

ORIGINAL ARTICLE

IL-5-overexpressing mice exhibit eosinophilia and altered wound healing through mechanisms involving prolonged inflammation

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Leucocytes are essential in healing wounds and are predominantly involved in the inflammatory and granulation stages of wound repair. Eosinophils are granulocytic leucocytes and are specifically regulated by interleukin-5 (IL-5), a cytokine produced by T helper 2 (Th2) cells. To characterize more clearly the role of the IL-5 and eosinophils in the wound healing process, IL-5-overexpressing and IL-5-deficient mice were used as models of eosinophilia and eosinophil depletion, respectively. Our results reveal a significantly altered inflammatory response between IL-5-overexpressing and IL-5 knockout mice post-wounding. Healing was significantly delayed in IL-5-overexpressing mice with wounds gaping wider and exhibiting impaired re-epithelialization. A delay in collagen deposition was observed suggesting a direct effect on matrix synthesis. A significant increase in inflammatory cell infiltration, particularly eosinophils and CD4⁺ cells, one of the main cell types which secrete IL-5, was observed in IL-5-overexpressing mice wounds suggesting that one of the main roles of IL-5 in wound repair may be to promote the infiltration of eosinophils into healing wounds. Healing is delayed in IL-5-overexpressing mice and this corresponds to significantly increased levels of eosinophils and CD4⁺ cells within the wound site that may contribute to and exacerbate the inflammatory response, resulting in detrimental wound repair.

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Wound healing is a complex process, vital for the maintenance and integrity of the skin.¹ Leucocytes are imperative in healing wounds and are predominantly involved in the inflammatory and granulation stages. Infiltration of macrophages,¹ neutrophils² and monocytes³ into wounds occurs in a distinctive order⁴ and while present in the wound they are responsible for phagocytosis of damaged tissue and invading pathogens, as well as secretion of chemo-attractants. In addition to these leucocytes, mast cells⁵ and eosinophils⁶ have also been implicated in the healing process. Eosinophils are granulocytic leucocytes normally present at low levels in the blood. Under normal conditions, eosinophils typically constitute 1–5% of white blood cells;^{7,8} however, the exact function of the eosinophil remains elusive. Eosinophils have phagocytic ability and are present in high numbers in asthma, allergic responses and parasitic infections,⁹ suggesting an involvement in the pathology or quite possibly in tissue homeostasis and repair.

The growth, differentiation, activation and survival of eosinophils are regulated by interleukin-5 (IL-5), a cytokine produced by T helper 2 (Th2) cells as well as by eosinophils and mast cells. Although earlier

studies suggested additional activities of IL-5 in regulating mouse B cells and antibody production, recent studies using an IL-5-deficient mouse strain indicate that the regulation of eosinophilia may be the only obligatory role for IL-5 in the adult mouse.¹⁰ Eosinophils have been recorded in the cutaneous wounds of mice, rabbits and hamsters, often in close proximity with fibroblasts.⁶ Interaction of eosinophils with fibroblasts, causing proliferation¹¹ and matrix production,¹² along with the accumulation of eosinophils in fibrotic disorders,¹³ suggests that eosinophils may influence the remodelling stages of wound healing.

Interleukin-5-deficient mice produced by gene targeting of embryonal stem cells appear normal and do not develop any obvious disease¹⁰ but are resistant to induction of experimental asthma¹⁴ and have impaired resistance to secondary infections with the helminth *Nippostrongylus brasiliensis*.¹⁵ IL-5-deficient mice have near normal levels of baseline eosinophils; however, these eosinophils are less likely to infiltrate into the areas of disease or infection.¹⁰ In contrast, IL-5-overexpressing mice have a more severe asthma phenotype and are highly resistant to primary infections with *N. brasiliensis* and *Strongyloides ratti*.^{16,17}

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Therefore, to more clearly characterize the role of IL-5 in the wound process, IL-5-deficient and IL-5 transgenic mice were used as models using an incisional wound healing model.

RESULTS

IL-5 transgenic mice have increased peripheral eosinophil numbers
Skin from IL-5 transgenic, wild-type and IL-5-deficient mice was assessed using real-time PCR for IL-5 gene expression. IL-5 transgenic mice had an approximately 40-fold increase in IL-5 gene expression compared to wild-type, while IL-5-deficient mice had a negligible amount of IL-5 (Figure 1a). Peripheral blood from the three mouse lines was assessed for eosinophils and results showed a 35-fold increase in eosinophils in the IL-5-overexpressing mice compared to wild-type and few circulating eosinophils were observed in the IL-5-deficient mice (Figure 1b).

IL-5 is increased in wounds of IL-5-overexpressing mice

Interleukin-5 was not detectable in unwounded skin from wild-type, IL-5 knockout or IL-5 transgenic mice (Figure 2a). No positive staining for IL-5 was observed at any time point in the IL-5 knockout mice confirming specificity of the IL-5 antibody. In wild-type mice, IL-5 levels increased in response to wounding peaking at 5 days and returning to baseline at 14 days post-wounding (Figures 2a and b). Positive IL-5 fluorescence was observed in inflammatory cells within the granulation tissue of the wound bed (Figure 2a). In the wounds of IL-5-overexpressing mice, IL-5 was greatly increased and unlike their wild-type counterparts this expression did not resolve but remained elevated even at 14 days post-wounding ($P < 0.001$ vs wild-type and IL-5-deficient mice) (Figures 2a and b).

Wound healing is delayed in IL-5-overexpressing mice

Representative macroscopic images of 0, 3, 5, 7 and 14 days wounds of IL-5-deficient, wild-type and IL-5-overexpressing mice are shown in Figure 3a. Planimetric analysis of surface wound area showed delayed healing in IL-5-overexpressing mice with significantly larger wounds observed when compared to wild-type and IL-5-deficient mice (Figure 3b). Histological analysis revealed that the distance between the epidermal wound margins was significantly larger in IL-5-overexpressing mice at 5 days post-injury, however by days 7 and 14 no significant differences were seen in any group (Figure 4b). Re-

epithelialization was calculated as the length of the new epithelium as a percentage of the total wound length and this was significantly delayed in IL-5-overexpressing mice. There was no significant difference in the percentage re-epithelialization in IL-5-deficient and wild-type mice (Figure 4c). By day 14, all experimental groups were 100% re-epithelialized (Figure 4c).

IL-5-overexpressing mice have elevated inflammatory wound infiltrates

The level of inflammation within the wounds was assessed semiquantitatively¹⁸ using haematoxylin and eosin sections. These were scored for the presence of leucocytes with a scale ranging from 1 (no or minimal infiltration) to 5 (abundant inflammatory infiltrate). Although no differences were observed between wild-type and IL-5-deficient mouse wounds at any time point examined (Figures 5a and b), there was at first a delay in inflammation at day 3 in wounds from IL-5-overexpressing mice, but this was followed by significantly increased inflammation at days 5 and 7 post-wounding (Figure 5b). By day 14, the inflammation had begun to resolve and there were no significant differences between the three groups (Figure 5b). The numbers of tissue eosinophils were counted in histological wound sections at days 3, 5, 7 and 14 post-wounding. In wild-type wounds, the numbers of tissue eosinophils were maximal at day 3, reducing to minimal levels by day 14. In contrast, wounds from IL-5-overexpressing mice had significantly elevated numbers of eosinophils, peaking at day 5 and these remained high through to day 14 (Figure 6).

IL-5-overexpressing mice have large CD4⁺ cell infiltration at day 5

Previous studies have revealed that depletion of T lymphocytes *in vivo* results in a decrease in wound strength.¹⁹ As Th2 cells are a major source of IL-5, immunohistochemistry was used to detect CD4⁺ cells in wounds. As day 5 had shown the largest differences in both wound measurements and eosinophil numbers, this point was chosen for more detailed analysis. IL-5-deficient mice were observed to have the lowest numbers of CD4⁺ cells within the wounds, whereas IL-5-overexpressing mice had significantly greater infiltration of CD4⁺ cells than wild-type mice (Figure 7b). CD4⁺ cells were predominantly located in the dermis adjacent to the wound site (Figure 7a). The numbers of B220⁺ cells were quantitated in IL-5-overexpressing, wild-type and IL-5-deficient wounds. No significant difference was

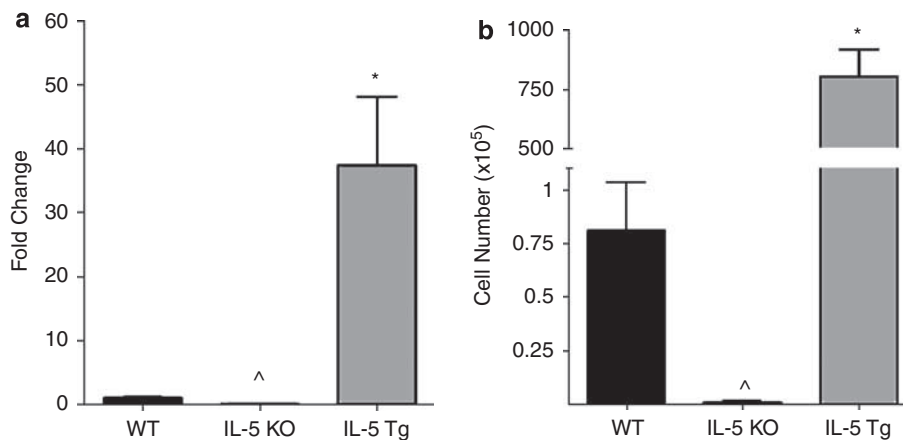


Figure 1 Interleukin (IL)-5 transgenic mice have increased IL-5 gene expression and peripheral eosinophil numbers. (a) The IL-5 levels from real-time quantitative PCR analysis in wild-type, IL-5 knockout and IL-5-overexpressing mice (b) circulating eosinophil numbers. * Represents significantly more than wild-type and IL-5 knockout mice and ^ represents significantly less than wild-type and IL-5-overexpressing mice ($P < 0.05$).

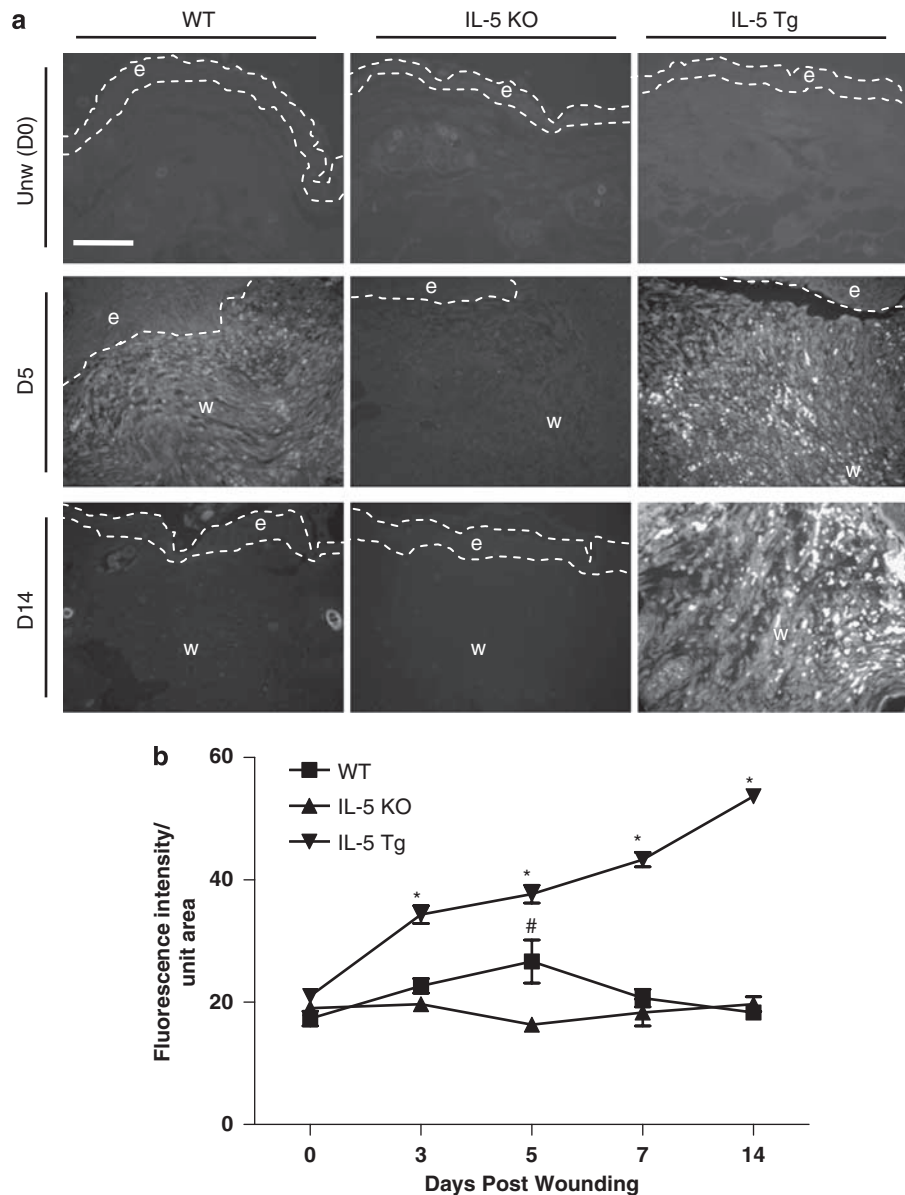


Figure 2 Interleukin (IL-5) levels are increased in wounds of IL-5 transgenic mice. **(a)** Representative images of wild-type, IL-5 knockout and IL-5 transgenic wounds immunostained with IL-5 at 0, 5 and 14 days post-injury. **(b)** Graph showing IL-5 fluorescence intensity per unit area in the wounds of IL-5-overexpressing mice, wild-type and IL-5-deficient cohorts at 0, 3, 5, 7 and 14 days post-wounding. Results represent means and s.e.m. $n=6$ wounds per mice group per time point. * Represents significantly greater than wild-type and IL-5 knockout mice wounds ($P<0.05$), # represents significantly greater than IL-5 knockout mice wounds ($P<0.05$). $n=6$ wounds per mice group. e, epidermis; w, wound; dotted line indicates position of epidermal/dermal connection. Magnification bar in **(a)**=100 μ m.

observed in B-cell expression between any of the groups assessed (Figure 7c), suggesting that although all groups have similar infiltration of B220⁺ cells, IL-5-overexpressing mice have a selective increase in CD4⁺ cells in their wounds.

Collagen deposition is impaired in IL-5-overexpressing mice

Total collagen production in IL-5-deficient, wild-type and IL-5-overexpressing wounds was assessed histologically using Masson's trichrome. Masson's trichrome selectively colours collagens green, and representative sections of IL-5-deficient, wild-type and IL-5-overexpressing wounds at 0, 5 and 14 days post-wounding are shown in Figure 8a. A 0–5 scoring system was used to semiquantitatively assess total collagen staining (Figure 8b). IL-5-overexpressing mice had

significantly reduced the levels of total collagen than wild-type and IL-5-deficient mice at day 5 post-wounding (Figure 8b) confirming the impaired healing of the IL-5-overexpressing mice. However, by day 7, no significant difference in collagen deposition was observed between any of the groups (Figure 8b).

DISCUSSION

This paper explores the role of IL-5 in a mouse model of incisional wound healing. Our results have revealed that healing is significantly delayed in IL-5-overexpressing mice with wounds gaping wider and exhibiting delayed re-epithelialization. A delay in collagen deposition was also observed, suggesting an effect on the synthesis of extracellular matrix. A significant increase in inflammatory cell infiltration,

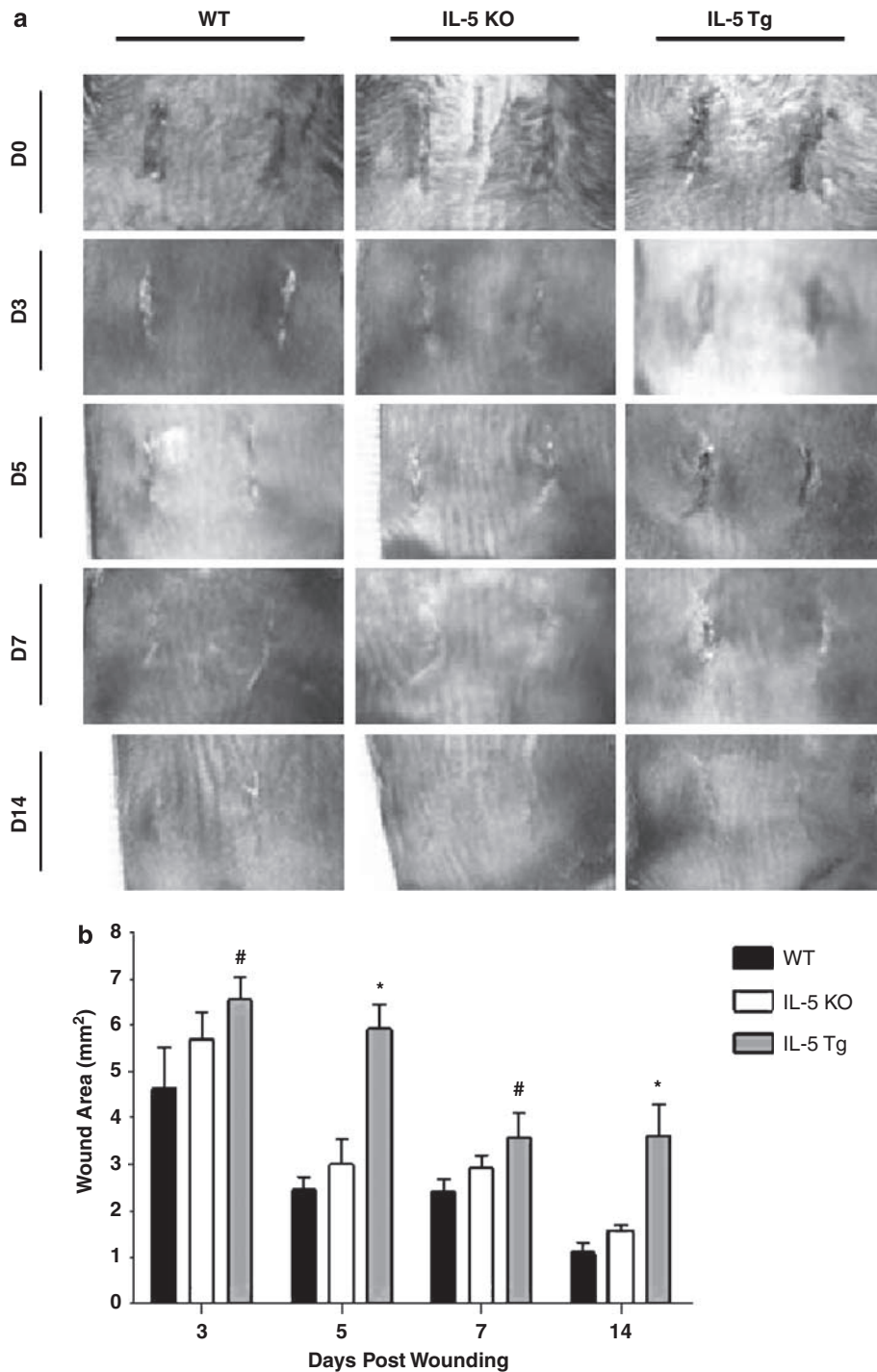


Figure 3 Wound healing is delayed in interleukin (IL)-5 transgenic mice. **(a)** Representative incisional wounds after 0, 3, 5, 7 and 14 days post-injury in IL-5-overexpressing wild-type and IL-5 knockout mice. **(b)** Graph showing surface wound area at 0, 3, 5, 7 and 14 days post-injury. Surface wound area was determined using planimetry measurements. * Denotes significantly different to wild-type and IL-5 knockout mice, # represents significantly different to values from wild-type mice ($P < 0.05$). Results represent mean \pm s.e.m. ($n=6$ wounds per group per time point).

particularly eosinophils and CD4⁺ cells, was observed in wounds from IL-5-overexpressing mice. In mice, the main activities of IL-5 are reported to be on eosinophils and B cells, whereas in humans IL-5 principally affects eosinophil lineage and its activities on B cells remain controversial.²⁰ Our results indicate that the recruitment of eosinophils and CD4⁺ cells to the wound site is influenced by the

activity of IL-5 and that both may contribute to impaired wound healing. Despite the observed delay in healing in IL-5-overexpressing mice, by 14 days post-wounding, there was no difference in any of the measured wound healing parameters between wild-type, IL-5 knockout or IL-5 transgenic mice, suggesting that the effect of IL-5 overexpression and increased eosinophilia on wound repair results in

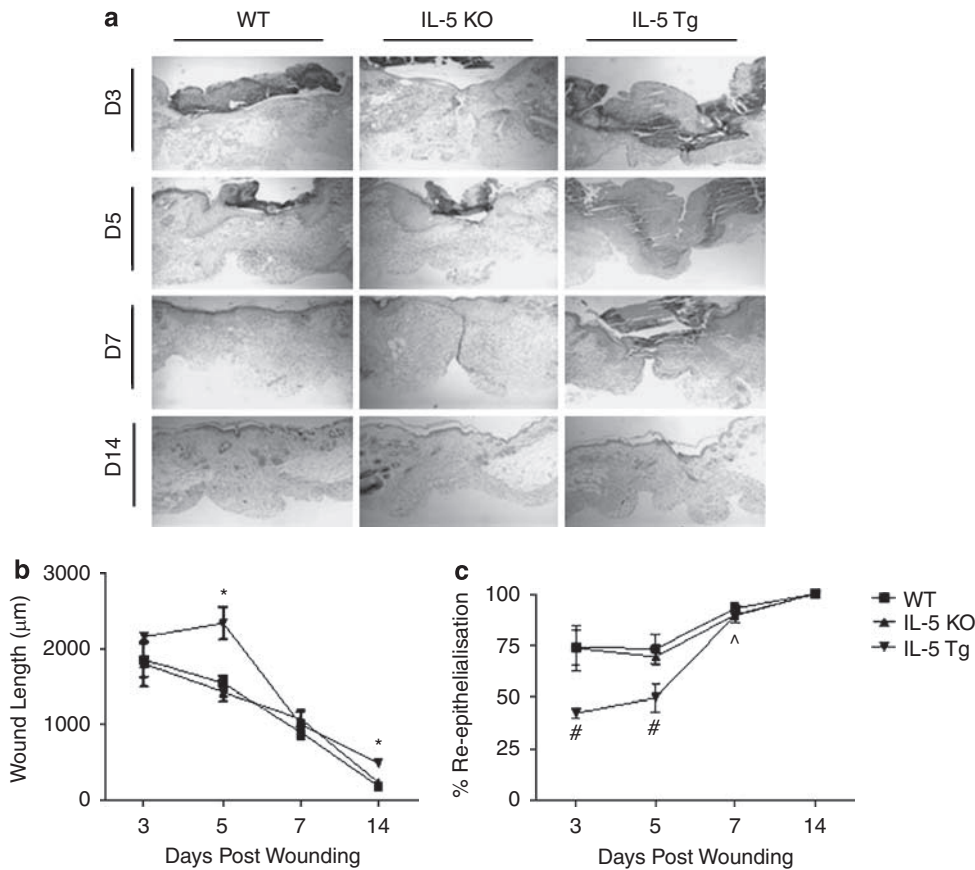


Figure 4 Microscopic analysis of interleukin (IL)-5 transgenic, wild-type and IL-5-deficient mouse wounds. (a) Representative haematoxylin and eosin-stained sections of full-thickness incisional wounds in IL-5-overexpressing wild-type and IL-5 knockout mice at 3, 5, 7 and 14 days post-injury. (b) Histological wound length was measured as the distance separating the epidermal edges of the wound. (c) Wound re-epithelialization was evaluated by measuring the length of neopidermis at days 3, 5, 7 and 14 days post-injury. * Denotes significantly different to wild-type and IL-5 knockout, # significantly less than wild-type and IL-5 knockout and ^ significantly less than wild-type mice ($P < 0.05$). Results represent mean \pm s.e.m. ($n = 6$ wounds per group per time point).

primarily a temporal delay and not an actual impairment in the quality of healing.²¹

The analysis of inflammatory cell infiltration into cutaneous wounds, demonstrates a heightened and prolonged response from eosinophils and CD4⁺ cells in IL-5-overexpressing mice. The significantly smaller inflammatory infiltrate seen on day 3 in wounds from IL-5-overexpressing mice is potentially a consequence of the delay in formation of extracellular matrix, as this is used as a base for migration.²² By days 5 and 7, a large inflammatory infiltrate rich in eosinophils was seen in wounds from IL-5-overexpressing mice. This was exaggerated relative to both wild-type and IL-5-deficient mice and may provide an explanation for the altered healing observed in the IL-5-overexpressing mice.

Although a larger number of eosinophils could be expected in the wounds of IL-5-overexpressing mice, simply due to the greater availability of eosinophils in the blood, this does not necessarily explain their persistence in the wounds. Apoptotic eosinophils may have been replaced at a high rate by newly recruited eosinophils and the overexpression of IL-5 may have prolonged eosinophil survival. The additional CD4⁺ cells recruited into wounds in IL-5-overexpressing mice may also have enhanced these processes. Eosinophils secrete chemoattractants, which perpetuate the inflammatory response, and this may account for the higher number of CD4⁺ cells seen in wound tissue from IL-5-overexpressing mice. T cells have previously been

demonstrated to accumulate in wounds during the formation of granulation tissue,²³ and this may be exacerbated by the enhanced recruitment of eosinophils. Links between T cells and hypereosinophilia,²⁴ an idiopathic disease often manifesting lesions of the skin and epithelia,²⁵ suggests that CD4⁺ cells may be important regulators of eosinophil activity in wound healing.

Interleukin-5 levels were increased in the wounds of wild-type mice peaking at day 5 post-wounding and returning to baseline levels by day 14, suggesting that it may play a role in the wound healing process potentially assisting in the chemoattraction of inflammatory cells to the wound site. IL-5 was significantly elevated in IL-5-overexpressing mice wounds and these levels did not resolve with time but remained elevated even at day 14 post-wounding. This increased expression of IL-5 in the wounds of IL-5-overexpressing mice coincides with the persistence of eosinophils and may contribute to their accumulation in the wound.

Previous studies have shown that healing is accelerated in hamsters wounds treated topically with IL-5 monoclonal antibody.²⁶ However, we saw no significant improvement in the rate of wound repair in IL-5-deficient mice compared to wild-type mice. This may be due in part to the localized application of neutralizing IL-5 antibodies that were directly injected in to the wound site to block specific wound-induced IL-5 functions as compared with systemic effects of gene knockout with the subsequent damping down of the entire IL-5 response. In the

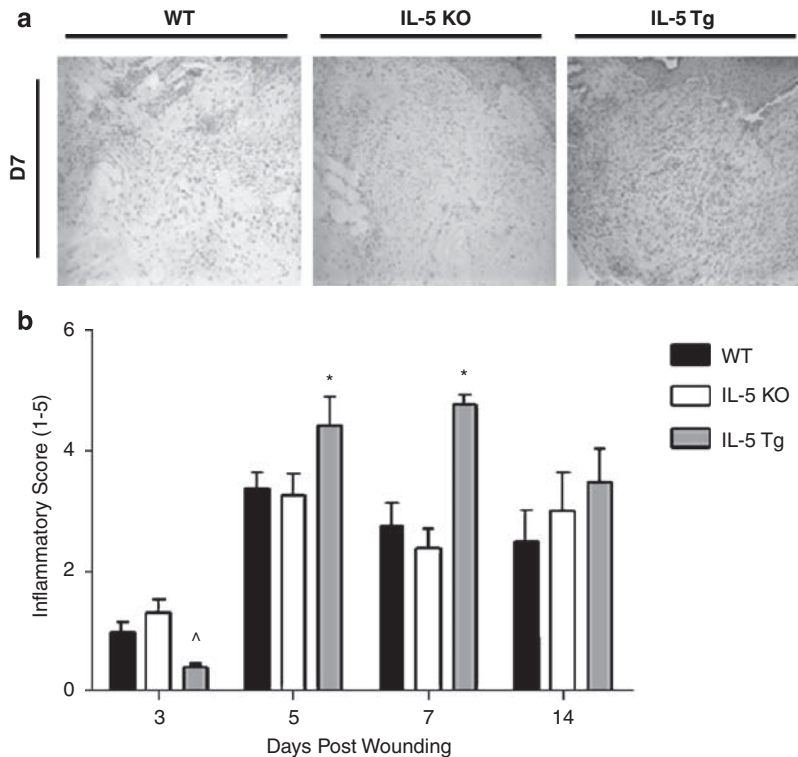


Figure 5 Interleukin (IL)-5 transgenic mice have elevated inflammatory infiltrate. (a) Representative haematoxylin and eosin-stained sections of full-thickness incisional wounds in IL-5-overexpressing, wild-type and IL-5 knockout mice at 7 days post-injury. The sections were scored for the presence of lymphocytes, plasma cells and polymorphonuclear cells with a scale ranging from 1 (no or minimal infiltration) to 5 (abundant inflammatory infiltrate). (b) Graph showing inflammatory cell scores for IL-5-overexpressing mice, wild-type and IL-5 knockout cohorts at 3, 5, 7 and 14 days post-wounding. * Represents significantly greater than wild-type and IL-5 knockout and ^ significantly less than wild-type and IL-5 knockout mice ($P < 0.05$). ($n = 6$ wounds per group per time point).

IL-5 gene knockout mice, eosinophils were still occasionally sighted in the wounds consistent with previous reports for other tissues,¹⁰ including the skin, lungs and gut of mice infected with parasites.^{15,27} Importantly, during the late stages of healing, eosinophil numbers in wounds were often equivalent in IL-5-deficient and wild-type mice, demonstrating that IL-5-deficient mice could still mobilize small numbers of fully functional eosinophils, similar to previous reports.²⁰ The presence of these eosinophils in IL-5-deficient wounds may have been sufficient to stimulate a normal wound response and help to explain the similarities in healing between wild-type and IL-5 knockout mice. To explore more specifically the full implications of eosinophil depletion in wound healing independently of IL-5, studies using GATA-1²⁸ or PHIL²⁹ mice, which are totally devoid of eosinophils^{15,28,29} would be useful.

The delayed healing observed in the IL-5-overexpressing mice would not often occur in natural physiological situations, but could potentially become more important when occurring in parallel with conditions known to cause eosinophilia in patients with asthma, allergy or parasitic infections. Asthma and allergy are increasing problems in the developed world, and parasitic infection are significant problems in third world countries, meaning that the development of therapies to reduce abnormally high eosinophils in wounds could be of benefit to aid the rate of wound healing and thereby reduce the possibility of wound infections.

With the greatly increased numbers of eosinophils in IL-5-overexpressing mice, it might have been expected that a longer lasting impairment in healing would occur particularly as high levels of eosinophils were still apparent in late time point wounds. This

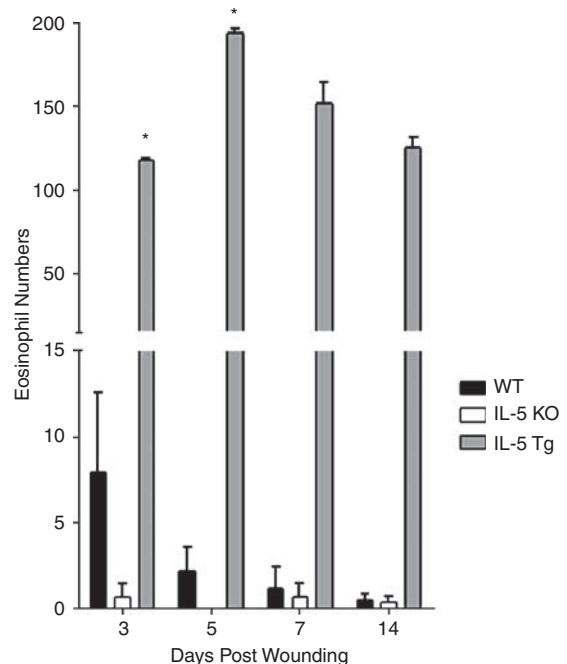


Figure 6 Eosinophil numbers in wounds peak at day 5 in interleukin (IL)-5 transgenic mice. Eosinophil numbers in wounds were quantified from haematoxylin and eosin-stained sections of wounds in IL-5-overexpressing mice, wild-type and IL-5 knockout cohorts. ($n = 6$ wounds per group per time point). * Represents significantly greater than IL-5 knockout and wild-type mice ($P < 0.05$).

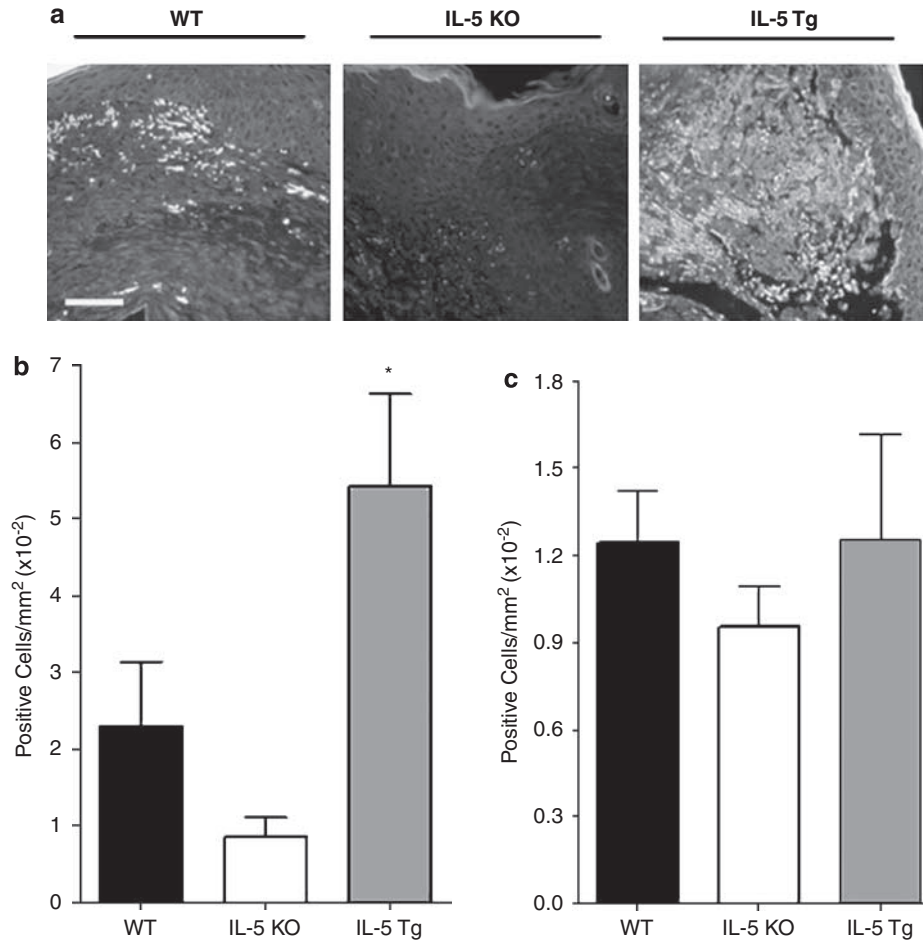


Figure 7 Interleukin (IL)-5 transgenic mice have large CD4⁺ cell infiltration at day 5. CD4⁺ cells were counted and normalized between sections by area. Representative examples of positively stained cells can be seen in (a) at $\times 20$ magnification. (b) Graph showing numbers of CD4⁺ cells per area at day 5 post-wounding. $n=6$ wounds per mice group. * Represents significantly greater than IL-5 knockout and wild-type mice ($P<0.05$). (c) Graph showing numbers of B220⁺ cells per area at day 5 post-wounding. $n=6$ wounds per mice group.

persistence of eosinophils in the wounds after the effects on healing have subsided could be a marker of the interaction of CD4⁺ cells and eosinophils in wounds. The high numbers of eosinophils seen at day 14 may in fact be new eosinophils that have been recruited by the increased numbers of CD4⁺ cells present in the wounds, rather than those initially recruited in the early stages of wound healing. CD4⁺ cells are known to induce the recruitment of eosinophils in the skin of mice,³⁰ and high numbers of CD4⁺ cells seen in the wounds of IL-5-overexpressing mice may indicate this form of involvement. The underlying mechanism involving eosinophils in wound healing remains to be elucidated; however, it could be similar to the role of eosinophils in other fibrotic conditions such as asthma.^{31,32} Degranulation of eosinophils may contribute to the recruitment of cells to the wound bed, or may introduce transforming growth factor- $\beta 1$ and - α , both of which are known to be vital contributors to the process of wound healing.³³

In summary, this study has investigated the role of differential IL-5 gene expression in incisional wound healing and has demonstrated an association between an altered inflammatory response including enhanced recruitment of eosinophils and CD4⁺ cells and delayed wound repair. The increased levels of eosinophils and IL-5 do not enhance wound healing and when present in excessive numbers have a

detrimental effect on the wound repair process. This knowledge may have particular significance in cases of healing of wounds in patients with an eosinophilia-related diseases.

METHODS

Antibodies

Monoclonal GK1.5 (CD4⁺) and B220 antibodies were produced from hybridomas obtained from the American Type Culture Collection (Manassas, VA, USA), Rabbit polyclonal anti-IL-5 antibody was obtained from AbD Serotec (Oxford, UK). Biotinylated horse anti-mouse IgG and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) were used in these studies.

Animal studies

All experiments were approved by the Adelaide Women's and Children's Hospital Animal Care and Ethics Committee and the University of Adelaide Animal Ethics Committee. Homozygous IL-5 deletion mice (IL-5 deficient) were generated on a C57BL/6 background by insertion of a neomycin-resistance region into the IL-5 gene, disrupting IL-5 production¹⁰ and backcrossed for 20 generations to the CBA strain.¹⁵ CBA/Ca Tg5C2 (IL-5 transgenic) mice were generated by inserting approximately 49 copies of the genomic mouse IL-5 gene under the control of the human CD2 dominant

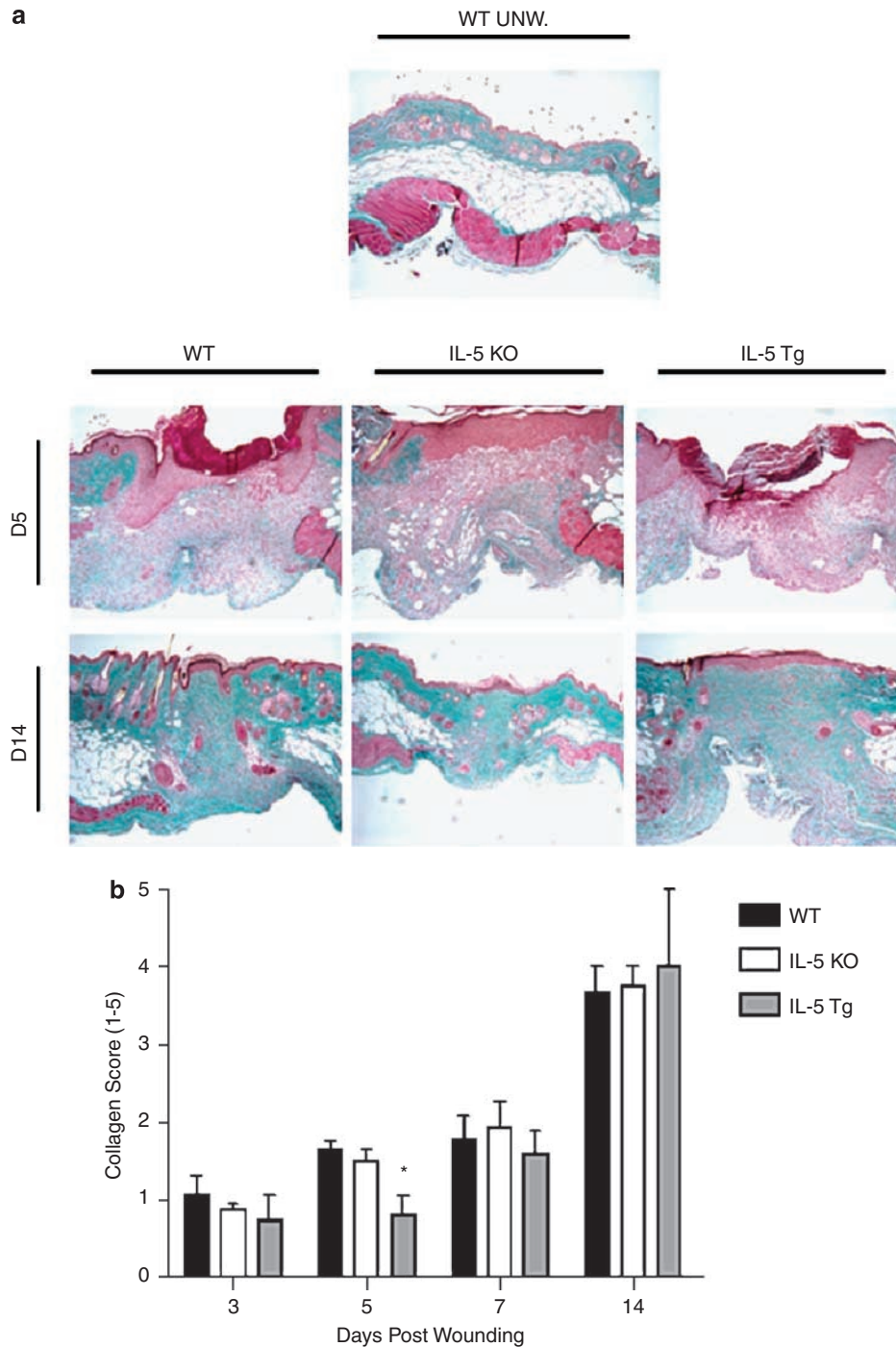


Figure 8 Collagen deposition is impaired in interleukin (IL)-5 transgenic mice. **(a)** Representative images of wounds stained with Masson's trichrome at 0, 3, 5, 7 and 14 days post-injury. **(b)** Masson's trichrome-stained wounds were scored for total collagen levels in wound beds of IL-5-overexpressing wild-type and IL-5 knockout mice by two independent blinded observers examining wound sections at day 0 (unwounded), 3, 5, 7 and 14 days post-injury. Results represent means and s.e.m. $n=6$ wounds per mice group per time point. * Represents significantly less than wild-type and IL-5 knockout mice ($P<0.05$).

control region and maintained and used as heterozygotes.³⁴ Age- and sex-matched wild-type litter mates were used as controls.

Murine surgical techniques

Sixteen-week-old IL-5-deficient, IL-5 transgenic and wild-type mice were anaesthetized with gaseous isoflurane, using 5% for induction, and 2% for maintenance and wounded as previously described.^{35,36} Briefly, two equidistant

1 cm full-thickness incisions were made through the skin and panniculus carnosus, using fine scissors on the flanks of the animals extending 3.5–4.5 cm from the base of the skull, 1 cm either side of the spinal column. The wounds were left to heal by secondary intention (that is, the wound edges were not closed by sutures). Digital photographs were taken of the wounds at 0, 3, 5, 7 and 14 days post-wounding. A ruler was aligned next to the wound to allow direct wound area and wound gape (midpoint of the 1 cm incision) measurements to be made. At 3, 5, 7 and 14 days, mice were euthanized using

CO₂ asphyxiation and cervical dislocation before harvesting of the wounds, which were then fixed in 10% buffered formalin and processed so that the midpoint of the wound was sectioned.

Peripheral eosinophils counts

Blood samples were collected from the tails of each mouse (approximately 15 µl per mouse), and WBC were enumerated and differentiated. The numbers of eosinophils were determined by microscopic analysis of Geimsa-stained blood smears.

Histology, immunohistochemistry and image analysis

Histological sections (4 µm) were cut from paraffin-embedded fixed tissue and stained with either haematoxylin and eosin or processed for immunohistochemistry using antigen retrieval according to the manufacturer's protocols (DAKO Corporation, Glostrup, Denmark). Following blocking with 6% normal horse serum for 1 h in phosphate-buffered saline, primary antibodies specific for IL-5 (2 µg ml⁻¹) were applied overnight in phosphate-buffered saline. GK1.5 (anti-CD4⁺) and B220⁺ antibodies were applied as neat hybridoma supernatants. Species-specific, biotinylated anti-immunoglobulin secondary antibodies (7.5 µg ml⁻¹) were then applied for 1 h and detected with CY3-conjugated streptavidin (1:200) (Sigma-Aldrich, St Louis, MO, USA). Fluorescence intensity per unit area was determined using AnalySIS software package (Soft Imaging System GmbH, Munster, Germany). The InSpeck Microscope Image Intensity Calibration Kits (Invitrogen, Carlsbad, CA, USA) were used to define fluorescence intensity levels for constructing calibration curves and evaluating sample brightness. Negative controls included replacing primary antibodies with normal rabbit IgG or normal mouse IgG. For verification of staining, nonspecific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

Masson's trichrome staining was performed by dewaxing and rehydrating sections and then incubating in Bouin's fixative for 30 min at 60 °C. Following this, sections were incubated in 0.5% Celestine Blue in 5% Iron Alum for 3 min, washed in water, then stained in Mayers haematoxylin for 3 min. Sections were then stained in Fuchsin Ponceau for 5 min, rinsed in water, incubated in 5% aqueous phosphotungstic acid for 10 min, stained in 2% Light Green in 1% acetic acid and then dehydrated and mounted. Sections were scored for the intensity and area of green staining on a blinded 1–5 scale.

Histological image analysis

Image analysis was performed using the ImageProPlus program (MediaCybernetics Inc., MD, USA). Wound size was determined by manually drawing below the epidermis or clot between the wound margins. The percentage of the wound that had re-epithelialized was determined by measuring the portions of the wound that were covered with epidermis as a percentage of the entire wound. The epithelial thickness was determined by measuring the average thickness between the outer keratinocyte layer and the basement membrane. The dermal gape was determined by measuring between the dermal wound margins. The level of inflammation within the wounds were randomly analyzed using semiquantitative five point scales.³⁷ Haematoxylin and eosin sections were scored for the presence of lymphocytes, plasma cells and polymorphonuclear cells with a scale ranging from 1 (no or minimal infiltration) to 5 (abundant inflammatory infiltrate). Blinded measurements of histological slides were performed by two independent assessors.

Real-time quantitative reverse transcription-PCR

Total RNA was extracted from wound samples using TRIzol reagent (Invitrogen), according to the manufacturers' protocols. Contaminating genomic DNA was removed using a DNA-free-kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 µg RNA using reverse transcriptase. cDNA together with specific primers were set up to a final concentration of 1× SYBR Green, 1× amplitaq PCR buffer, 3 mM MgCl₂, dNTPs (200 µM each), 0.9 µM of primers (forward and reverse), 1.25 Units AmpliTaq Gold DNA polymerase in 25 µl H₂O. Real-time quantitative PCR reactions were run with an initial 95 °C step for 15 min to activate the Taq buffer, then 35 cycles of: denaturation (95 °C for 30 s), annealing (60 °C for 30 s) and elongation (72 °C for 30 s). Primers used for real-time quantitative PCR analysis are as follows: CypA forward 5'-GG

TTGGATGGCAAGCATGTG-3' and reverse 5'-TGCTGGTCTTGCCATTCC TG-3', IL-5 forward 5'-CCACACTTCTCTTTTGGCG-3' and reverse 5'-TC ACCGAGCTCTGTTGACAA-3'. Cycle threshold values for IL-5 were normalized firstly to CypA and then to wild type to value for fold change.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test or an analysis of variance. For data not following a normal distribution, the Mann-Whitney *U*-test was performed. A *P*-value of less than 0.05 was considered significant.

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