

Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8)

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ABSTRACT Matrix metalloproteinases (MMPs) have been implicated in numerous tissue-remodeling processes. The finding that mice deficient in collagenase-2 (MMP-8) are more susceptible to develop skin cancer, prompted us to investigate the role of this protease in cutaneous wound healing. We have observed a significant delay in wound closure in *MMP8*^{-/-} mice and an altered inflammatory response in their wounds, with a delay of neutrophil infiltration during the first days and a persistent inflammation at later time points. These changes were accompanied by alterations in the TGF- β 1 signaling pathway and by an apoptosis defect in *MMP8*^{-/-} mice. The delay in wound healing observed in *MMP8*^{-/-} mice was rescued by bone marrow transplantation from wild-type mice. Analysis of other MMPs showed that *MMP8*^{-/-} mice had a significant increase in the expression of MMP-9, suggesting that both proteases might act coordinately in this process. This possibility was further supported by the novel finding that MMP-8 and MMP-9 form specific complexes *in vivo*. Taken together, these data indicate that MMP-8 participates in wound repair by contributing to the resolution of inflammation and open the possibility to develop new strategies for treating wound healing defects.—Gutiérrez-Fernández, A., Inada, M., Balbín, M., Fueyo, A., Pitiot, A. S., Astudillo, A., Hirose, K., Hirata, M., Shapiro, S. D., Noël, A., Werb, Z., Krane, S. M., López-Otín, C., Puente, X. S. *FASEB J.* 21, 2580–2591 (2007)

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PROTEASES PERFORM A WIDE VARIETY of functions in numerous physiological and pathological conditions, including development, cell migration, inflammation, and apoptosis (1, 2). Members of the matrix metalloproteinase (MMP) family of enzymes have been implicated in multiple processes that require extracellular matrix remodeling (3–5). Skin wound healing is a complex process in which the presence of a coordinated proteolytic activity is necessary to cleave collagens and other extracellular

matrix proteins, to replace the provisional fibrin matrix and to allow keratinocyte migration (6, 7). The relevance of MMPs in skin wound healing was first demonstrated after the finding that MMP inhibitors severely impair this process in different animal models (7–9). Similarly, *Coll1a1*^{tm1Jae} mice, which are considered as a multiple MMP-deficient model as the main site of collagen I cleavage has been mutated, also exhibit a clear delay in cutaneous wound repair, underscoring the importance of collagenases in this process (10). However, studies with mutant mice deficient in specific MMPs possessing collagenolytic activity have failed to reveal significant defects in this process. Thus, wound closure is unaltered in mice deficient in MT1-MMP, a membrane-bound metalloproteinase that activates proMMP-2 and also exhibits collagenolytic activity (11–13). Likewise, mutant mice lacking collagenase-3 (MMP-13), a potent enzyme strongly overexpressed during the initial steps of wound healing, do not exhibit any apparent abnormality in the process of skin repair (14).

Collagenase-2 (MMP-8) is mainly produced by neutrophils, where it is concentrated in secretory granules that are degranulated on neutrophil activation. To date, the biological functions of this neutrophil collagenase are uncertain, although it is implicated in a variety of tissue remodeling processes associated with inflammatory conditions or in the course of cutaneous wound healing (15–19). The relevance of MMP-8 in wound repair is suggested by the finding that this neutrophil protease is the main collagenase produced in healing wounds as well as in nonhealing ulcers (19, 20). Interestingly, analysis of MMP expression patterns in wounds from *MMP13*^{-/-} has shown an increased production of MMP-8 at the wound site. Therefore, this suggests that the lack of MMP-13 may be functionally

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compensated by an enhanced expression of MMP-8, finally resulting in normal healing (14). Taken together, these results indicate that MMP-8 participates in the process of skin wound healing, and its presence might be necessary for this process to be completed. However, beyond these correlative observations, to date no information is available about the precise functional role of this enzyme in cutaneous wound repair. In this study, we have taken advantage of mutant mice deficient in MMP-8 to study its role in skin wound healing.

MATERIALS AND METHODS

Animals

MMP8^{-/-} mice of a mixed C57BL/6J/129 background (21) were used, and wild-type littermates served as controls. The generation of *MMP9*^{-/-} mice in a C57BL/6J background has been previously described (22). All experiments were performed with 8–12 wk old male mice, and wild-type littermates were used as controls. All mice were kept under specific pathogen-free conditions, and the experiments were conducted in accordance with the guidelines of the Committee on Animal Experimentation of the Universidad de Oviedo, Oviedo-Spain.

Wound model

Skin wounds were performed as described previously (23). Briefly, mice were anesthetized by isoflurane inhalation, and after shaving the dorsal hair and cleaning the exposed skin with 70% ethanol, full-thickness excisional skin wounds were performed on either side of the dorsal middle line using an 8 mm biopsy-punch (Accuderm, Ft. Lauderdale, FL, USA). Usually, two wounds were made on the same animal, and healing was monitored by taking photographs at the indicated time points. Wound area was calculated for each time point, and wound closure was expressed as percentage of recovery with respect to the initial wound area. For biochemical and histological experiments, animals were sacrificed by cervical dislocation, and wounds and surrounding area were harvested and snap-frozen for biochemical analysis, or fixed for histological analysis.

Bone marrow transplantation

Bone marrow transplantation experiments were performed as described previously (24). Briefly, wild-type or *MMP8*^{-/-} mice were treated with 25 mg/Kg of busulfan for 4 days, followed by injection of 200 mg/Kg of cyclophosphamide. Twenty-four hours later, bone marrow was collected from the femurs of donor mice, and 4×10^6 cells were injected in recipient animals via the lateral tail vein. Six weeks later the engraftment was evaluated by immunofluorescence in blood samples using a neutrophil-specific antibody against Ly-6G/Gr-1 (Pharmingen, La Jolla, CA, USA), and an antibody against MMP-8 (21). Transplant efficiency was more than 90% in all cases.

Histological analysis and *in situ* hybridization

Wound tissues were fixed overnight in 4% paraformaldehyde buffered with phosphate-buffered saline (PBS) (pH 7.4) and embedded in paraffin. Sections (4 μ m width) were subjected

to hematoxylin and eosin staining. For the *in situ* hybridization experiments, skin samples were fixed overnight in 4% paraformaldehyde at 4°C, then dehydrated in ethanol and embedded in paraffin. Sections (5 μ m) were hybridized to ³⁵S-labeled antisense *MMP13* and *MT1-MMP* riboprobes as described previously (25). Slides were exposed to photographic emulsion at 4°C for 6 days, then developed, fixed, and cleared. Sections were counterstained with 0.02% toluidine blue. Sections hybridized with a labeled-sense riboprobe were used as negative controls. No positive hybridization signal was found in negative controls. Bright-field and dark-field images were captured with SPOT digital camera. Sense or antisense ³⁵S-uridine triphosphate-labeled RNA probes were synthesized from the corresponding linearized DNA using the appropriate RNA polymerases. Detection of apoptotic cells in wound sections was performed using a fluorescent terminal transferase dUTP nick end-labeling (TUNEL) kit (Chemicon, Temecula, CA, USA) following manufacturer's instructions. TUNEL-staining was visualized using a fluorescent microscope and photographed with a CCD camera.

Analysis of re-epithelialization

Re-epithelialization was quantified as described previously (26). Briefly, wound sections were stained with hematoxylin and eosin, and wound width and distance between the leading edges of keratinocyte migration was measured. Re-epithelialization was calculated as the ratio between the distance covered by the epithelium and the distance between wound ends.

Gelatin zymography and Western blot analysis

MMP-2 and MMP-9 activity was analyzed by gelatin zymography as described previously (27). Basically, wound extracts were homogenized in a modified RIPA lysis buffer (0.1 M Tris/HCl, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, pH 7.4) containing complete protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany), and protein content was quantified using the BCA method (Pierce, Rockford, IL, USA). Protein (10 μ g) were separated in a 8% SDS-PAGE gel containing 0.2% gelatin. Gels were washed twice for 30 min with 2.5% Triton X-100 and incubated at 37°C for 4 h in the presence of 10 mM Tris (pH 8.0) and 5 mM CaCl₂ and stained with Coomassie brilliant blue to reveal gelatinolytic bands. Bone marrow cells were obtained from the femurs from 8-wk-old male mice, resuspended at 10^7 cells/ml in PBS, and 10^6 cells were degranulated by treatment with TPA (1 μ M) or fMLP (1 μ M). To analyze skin wound extracts by Western blot, 20 μ g of protein were analyzed using rabbit IgGs against murine collagenase-2/MMP-8, pSmad2, pSmad3, Smad4, TGF- β RI, and actin, or sheep antibodies against murine MMP-9 (provided by G. Murphy, University of Cambridge, UK). For the detection of pSmad3, wound lysates were immunoprecipitated with a rabbit antibody against Smad2/3 (Cell Signaling, Beverly, MA, USA) and protein A/G-sepharose. We used secondary antibodies, donkey antibody to rabbit and goat antibody to sheep IgGs coupled to peroxidase, at 1:20,000 dilution. We developed the reaction with Supersignal Chemiluminescent Substrate (Pierce). For the detection of MMP-8/MMP-9 complexes, samples were run unreduced, otherwise samples were reduced with 2-mercaptoethanol.

Measurement of cytokines and chemokines by ELISA

Wound samples were homogenized in PBS containing 0.1% SDS, 1% Nonidet P-40, and 5 mM EDTA, pH 7.4, containing

complete protease inhibitor mixture, and cell debris were cleared by centrifugation at 13,000 *g* for 15 min at 4°C. Levels of TGF-β1, KC, and MIP-2 proteins in wound extracts were quantified using commercial ELISA kits (R&D Systems and Promega, Madison, WI, USA) according to the manufacturers' instructions. Total protein concentration was measured using the BCA assay as above, and the data were expressed as picograms of chemokine per milligram of sample.

Statistical analysis

Statistical differences were determined using the Student's *t* test or analysis of variance. All data are presented as the mean ± SEM.

RESULTS

Loss of MMP-8 impairs wound healing in the skin

The ability of collagenases to degrade different components of the extracellular matrix, together with previous observations showing that MMP-8 is the predominant collagenase in healing wounds (19, 20), prompted us to investigate whether this neutrophil proteinase participates in the process of cutaneous wound repair. We used *MMP8*^{-/-} mice and their corresponding wild-type littermate controls, and performed full-thickness skin excisional wounds as described under Materials and Methods. After monitoring the wound healing process at different time points, we observed that wound closure in *MMP8*^{-/-} mice was significantly delayed when compared to wild-type littermates (Fig. 1A, B). This delay was observed from the first day and

was sustained throughout the healing process, resulting in the complete closure of the wound 13–14 days after injury in *MMP8*^{-/-} animals, in comparison to wild-type animals, in which healing was completed by days 8 and 9. These data indicate that MMP-8 participates in skin wound closure.

To further evaluate the participation of MMP-8 during the wound healing process, we analyzed the presence of this protease in the wound area; we detected MMP-8 in wild-type animals as soon as 24 h after wounding, and it was present in the wound area up to 7 days after injury, while MMP-8 could not be detected in uninjured skin (Fig. 1C and data not shown). These expression data, together with the observation that the absence of MMP-8 results in wound closure delay, indicate that this neutrophil enzyme plays a nonredundant functional role during skin wound healing.

Delayed re-epithelialization at the wound sites in *MMP8*^{-/-} mice

Re-epithelialization is a crucial event during the wound healing process, allowing the formation of a thick and hyperproliferative epithelium as well as granulation tissue that covers the wound (28, 29). The observed delay in skin wound healing in *MMP8*^{-/-} mice prompted us to perform a detailed analysis of the re-epithelialization process as shown in Fig. 2. Three days after injury, re-epithelialization was significantly slower in *MMP8*-deficient animals compared with wild-type littermates. In addition, it was exclusively limited to the wound margins, while in wild-type animals re-epithelialization occurred at a normal rate, and by day

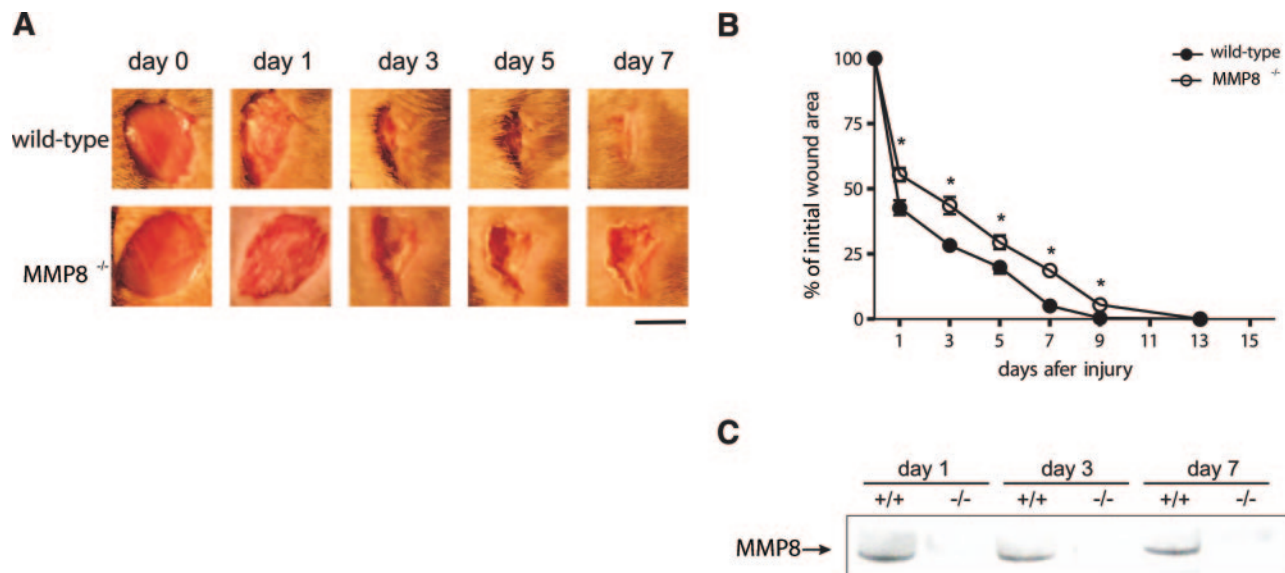


Figure 1. Loss of MMP-8 results in delayed wound healing. Excisional wounds were performed in *MMP8*^{-/-} mice and wild-type littermates, and the healing process was monitored at different time points. *A*) Representative images of wounds from wild-type and *MMP8*^{-/-} mice at different time points. Wild-type mice completely healed the wound area between 8 and 9 days after injury. However, mice lacking *MMP8* showed a delay in this process, and complete healing was not observed until 13–15 days postinjury. Scale bar: 5 mm. *B*) Wound area was quantified every two days and expressed as the percentage of wound closure for each genotype (*n*=8; **P*<0.05). *C*) Western blot analysis of skin extracts from wild-type (+/+) and *MMP8*^{-/-} (-/-) animals at different time points after injury. MMP-8 was detected in injured skin in wild-type animals from days 1 to 7 after injury.

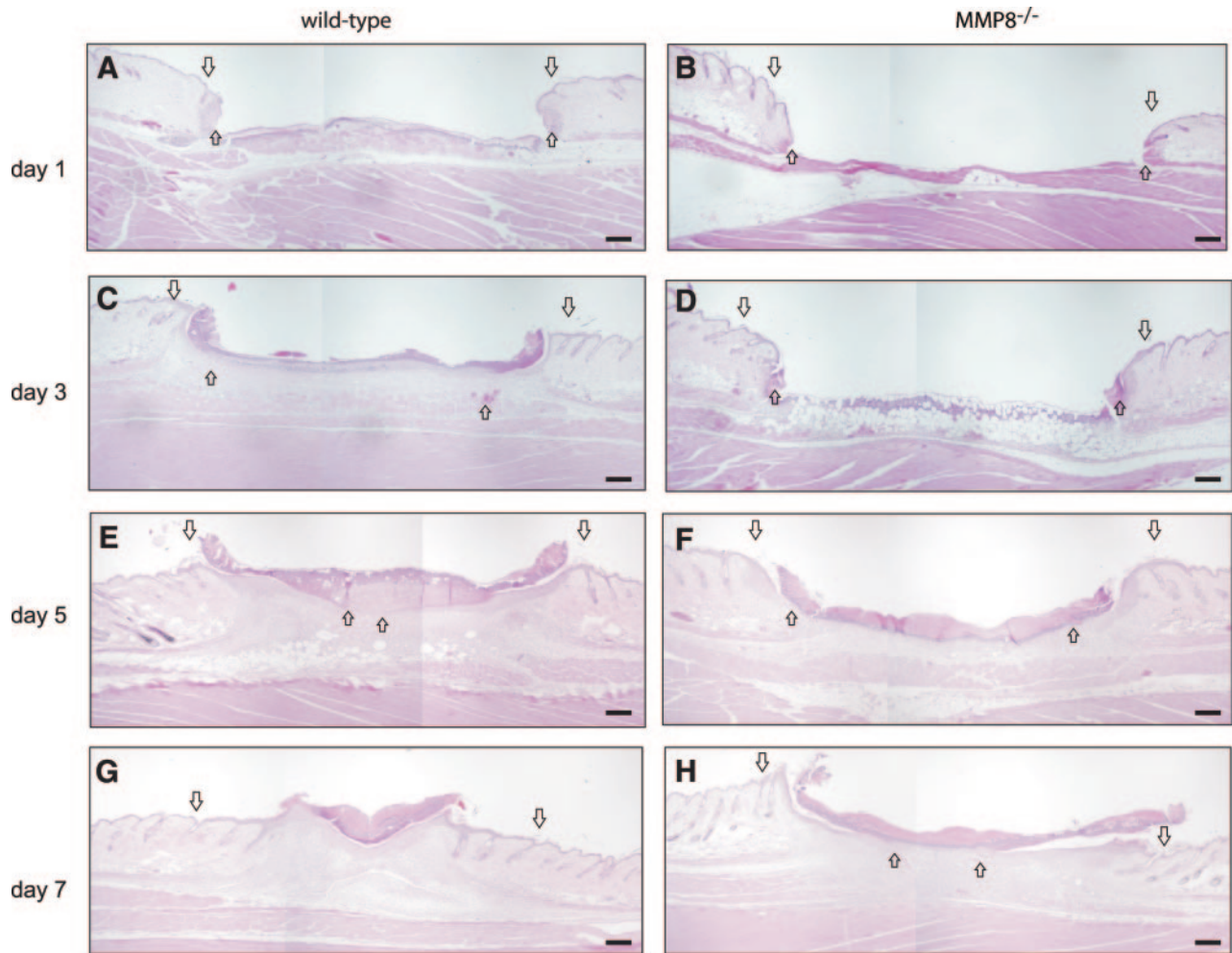


Figure 2. Re-epithelialization is delayed in *MMP8*^{-/-} wounds. Wound tissue was obtained from wild-type and *MMP8*^{-/-} mice at different time points, and histological analysis was performed to examine re-epithelialization. *A–H*) Representative hematoxylin and eosin-stained tissue sections illustrating the effect of *MMP8* deletion in granulation tissue development. Wound ends are indicated by big arrows, and the leading edge of keratinocyte migration is indicated by small arrows. Scale bars: 200 μ m.

3 ~30% of the wound was already re-epithelialized (Fig. 2*A–D*). The delay in re-epithelialization continued throughout the wound-healing process, with the greatest differences being observed at day 5, in which re-epithelialization was starting in *MMP8*^{-/-} mice, covering only about one-third of the wound area, whereas this process was almost completed in wild-type controls (Fig. 2*E, F*). Finally, by day 7, re-epithelialization was completed in control mice but it was still partial in *MMP8*^{-/-} animals, indicating that these animals have a significant delay in this process (Fig. 2*G, H*).

Neutrophil recruitment is impaired in *MMP8*^{-/-} wounds

A characteristic feature following skin injury is the rapid release of proinflammatory cytokines and chemokines, including TNF- α , KC/CXCL1, MIP-2/CXCL2, and LIX/CXCL5, which promote the migration of a variety of inflammatory cells into the wound site (30). During this inflammatory phase, neutrophils and mac-

rophages infiltrate the wound area, preventing the growth of pathogens and contributing to the healing process. As *MMP-8* is produced mainly by neutrophils, we investigated whether neutrophil infiltration was altered in skin wounds from *MMP8*^{-/-} mice. To determine the presence of neutrophils in the wound site, we performed histological analysis of wounded tissue at different time points. Similar to previous reports, neutrophil recruitment into the wound site started a few hours after injury in wild-type animals, reaching a maximum 2 days postinjury, and declining progressively afterward, returning to basal levels 3–5 days after injury (Fig. 3*A, B*). However, *MMP8*^{-/-} mice showed a different pattern of inflammatory response, which could be divided into two different phases. During the first phase of inflammation, the influx of neutrophils into the wound site was delayed in *MMP8*^{-/-} mice when compared to wild-type animals (Fig. 3*B*). In contrast with this reduced neutrophil recruitment observed during the first phase of healing, in a later phase, the number of neutrophils was significantly

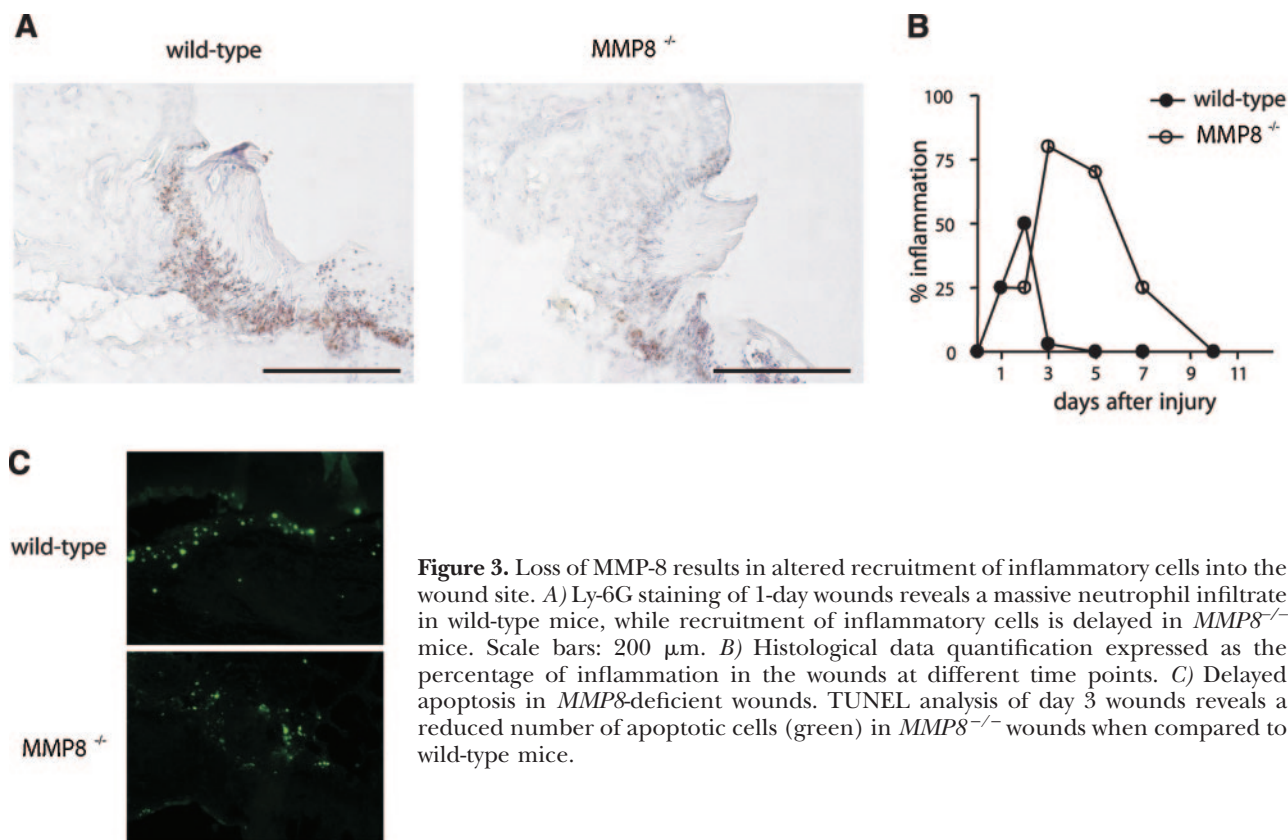


Figure 3. Loss of MMP-8 results in altered recruitment of inflammatory cells into the wound site. *A*) Ly-6G staining of 1-day wounds reveals a massive neutrophil infiltrate in wild-type mice, while recruitment of inflammatory cells is delayed in $MMP8^{-/-}$ mice. Scale bars: 200 μ m. *B*) Histological data quantification expressed as the percentage of inflammation in the wounds at different time points. *C*) Delayed apoptosis in $MMP8$ -deficient wounds. TUNEL analysis of day 3 wounds reveals a reduced number of apoptotic cells (green) in $MMP8^{-/-}$ wounds when compared to wild-type mice.

increased in $MMP8^{-/-}$ wounds (Fig. 3*B*), indicating that the resolution of inflammation was delayed in $MMP8$ -deficient mice. On the one hand, these results suggest that the defect in neutrophil emigration to the site of injury could be due to a failure to produce the inflammatory signals necessary for neutrophil recruitment to the wound area. On the other hand, the delay in wound healing and re-epithelialization observed in $MMP8^{-/-}$ mice could be due to a defect in the inflammatory response that accompanies the healing process, either through the delay in neutrophil recruitment to the wound site or to the persistent inflammation in these mutant animals.

Sustained inflammation in $MMP8^{-/-}$ wounds is accompanied by a delay in neutrophil apoptosis

Inflammation plays an important role during the wound healing process. In fact, recruitment of inflammatory cells during the first hours after injury is necessary to release different molecules necessary for matrix remodeling and keratinocyte migration, as well as to prevent pathogen growth. However, after this initial response, which is mainly mediated by neutrophils, resolution of inflammation is required for the healing process to proceed. Failure to resolve this inflammatory response can impair the healing process (31). $MMP8^{-/-}$ skin wounds were characterized by an increased inflammation, and neutrophils were still present in skin wounds 5 and 7 days after injury (Fig. 3*B*). In contrast, it is well established that during normal wound healing neutrophils

have already died by apoptosis and are cleared by macrophages at these later time points (31). Based on these findings, we investigated whether the increased inflammation observed in $MMP8^{-/-}$ wounds was due to a defect in neutrophil apoptosis. We analyzed the presence of apoptotic cells in the wound area in wild-type and $MMP8^{-/-}$ mice. We observed that the number of apoptotic cells determined by TUNEL assays was significantly reduced in $MMP8^{-/-}$ wounds when compared with wild-type wounds (Fig. 3*C*). These results reflect a defect in apoptosis in $MMP8$ -deficient animals and are reminiscent of previous studies in an allergen-induced model of asthma (32), suggesting that the persistent inflammation observed in $MMP8^{-/-}$ skin wounds is likely due to a delay in neutrophil apoptosis.

$MMP8$ deficiency alters the TGF- β 1 signaling pathway during cutaneous wound healing

To identify molecular pathways that could be responsible for the altered inflammatory response and delay in wound healing observed in $MMP8^{-/-}$ animals, we analyzed the levels of different cytokines and growth factors previously implicated in wound healing and inflammation, including TNF- α , TGF- β 1, IL-1 β , KC, or MIP-2 (30). Changes in TGF- β 1, as well as in its intracellular effectors, members of the Smad family of transcription factors have been previously associated with altered ability to heal skin wounds (33, 34). $MMP8^{-/-}$ mice showed reduced levels of active TGF- β 1 at days 3 and 5 when compared to wild-type animals

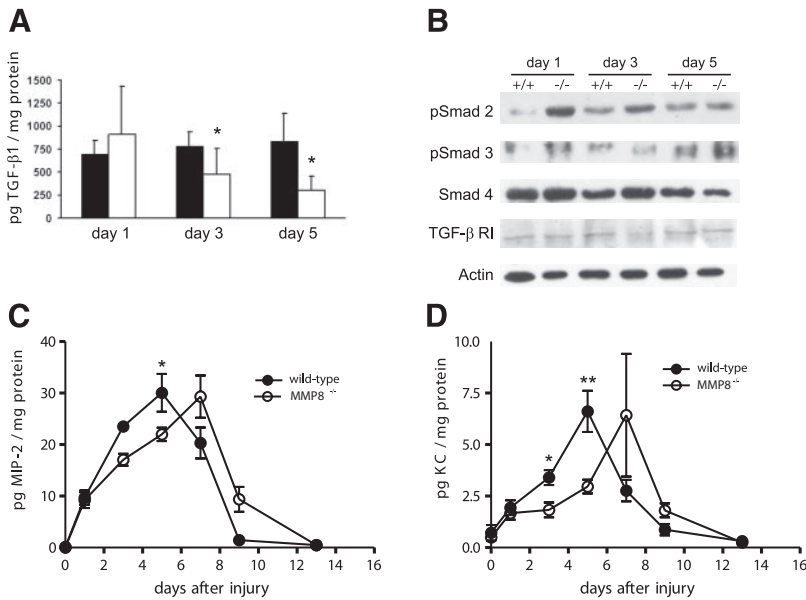


Figure 4. TGF-β1 signaling is altered in *MMP8*^{-/-} wounds. *A*) ELISA for active TGF-β1 in wound extracts from wild-type (closed bars) and *MMP8*^{-/-} mice (open bars) at different time points (*n*=3; **P*<0.05). *B*) Western blot analysis of protein extracts from wild-type and *MMP8*^{-/-} wounds for pSmad2, pSmad3, Smad4, TGF-β RI, and actin. pSmad3 was detected after immunoprecipitation of Smad3 from wound homogenates. *C, D*) ELISA analysis of the proinflammatory chemokines MIP-2 and KC shows that there is a delay in the production of these chemokines during the wound healing process (*n*=4; **P*<0.05, ***P*<0.01).

(Fig. 4A). In keeping with this, pSmad2 was significantly increased in *MMP8*^{-/-} wounds when compared to wild-type mice, and a moderate increase in pSmad3 was also observed at 24 h, while TGF-β receptor I or total Smad4 levels did not change (Fig. 4B). These data suggest that the reduced levels of active TGF-β1, together with the increased activation of Smad2 and/or Smad3 might contribute to the observed differences in neutrophil infiltration in these animals, as well as to the delay in wound healing, as these processes have been shown to be dependent on TGF-β1 signaling (33, 34). We did not find significant changes in the levels of the proinflammatory cytokines TNF-α or IL-1β (data not shown). However, analysis of KC and MIP-2 in wound extracts revealed that while both chemokines reached maximum levels 5 days postinjury in wild-type mice, *MMP8*^{-/-} animals showed a delay of two days in reaching their maximum levels (Fig. 4C, D). The delayed expression of KC and MIP-2 could be responsible for the persistence of inflammation in *MMP8*^{-/-} mice, contributing to the overall delay in wound healing observed in these animals.

Bone marrow transplantation rescues the wound healing defect in *MMP8*^{-/-} mice

The increased inflammation observed in *MMP8*^{-/-} wounds, and the fact that MMP-8 is confined to neutrophils, suggest that the wound healing defect in these animals is attributable to these inflammatory cells. To test this hypothesis, we asked whether bone marrow transplantation could rescue the healing defect observed in *MMP8*^{-/-} mice. We transplanted *MMP8*^{-/-} mice with bone marrow from wild-type donor mice, and *vice versa*. Six weeks later, engraftment efficiency was found to be more than 90% in all cases (Fig. 5). To investigate the wound healing ability of transplanted mice, excisional skin wounds were performed as above, and wound healing was followed until complete closure. Interestingly,

bone marrow transplantation was sufficient to rescue the wound healing delay observed in *MMP8*^{-/-} animals. In fact, wound healing in *MMP8*^{-/-} mice transplanted with wild-type bone marrow was similar to that observed in wild-type animals, with more than 50% of the wound closed after day 1, and achieving complete wound closure in 9 days (Fig. 5B). Furthermore, wild-type animals transplanted with bone marrow from *MMP8*^{-/-} donors showed a delay in the wound healing process similar to that observed in *MMP8*^{-/-} animals (Fig. 5B), achieving complete closure in 13 days. These results demonstrate that the healing defect observed in *MMP8*^{-/-} mice is solely due to the absence of MMP-8 in bone marrow-derived inflammatory cells and suggest that this metalloprotease has a positive effect during skin wound healing and contributes to resolve the inflammation associated with this process.

Altered expression of MMP-9 and MMP-13 in wounds from *MMP8*^{-/-} mice

It is well established that a dramatic remodeling of the extracellular matrix takes place during the wound healing process. These remodeling events are largely mediated by an array of proteolytic enzymes, which are secreted by inflammatory cells or keratinocytes, and facilitate migration of keratinocytes to cover the wound area (3). MMPs can cleave virtually all protein components of the extracellular matrix, and treatment with an MMP inhibitor delays the wound healing process (7, 8), suggesting that MMPs contribute to this process, either independently or coordinately. We next examined whether other MMPs potentially implicated in the wound healing process were altered in the absence of *MMP8*. By *in situ* hybridization (Fig. 6A), expression of *MMP13* and *MT1-MMP* (*MMP14*) was detected in wild-type as well as in *MMP8*^{-/-} wounds. No differences were observed in the distribution of *MMP14* transcripts (Fig. 6A). The expression pattern of *MMP13* was clearly

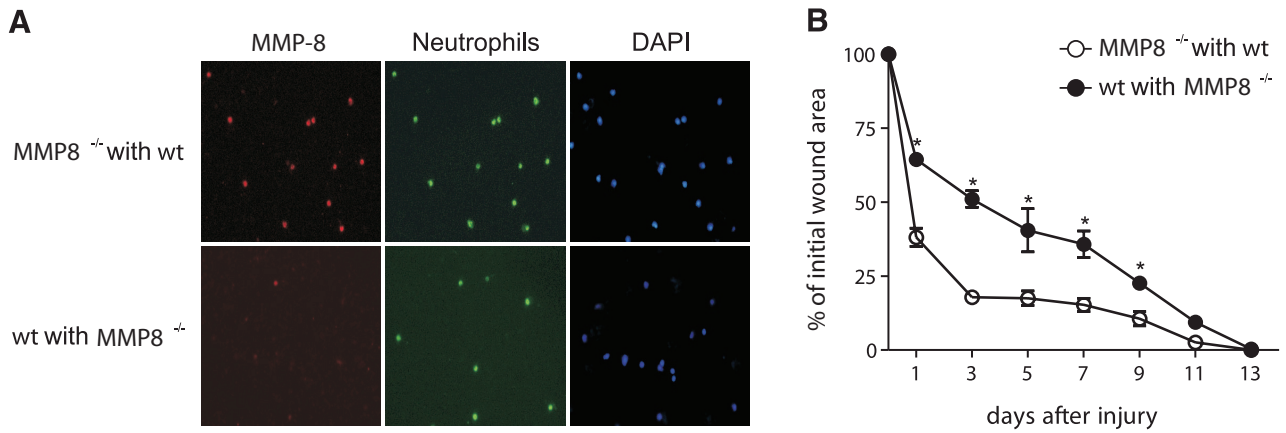


Figure 5. Bone marrow transplantation rescues the wound healing defect observed in *MMP8*^{-/-} mice. Wild-type mice and *MMP8*^{-/-} animals were transplanted with bone marrow from *MMP8*^{-/-} or wild-type mice, respectively. *A*) Immunofluorescence analysis of peripheral blood cells from these animals stained with specific antibody against MMP-8 (red) and the neutrophil-specific antigens (Ly6G, green). Cells were counterstained with DAPI (blue). *B*) Excisional wounds were performed in either *MMP8*^{-/-} mice transplanted with bone marrow from wild-type mice (open bars), or in wild-type littermates transplanted with *MMP8*^{-/-}-derived bone marrow (closed bars). Wound closure was monitored at different time points and expressed for each condition and genotype as the percentage of wound closure ($n=7$; $*P<0.05$).

altered in *MMP8*-deficient animals, where a more restricted distribution was observed compared to that in wild-type mice (Fig. 6A). Because *MMP13* transcripts were associated with the leading edge of the re-epithelialization layer, this suggests that the differences observed in the expression pattern are likely the result of the delay in re-epithelialization in *MMP8*-deficient animals. It should be noted that MMP-8 and MMP-13 could compensate for each other, as a recent report hypothesized that MMP-8 compensates for the lack of MMP-13 during the wound healing process in *MMP13*^{-/-} mice (14).

We next examined the MMP-2 (gelatinase A) and MMP-9 (gelatinase B) levels in wounds from *MMP8*^{-/-} mice by gelatin zymography. As can be seen in Fig. 6B, both metalloproteinases were detected at significant amounts in samples derived from wild-type and *MMP8*^{-/-} mice. MMP-2 was present in skin wound extracts as soon as 24 h postinjury, however at these initial stages the 72 kDa proMMP-2 was mainly detected (Fig. 6B). By day 3, activated MMP-2 was detected as a 62 kDa band in wound extracts from both wild-type and *MMP8*^{-/-} mice. The absence of significant changes suggests that MMP-2 does not contribute to the wound healing defect observed in *MMP8*-deficient animals. Interestingly, in the absence of MMP-8, there was increased MMP-9 in the wounded area 3 days after injury, compared with wild-type animals (Fig. 6B, C). This enhanced production of MMP-9 might be the result of the accumulation of neutrophils in these *MMP8*^{-/-} animals, and/or a compensatory *MMP9* up-regulation.

MMP-8 and MMP-9 form specific complexes *in vivo*

To determine if MMP-9 production was increased in *MMP8*^{-/-} neutrophils, we compared the MMP-9 released from bone marrow cells obtained from

MMP8^{-/-} and wild-type mice. Degranulation of the same number of bone marrow cells (10^6) was induced by treatment with TPA, and secreted MMP-9 was analyzed by gelatin zymography (Fig. 7A). We observed no difference in the intensity of the of the 92 kDa gelatinolytic band released from induced bone marrow cells from *MMP8*^{-/-} and wild-type mice. What we did find was the absence of a relatively low intensity gelatinolytic band (~150 kDa) present in extracts from cells from wild-type bone marrow but absent in extracts from *MMP8*^{-/-} bone marrow (Fig. 7A). As MMP-9 has been shown to form homodimers as well as stable complexes with proteins like neutrophil gelatinase-associated lipocalin (Ngal) (35), we reasoned that this band could represent a complex containing MMP-9 and another protein absent in *MMP8*^{-/-} mice. Western blot analysis of TPA-treated bone marrow supernatants with anti-MMP-9 specific antibodies revealed that the 150 kDa complex possessing gelatinolytic activity contained MMP-9 (Fig. 7B). Surprisingly, incubation of the same blot with an antibody against murine MMP-8 not only resulted in the detection of its 60 kDa band, but also in the detection of a specific signal associated with this 150 kDa complex (Fig. 7C), suggesting that this band is likely a complex containing MMP-8 and MMP-9.

To further confirm that MMP-8 forms a stable complex with MMP-9, we obtained bone marrow cells from *MMP9*^{-/-} mice, and these cells were analyzed by Western blot with specific antibodies against MMP-8. As expected, we detected a 60 kDa band corresponding to this MMP-8, but in the absence of MMP-9, the additional 150 kDa band containing MMP-8 was no longer detected, confirming that this high molecular weight complex is formed by the interaction between MMP-8 and MMP-9 (Fig. 7C). These results represent the first observation that MMPs can form heterodimeric complexes *in vivo* when secreted by neutrophils and open the possibility

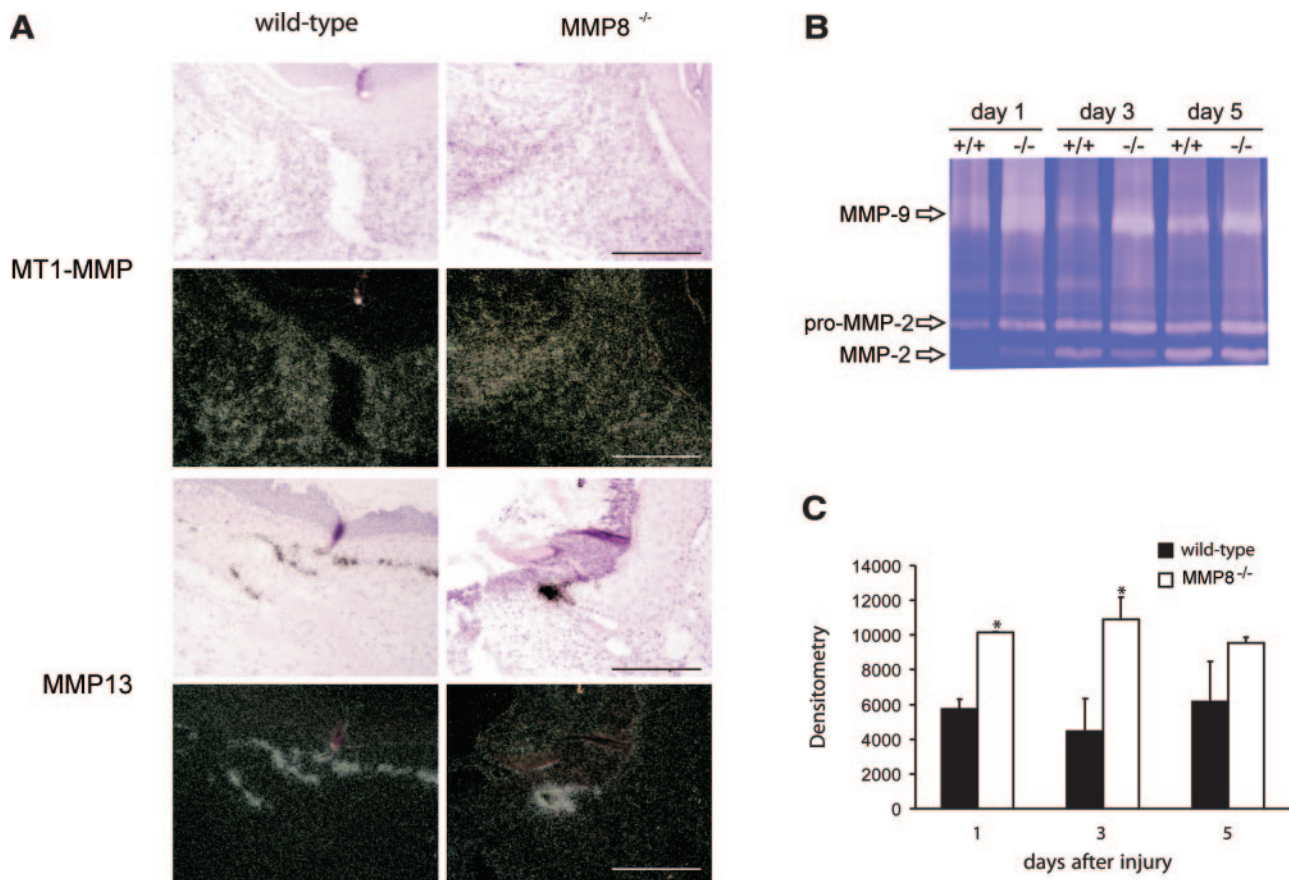


Figure 6. Altered expression of MMPs in $MMP8^{-/-}$ wounds. **A)** Representative *in situ* hybridizations of day 5 wounds with specific probes for *MT1-MMP/MMP14* and *MMP13* in wild-type and $MMP8^{-/-}$ mice. Top panels show dark-field images and bottom panels the corresponding bright-field images. Differences in the expression pattern of *MMP13* could be observed between wounds from wild-type and $MMP8$ deficient mice. Cells expressing *MMP13* are confined to keratinocytes of the leading wound edge in both genotypes, but due to the delay in re-epithelialization in $MMP8^{-/-}$ wounds, the distribution pattern of *MMP13* expression is broader in wild-type wounds. Scale bars: 200 μ m. **B)** Gelatin zymography of wound extracts reveals that MMP-9 production at the wound site is increased in $MMP8^{-/-}$ animals, while production of MMP-2 does not show major differences. **C)** Densitometric quantification of MMP-9 gelatinolytic bands shown in **B** ($n=3$; $*P<0.05$).

to perform additional studies to uncover the functional relevance of this newly identified complex.

DISCUSSION

MMPs are widely associated with processes in which extensive remodeling of the extracellular matrix is required (3, 4). Indeed, MMPs, including the three collagenases (MMP-1, MMP-8, and MMP-13), as well as MMP-2, -3, -9, -19, -26, and MMP14/MT1-MMP, have been shown to be expressed at wound sites during the healing process (14, 36–40). However, although blockade of their activity with MMP inhibitors delays wound healing (7, 8), all MMP-deficient animals previously described do not show major abnormalities in wound healing in the skin with the exception of *MMP3* (stromelysin-1)-deficient animals, which show a defect in wound contraction (11, 14, 41, 42). This raised the question of whether the critical MMP had not been determined or whether no one MMP was essential for the wound healing. The recent availability of mutant

mice deficient in *MMP8* has allowed us to investigate its individual contribution to the wound healing process (21). In the present study, we demonstrate the functional relevance of this enzyme in cutaneous wound repair by showing that $MMP8^{-/-}$ mice exhibit a significant delay in wound closure and re-epithelialization. We also show that this delay is associated with marked alterations in the inflammatory response characteristic of the wound healing process. Moreover, this healing defect can be completely rescued by transplantation of bone marrow from wild-type animals to $MMP8^{-/-}$ mice, demonstrating that *MMP8* derived from inflammatory cells is necessary for the wound healing process to proceed normally.

The alterations observed in $MMP8^{-/-}$ mice differentially affect the two distinct phases of the cutaneous repair process. Thus, during the first 48 h after wounding, recruitment of inflammatory cells to the wound site is delayed in $MMP8^{-/-}$ mice, suggesting that inflammatory cells are unable to extravasate into the wound site. This could be due to an inability to degrade type I collagen, or to impaired production or mobilization of

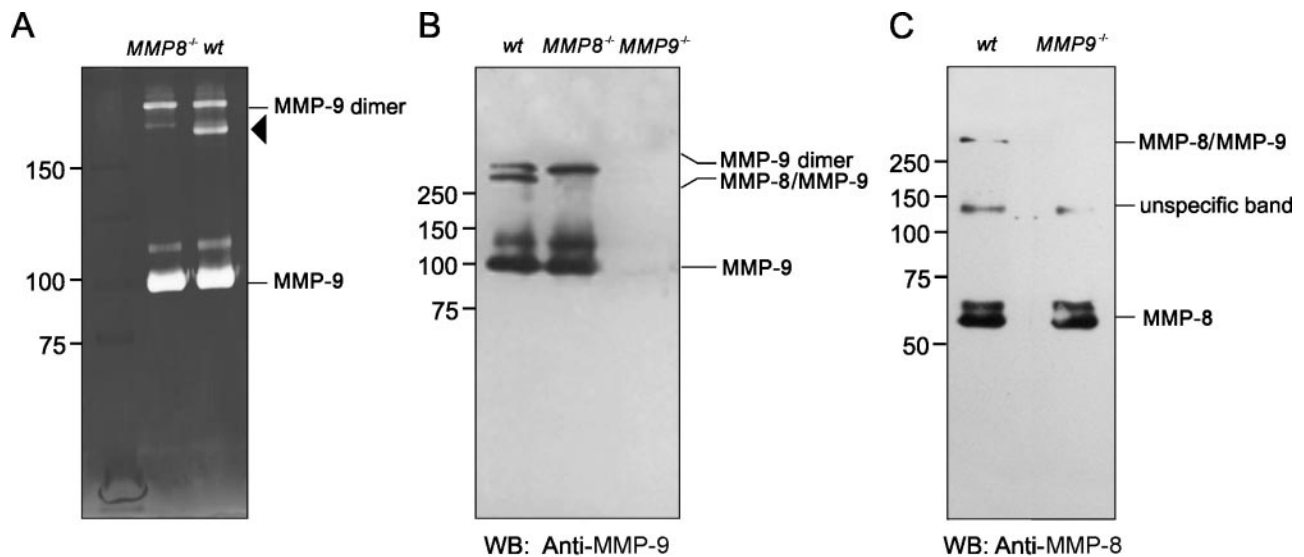


Figure 7. MMP-8 forms specific complexes with MMP-9. Bone marrow cells (10^6) from the indicated genotypes were treated with TPA to induce degranulation, and the presence of MMP-9 or MMP-8 was detected by gelatin zymography or Western blot. *A*) Gelatin zymography of supernatant from TPA-treated bone marrow cells reveals the presence of similar amounts of MMP-9 activity in wild-type and *MMP8*^{-/-} mice. A high molecular mass gelatinolytic band (arrowhead) is present in wild-type cells but absent in *MMP8*-deficient cells. *B*) Western blot analysis of supernatant from TPA-treated bone marrow cells with antibodies against murine MMP-9 confirms that MMP-9 forms part of the high-molecular-mass gelatinolytic band absent in *MMP8*^{-/-} cells. No hybridization bands are detected in the supernatant of *MMP9*^{-/-} cells. *C*) Western blot analysis of the same supernatants as above using an antibody against murine MMP-8 shows that the high-molecular-mass gelatinolytic band contains MMP-8, and the complex is specifically formed between MMP-8 and MMP-9, as the complex is not present in *MMP9*^{-/-} cells.

proinflammatory signaling molecules in *MMP8*^{-/-} animals. However, a failure of *MMP8*^{-/-} inflammatory cells to transmigrate to the wound site would be expected to result in an accumulation of inflammatory cells in surrounding blood vessels, an effect that we did not observe in *MMP8*^{-/-} wounds.

The mechanism by which the delayed infiltration of neutrophils into the wound area retards the repair process in *MMP8*^{-/-} mice appears to be dependent on TGF- β 1 signaling, a central regulator of inflammation and wound healing. In fact, animals lacking Smad3 show accelerated wound healing and reduced local inflammation, while overexpression of Smad2 in the epidermis under the control of the keratin-14 promoter has been shown to delay wound healing (33, 34), indicating that these proteins are critical for normal healing in the skin. In this regard, our results show reduced levels of active TGF- β 1 and an increased activation of Smad2 and moderate Smad3 phosphorylation in *MMP8*^{-/-} mice when compared to wild-type animals from day 1 to day 3 postinjury, suggesting that this altered signaling pathway could be responsible for the abnormal inflammatory response and the delay in re-epithelialization and wound healing observed in these mutant mice. Additionally, we observed a delay of 2 days in the expression of the chemokines KC and MIP-2 in *MMP8*^{-/-} wounds. However, this was evident later in the wound healing process around day 5, suggesting that these chemokines are not responsible for the initial defects observed in these animals. These results are in agreement with previous observations in TNF- α -induced lethal hepatitis in *MMP8*^{-/-} mice, in

which leukocyte infiltration is also impaired during the first hours (43), and confirm the importance of MMP-8 in neutrophil recruitment *in vivo*.

In marked contrast with the observed delay in the earliest steps of the wound healing process in *MMP8*^{-/-} mice, a sustained inflammatory response was found in wounds from these mice at later steps. This phase of healing, extending from days 3 to 9, is characterized by a massive apoptotic death of neutrophils, which are then phagocytosed by invading macrophages (31). Accordingly, the sustained inflammation exhibited by *MMP8*^{-/-} wounds is likely due to a defect in neutrophil apoptosis, since we observed decreased numbers of apoptotic cells in *MMP8*^{-/-} wounds. The apoptosis defect observed during wound healing in *MMP8*^{-/-} mice also confirms and extends previous findings showing a reduction in neutrophil apoptosis after induction of asthma in these mutant mice (32). This finding also suggests that MMP-8 might be implicated in other inflammatory diseases in which defective apoptosis and persistent inflammation is observed (43, 44).

Although previous reports had shown that MMP-8 expression was associated with healing wounds as well as with nonhealing ulcers, thereby suggesting that this enzyme might impair wound repair (20, 36), our data indicate that it plays an overall beneficial effect and is necessary for the healing process to proceed normally. The association between MMP-8 and nonhealing ulcers, such as those present in diabetic patients (45), might just reflect the sustained inflammatory response associated with these processes. The participation of

MMPs in wound healing has been traditionally linked to their ability to cleave different components of the extracellular matrix. In this sense, although MMP-8 was first discovered as a collagenolytic enzyme, our results suggest it has additional functions independent of its collagen degrading activity. In fact, loss of *MMP8* results in complete healing of skin wounds 13–15 days postinjury, 5 days later than in wild-type mice, indicating that its activity is necessary for this process to proceed normally, but is dispensable for wound closure, as this process is completed in *MMP8*^{-/-} animals. Similarly, re-epithelialization is delayed in these mice, being complete about two days later than in their wild-type littermates.

As extensive remodeling of the extracellular matrix occurs during the re-epithelialization phase, we cannot rule out the possibility that the absence of collagenolytic activity provided by MMP-8 might affect remodeling of the extracellular matrix. However, due to the presence of numerous proteases released by keratinocytes, fibroblasts, and inflammatory cells in the wound area, it is possible that other extracellular matrix-cleaving proteases might compensate the loss of MMP-8, thus allowing the wound healing process to be completed despite its absence. When we examined the status of other members of the MMP family that have been previously associated with wound healing, we found that the expression of *MMP13* (collagenase-3) and *MMP9* showed some differences between wild-type and *MMP8*^{-/-} wounds. *MMP13* was highly expressed during the early phase of wound healing at the keratinocyte migration leading edge in wild-type animals (27), but its distribution pattern in *MMP8*^{-/-} wounds was more confined to the leading edge, in agreement with the smaller size of the edge. These observations suggest that the absence of MMP-8 might be partially compensated by an altered expression of *MMP13*. The expression of *MMP13* may be increased by the presence of unprocessed type I collagen in skin wounds (27), as shown for animals containing the collagenase-resistant collagen Col1a1^{tm1Jae}. This suggests that the lack of MMP-8, which preferentially cleaves type I collagen (46), may lead to the accumulation of unprocessed type I collagen and therefore to the increased expression of *MMP13*. Although the altered expression of *MMP13* in *MMP8*^{-/-} wounds could compensate the lack of MMP-8, *MMP13*^{-/-} animals do not show defects in wound healing (14). However, *MMP13*^{-/-} mice show enhanced expression of *MMP8* in the wound area (14), confirming the importance of the latter neutrophil enzyme in the wound healing process. These results, together with the observations presented herein, suggest that functional compensatory mechanisms between MMP-8 and MMP-13 might allow the completion of the wound healing process in the absence of either protease. Generation of double knockout mice deficient for both collagenases could contribute to clarify this possibility. Unfortunately, the genes encoding these enzymes are closely linked in a small region of mouse chromosome 9A1 (47, 48), thereby preventing

the generation of double deficient mice by simply breeding of animals deficient in each protease.

In this study, we also observed that *MMP8*^{-/-} wounds show an up-regulation of MMP-9. This effect was specific to the healing process and not the result of a general increase of this protease in *MMP8*^{-/-} mice, because bone marrow cells from wild-type and *MMP8*^{-/-} mice secreted similar amounts of MMP-9. Previous reports have shown that animals deficient in *MMP9* show an enhancement in wound healing, indicating that this protease inhibits the rate of wound closure in cornea and skin (41). Together, these data suggest that the increased secretion of MMP-9 might contribute to the healing defects observed in *MMP8*^{-/-} animals. Interestingly, the relationship between both proteases is further illustrated by the novel finding that MMP-8 and MMP-9 form specific complexes *in vivo*. The functional relevance of this complex in the biological activity of both proteases is currently under investigation. In this regard, it is interesting to notice that recent reports have shown that MMP-8 binding to the neutrophil cell membrane prevents inactivation of this protease by tissue inhibitors of metalloproteases-1 and -2 (TIMP-1 and -2) (49). Similarly, interaction of MMP-9 with neutrophil gelatinase-associated lipocalin (Ngal) protects MMP-9 from degradation, which results in an overall increase in MMP-9 enzymatic activity (50). The finding that MMP-8 and MMP-9 form protein complexes *in vivo* raises the possibility that this complex might represent an additional regulatory mechanism by which the activity of these MMPs is controlled, or by which both proteases could act coordinately to process specific substrates.

This work provides the first causal evidence that MMP-8 is necessary for the healing of skin wounds, as its absence results in a significant delay in wound closure. The defects in wound healing and re-epithelialization observed in *MMP8*^{-/-} mice are due to an altered inflammatory response that can be fully restored by performing a bone marrow transplantation using wild-type donors. These results confirm and extend the importance of this metalloproteinase in the regulation of inflammation. Furthermore, we have shown that MMP-8 may function in a coordinate fashion with MMP-9 during the wound healing process, as both proteases form stable and specific complexes *in vivo*. Finally, these data might shed insights into the biological function of MMP-8, and the mechanisms implicated in the protective effect of this neutrophil enzyme against skin tumor progression. EJ

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