# Large induction of the chemotactic cytokine RANTES during cutaneous wound repair: a regulatory role for nitric oxide in keratinocyte-derived RANTES expression

Stefan FRANK[1], Heiko KÄMPFER, Christian WETZLER, Birgit STALLMEYER and Josef PFEILSCHIFTER

Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

We investigated the role of NO on expressional regulation of the chemotactic cytokine RANTES (**r**egulated upon **a**ctivation, **n**ormal **T**-cell **e**xpressed and **s**ecreted) during tissue regeneration using an excisional wound-healing model in mice. Wound repair was characterized by a large and sustained induction of RANTES expression, and inhibition of inducible nitric oxide synthase (iNOS) during repair only slightly decreased RANTES expression levels. Immunohistochemical analysis revealed keratinocytes of the wound margins and the hyperproliferative epithelium to be the main RANTES-expressing cell type within the wound. Therefore we analysed the regulation of RANTES expression *in vitro* in cultured human keratinocytes of the cell line HaCaT. Here we demonstrate that NO very efficiently suppressed interleukin-1$\beta$- and tumour-necrosis-factor-$\alpha$-induced RANTES expression in keratinocytes. Furthermore, down-regulation of cytokine-induced RANTES mRNA in keratinocytes was dependent on endogenously produced NO, as inhibition of the co-induced iNOS by L-$N^G$-monomethyl-L-arginine increased cytokine-triggered RANTES expression in the cells. Moreover, we observed strongest RANTES-immunopositive labelling in epithelial areas which were characterized by a NO-mediated low cellularity. Thus our data implicate NO as a negative regulator of RANTES expression during wound repair *in vivo*, as decreased numbers of keratinocytes observed in the absence of wound-derived NO might compensate for the high levels of RANTES expression which are associated with normal repair.

## INTRODUCTION

The process of tissue regeneration after injury is characterized by four overlapping phases involving haemostasis, inflammation, proliferation and the remodelling phase of repair. Cutaneous injury initiates re-epithelialization and is followed by granulation-tissue formation. Central to this latter process is the infiltration of macrophages, ingrowth of fibroblasts, matrix deposition, and angiogenesis into the regenerating area [1]. Inflammation, re-epithelialization, and granulation-tissue formation are driven in part by a complex mixture of growth factors and cytokines that are released into the area of injury. Besides these protein-type factors and mitogens, an important role for the process of cutaneous wound repair has been demonstrated for NO. Wound closure was delayed in inducible nitric oxide synthase (iNOS)-deficient mice [2], and a functionally active iNOS has been shown to be a crucial prerequisite for keratinocyte proliferation and high-level expression of vascular endothelial growth factor during repair [3–5].

It is well known that the early inflammatory phase of repair is characterized by the infiltration of neutrophils and macrophages which are attracted into the wound site by chemotactic factors. These immune cells have been shown to represent an important source of growth factors or cytokines, and hence both cell types are central to the process of tissue regeneration [6–9]. Thus the regulated attraction of leucocytes is essential to ensure a well-ordered inflammatory process that initiates tissue repair. Important to this process are chemokines which share important structural features and the ability to attract leucocytes [10,11]. Two families of chemokines, which contain four cysteine residues in their amino acid sequence, have been characterized extensively: the $\alpha$- (or CXC-) chemokines (the first two cysteine residues are separated by one amino acid) and the $\beta$- (or CC-) chemokines (the first two cysteine residues are adjacent to each other). $\alpha$- and $\beta$-chemokines exhibit functional diversity, as $\alpha$-chemokines are chemotactic for neutrophils and lymphocytes, whereas $\beta$-chemokines act on monocytes, activated T-cells, eosinophils and basophils [10,11]. The observed cell-specificity is mediated by CXC- and CC-chemokine receptors, which only recognize the chemokines of the corresponding subfamily [12].

Most chemokines are expressed under pathological conditions by resident tissue cells and infiltrating leucocytes [13]. Since macrophages are key players in the process of granulation-tissue formation and, therefore, tissue regeneration, we investigated the role of RANTES (**r**egulated upon **a**ctivation **n**ormal **T**-cell **e**xpressed and **s**ecreted) during cutaneous wound repair. RANTES is a member of the $\beta$-chemokine subfamily and thus acts as a chemotactic signal attracting monocytes into the wound site. Although enhanced production of RANTES has been described for inflammatory diseases of the skin, including atopic dermatitis and psoriasis [14–17], the regulation of this prototypical $\beta$-chemokine still remains unclear for the process of tissue regeneration.

In the present study we investigated the expression pattern of RANTES during the highly dynamic process of cutaneous wound repair. We provide evidence that keratinocytes are the main

Abbreviations used: iNOS, inducible nitric oxide synthase; L-NIL, L-$N^6$-(1-iminoethyl)lysine; L-NMMA, L-$N^G$-monomethyl-L-arginine; RANTES, **r**egulated upon **a**ctivation, **n**ormal **T**-cell **e**xpressed and **s**ecreted; TNF, tumour necrosis factor; IL, interleukin; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; GSNO, *S*-nitrosoglutathione; KGF, keratinocyte growth factor; IFN-$\gamma$, interferon-$\gamma$; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hyperproliferative epithelium/epithelia.

[1] To whom correspondence should be sent at the following address: Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany (e-mail S.Frank@em.uni-frankfurt.de).

producers of RANTES during repair. Furthermore, our data suggest that cytokine-induced RANTES expression might be down-regulated by wound-derived NO in keratinocytes.

## EXPERIMENTAL

### Inhibitor treatment of mice

Mice were wounded as described below. As described recently, female Balb/c mice (3 months old) were injected intraperitoneally twice a day at 07.00 h and 19.00 h with 2.5 mg of L-$N^6$-(1-iminoethyl)lysine (L-NIL) in 0.5 ml of PBS per injection for 13 days [4,5]. L-NIL represents a highly selective inhibitor of iNOS enzymic activity [18]. Nitrite accumulation in wound lysates, measured as a read-out for iNOS enzymic activity, was clearly inhibited to control levels in L-NIL-treated animals [4,5]. Control mice were injected with PBS. L-NIL was from Alexis Corporation (Grünberg, Germany).

### Wounding and preparation of wound tissues

To examine RANTES expression during the wound-healing process, six full-thickness wounds were created on each animal, and skin biopsy specimens from four animals were obtained 1, 3, 5, 7 and 13 days after injury. To investigate the effect of L-NIL on RANTES expression, wounds from four L-NIL-treated mice were obtained 1, 3, 5, 7 and 13 days after injury. Mice were anaesthetized with a single intraperitoneal injection of Ketamin (80 mg/kg body weight)/Xylazin (10 mg/kg body weight). The hair on the back of these mice was shaved off, and the back was subsequently wiped with 70 % ethanol. Six full-thickness wounds (approx. 4–6 mm in diameter, 3–4 mm apart) were made on the backs of these mice by excising the skin and the underlying panniculus carnosus. The wounds were allowed to dry to form a scab. An area 7–8 mm in diameter which included the scab and the complete epithelial margins was excised at each time point. As a control, a similar amount of skin was taken from the backs of four non-wounded mice. For every experimental time point, the wounds from four animals ($n = 16$ wounds) and the non-wounded back skin from four animals respectively were combined, frozen immediately in liquid nitrogen and stored at $-80$ °C until used for RNA isolation. All animal experiments were carried out according to the guidelines of, and with the permission from, the local government of Hessen.

### Immunohistochemistry

Mice were wounded as described above. Animals were killed at day 1, 3 and 5 after injury. Complete wounds were isolated from the middle of the back, bisected, and frozen in tissue freezing medium. Frozen sections, 6 $\mu$m in thickness, were fixed with acetone and treated for 10 min at room temperature with 1 % $H_2O_2$ in PBS to inactivate endogenous peroxidases. They were subsequently incubated for 60 min at room temperature with a polyclonal antiserum against human RANTES (PeproTech, Frankfurt, Germany) (1:50 diluted in PBS/0.1 % goat serum albumin). The slides were subsequently stained with the avidin–biotin–peroxidase complex system from Santa Cruz (Heidelberg, Germany) using 3-amino-9-ethylcarbazole as a chromogenic substrate. After development, they were rinsed with water, counterstained with haematoxylin (Sigma, Deisenhofen, Germany) and mounted.

### Cell culture

The human keratinocyte cell line HaCaT [19] was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal-calf serum (FCS). Under these culture conditions the keratinocytes remain proliferative and undifferentiated. For the RANTES-induction experiments, cells were grown to confluency without changing the medium and rendered quiescent by a 24 h incubation in serum-free DMEM. Cells were then incubated for various periods in fresh DMEM containing serum, growth factors, cytokines or reagents for the indicated time periods. Aliquots of cells and cell-culture supernatants were harvested before, and at different time points after, treatment with these agents and used for RNA isolation or ELISA respectively. Each experiment was done in triplicate. FCS and DMEM were purchased from Gibco Life Technologies, Inc. (Eggenstein, Germany), growth factors and cytokines were from Roche Biochemicals (Mannheim, Germany), L-$N^G$-monomethyl-L-arginine (L-NMMA) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) were from Alexis Inc. (Grünberg, Germany).

### ELISA

A 10 ml portion of keratinocyte-conditioned cell-culture supernatants from the individual experimental time points was pooled and cleared by centrifugation. A 100 $\mu$l sample of the cell-culture supernatants was subsequently analysed for the presence of immunoreactive RANTES protein by ELISA using the Quantikine human RANTES kit (R&D Systems, Wiesbaden, Germany) as described by the manufacturer.

### RNA isolation and RNase protection analysis

RNA isolation was performed as described [20]. A 30 $\mu$g sample of total RNA from wounded or non-wounded skin, or 20 $\mu$g of RNA from the cell-culture experiments, respectively, was used for RNase protection assays. RNase protection assays were carried out as described previously [21]. All protection assays were carried out with at least three different sets of RNA from independent wound-healing or cell-culture experiments. Protected fragments were analysed by PhosphoImager (Fuji, Straubenhardt, Germany) analysis of the radiolabelled gels.

### Probe cDNAs

The murine and human RANTES cDNA probes and the murine interleukin (IL)-$\beta$ and tumour-necrosis-factor (TNF)-$\alpha$ cDNA probes respectively were cloned by PCR. The cloned cDNA fragments correspond to nucleotides 40–298 (for murine RANTES), nucleotides 27–279 (for human RANTES), nucleotides 481–759 (for murine IL-1$\beta$) or nucleotides 541–814 (for murine TNF-$\alpha$) of the published sequences [22–25].

### S-Nitrosoglutathione (GSNO) synthesis

GSNO was synthesized as described previously [26]. Briefly, glutathione was dissolved in 0.625 M HCl at 0 °C to a final concentration of 625 mM. An equimolar amount of $NaNO_2$ was added and the mixture was stirred at 0 °C for 40 min. After the addition of 2.5 vol. of acetone, stirring went on another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80 % acetone, two times with 100 % acetone, and finally three times with diethyl ether and was dried under vacuum. GSNO was characterized by u.v. spectroscopy.

### Statistical analysis

Data are shown as means $\pm$ S.D. The results are presented either as $x$-fold induction compared with the unstimulated control

(100%), or as mean concentrations (pg/ml). Results were analysed by unpaired Student's $t$ test on raw data using Sigma Plot (Jandel Scientific, Erkrath, Germany).

## RESULTS

### Expression of RANTES is highly induced during wound repair

As NO has been shown to represent an important mediator during wound repair [2–5], we were interested in whether expression levels of RANTES might be influenced by NO during wound healing. Therefore we first investigated the time course of RANTES mRNA expression during this process. We isolated total RNA from full-thickness excisional wounds at different intervals after injury from PBS-treated control mice and L-NIL-injected mice and performed RNase protection assays. RNA from two independent wound-healing experiments were analysed for RANTES expression. For the RNase protection assays shown in Figure 1, note that every experimental time point represents 16 wounds ($n = 16$) isolated from four animals. As shown in Figure 1 ('ctrl skin'), we could detect a low level of basal expression of RANTES mRNA in normal back skin. Upon injury, a strong and rapid increase in RANTES mRNA expression was observed within 24 h after wounding. We detected constantly high levels of RANTES mRNA (20–30-fold compared with control) between day 1 and 7 after injury. RANTES mRNA induction reached a peak at day 3 of the repair process. RANTES mRNA levels remained elevated even at the late phase of repair (day 13), when all wounds were completely re-epithelialized, but the granulation tissue still revealed a high cellularity (results not shown). Notably, a comparable time course of RANTES expression was seen in L-NIL-treated animals, although RANTES mRNA levels seemed to be decreased at day 5 and 7 after wounding (Figure 1B). Note that every experimental time point in Figure 1(B) represents a total of 32 wounds ($n = 32$) from two independent wound-healing experiments.

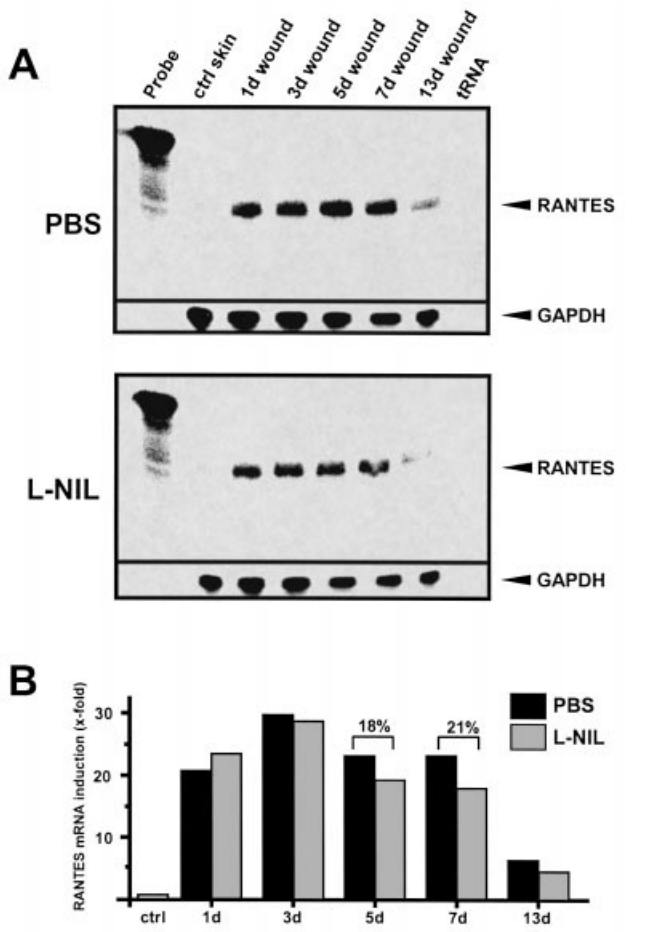


**Figure 1 Large induction of RANTES mRNA expression during wound repair**

(**A**) Total cellular RNA (30 $\mu$g) from normal and wounded skin from PBS-treated control mice and L-NIL-treated mice (as indicated) was analysed by RNase protection assay for the expression of RANTES. A total of 16 wounds ($n = 16$) from the backs of four animals were excised for each experimental time point and used for RNA isolation. The time after injury is indicated at the top of each lane. Control skin refers to non-wounded skin of normal mice. A 1000 c.p.m. portion of the hybridization probe was added to the lane labelled 'Probe'. A 50 $\mu$g sample of tRNA was hybridized as a negative control. Expression of GAPDH mRNA is shown as a loading control for both experiments (lower panels). The degree of RANTES mRNA induction, as assessed by PhosphoImager (Fuji) analysis, of the radiolabelled gels from two independent wound healing experiments is shown schematically in (**B**). Note that every experimental time point represents 32 wounds ($n = 32$ wounds) from eight animals from two independent wound-healing experiments. The percentages of decreased RANTES expression in L-NIL-treated mice are given above in the square brackets.

### Expression of RANTES protein in 1-, 3- and 5-day mouse wound tissue

As a next step we examined whether the observed increase of RANTES mRNA expression after skin injury correlates with the presence of immunoreactive RANTES protein in wounds. For this purpose we investigated the localization of RANTES during wound healing using sections from 1-, 3- and 5-day full-thickness mouse wounds. Sections were stained with a monospecific, polyclonal antibody against RANTES protein. In 1-day wounds, a remarkably strong expression of RANTES was seen in the epidermis and hair follicles directly adjacent to the wound site (Figure 2A). Keratinocytes had started to proliferate and migrate towards the area of injury, as the epidermis at the wound margins already appeared to be increased in diameter. At this very early stage of repair, when formation of the hyperproliferative epithelium (HE) has not yet been initiated, those keratinocytes located at the wound margins were observed to be strongly labelled with the anti-RANTES antibody. Furthermore, a second population of labelled cells was located directly within the wounded area at this early stage of repair. These cells are most likely to represent infiltrating immune cells. Although polymorphonuclear neutrophils are the first cells invading the wound area, we could not observe a labelling of those cells which are characterized by a segmented nucleus after counterstaining with haematoxylin, which is a typical morphological feature of polymorphonuclear leucocytes. For these reasons, the particularly strongly labelled infiltrating cells might represent macrophages which accumulate at the wound site following the preceding neutrophils. In 3-day wounds, localization of RANTES expression did not change, as keratinocytes of the thickened epidermis adjacent to the wound expressed RANTES protein (Figure 2B). Remarkably, with this antibody, immunopositive signals were obtained in keratinocytes, particularly within the developing HE at the wound margins, whereas epidermal keratinocytes distant from the wound remained unlabelled (Figure 2B). Finally, we demonstrated keratinocytes of the large HE to be the major source of RANTES protein in 5-day wounds (Figure 2C), as the underlying granulation tissue revealed almost no labelled cells. Weakly stained cells within the granulation tissue seem to represent migrating fibroblasts and infiltrating mononuclear immune cells and, therefore, most likely macrophages (Figure 2C).

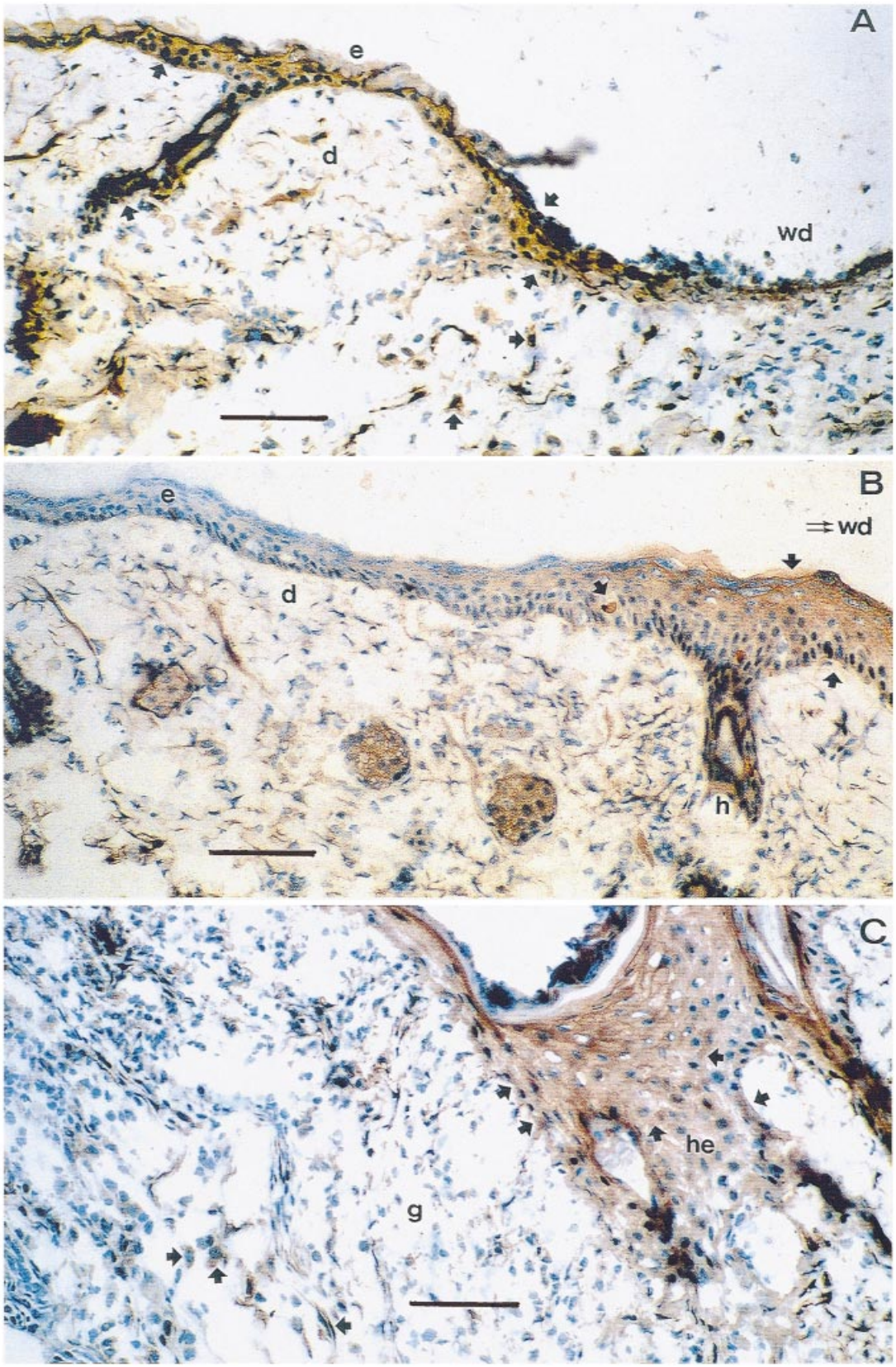


**Figure 2 Expression of RANTES protein in 1-, 3- and 5-day mouse wounds**

(**A** and **B**) Frozen serial sections from representative 1- or 3-day wounds isolated from PBS-injected control mice. (**C**) Frozen serial section from a representative 5-day wound isolated from L-NIL-treated mice. Sections were incubated with a monospecific polyclonal antibody directed against human RANTES and stained with the avidin–biotin–peroxidase complex system using 3-amino-

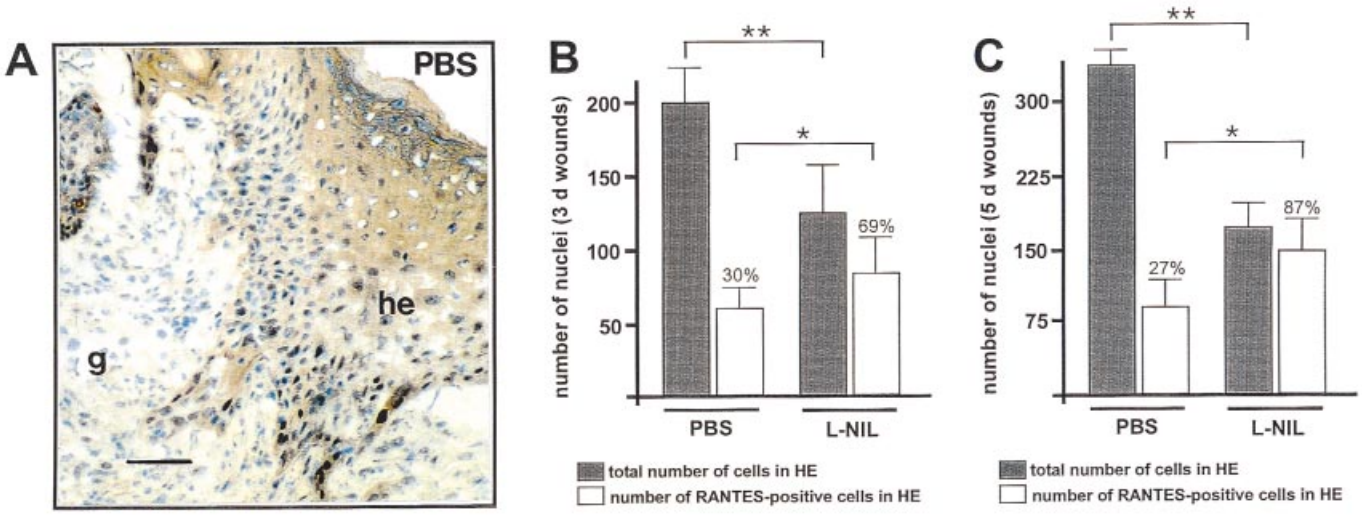


**Figure 3 RANTES expression correlates with cellularity of the epithelial wound margins**

(**A**) Frozen serial section from a representative 5-day wound isolated from PBS-injected control mice. The section was incubated with a monospecific polyclonal antibody directed against human RANTES and stained with the avidin–biotin–peroxidase complex system using 3-amino-9-ethylcarbazole as a chromogenic substrate. Nuclei were counterstained with haematoxylin. g, granulation tissue; he, HE. Low-magnification photographs (71 ×) of frozen serial sections of 3-day wounds (**B**) or 5-day wounds (**C**) were analysed for total keratinocyte cell numbers within whole HE from PBS-treated control mice and L-NIL-injected mice ($n = 3$) as indicated (grey bars). This was done by counting haematoxylin-stained keratinocytes. The same HE were analysed for the RANTES-expressing proportion of keratinocytes (white bars) for PBS-treated, or L-NIL-treated mice as indicated. This was done by counting keratinocyte numbers of strongly RANTES-labelled areas of the HE. Data are expressed as total number of keratinocyte nuclei, or RANTES-positive keratinocytes ± S.D. ($n = 3$). $^{**}P < 0.01$; $^{*}P < 0.05$ as indicated above the square brackets; percentages mean compared with total keratinocyte numbers as indicated.

### NO-mediated differences in epithelial cellularity are associated with RANTES expression

Very recently we demonstrated that inhibition of iNOS during repair was directly associated with a strong decrease in keratinocyte proliferation [5]. A clearly diminished number of keratinocytes within the HE of L-NIL-treated mice was observed compared with the situation found in PBS-treated animals (see Figures 3B and 3C). Note that, in contrast with the situation observed for L-NIL-treated mice (Figure 2C), the HE in PBS-treated control mice were formed by a dense mass of keratinocytes (Figure 3A). Moreover, the RANTES-specific staining appeared to be stronger in those proliferative epithelia, that are characterized by an apparently low cellularity. To assess this observation further, we analysed frozen serial sections from 3- and 5-day wounds ($n = 3$ wounds from three independent animals) of PBS- or L-NIL-treated mice respectively. For this reason we counted haematoxylin-stained nuclei within the HE to determine total keratinocyte numbers. Furthermore, we counted haematoxylin-stained nuclei of the strongly RANTES-immunolabelled areas to assess total numbers of RANTES-expressing cells within the epithelia. As shown in Figures 3(B) and 3(C), total numbers of keratinocytes (grey bars) within the HE were clearly diminished in L-NIL-treated mice. This is consistent with our previous observations [5]. However, RANTES expression appeared to be directly associated with NO-mediated differences in epithelial cellularity. We observed markedly diminished numbers of strongly immunolabelled cells (white bars) in epithelia of PBS-treated control mice compared with L-NIL-injected mice (see Figure 3B). The observed effect was even more pronounced in 5-day wounds, where the differences in keratinocyte cell numbers were further enhanced after inhibition of iNOS enzymic activity (see Figure 3C).

### Induction of RANTES expression by NO, serum growth factors and keratinocyte growth factor (KGF)

Since we have observed a potential regulatory role of NO for keratinocyte-derived RANTES expression during skin repair, we used the human keratinocyte cell line HaCaT [19] as an *in vitro* system to investigate the regulation of RANTES expression. We first tested the potency of the NO-donating agent GSNO to modulate serum-mediated RANTES expression in keratinocytes. Remarkably, GSNO (500 $\mu$M), 10% FCS and the purified growth factors epidermal growth factor (10 ng/ml), platelet-derived growth factor (10 ng/ml) and transforming growth factor $\beta$1 (10 ng/ml) alone or a combination of FCS or growth factors given simultaneously with GSNO did not induce RANTES expression in keratinocytes *in vitro* (results not shown). Also, for KGF (10 ng/ml), which has been shown to be highly expressed during repair [21], we could not detect an induction of RANTES expression in keratinocytes (results not shown).

9-ethylcarbazole as a chromogenic substrate. Nuclei were counterstained with haematoxylin. A 1-day wound is shown in (**A**) (PBS-treated), the wound margins of a 3-day wound in (**B**) (PBS-treated) and the 5-day wound in (**C**) (L-NIL-treated). Scale bars are 50 $\mu$m for (**A**)–(**C**). Strongly immunopositive signals within the sections are indicated with arrows. d, dermis; e, epidermis; g, granulation tissue; h, hair follicle; he, HE; wd, wound site. In this Figure and in Figure 3 the brownish colour represents the anti-RANTES immunoreactivity, whereas the blue colour results from counterstaining the tissue with haematoxylin, which stains the nuclei.

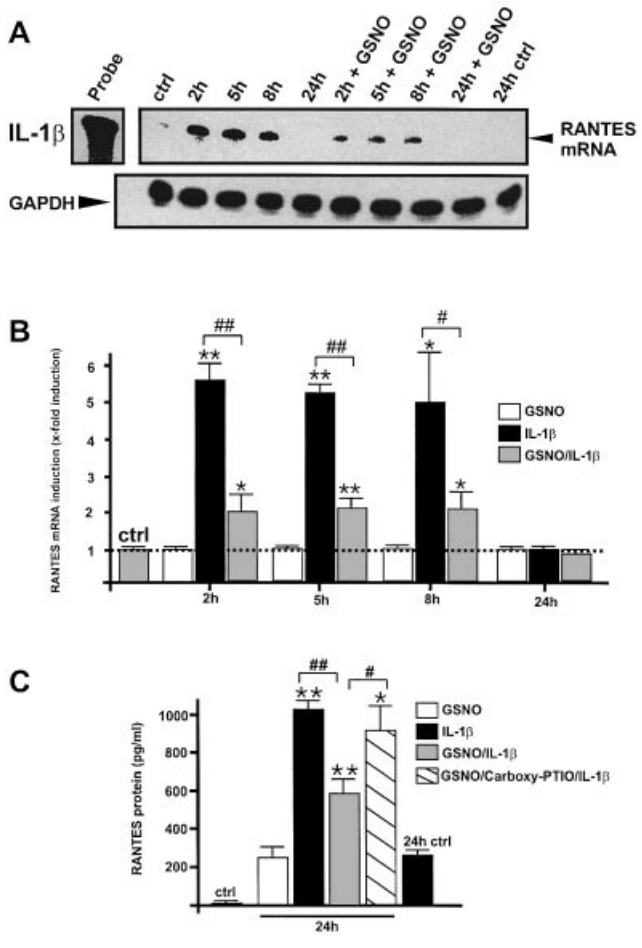


**Figure 4 GSNO suppresses IL-1$\beta$-induced RANTES mRNA and protein expression in keratinocytes**

(**A**) Serum-starved keratinocytes were stimulated for different time periods with IL-1$\beta$ (2 nM) in the presence or absence of 500 $\mu$M GSNO as indicated. A 20 $\mu$g portion of total cellular RNA from these cells was analysed by RNase protection assay for the expression of RANTES mRNA (**A**, upper panel). Simultaneous GAPDH hybridization of total cellular RNA from the same experiment was used as a loading control (**A**, lower panel). A 1000 c.p.m. portion of the hybridization probe was used as a size marker. The degree of RANTES mRNA induction as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gels is shown schematically in (**B**). Data are expressed as *x*-fold induction of unstimulated control. Mean percentage changes in RANTES mRNA levels $\pm$ S.D. are shown ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control. $\#P < 0.05$; $\#\#P < 0.01$ compared with the conditions as indicated above the square brackets. The total amount of RANTES-specific proteins in IL-1$\beta$- or IL-1$\beta$/GSNO-stimulated keratinocyte cell-culture supernatants as determined by ELISA are shown in (**C**). NO-dependency of GSNO-mediated effects is demonstrated by reversal of GSNO-mediated effects by the NO-scavenging reagent carboxy-PTIO (500 $\mu$M). Data are expressed as mean concentrations $\pm$ S.D. ($n = 3$). $^{**}P < 0.01$ compared with the 24 h control. $\#\#P < 0.01$ compared with the conditions as indicated above the square brackets.

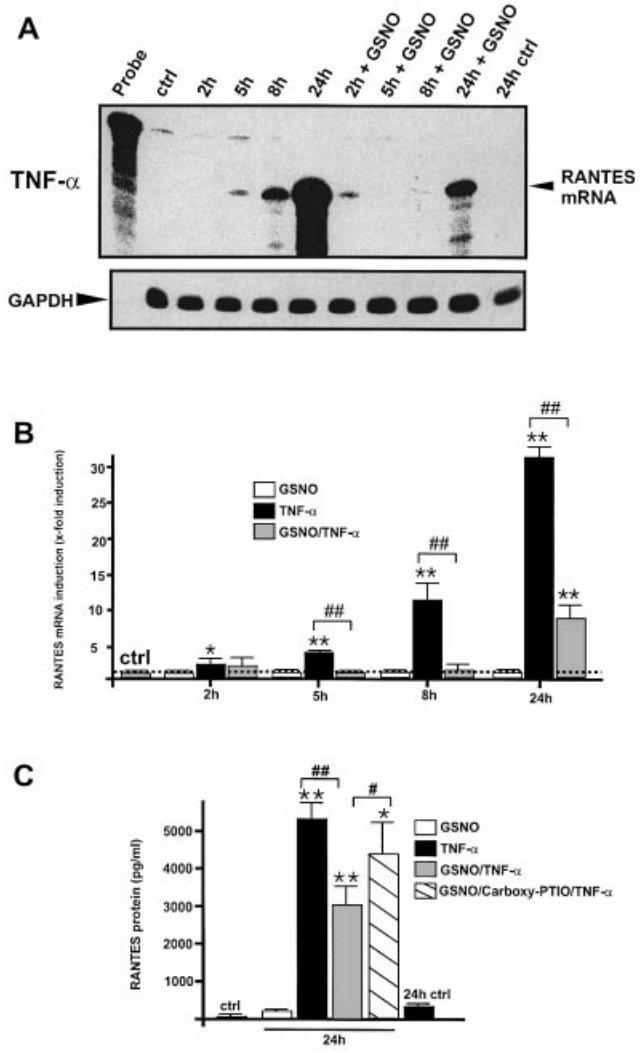


**Figure 5 GSNO decreases TNF-$\alpha$-mediated RANTES mRNA and protein expression in keratinocytes**

(**A**) Keratinocytes were rendered quiescent by serum starvation and subsequently treated with TNF-$\alpha$ (2 nM) in the presence or absence of 500 $\mu$M GSNO for different time points as indicated. A 20 $\mu$g portion of total cellular RNA from these cells were analysed by RNase protection assay for the expression of RANTES mRNA (**A**, upper panel). GAPDH hybridization of the same set of RNA was used as a loading control (**A**, lower panel). The degree of induction as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gels is shown schematically in (**B**). Data are expressed as *x*-fold induction of unstimulated control. Mean percentage change in RANTES mRNA levels $\pm$ S.D. are shown ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control. $\#\#P < 0.01$ compared with the conditions as indicated by the brackets. The total amount of RANTES-specific proteins in TNF-$\alpha$- or TNF-$\alpha$/GSNO-stimulated keratinocyte cell-culture supernatants as determined by ELISA are shown in (**C**). NO-dependency of GSNO-mediated effects is demonstrated by reversal of GSNO-mediated effects by the NO-scavenging reagent carboxy-PTIO (500 $\mu$M). Data are expressed as mean concentrations $\pm$ S.D. ($n = 3$). $^{**}P < 0.01$ compared with the 24 h control. $\#\#P < 0.01$ compared with the conditions indicated by the brackets.

### Cytokine-induced RANTES expression is suppressed by GSNO

Since the infiltration of immune cells is another crucial event in wound repair, we tested the ability of cytokines produced by these cells to induce RANTES expression in the presence or absence of the NO-donor GSNO. As shown in Figure 4, IL-1$\beta$ rapidly induced RANTES mRNA levels (5–6-fold) within 2 h of stimulation. Elevated levels of RANTES mRNA could be observed for 8 h. The induction of RANTES mRNA by IL-1$\beta$ was only transient, as RANTES mRNA could not be detected after 24 h of stimulation (Figures 4A and 4B). IL-1$\beta$-mediated RANTES mRNA induction was dramatically decreased in the presence of 500 $\mu$M GSNO. This down-regulatory effect of GSNO (60–70 % decrease in IL-1$\beta$-induced RANTES mRNA levels) could be observed for all time points where RANTES mRNA levels are elevated after IL-1$\beta$ stimulation (Figures 4A and 4B). This down-regulation of IL-1$\beta$-induced RANTES

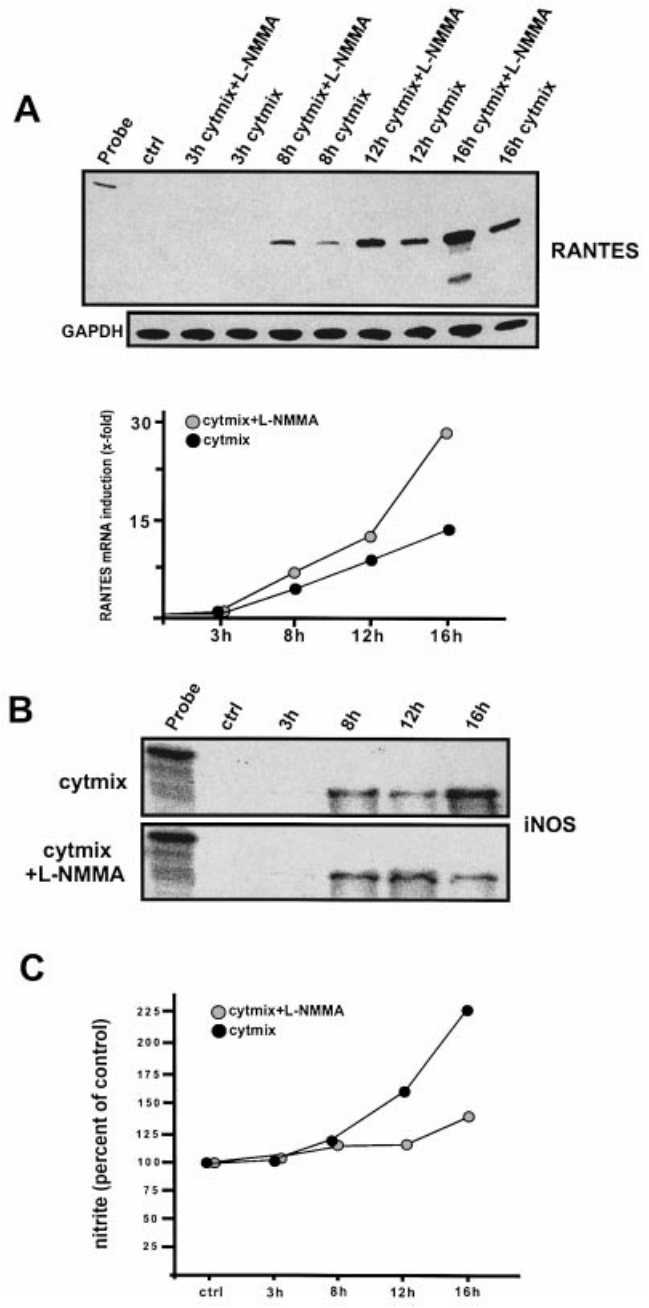


**Figure 6 Inhibition of endogenously produced NO triggers an enhanced expression of cytokine-induced RANTES mRNA**

(**A**) Keratinocytes were rendered quiescent by serum starvation. They were subsequently stimulated using a combination of IL-1$\beta$ (2 nM), TNF-$\alpha$ (2 nM) and IFN-$\gamma$ (100 units/ml) ('cytmix') in the presence or absence of the NOS inhibitor L-NMMA (2 mM) for 3, 8, 12 and 16 h as indicated. A 20 $\mu$g portion of total cellular RNA from these cells was analysed by RNase protection assay for RANTES mRNA expression. The L-NMMA-mediated increase in RANTES mRNA as assessed by PhosphoImager (Fuji) analysis of the radiolabelled gel is shown in the lower panel. One representative experiment is shown. (**B**) The same set of RNA was simultaneously analysed for iNOS mRNA expression. (**C**) Nitrite accumulation in the supernatants from the same cell-culture experiment was measured as a readout for iNOS enzymic activity and the inhibitory potency of L-NMMA.

expression by GSNO was not due to a loss in cell viability, as proved by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as a control (Figures 4A and 5A) and determining cell viability using the Trypan Blue method (results not shown). The observed suppressive effect of GSNO at the mRNA level is clearly followed by a decrease in RANTES protein secretion into the cell-culture supernatants (Figure 4C).

Figure 5 demonstrates the situation for TNF-$\alpha$-stimulated keratinocytes. TNF-$\alpha$ was a very potent inducer of RANTES mRNA and protein expression in keratinocytes. In contrast with IL-1$\beta$, TNF-$\alpha$ mediated a long-lasting RANTES mRNA expression, with the highest levels observed after 24 h of stimulation. Furthermore, RANTES mRNA levels increased about 30-fold (Figure 5B) compared with the 5-fold less induction by IL-1$\beta$ (Figure 4B). Interestingly, GSNO (500 $\mu$M) is able to suppress TNF-$\alpha$-mediated RANTES mRNA nearly completely within the first 8 h of induction. After 24 h, TNF-$\alpha$-treated cells revealed a 70–80 % decrease in RANTES mRNA levels in the presence of GSNO (500 $\mu$M) compared with cells stimulated with TNF-$\alpha$ alone. These results could be confirmed at the protein level, as TNF-$\alpha$-mediated the secretion of large amounts of RANTES protein (5400 pg/ml) into the cell-culture supernatants that could be diminished to about 50 % after simultaneous treatment of cells with GSNO (Figure 5C). Note that, as shown in Figure 4(C) and Figure 5(C) (hatched bars), the GSNO-mediated decrease in IL-1$\beta$-, or TNF-$\alpha$-induced RANTES protein could be reversed, at least partially, by simultaneously added carboxy-PTIO (500 $\mu$M). Carboxy-PTIO represents a water-soluble and stable NO scavenger that shows antagonistic action against the free NO radical. Carboxy-PTIO was found to react with NO in a stoichiometric manner [27]. Thus inhibition of GSNO-mediated down-regulation of IL-1$\beta$-, or TNF-$\alpha$-induced RANTES expression by carboxy-PTIO indicated that the observed effects were indeed mediated by the NO moiety released from GSNO. By contrast, we were not able to detect an induction of RANTES mRNA and protein after stimulation with interferon-$\gamma$ (IFN-$\gamma$), even after 24 h of treatment (results not shown). This negative result was not due to loss of a functional receptor, as the HaCaT cell line used indeed responds to a IFN-$\gamma$ stimulus with induction of gene expression [28].

### Cytokine-induced RANTES mRNA expression is counter-regulated by endogenously produced NO

Recently we demonstrated that keratinocytes of the wound margins and the HE express iNOS during repair [3]. Since exogenously added NO down-regulated IL-1$\beta$- and TNF-$\alpha$-induced RANTES expression in keratinocytes, we investigated whether endogenously produced NO has the potency to influence RANTES expression in these cells. As iNOS and subsequent nitrite accumulation in the cell culture supernatants could be induced in keratinocytes only by a combination of cytokines [28], we stimulated the cells using a 'cytmix' (2 nM IL-1$\beta$/2 nM TNF-$\alpha$/100 units/ml IFN-$\gamma$) to co-induce iNOS and RANTES expression. As shown in Figures 6(A) and 6(B), the cytmix co-induced RANTES and iNOS expression in the cells. Addition of L-NMMA (2 mM), a potent inhibitor of all NOS isoenzymes, further enhanced the induction of cytokine-induced RANTES mRNA (Figure 6A). Therefore cytokine-induced RANTES mRNA levels appeared to be counter-regulated by a co-induced and functionally active iNOS. Inhibition of NOS enzymic activities could be demonstrated by measuring nitrite levels in the cell-culture supernatants of the same experiment (Figure 6C). These data suggest that both exogenous and endogenous NO are negative regulators of RANTES expression in keratinocytes.

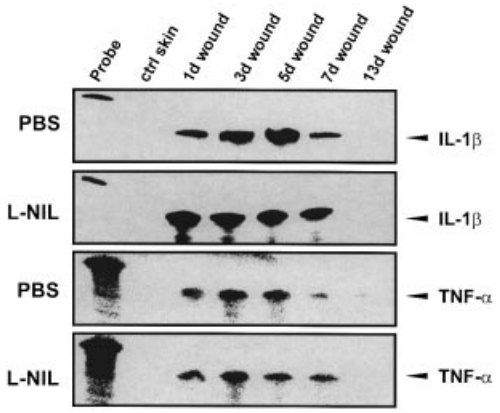


**Figure 7 IL-1$\beta$ and TNF-$\alpha$ are highly induced during wound healing in L-NIL-treated mice**

Total cellular RNA (30 $\mu$g) from normal and wounded skin from PBS-treated control mice and L-NIL-treated mice (as indicated) was analysed by RNase protection assay for the expression of the RANTES-inducing inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ as indicated. A total of 16 wounds ($n = 16$) from the backs of four animals were excised for each experimental time point and used for RNA isolation. The time after injury is indicated at the top of each lane. Control skin refers to non-wounded skin of normal mice. A 1000 c.p.m. portion of the hybridization probe were added to the lane probe. One representative experiment is shown.

### Sustained high level expression of IL-1$\beta$ and TNF-$\alpha$ in L-NIL-treated animals

Exogenously applied as well as endogenously produced NO is most likely to negatively regulate cytokine-induced RANTES expression in keratinocytes. As L-NIL-treated mice, which are characterized by a clearly diminished number of wound keratinocytes [5], are most likely to compensate for high-level expression of RANTES in the absence of a functionally active iNOS, we had to ensure the presence of essentially those cytokines during NO-deficient repair that were able to potently induce RANTES in keratinocytes. IL-1$\beta$ and TNF-$\alpha$, respectively, which have been proved to be potent RANTES-inducing cytokines in keratinocytes *in vitro*, were strongly expressed in L-NIL-injected animals (Figure 7). Notably, IL-1$\beta$ and TNF-$\alpha$ mRNAs even appeared to be slightly elevated at day 7 post-wounding.

## DISCUSSION

The wound healing process, which is initiated upon injury to overcome tissue damage, represents a highly dynamic process. Resident cells and invading cell types have to develop a complex system of intercellular communication to ensure an at least partial reconstruction of the injured tissue. For these reasons, protein-type growth factors, mitogens and cytokines are central to this process in orchestrating tissue regeneration [1,29]. Besides these protein-type factors, a small diffusible radical gas, namely NO, has been shown to be another key player within this highly ordered intercellular communication during wound healing. Expression of iNOS is largely induced during the inflammatory phase of repair [3], indicating a role for NO in tissue regeneration, as iNOS-deficient mice are characterized by a severe delay in wound closure [2]. These data implicate a potential role of iNOS for a normal healing process, but the molecular mechanisms underlying NO action during repair remain largely unknown. Very recently we were able to demonstrate that regulation of keratinocyte proliferation, and, moreover, keratinocyte-derived vascular-endothelial-growth-factor expression, was severely impaired when iNOS enzymic activity was inhibited during the repair process [4,5]. These data implicate a regulatory role for NO as a mediator molecule controlling epithelial actions during repair. As keratinocytes of the developing HE at the wound margins were major producers of RANTES, a negative regulatory role of NO for epithelially derived RANTES expression might be implicated during repair. This suggestion is supported by the observation that wounds isolated from L-NIL-treated mice were characterized by RANTES expression levels that were maintained at nearly those elevated levels observed in PBS-injected control animals. As immunohistochemistry revealed keratinocytes of the developing HE at the wound margins to be the major source of RANTES during repair, our findings suggest that the clearly diminished number of wound keratinocytes observed during tissue repair in L-NIL-treated mice [5] (and the present study) might compensate for high-level RANTES expression, since a counter-regulatory effect of exogenous (derived from macrophages) and endogenous NO (keratinocytes) is neutralized by the functional inhibition of iNOS. Thus we observed an expression pattern for RANTES which is most likely associated with the NO-mediated differences in cellularity of the developing epithelia, as the strongest expression of RANTES was observed in those epithelial areas which were characterized by a low cellularity (especially in L-NIL-treated mice). The observed counter-regulatory mechanism appeared to be even more pronounced at those stages of repair when keratinocytes at the wound margins develop into major producers of RANTES during repair (days 5 and 7 after injury).

As a next step, we assessed a potential role of NO in mediating a negative regulatory mechanism in keratinocyte-derived RANTES production using the human keratinocyte cell line HaCaT [19]. Consistent with the finding that a variety of cell types, including keratinocytes, is able to secrete chemokines as a response to the early proinflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ [16,17,30], HaCaT keratinocytes responded to IL-1$\beta$, or TNF-$\alpha$ with the production of RANTES. Remarkably, serum growth factors and KGF, which are present during the initial phase of repair [21,29], did not induce RANTES expression in the cells. In line with our *in vivo* observations, interestingly, IL-1$\beta$- and TNF-$\alpha$-induced RANTES expression could be suppressed by NO *in vitro*. Remarkably, TNF-$\alpha$ mediated a strong induction of RANTES in keratinocytes, whereas IL-1$\beta$ was much less potent. This observation further supports our suggestion that RANTES expression might be correlated with NO-mediated keratinocyte proliferation. After addition of TNF-$\alpha$, which is well known to be cytostatic for keratinocytes [31], we could observe large amounts of RANTES mRNA and protein after stimulation. By contrast, the keratinocyte mitogen IL-1$\beta$ [32] mediated only a moderate expression of RANTES compared with TNF-$\alpha$. Furthermore, one might speculate that the observed differences might be due to a TNF-$\alpha$-inducible factor which further contributes to, and enhances, TNF-$\alpha$-triggered RANTES production. Possibly, the co-induced factor might be under the regulatory control of NO, thus amplifying the differences observed for NO-mediated down-regulation of TNF-$\alpha$- or IL-1$\beta$-mediated RANTES expression. Moreover, NO has the potency to interfere with cytokine-activated signal-transduction pathways, as the presence of either exogenous NO, or endogenously produced NO, markedly decreased cytokine-triggered RANTES expression. Until now, however, cellular targets of NO action have been poorly defined and have not been clearly elucidated [33].

Our observation might resemble a feedback regulatory mechanism which is mediated by NO during the repair process. Neutrophils, although expressing iNOS [3,34], are only a minor

source of NO production during healing [34]. Thus RANTES might be induced efficiently by neutrophil-derived IL-1$\beta$ and TNF-$\alpha$ in keratinocytes located at the wound margins. As a next step, monocytes start to infiltrate the wound, particularly as a result of RANTES expression and release, subsequently differentiating into activated macrophages. Activated wound macrophages express iNOS [3,34] and produce large amounts of NO, which is released into the wound fluid [34]. For these reasons, one could speculate that macrophage-derived NO might down-regulate RANTES expression in keratinocytes, and, thus, control an overshooting of macrophage accumulation at the wound site. As inflammatory cytokines are potent inducers of iNOS in keratinocytes [28], as well as keratinocytes of the wound margins and the HE are known to express iNOS [3], endogenously produced NO might further contribute to the observed down-regulation of RANTES expression in these cells. This NO-driven counter-regulatory mechanism is further supported by the observation that high expression levels of RANTES-inducing cytokines IL-1$\beta$ and TNF-$\alpha$, which have been shown to be present during normal repair [9], were not down-regulated in wounds from L-NIL-treated animals. Two very recent reports further strengthen the observed function of NO as a negative regulator of RANTES expreon. The anti-arthritic effects of an acyclic nucleoside phosphonate in a rat model of adjuvant-induced arthritis were coupled to substantially reduced nitrite and nitrate levels in the serum which were directly associated with greatly enhanced systemic levels of RANTES [35]. Additionally, using a rat model of endotoxic shock, it has been shown that supplementation of L-arginine increased the release of glomerular nitrite and simultaneously diminished glomerular RANTES expression after injection of bacterial lipopolysaccharide. Inhibition of the L-arginine/NO pathway subsequently increased glomerular RANTES mRNA expression and the number of infiltrating macrophages [36].

In summary, our data suggest that neutrophil-derived IL-1$\beta$ and TNF-$\alpha$ initiate expression of RANTES in keratinocytes during early wound repair. Furthermore, our findings suggest that NO might be involved in the regulation of macrophage infiltration into the wound site by down-regulating the expression of macrophage-attracting RANTES protein in keratinocytes.

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