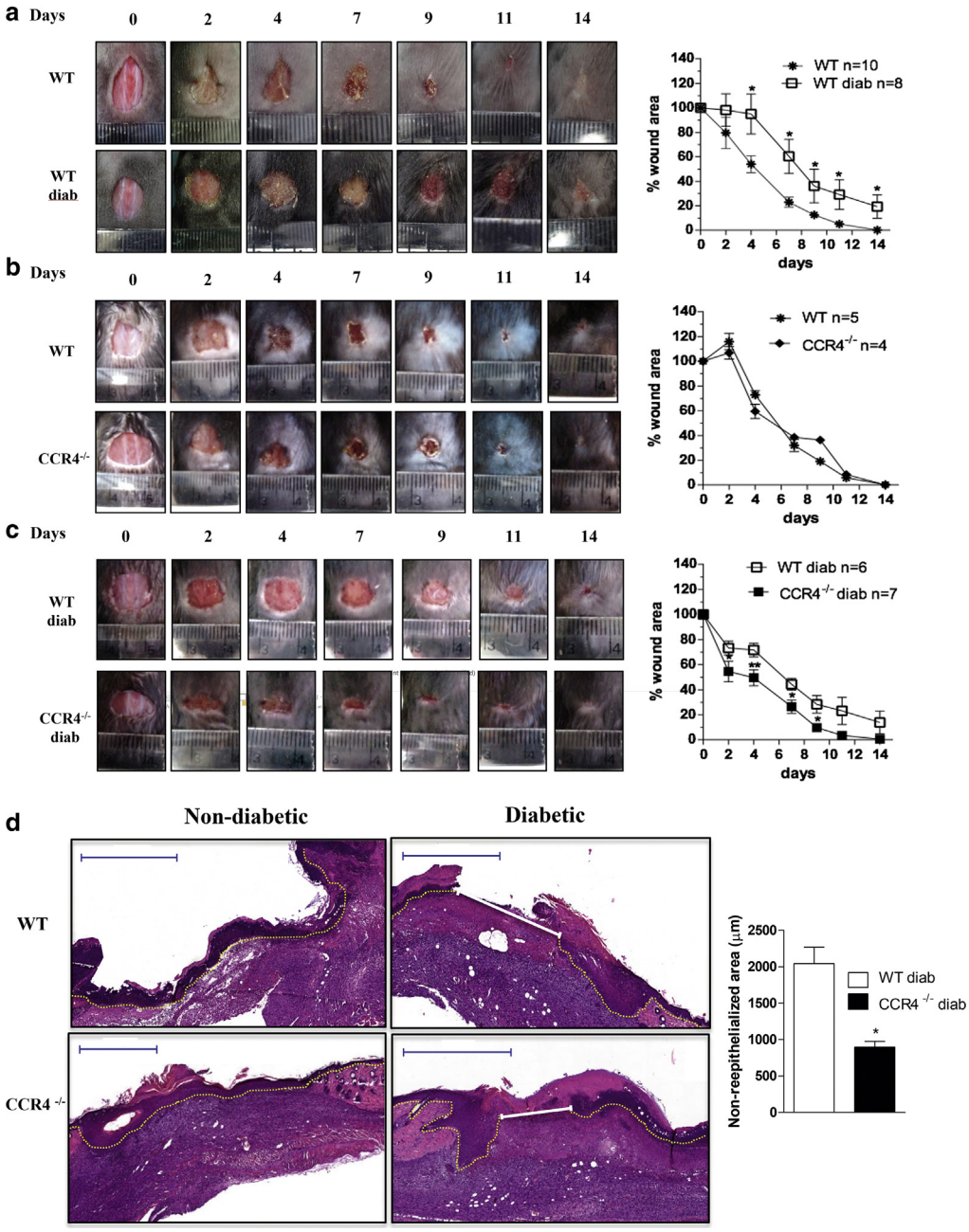


Figure 1. CCR4$^{-/-}$ diabetic mice accelerate wound healing. Mice were submitted to excisional full-thickness wounding, and open wound area was measured at different times. The wounds were measured at days 0, 2, 4, and 7. The area at day 0 was considered 100%, and the subsequent areas were proportional (%) to the initial area. (**a**) Representative images and graph of WT diabetic and non-diabetic mice, (**b**) WT and CCR4$^{-/-}$ non-diabetic mice, and (**c**) WT and CCR4$^{-/-}$ diabetic mice. (**d**) Midline sections of WT and CCR4$^{-/-}$ non-diabetic and diabetic wounds stained with hematoxylin and eosin at day 7. Scale bars = 1,000 µm. The graph depicts the quantification of non–re-epithelialized area between edges of migrating epithelial lips. Data are expressed as mean ± standard error of the mean. $*P < 0.05$ and $**P < 0.01$ by Student *t* test, compared to WT or WT diabetic mice; n = 4–10. WT, wild-type.

Guo and DiPietro, 2010; Singer and Clark, 1999). In the proliferative phase, fibroblasts proliferate and differentiate, the rate of collagen synthesis increases, and re-epithelialization and angiogenesis occur (Acosta et al., 2008; DiPietro, 1995; Singer and Clark, 1999). The remodeling phase comprises proliferation and protein synthesis interruption, with marked dynamic collagen remodeling (Guo and DiPietro, 2010; Singer and Clark, 1999). These phases are very well coordinated and integrated, although if something interferes in this process, it could impair or delay the tissue repair (Guo and DiPietro, 2010). A better understanding of the cell subtypes and mediators involved in wound healing may contribute to the discovery of new therapeutic targets for acute and chronic wound treatment.

Some chemokines and their receptors have important functions in wound healing, contributing to the regulation of epithelialization, tissue remodeling, and angiogenesis (Iellem et al., 2001). The chemokine receptor CCR4 and its ligands, CCL17 (TARC/thymus and activation-regulated chemokine) and CCL22 (MDC/macrophage-derived chemokine), are involved in the recruitment of regulatory T cells (Tregs) from/to inflamed skin (Baekkevold et al., 2005; Iellem et al., 2001; Kato et al., 2011; Wang et al., 2010). Tregs are characterized as a subpopulation of CD4^{+}CD25^{+} cells that constitutively express the transcription factor Foxp3^{+}, representing a unique and pivotal T cell subpopulation in immune surveillance (Fontenot et al., 2003; Hori et al., 2003). T cells migrate into wounds during the proliferative phase (Guo and DiPietro, 2010); however, the role of Tregs specifically in wound repair is not completely understood. Thus, this work aims to describe the role of CCR4 and Tregs in diabetic wound healing.

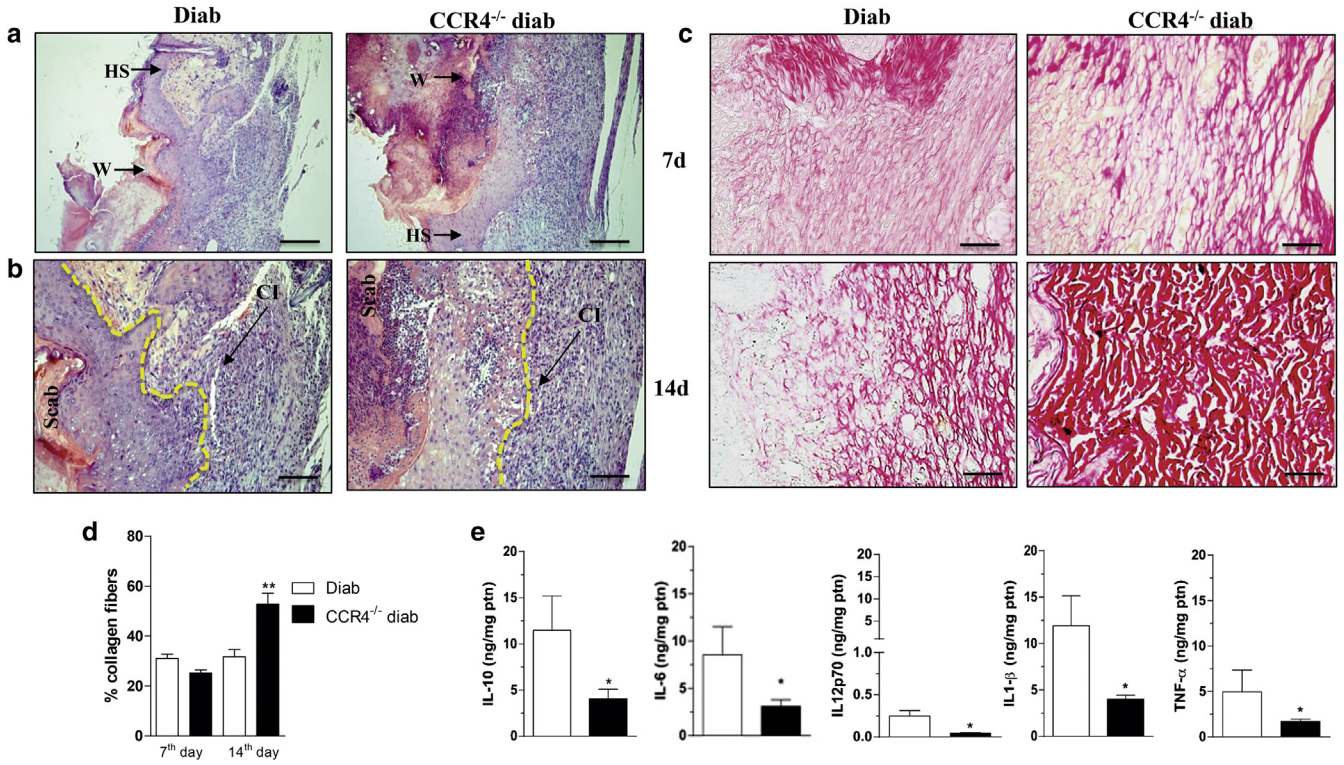


Figure 2. CCR4$^{-/-}$ diabetic mice increase collagen deposition and reduce cytokine in the wound. (**a, b**) Wounds were harvested from wild-type and CCR4$^{-/-}$ diabetic mice at day 7 after wounding and stained with hematoxylin and eosin. Scale bars: (**a**) 200 μm, (**b**) 100 μm. (**c, d**) Collagen deposit in wounds at day 7 and 14 stained with Picro Sirius and bar graph. Scale bar = 50 μm. $^{*}P < 0.05$ by Student *t* test, compared to wild-type diabetic mice, day 14 and $^{\#}P < 0.05$ compared to CCR4$^{-/-}$ diabetic mice, day 7 by one-way analysis of variance, followed by the Bonferroni post-hoc test; n = 5. (**e**) Cytokine levels at day 2 after wounding. $^{*}P < 0.05$ by Student *t* test; n = 4–6. All data are expressed as the mean ± standard error of the mean. CI, cellular infiltrate; HS, healthy skin; W, wound.

RESULTS

CCR4 contributes to diabetic wounds

To investigate the role of CCR4 and Tregs in wound healing, wild-type (WT) and CCR4 knockout (CCR4$^{-/-}$) mice were subjected to the full-thickness wound model. Initially, to validate our model, the wound healing was evaluated in WT and alloxan-induced diabetic WT mice (diabetic) over 14 days. Diabetic mice presented with a delay in wound healing compared to WT mice (Figure 1a). WT and CCR4$^{-/-}$ mice were also subjected to a full-thickness wound model, and no significant difference was observed in the healing process (Figure 1b). However, when diabetic, the CCR4$^{-/-}$ mice presented with an accelerated wound healing process compared to the diabetic mice at days 2, 4, 7, and 9 (Figure 1c). The histomorphometric analysis of non-reepithelialized area between edges of migrating epithelial lips confirmed more efficient healing process in CCR4$^{-/-}$ diabetic mice (Figure 1d). These data suggest that CCR4 plays a detrimental role in wound healing.

CCR4 retards the tissue formation of diabetic wounds

Cellular infiltrate was evaluated from images of each animal obtained at day 7. Cellular infiltrate in the wound bed was abundant in the diabetic and CCR4$^{-/-}$ diabetic mice, but slightly increased in the latter group (Figure 2a, 2b). Collagen deposition was evaluated by Picro Sirius Red staining in wound tissues samples obtained at days 7 and 14. CCR4$^{-/-}$ diabetic mice presented more collagen deposition only at day 14 compared with the diabetic mice. Interestingly, CCR4$^{-/-}$ diabetic mice presented with twice as much collagen deposition at day 14 compared to day 7 (Figure 2c, 2d), suggesting that the new tissue formed in the CCR4$^{-/-}$ diabetic mice is probably more organized than that formed in the diabetic mice.

Wounds homogenates from CCR4$^{-/-}$ diabetic mice presented with reduced amounts of all cytokines evaluated (IL-1β, IL-6, IL-10, IL-12p70, and TNF-α) in comparison to the diabetic mice at day 2, but still presented significant cytokine levels in the lesion (Figure 2e). At days 4 and 7 after wounding, cytokine levels were reduced, therefore, similar between groups (data not shown). Levels of MCP-1 were similar between groups at all time points analyzed. We also analyzed cytokines levels in the lesions of WT and CCR4$^{-/-}$ mice at days 2, 4, and 7, but no difference was observed between groups, despite the fact that significant amounts were detected in the wounds (data not shown).

Because the histologic analysis at day 7 revealed slightly more leukocyte accumulation in the wounds of the CCR4$^{-/-}$ diabetic mice than in the diabetic mice, we investigated the leukocyte profile by flow cytometry. Percentage of CD11b^{+}Ly6G^{+} (neutrophils) and CD11b^{+}CD11c^{+} (macrophages and dendritic cells) cell population were similar in the draining lymph nodes (dLNs) and in the wounds of the diabetic and CCR4$^{-/-}$ diabetic mice at day 7 (Figure 3a, 3b). The absolute number of these populations was also similar between groups in the dLNs and in the wounds. Due to the

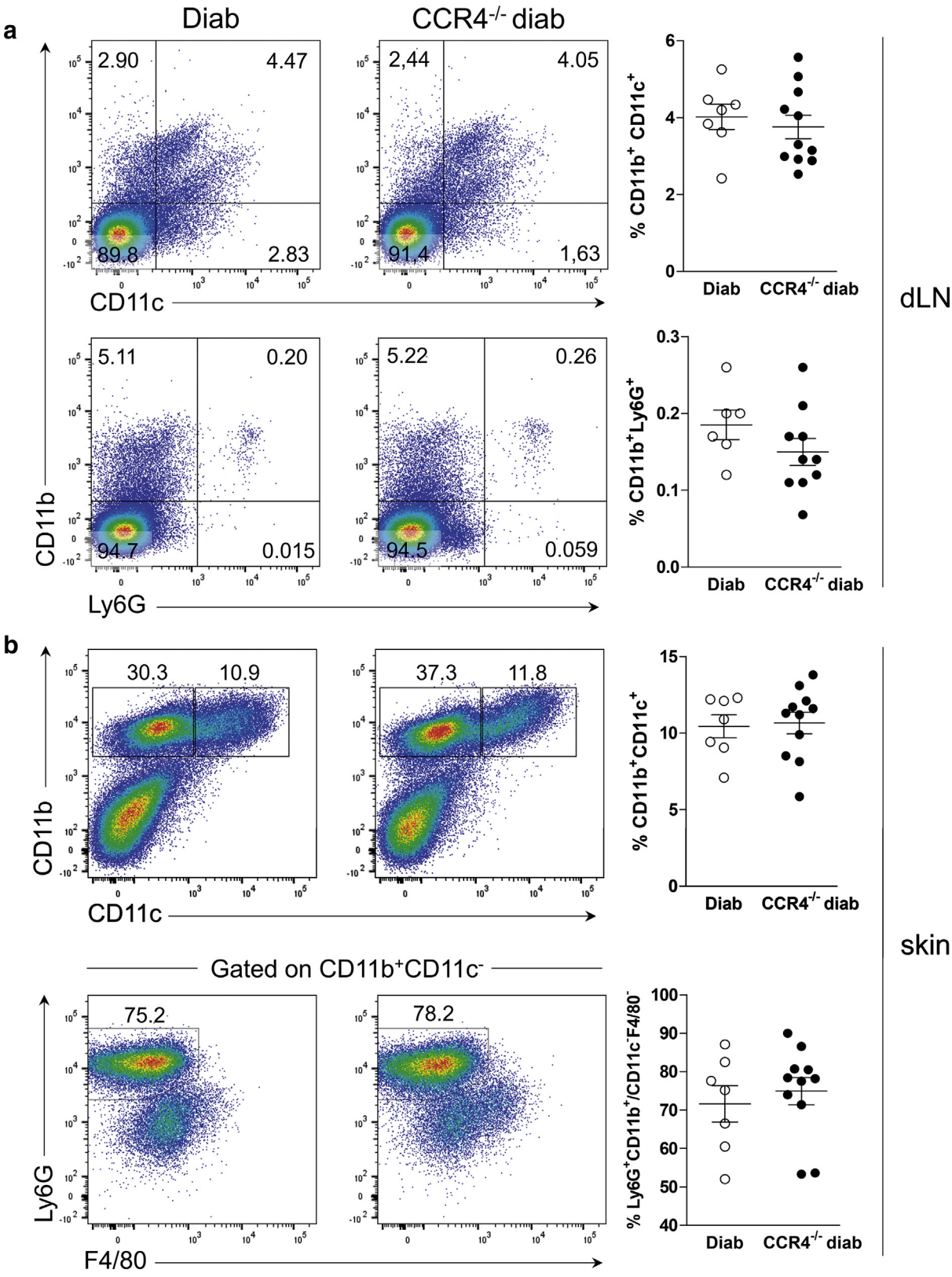


Figure 3. CD11b$^+$Ly6G$^+$ and CD11b$^+$CD11c$^+$ cell infiltration in the wound does not change in CCR4$^{-/-}$ diabetic mice. dLNs and wounds were harvested at day 7 after wounding and the cell suspensions were analyzed by flow cytometry. (**a**) Dot plots (left) and scatter dot plot graphs (right) show CD11b$^+$CD11c$^+$ (top row) and CD11b$^+$Ly6G$^+$ (bottom row) cell frequencies in dLNs. (**b**) Dot plots (left) and scatter dot plot graphs (right) show CD11b$^+$CD11c$^-$ and CD11b$^+$CD11c$^+$ (top row) cell frequencies in wounded skin area. The percentage of Ly6G$^+$F4/80$^-$ leukocytes was determined in the CD11b$^+$CD11c$^-$ gate (bottom row). Data are expressed as the mean ± standard error of the mean. $^{**}P < 0.01$ by Student *t* test; n = 7–11. dLN, draining lymph node.

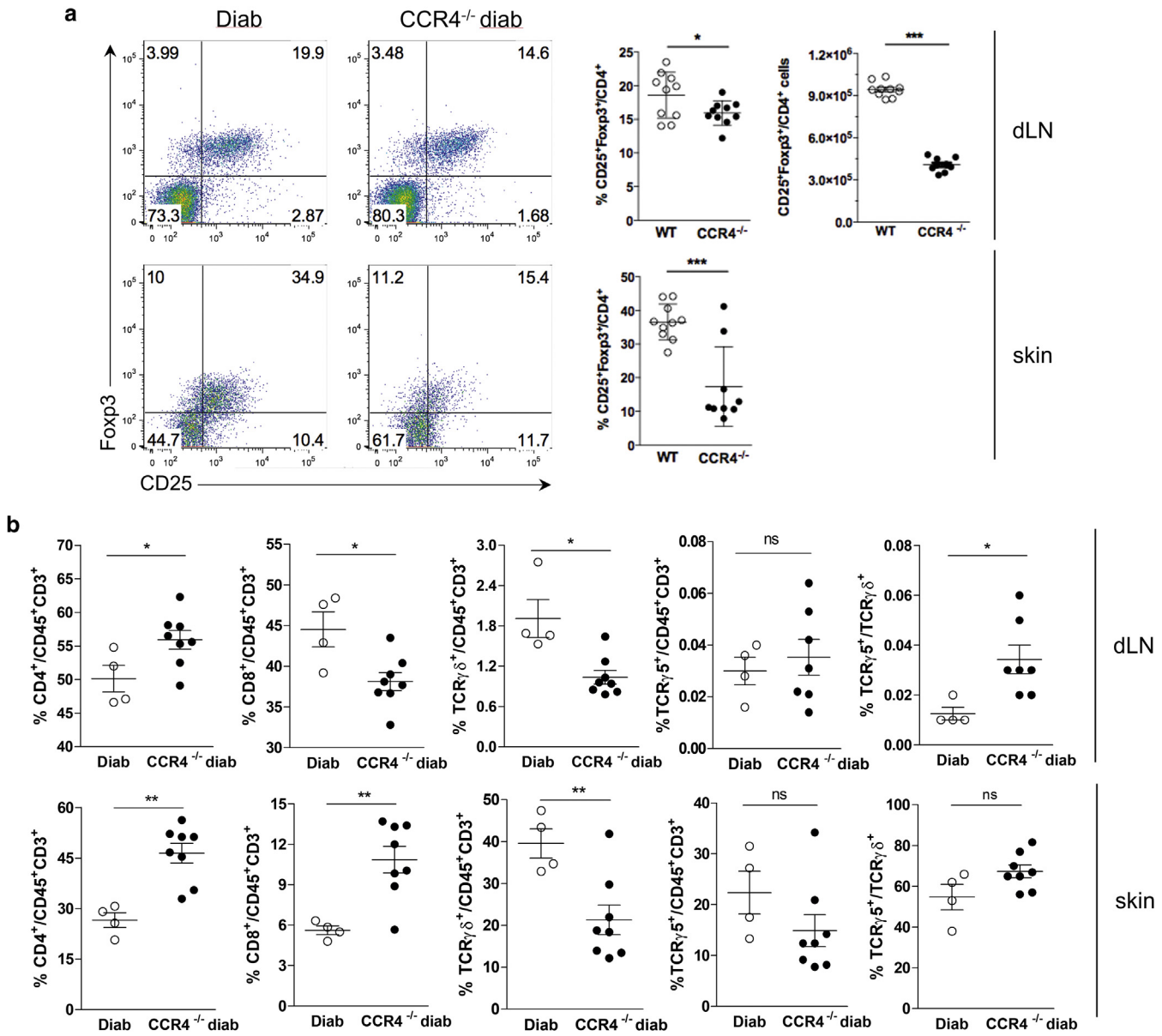


Figure 4. CCR4$^{-/-}$ diabetic mice present with a reduced regulatory T cell population and consequently altered T cell migration in the wound bed and in the dLNs. dLNs and wounds were harvested at day 7 after wounding and the cell suspensions were analyzed by flow cytometry. (**a**) Dot plots (left) and scatter dot plot graphs (right) show CD25$^+$Foxp3$^+$ cell frequencies. (**b**) Scatter dot plot graphs show CD4$^+$, CD8$^+$, TCRγδ$^+$, and TCRγ5$^+$ populations. Data were expressed as mean ± standard error of the mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Student *t* test; n = 4–8. dLN, draining lymph node.

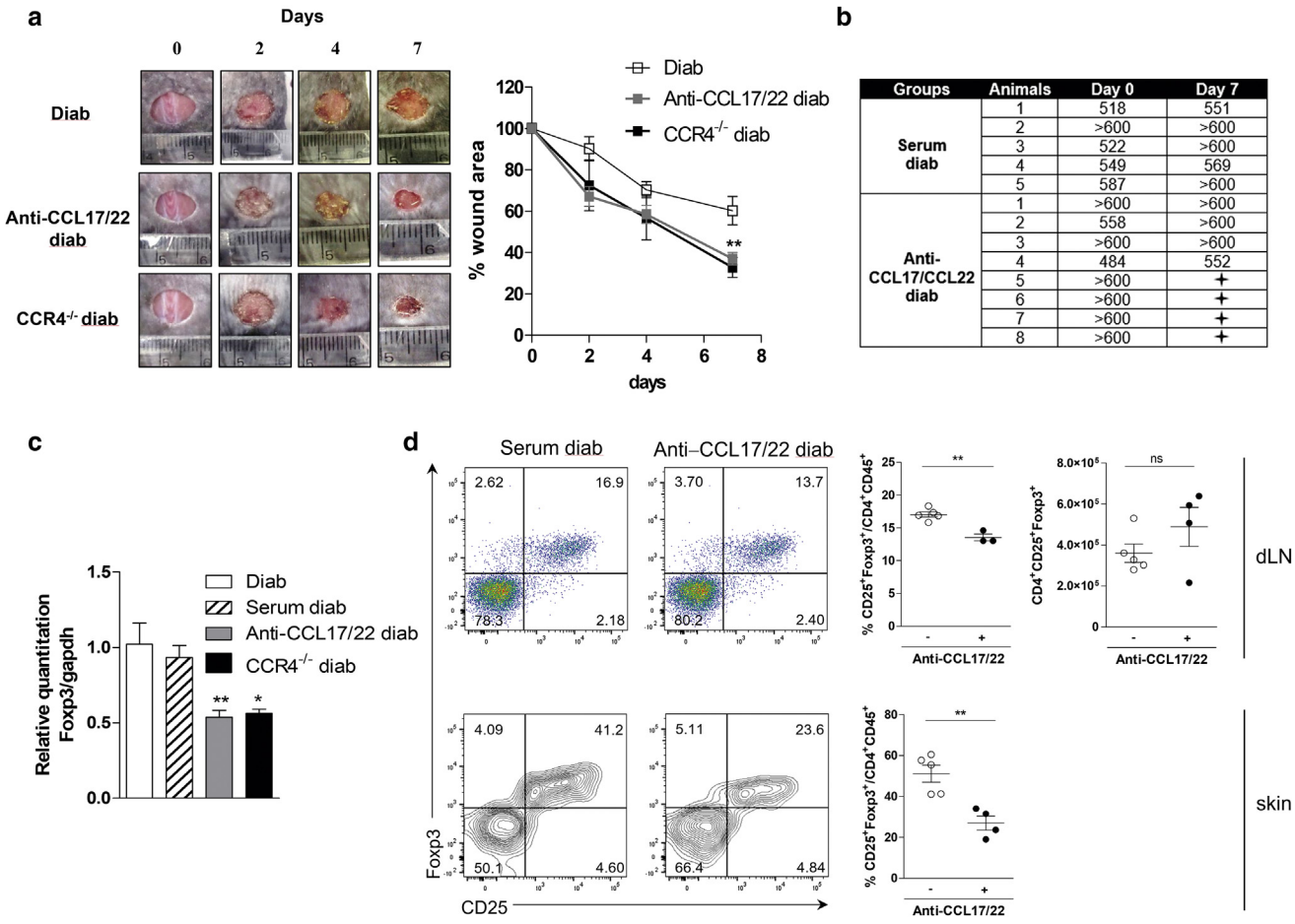
leukocyte enrichment from skin samples, absolute number in the skin was not evaluated.

CCR4 knockout diabetic mice have fewer Tregs in the skin wound and dLNs

As CCR4 and their ligands are involved in the migration of Tregs from/to the inflammatory skin, we quantified Tregs both in the dLNs and in the wounds of diabetic and CCR4$^{-/-}$ diabetic mice. The percentage and the absolute number of CD25$^+$Foxp3$^+$Treg was significantly decreased in the dLNs of CCR4$^{-/-}$ diabetic mice at day 7 when compared to diabetic counterparts. Such a difference is even more robust when the frequencies of CD25$^+$Foxp3$^+$ Tregs were determined in the wounded skin (Figure 4a). Besides Tregs, we also observed that CD4$^+$ and CD8$^+$ T cells were increased, while γδ T cells were reduced in the CCR4$^{-/-}$ diabetic wounds (Figure 4b). TCRγ5 T cells, a subset of γδ T cells resident in the skin, did not change the percentage in the wounds between groups. In the dLNs, the percentage and absolute number of CD4$^+$ and γδ T cells showed the same pattern observed in the wounds. Percentage of CD8$^+$ T cells reduced in dLNs, but did not change as absolute number; and the percentage of TCRγ5 T cells increased in CD45$^+$CD3$^+$ population (Figure 4b and data not shown). These data suggest that a reduction of Treg migration toward wound site, due to CCR4 deficiency, seems to modulate other T cells in the skin, which may contribute to wound healing.

Tregs retard wound healing in diabetic mice

To investigate the contribution of the CCL17 and CCL22 chemokines, which are both involved in Treg activation and chemotaxis, we used neutralizing antibodies in vivo.



Groups	Animals	Day 0	Day 7
Serum diab	1	518	551
	2	>600	>600
	3	522	>600
	4	549	569
	5	587	>600
Anti-CCL17/CCL22 diab	1	>600	>600
	2	558	>600
	3	>600	>600
	4	484	552
	5	>600	+
	6	>600	+
	7	>600	+
	8	>600	+

Figure 5. Anti-CCL17/22 administration accelerates wound healing in diabetic mice. (**a**) Representative images and graph of wound healing of mice subjected to excisional full-thickness wounding. (**b**) Glycemia of anti-CCL17/CCL22–treated and non-treated diabetic mice at days 0 and 7 after wounding. (**c**) Wounds were collected at day 7 and the samples were analyzed by reverse transcriptase PCR for Foxp3 transcripts. Data are expressed as the mean ± standard error of the mean. $^{*}P < 0.05$ compared to diabetic mice and $^{\#}P < 0.05$ compared to control serum diabetic mice; n = 6–9. (**d**) The frequencies of $CD25^{+}Foxp3^{+}$ regulatory T cells were determined by flow cytometry in cell suspensions obtained from the dLNs or from the wounds at day 7 after wounding. Data are expressed as mean ± standard error of the mean by Student *t* test; n = 4–5. dLN, draining lymph node.

Anti-CCL17/22 injected WT diabetic mice healed faster than those mice that did not receive the immunoglobulins. The healing profiles of anti-CCL17/22–injected WT diabetic mice and untreated $CCR4^{-/-}$ diabetic mice were similar (Figure 5a). It is important to state that diabetic mice treated with anti-CCL17/22 presented with higher glycemia and 50% mortality, confirming the protective role of Tregs in diabetogenesis (Figure 5b). Even though diabetes status was the worst, these mice healed better. A decreased amount of Foxp3 transcripts, indicative of fewer Tregs, was detected by reverse transcriptase PCR (RT-PCR) in the wound bed of both anti-CCL17/22–injected diabetic mice and untreated $CCR4^{-/-}$ diabetic mice (Figure 5c). Corroborating, the percentage of $Foxp3^{+}$ cells in the skin and dLNs of anti-CCL17/22–treated diabetic mice was reduced compared to diabetic mice (Figure 5c). These data suggest that CCR4 and their ligands are involved in Treg migration to the wound site, highlighting a detrimental role of Tregs in wound healing in diabetes.

To confirm the effect of Tregs during wound healing, we performed a traditional Treg cell depletion protocol, consisting of systemic treatment with anti-CD25 two days before wounding. Confirming our hypothesis, depletion of $CD25^{+}$ cells improved wound healing in diabetic mice (Figure 6a). Anti-CD25–treated diabetic mice showed decreased Treg populations in the wound bed at day 7, evaluated by RT-PCR (Figure 6b). These data reinforced the detrimental role of Tregs in wound healing in diabetic mice.

DISCUSSION

Menke et al. (2007) defined chronic wounds as lesions that have failed to progress through the normal stages of healing, remaining at a state of pathologic inflammation that results in a delayed healing process. In this context, it is important to understand and develop new strategies to treat chronic wounds.

In this study, we confirmed that diabetic mice had a delay in the skin wound healing process compared to WT. We demonstrated that the absence of CCR4, as well as ligands CCL17 and CCL22, contributed to a reduction of Treg migration to the skin lesion, correlating with an accelerated wound healing process. The same profile was observed in anti-CD25–injected diabetic mice, reinforcing the negative role of Tregs in the wound site for the healing process.

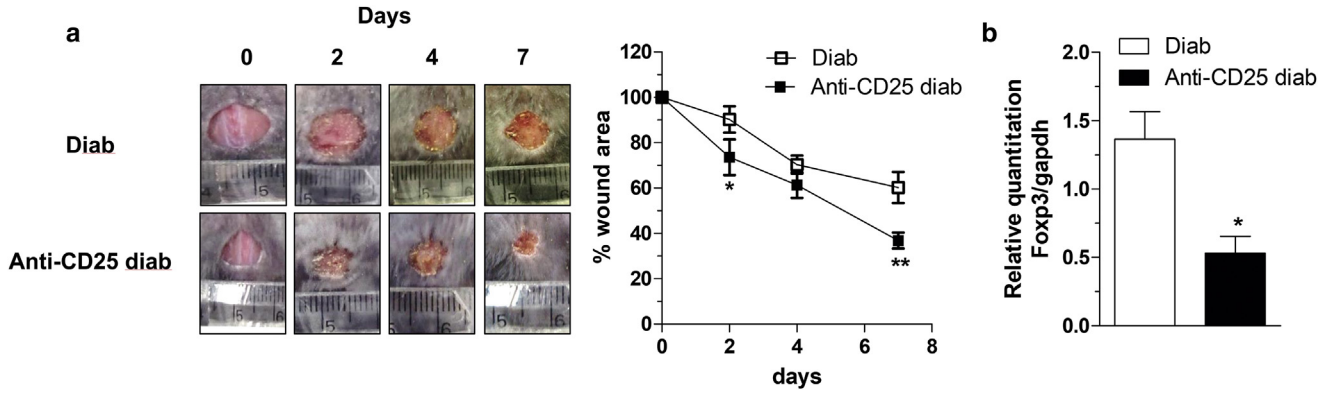


Figure 6. Anti-CD25 administration accelerates wound healing in wild-type diabetic mice. (**a**) Representative images and graph of wild-type and anti-CD25−injected diabetic mice subjected to excisional full-thickness wounding. (**b**) Reverse transcriptase PCR for Foxp3 transcripts in wounds collected at day 7 from wild-type and anti-CD25−injected diabetic mice. Data are expressed as the mean ± standard error of the mean. $*P < 0.05$ and $**P < 0.01$; n = 6–9.

Little is known about the involvement of CCR4 in wounds, but the literature provides some information about its role in other inflammatory disorders. Lehtimaki et al. (2012) demonstrated in an oxazolone-induced contact hypersensitivity model that the absence of CCR4 resulted in an enhanced inflammatory response. In the same study, $CCR4^{-/-}$ mice presented with increased ear swelling, inflammatory cell infiltration, and a higher expression of inflammatory cytokines in the skin compared with WT mice. Thus, the absence of CCR4 contributes to at least a better and more effective innate response. On the other hand, Kroeze et al. (2012) also demonstrated that CCR4 was expressed in human skin and that CCL22 induced keratinocyte migration in vitro, which could favor wound healing. In addition, Kato et al. (2011) observed that CCL17 induced fibroblast migration to skin lesions, accelerating wound healing in mice. Our data corroborate the modulatory effect of CCR4 because $CCR4^{-/-}$ diabetic mice healed faster.

Our hypothesis was that recruited $CCR4^{+}$ Tregs would impair wound healing in diabetic mice due to their inhibitory effect on inflammation by modulating cell recruitment and activation. The early inflammatory response is mandatory for the healing process. In a different model, Cavassani et al. (2006) demonstrated that a *Paracoccidioides brasiliensis*−infected skin lesion in human was aggravated by the presence of $CCR4^{+}$ Tregs in the lesion, which corroborates our data. Tregs represent approximately 5−10% of the total resident T cells in human skin and, as the skin's active resident memory, Tregs persist in the skin and present suppressive activity to autoimmune responses (Clark and Kupper 2007; Seneschal et al., 2011; Zhou et al., 2015). Nevertheless, during inflammation, these cells may proliferate and accumulate at the inflammatory site and negatively modulate the innate and acquired immune response (Clark and Kupper 2007; Rosenblum et al., 2011; Zhou et al., 2015). In accordance, in our work, the reduction of Tregs via CCR4 deficiency or anti-CD25 or anti-CCL17/22 treatment accelerated the wound healing. Indeed, it is important to consider the severe diabetes observed in anti-CCL17/22−treated mice due to the reduced number of systemic Tregs. However, our aim with the Treg systemic reduction strategy was just to characterize the effect of Tregs in the wounds of diabetes mice. Nevertheless, we do not rule out local treatment to modulate Treg number, which seems to be promising.

Moreover, $CCR4^{-/-}$ diabetic mice presented an increase of $CD4^{+}$ and $CD8^{+}$ T cells and a decrease of γδ T cells, which may contribute to skin homeostasis and immune surveillance. $CD4^{+}$ T cells release IL-22, IL-23, and IL-26 responsible for antimicrobial properties; skin barrier, such as keratinocytes and cellular differentiation; and survival (Cruz et al., 2018). Vγ5 T lymphocytes comprise the subset of γδ T cells that populate mouse skin and play an important role as immunomodulator; CCR4 is expressed by Vγ5 T lymphocytes, but this receptor is not important for skin homing. Vγ5 T cells seem to increase in γδ T cell population in the skin, although it seems to be reduced in T cell population of $CCR4^{-/-}$ diabetic mice. Its permanence in the wound suggests a relevant immune-modulatory role in a diabetic scenario. Interestingly, the absence of CCR4 also impacted on T cell populations in the dLNs of diabetic mice, suggesting an activation of acquired immune response relevant for immune surveillance and wound healing.

Even though the neutrophil and $CD11b^{+}/CD11c^{+}$ cells infiltration in the wound was not different between diabetic and $CCR4^{-/-}$ diabetic mice at day 7, we cannot rule out differences in cell activation or subpopulations between groups. Moreover, it was postulated that specific neutrophil and macrophage subtypes could play a beneficial role in wound healing (Brancato et al., 2011; Klinkert et al., 2017; Soehnlein et al., 2017). However, the characterization of these cells subtypes was not in the scope of this work.

Collagens fibers are the major extracellular matrix proteins, which are important for new tissue formation. It is produced mainly by myofibroblasts, the cells responsible for wound contraction and matrix protein deposition. We found increased collagen fiber deposits in $CCR4^{-/-}$ diabetic mice, as well as reduced levels of IL-6, IL-12, IL-1β, TNF-α, and IL-10, events that favor wound healing. Our data are in accordance with Eming et al. (2007), who also showed increased collagen deposits and augmented myofibroblast differentiation in $IL\text{-}10^{-/-}$ mice. Wound healing depends on the inflammatory response and requires a balance between anti-inflammatory and pro-inflammatory cytokines. Barrientos et al. (2008) showed that a high level of TNF-α at

the injury site was predictive of failure in the closure of the lesion. Thus, the balance of cytokine profile and cellular infiltration in skin wounds is critical to promote epithelialization and contraction of wound tissue.

In summary, our results show that the CCR4 chemokine receptor contributes negatively to the wound healing process. The deleterious effect of CCR4 may be due in part to the recruitment of Tregs to the wound site. The present data can be useful for the future development of novel therapeutic strategies for acute and chronic wound treatments targeting CCR4 and Tregs locally.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice weighing 20–25 g were obtained from Oswaldo Cruz Foundation Animal Facility (Fiocruz, RJ, Brazil). Animals were kept at a constant temperature (25°C) under a 12-hour light/dark cycle with free access to food and water. All experiments were conducted in accordance with the ethical guidelines of the Institutional Animal Care Committee of the Health Science Center of the Federal University of Rio de Janeiro (protocol code: DFBCICB028).

Experimental model of type 1 DM

Type 1 DM induction was performed by a single dose of alloxan (65 mg/kg, intravenously). Animals were fasted for 8 hours before the injection. After 7 days, blood glucose was measured by an Accu-Check Active glucometer (Roche Diagnostics, São Paulo, Brazil) to confirm hyperglycemia. Animals presenting with blood glucose >350 mg/dl were considered diabetic. In another set of experiment, the blood glucose was also measured at day 0 or day 7 after wounding.

Full-thickness excisional wound model

Full-thickness excisional wounds were performed as described previously (Wong et al., 2011). Briefly, mice were anesthetized with ketamine (112.5 mg/kg, intraperitoneally) and xylazine (7.5 mg/kg, intraperitoneally), their dorsal surfaces were shaved and cleaned, and a full-thickness excision wound was made with a biopsy punch of 10 mm in diameter. After surgery, the animals were kept in boxes and monitored for 14 days. During the experiment, the mice had free access to food and water and were kept at 25°C. Wound skin samples were collected at days 2, 4, 7, and 14 for future analysis.

Morphometric analysis

Wound areas were measured at days 0, 2, 4, 7, 9, 11, and 14. Digital photos were obtained at a distance of 10 cm from the wounds, and the wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD). Areas obtained at day 0 were considered 100%, and the subsequent measurements were calculated as a percentage of the initial wound area. The graphs represent the mean ± standard error of mean of all animals analyzed in each group.

Histologic procedures

Skin tissue samples were fixed with buffered formalin (10%; Sigma Aldrich, St. Louis, MO) for 48 hours, dehydrated in ethanol, clarified in xylene, and embedded in paraffin. Five-micrometer-thick slices were prepared for hematoxylin and eosin or Picro Sirius Red staining.

Total collagen quantification

Total collagen was quantified in samples stained by Picro Sirius Red. The analysis was carried out using a computerized system for image analysis comprising a digital camera coupled to a microscope (Olympus DP72; Olympus, Tokyo, Japan). The captured images were obtained using the CellSens Standard software (Olympus).

Flow cytometry

Flow cytometry of wound tissues was performed as described previously by Brubaker et al. (2011). Briefly, wounds obtained from individual skin samples were digested by dispase enzyme solution (0.375 mg/ml; Roche Diagnostics) and enzyme cocktail (Sigma Aldrich). Cells (10^6 cells/ml) obtained from each mouse were resuspended in phosphate buffered saline and blocked with Fc block (BD Biosciences Pharmingen, San Jose, CA). Different cell populations were identified using the corresponding antibodies: Ly6G-Alexa647, CD11b-FITC, CD11c-PECy5 and F4/80-PE (neutrophils and mononuclear cells), CD45-allophycocyanin-Cy7, CD4-PECy7, CD25-allophycocyanin and Foxp3-PE or CD45-allophycocyanin-Cy7, CD4-PECy7, CD3-BV421, CD8-peridinin chlorophyll protein complex, TCRγδ-PE, and TCRγ5-FITC (CD4 and CD8 T cells, γδ T cells, and Tregs). For all experiments was used Live/Dead staining (BD Biosciences). Lymphocyte populations were analyzed in the skin and draining inguinal, axillary, and brachial lymph nodes. For Treg analysis in the skin, samples were enriched by Percoll gradient for mononuclear cells. Samples were acquired by BD FACS Canto II (BD Biosciences) and then analyzed by FlowJo software (FlowJo LLC, Ashland, OR). We analyzed according to the following strategy for T cell: (i) forward scatter and side scatter plots show a gated region representing leukocytes; (ii) gated region of live cells; (iii) gated region of $CD45^+CD3^+$ cells; and (iv) dot plot of $CD4^+$, $CD8^+$, or $TCR\gamma5^+$ $TCR\gamma\delta^+$ population; and for Treg: (i) forward scatter and side scatter plots show a gated region representing leukocytes; (ii) gated region of live cells; (iii) gated region of $CD45^+CD4^+$ cells; and (iv) dot plot of $CD25^+$, Foxp3 (data not shown).

Cytokine quantification

Wound tissues were collected and homogenized in phosphate buffered saline containing protease inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). Cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 were quantified by flow cytometry using the Cytometric Bead Array Mouse Inflammation kit (BD Biosciences) according to the manufacturer's procedures. Samples (50 μl) were sorted by FACSCalibur flow cytometer (BD Bioscience) and analyzed by FCAP Array software (BD Biosciences). IL1-β quantification was performed in the supernatants by an ELISA assay kit (PeproTech, Rocky Hill, NJ and R&D Systems, Inc, Minneapolis, MN) according to the manufacturer's procedures. All cytokines levels were normalized by skin protein concentrations, which were quantified by the Bradford method.

Anti-CCL17 and anti-CCL22 administration

Diabetic mice were divided into two groups that received 1-ml intraperitoneal injections of anti-CCL17 and anti-CCL22 serum or unspecific serum, at 1×10^6–1×10^7 IgG titration, 1 week before wounding. The wound lesion was evaluated until day 7, and samples were also collected for Foxp3 mRNA quantification by RT-PCR.

$CD4^+CD25^+$ T cell depletion

Diabetic mice received intraperitoneal injections of 125 μg (100 μl intraperitoneal) of anti-CD25 mAb PC61 or phosphate buffered saline 2 days before wounding. This procedure produced an 80%

transient depletion of $CD25^+$ cells for approximately 4 weeks in normal mice (Darasse-Jèze et al., 2006). Wound healing was evaluated for 7 days and wound sample were also collected for RT-PCR and flow cytometry.

Total RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from wound tissue collected at day 7 using TRIzol reagent following the manufacturer's procedures. One microgram of RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Quantitative real-time RT-PCR was performed with the SYBR-green fluorescence quantification system. PCR cycling parameters were 95°C (10 minutes) and then 40 cycles of 95°C (30 seconds), 60°C (1 minute), followed by the standard denaturation curve. Primer sets were as follows: RPL13a forward (F): 5′GAGGTCGGGTGGAAGTACCA; RPL13a reverse (R): 5′TGCATCTTGGCCTTTTCCTT; foxp3 (F): 5′AGCAGTGTGGACCGTAGATGA; foxp3 (R): 5′GGCAGGGATTGG AGCACTT. Foxp3 to rpl13a relative expression was calculated using the comparative Ct method.

Statistical analysis

The nonparametric Student *t* test or one-way analysis of variance, followed by the Bonferroni post-hoc test, was applied in all analysis. The level of significance for significant difference between groups was set at $^*P < 0.05$ and $^{**}P < 0.01$. All data were expressed as the mean ± standard error of the mean. All statistical analysis were made with GraphPad Software (La Jolla, CA).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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