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Keratinocyte arginase 1 regulates proinflammatory responses and drives re-epithelialization via lipocalin 2

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Abstract

Background Chronic skin wounds exhibit impaired re-epithelialization due to defective keratinocyte migration and excessive inflammatory signals. Arginase 1 (ARG1) is an enzyme expressed by keratinocytes that is important for skin wound healing. However, the mechanisms underpinning keratinocyte ARG1 function in wound closure are not fully understood.

Objectives To investigate the role of ARG1 in keratinocyte wound closure and inflammatory responses.

Methods *In vitro* two-dimensional wounding assays using ARG1-inhibited keratinocytes were used to explore the function of ARG1 in keratinocyte scratch closure. ARG1 was also assessed in human skin wounds.

Results ARG1 was strongly expressed in the perilesional regions of acute wounds, but its expression was dysregulated in chronic ulcers, with high expression in the upper layers of the hyperplastic epidermis. ARG1 inhibition in keratinocytes led to a significant decrease in two-dimensional scratch closure, augmented interleukin-1 family- and tumour necrosis factor-α-driven proinflammatory signalling, and significant downregulation of lipocalin 2 (LCN2). LCN2 expression was partially dependent on Janus kinase/signal transducer and activator of transcription signalling. Keratinocyte LCN2 neutralization led to delayed scratch wound closure, while LCN2 expression in response to bacterial challenge was impaired upon ARG1 inhibition. ARG1 metabolic products, putrescine and urea, could rescue keratinocyte migration and LCN2 expression in ARG1-inhibited cells.

Conclusions ARG1 plays a major role in keratinocyte re-epithelialization, regulating inflammation and LCN2 production in sterile and infected wound conditions. Dysregulated expression in chronic ulcers may reflect altered enzymatic capacity to drive re-epithelialization or attempts to reduce inflammation and promote antimicrobial function. In culture, impaired wound responses of ARG1 inhibited cells can be rescued by its downstream products putrescine and urea, highlighting the complexity of ARG1 control of wound healing. Manipulation of the ARG1 pathway may have the potential to be used for the management of skin conditions such as infected chronic lesions; however, further study of the pathway is needed to fully understand the role of ARG1 in chronic wounds.

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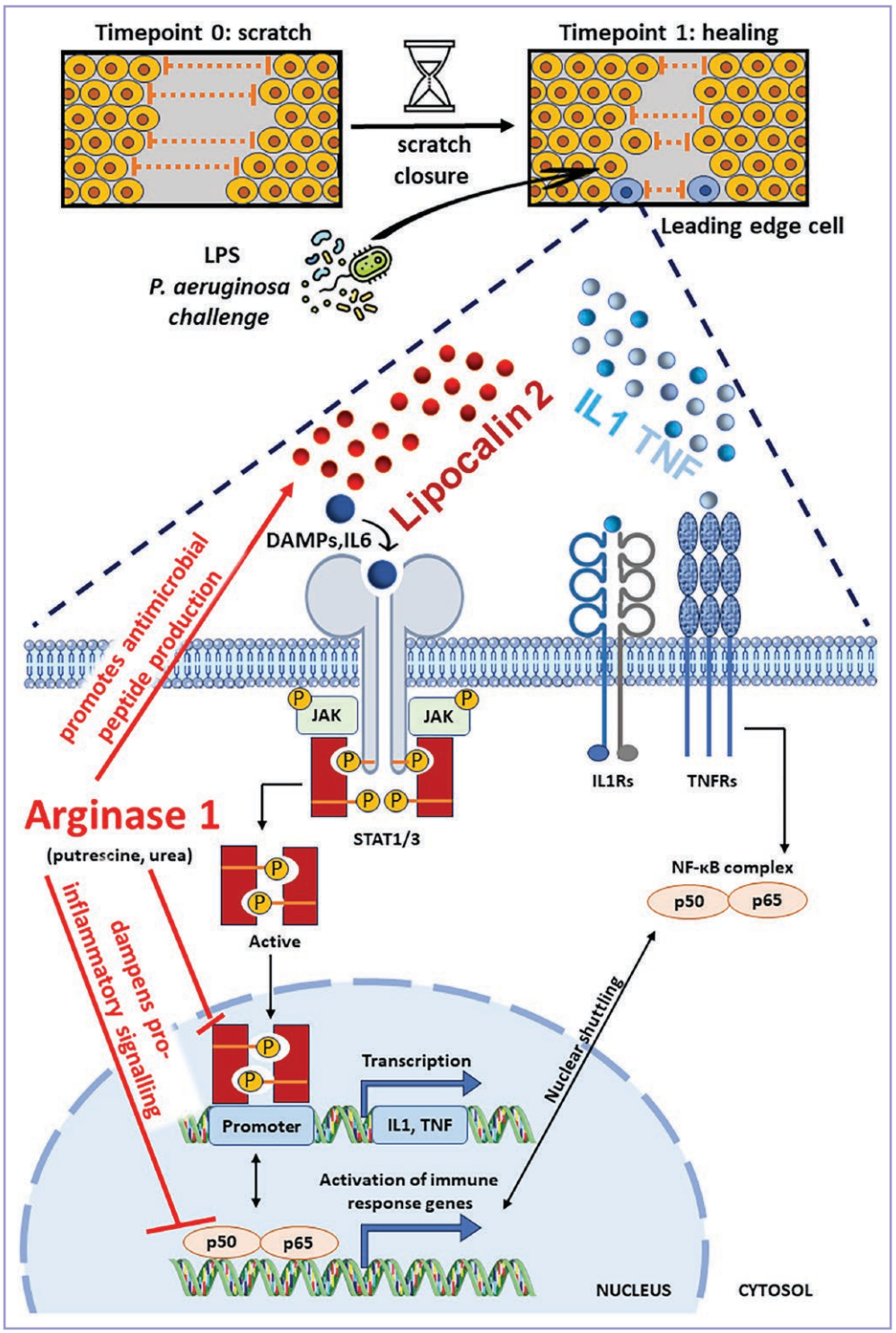
Graphical Abstract



Lay summary

Non-healing skin wounds (or 'ulcers') are serious skin conditions. They are more common as a person ages. A common feature of ulcers is a delay in the closure of the damaged uppermost layer of the skin. These wounds are highly inflamed and prone to infection, which delays healing. We have shown before that a protein called arginase 1 ('ARG1' for short) is needed in wound closure. How it does this is unknown.

In this study, we aimed to find out the role of ARG1 in wound closure and inflammation. We did this using skin cells grown in the lab and by analysing human skin samples. ARG1 was found only in the top layers in normal wounded skin. However, in non-healing skin ulcers, its production was more dispersed. The production of ARG1 in skin cells grown in the lab increased as the cells moved to close a scratch between the cells. Blocking ARG1 impaired scratch closure. However, this was reversed by adding products that can activate ARG1. Inhibiting ARG1 activity increased the production of inflammatory products. In contrast, the production of another protein called lipocalin 2 ('LCN2' for short) that helps fight microbial infection was dependent on ARG1. LCN2 also directly helped promote skin scratch closure.

In conclusion, we found that ARG1 helps skin wound closure, at least in part, by promoting the production of LCN2. LCN2 also contributes to antimicrobial responses and may compensate for reduced inflammatory activity. ARG1 is expressed highly but in a disordered pattern in skin ulcers. This suggests that understanding and manipulating ARG1 activity might be helpful in treating ulcers.

What is already known about this topic?

- Arginase 1 (ARG1) is needed for effective re-epithelialization and timely wound closure, but the mechanism by which it does this is unknown.
- Inhibition of ARG1 and reduced expression of ARG1 is linked with delayed wound healing.

What does this study add?

- ARG1 is required for keratinocyte scratch closure and multifunctional antimicrobial peptide expression via its metabolic products putrescine and urea.
- The antimicrobial peptide lipocalin 2 contributes to keratinocyte scratch closure and microbe stimulation responses in an ARG1-dependent manner.

What is the translational message?

- Supplementation with the ARG1 metabolites putrescine and/or urea might contribute to the management of conditions with aberrant re-epithelialization such as chronic skin ulcers.

Cutaneous wound healing involves multiple stages and cell types to ensure wound closure.[1] Older patients and those with comorbidities such as diabetes are prone to developing wounds that fail to heal.[2] Chronic wounds exhibit persistent inflammation and a failure to re-epithelialize, and are susceptible to infection, subsequent sepsis and amputations.[2,3] The increasing incidence of chronic wounds has been described as a silent epidemic, affecting the quality of life of > 40 million people worldwide.[3]

Re-epithelialization is paramount for effective wound healing. We previously identified that epidermal arginase is necessary for keratinocyte wound healing responses,[4] but the mechanisms were not fully elucidated. Arginase 1 (ARG1) competes with nitric oxide synthase (NOS) for the amino acid L-arginine.[5] ARG1 activity drives the breakdown of L-arginine to urea, L-ornithine, L-proline and polyamines.[6] Although urea can enhance keratinocyte barrier function,[7] its contribution to keratinocyte re-epithelialization is unclear. Ornithine decarboxylase 1 (ODC1) and adenosyl methionine decarboxylase 1 (AMD1) are rate-limiting enzymes involved in polyamine synthesis downstream of ARG1 activity.[8] The polyamines putrescine, spermidine and spermine are ubiquitous, naturally occurring polycations that increase in response to wound-related cellular damage,[9,10] and are required for effective re-epithelialization via AMD1.[11] Ornithine aminotransferase (OAT)-driven L-proline production downstream of arginase activity enhances keratinocyte migration and subsequent wound re-epithelialization,[12] suggesting multiple aspects of L-arginine metabolism by the arginase pathway contribute to effective barrier repair.

Low-level inflammation is required for effective healing, yet protracted inflammation contributes to impaired re-epithelialization and wound chronicity.[13] Cytokines contribute to inflammatory processes and are tightly regulated via signalling pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway.[14,15] Prolonged JAK/STAT activity can drive chronic inflammation contributing to refractory wounds.[16] Keratinocytes also express lipocalin 2 (LCN2), a multifunctional protein with anti-inflammatory and antimicrobial properties that has been demonstrated to be important for effective healing.[17] LCN2 has also been shown to activate the JAK/STAT signalling

pathway.[18] The potential involvement of ARG1 signalling in keratinocyte JAK/STAT regulation, cytokine and LCN2 expression is unknown.

We investigated the role of keratinocyte ARG1 in the wound healing process. ARG1 is rapidly upregulated in keratinocytes postwounding and this upregulation is necessary for cell migration – at least partially – due to polyamine and urea metabolism. ARG1 metabolism inhibits proinflammatory cytokines while promoting LCN2 production, which, in turn, plays a dual role in driving re-epithelialization and inflammatory responses.

Materials and methods

Skin specimens

Human skin specimens were obtained from six adult patients following surgery (Table S1; see Supporting Information).

Histology and immunofluorescence

Histology sections prepared from formalin-fixed paraffin-embedded human skin or scratched keratinocyte monolayers were immunofluorescently stained with primary antibodies against ARG1 (GTX109242; GeneTex, San Antonia, TX, USA), keratin 1 (MA1-35367; ThermoFisher Scientific, Waltham, MA, USA), lamin B1 (ab16048; Abcam, Cambridge, UK) and RELA/NFκB p65 (sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Alexa Fluor 488 goat antirabbit (A11070; Invitrogen, Carlsbad, CA, USA) or 594 goat antirabbit (A11012; Invitrogen) secondary antibodies. Slides were counterstained with 4′,6-diamidino-2-phenylindole (D523; Dojindo Molecular Technologies, Munich, Germany), mounted and visualized (Appendix S1; see Supporting Information).

Cell culture

The N/TERT-1 human immortalized keratinocyte cell line was cultured in Gibco™ Keratinocyte SFM (17005042; ThermoFisher Scientific) supplemented with 0.2 ng mL^{-1} Gibco human recombinant Epidermal Growth Factor [EGF 1-53; 37000015 (ThermoFisher Scientific)], 25 μg mL^{-1} Bovine Pituitary Extract (37000015; ThermoFisher Scientific) and 0.3 mmol L^{-1} $CaCl_2$ (ALFA12312.36; Alfa Aesar, ThermoScientific Chemicals, Waltham, MA, USA) (Appendix S1).[19,20]

In vitro two-dimensional keratinocyte scratch assay

Confluent keratinocytes on ImageLock 96-well microplates (Essen BioScience, Ann Arbor, MI, USA) were scratched using a 96-pin wound maker (Essen BioScience). Before scratching, cells were treated with putrescine [100 μmol L^{-1}; P5780 (Sigma-Aldrich, St. Louis, MO, USA)] or urea [100 μg mL^{-1}; 15505-050 (Invitrogen)]. After 24 h of putrescine or urea pretreatment, nor-NOHA (NN), an ARG1 inhibitor [50 μmol L^{-1}; 10006861 (Cayman Chemicals, Ann Arbor, MI, USA)], or lipopolysaccharide [LPS (10 μg mL^{-1}; L2630 (Sigma-Aldrich)] were added for 24 h prescratch. For other experiments, cells were incubated with antibodies against LCN2 (ab125075; Abcam) and LPS or the JAK/STAT inhibitor tofacitinib on scratch. Cells were either harvested in TRIzol reagent (15596026; Invitrogen) for RNA analysis or RIPA buffer (89900; Thermo Fisher Scientific) for Western blot.

Cytokine array

A Luminex® (ThermoFisher Scientific) analysis of 48 cytokines from cell culture supernatants was performed using a multiplex platform (Table S2; see Supporting Information).

Western blot

Protein was resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. ARG1 (GTX109242; GeneTex), β-actin (A5441; Sigma-Aldrich), interleukin (IL)-1α (AF-200-NA; bio-techne®, Minneapolis, MN, USA), IL-1β (sc-7884; Santa Cruz), LCN2 (ab125075; Abcam), STAT1 (#9172; Cell Signalling Technology, Danvers, MA, USA), phosphorylated (p)-STAT1 (EPR3146; Abcam), STAT3 (sc-8019; Santa Cruz) and p-STAT3 (sc-8059; Santa Cruz) primary antibodies were used followed by incubation with horseradish peroxidase-labelled secondary antibodies.

Measurement of amino acid levels

Cell counts were determined prior to cell lysis using an EVE™ automated cell counter (NanoEntek, Waltham, MA, USA). Amino acid measurements were made by liquid chromatography mass spectrometry, analysed using Skyline software (https://skyline.ms/project/home/software/skyline/begin.view) and normalized to cell counts.[21,22]

Statistical analysis

Data were expressed as mean (SEM). Student's *t*-tests, one-way ANOVA with Tukey's or Dunnett's post hoc test, and two-way ANOVA tests were performed using Prism version 10 (GraphPad, La Jolla, CA, USA). A P-value < 0.05 was considered to be statistically significant. Experiments were performed in triplicate, at a minimum.

Results

Arginase 1 is increased in epidermal cells of chronic ulcers

ARG1 expression was assessed using immunostaining of human incisional acute lesions and pressure ulcer tissue (Table S1). A distinct suprabasal ARG1 localization was noted in acute wounds (Figure 1a), corroborating prior data.[4] In contrast, ARG1 was more diffusely expressed and highest in the upper layers of the thickened epidermis from the perilesional region of chronic ulcers (Figure 1a), similar to increased levels of global arginase previously reported in chronic wounds.[23,24] In addition, ARG1 protein levels and *ARG1* mRNA levels were significantly increased in *in vitro* keratinocyte scratch assays 24 h postwounding

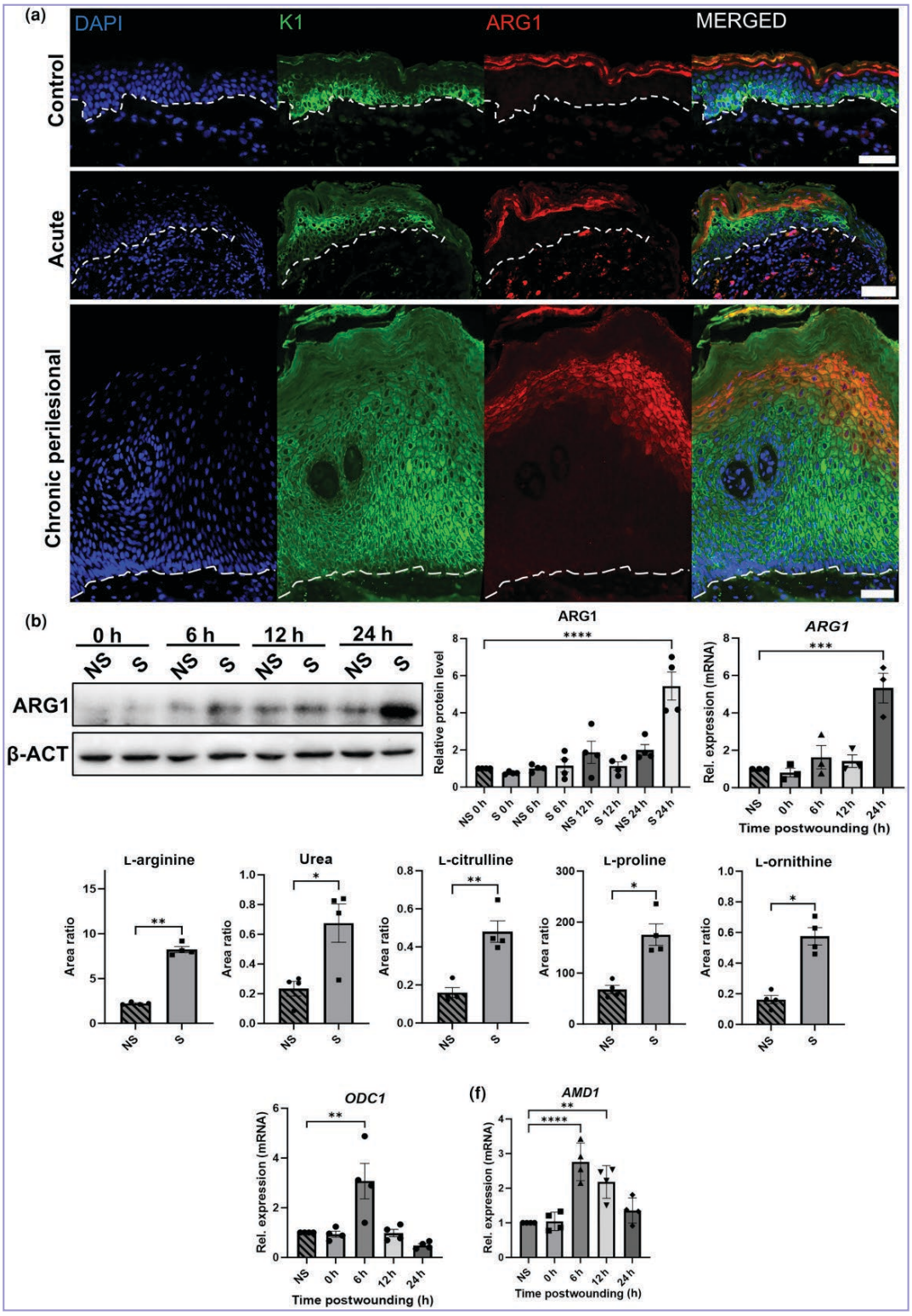


Figure 1 Arginase 1 (ARG1) is highly expressed in wounded keratinocytes of the epidermis. (a) Representative immunofluorescence staining demonstrating keratin 1 (K1; green) and ARG1 (red) expression in unwounded control human skin, as well as acute and chronic pressure lesions; nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and epidermal–dermal junctions are marked by white dotted lines (scale bars = 100 μm). (b) Western blot illustrating ARG1 protein expression and quantification in scratch-wounded N/TERT-1 cell monolayers; β-actin (β-ACT) was used as a loading control. (c) Quantitative polymerase chain reaction (qPCR) mRNA-level analysis of *ARG1* in scratch-wounded N/TERT-1 cell monolayers; mRNA expression was normalized to the housekeeping gene *RPLP0.* (d) Mass spectrometry quantification of L-arginine, urea, L-citrulline, L-proline and L-ornithine in *in vitro* scratched N/TERT-1 keratinocytes. Cells were harvested for analysis 24 h after wounding. Amino acid levels were normalized to cell counts. (e) qPCR mRNA-level analysis of *ODC1* and *AMD1* in i*n vitro* scratched N/TERT-1 keratinocytes. mRNA expression was normalized to *RPLP0.* For all experiments, the results represent the mean (SEM) of at least three independent biological replicates. ns, not significant (i.e. $P > 0.05$); NS, not scratched; S, scratched. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; **** $P < 0.001$.

(Figure 1b, c). Collectively, these data show that ARG1 is upregulated on wounding.

L-Arginine metabolism increased upon keratinocyte scratch wounding

L-Arginine pathway metabolite levels and associated enzymes were assessed in keratinocyte scratch assays [Figure 1d,e; Figure S1 (see Supporting Information)]. Alternative L-arginine metabolism by NOS is dampened, demonstrated by marginally decreased *NOS2* mRNA (Figure S1b), in line with significantly increased L-arginine availability, 24 h postwounding (Figure 1d). ARG1 and NOS2 metabolic products (urea and L-citrulline, respectively) significantly increased postscratch (Figure 1d). Although *OAT* mRNA levels were unchanged in scratched keratinocytes (Figure S1b), L-proline (downstream of OAT activity) levels were significantly increased (Figure 1d). The availability of L-ornithine increased in scratched keratinocytes (Figure 1d), while the mRNA of downstream enzymes involved in its metabolism, *ODC1* and *AMD1*, were significantly upregulated (Figure 1e,f). Putrescine, the direct metabolic product from L-ornithine metabolism by the enzyme ODC1, was not significantly increased upon scratch (Figure S1b), likely due to its conversion to the higher polyamines spermidine and spermine. These data show that the arginase pathway and its downstream metabolites are dynamically regulated on wounding.

Arginase 1 deficiency-related delay in keratinocyte scratch closure is rescued by putrescine and urea

We have previously shown that arginase is important for wound healing;[4] therefore, the involvement of polyamine production downstream of ARG1 was investigated in scratch closure (Figure S2; see Supporting Information). Putrescine treatment upon ARG1 inhibition via NN in scratched keratinocytes led to partial rescue of the delayed scratch phenotype (Figure S2a,b), as previously shown,[4] supporting a role for polyamines in keratinocyte migration.[11] Moreover, exogenous urea fully rescued the NN-related delay in keratinocyte scratch closure (Figure S2a,c). Collectively, this suggests that ARG1 drives keratinocyte scratch closure on wounding by promoting putrescine and urea.

Re-epithelialization depends on a balance between cell migration and proliferation.[25] Cell numbers were unchanged in keratinocyte monolayers treated with NN for 24 h before scratch (Figure S2d). Moreover, the scratch-induced increase of the proliferation marker *MKI67* mRNA levels were unaffected by ARG1 inhibition (Figure S2e), suggesting that ARG1 has little impact on keratinocyte proliferation during *in vitro* scratch wounding.

Arginase 1 inhibits proinflammatory cytokines on wounding

Cytokines such as IL-1α and IL-1β have been linked to epithelial cell migration;[26,27] therefore, we assessed ARG1 function in cytokine expression and signalling. *IL1A* mRNA and protein levels significantly increased in scratched keratinocytes (Figure 2a,b), with protein expression further increasing upon ARG1 inhibition via NN (Figure 2b). Similar data were observed with *IL1B* mRNA levels and pro-IL-1β protein (Figure 2c,d). Keratinocyte secretion of activated IL-1β was not further enhanced by ARG1 inhibition, despite a significant increase on scratch (Figure S3a; see Supporting Information). No significant change was noted in *IL1R1*, *IL1R2* or *IL1R3* with NN treatment (Figure S3b). The mRNA levels of *IL36G* and *TNFA* were upregulated in scratched keratinocytes and further upregulated upon ARG1 inhibition (Figure 2e). A trend of increased proinflammatory gene expression was also noted in the 3-day neoepidermis of a keratinocyte-specific ARG1-null (*K14cre:Arg1*$^{fl/fl}$) murine wound model vs. Cre counterparts (Figure S3c,d). Furthermore, subcutaneous administration of NN *in vivo* led to higher *IL1B* and *TNFA* mRNA in 3-day murine wounds vs. controls, although this was not statistically significant (Figure S3e,f).

Keratinocyte proinflammatory responses can be modulated via a JAK/STAT-dependent signalling mechanism. *IL1A* mRNA levels decreased both at 6 h and 24 h postscratch in tofacitinib-treated keratinocytes (Figure 3a,b). Tofacitinib-dependent JAK inhibition significantly downregulated *IL1B* mRNA expression 6 h postinjury in scratched keratinocytes, and *TNFA* levels were significantly reduced 24 h postwounding (Figure 3a,b). ARG1 inhibition in tofacitinib-treated cells significantly increased *IL1A* and *IL1B* at 24 h postwounding (Figure 3b), suggesting that STAT signalling downstream of JAK can – at least partially – drive keratinocyte cytokine production when ARG1 activity is reduced. Indeed, both p-STAT1 and p-STAT3 were increased in *in vitro* scratched keratinocytes and p-STAT3 further increased upon NN treatment (Figure 3c,d). However, no changes in STAT phosphorylation status were noted upon putrescine or urea treatment in ARG1-inhibited keratinocytes (Figure 3c,d). Increased production of the cytokine IL-6, known to induce STAT3 signalling, was noted in NN-treated cells (Figure 3e), in line with increased p-STAT3, whereas interferon-γ, known to promote STAT1 signalling, was unchanged (Figure 3f). These results suggest that, upon wounding, keratinocyte *IL1A* and *IL1B* are upregulated in a p-STAT1/3-related manner and that ARG1 dampens this response – at least in part – through a reduction in p-STAT3 levels.

Many cytokines, including IL-1α, IL-1β and tumour necrosis factor (TNF)-α,[28–30] promote nuclear factor-κB (NF-κB) subunit p65 activation and nuclear localization; therefore, p65 expression was assessed (Figure S4; see Supporting Information). In line with enhanced expression of IL-1α, IL-1β and TNF-α postscratch (Figure 2), *RELA* mRNA was increased on scratch and this was not affected by NN treatment (Figure S4a). There was greater nuclear localization of p65 on scratch, which was further increased in NN-treated cells (Figure S4b,c). Thus, our data suggest that inhibition of ARG1 leads to an upregulation of cytokines, resulting in increased proinflammatory NF-κB activation which, in turn, enhances autocrine and paracrine inflammatory responses. This could support an immunoregulatory role for keratinocyte arginase in the wound response, whereby ARG1 dampens the production of cytokines that can promote inflammation.

Arginase 1 increases lipocalin 2 expression and activity in scratch-wounded keratinocytes

LCN2 is a multifunctional protein, known as a modulator of inflammation and iron homeostasis.[31] *LCN2* mRNA

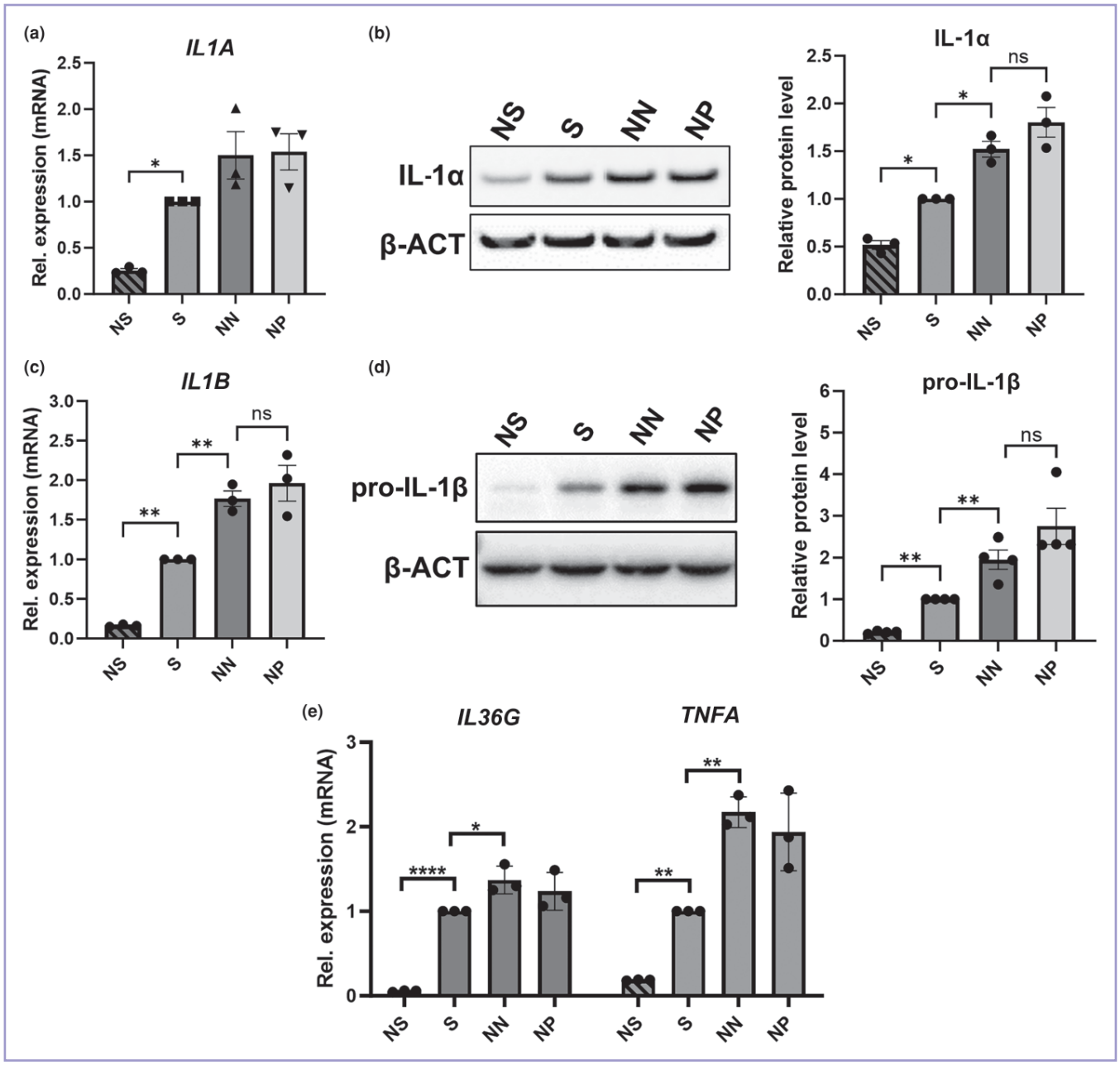


Figure 2 Arginase 1 (ARG1) inhibition leads to an increase in proinflammatory cytokine production. (a) Quantitative polymerase chain reaction (qPCR) mRNA-level analysis of *IL1A* 6 h postwounding in *in vitro* scratched N/TERT-1 cells treated with the ARG1 inhibitor nor-NOHA (NN) and the polyamine putrescine (NP). (b) Representative Western blot images illustrating interleukin (IL)-1α expression and quantification 24 h postwounding in scratch-wounded N/TERT-1 cell monolayers treated with NN and NP. (c) qPCR mRNA-level analysis of *IL1β* 6 h postwounding in scratch-wounded N/TERT-1 cell monolayers treated with NN and NP. (d) Representative Western blot images illustrating IL-1β expression and quantification 6 h postwounding in scratch-wounded N/TERT-1 cell monolayers treated with NN and NP; β-actin (β-ACT) was used as a loading control. (e) qPCR mRNA-level analysis of *IL36G* and *TNFA* at 6 h postwounding in scratched N/TERT-1 cell monolayers treated with NN and NP. mRNA expression was normalized to the housekeeping gene *RPLP0*. For all experiments, results represent the mean (SEM) of at least three independent biological replicates. ns, not significant (i.e. $P > 0.05$); NS, not scratched; S, scratched. *$P < 0.05$; **$P < 0.01$; ****$P < 0.001$.

significantly increased in line with *ARG1* levels 24 h post-scratch in keratinocytes (Figure 4a). Expression of *LCN2* mRNA was reduced on ARG1 inhibition via NN in scratched keratinocytes and rescued via exogenous putrescine (Figure 4b). LCN2 protein levels decreased upon ARG1 inhibition; this was rescued with putrescine but not with urea (Figure 4c). Moreover, *LCN2* mRNA was significantly downregulated in subcutaneous NN-treated *in vivo* 3-day murine wounds (Figure 4d). Tofacitinib treatment in scratched keratinocytes significantly downregulated *LCN2* mRNA levels both at 6 h and 24 h postwounding (Figure 4e), suggesting that LCN2 expression might be driven – at least in part – by JAK/STAT signalling.

LCN2 inhibition via specific monoclonal antibodies significantly hindered keratinocyte scratch closure (Figure 4f). As LCN2 showed ARG1 dependence, the effect of the

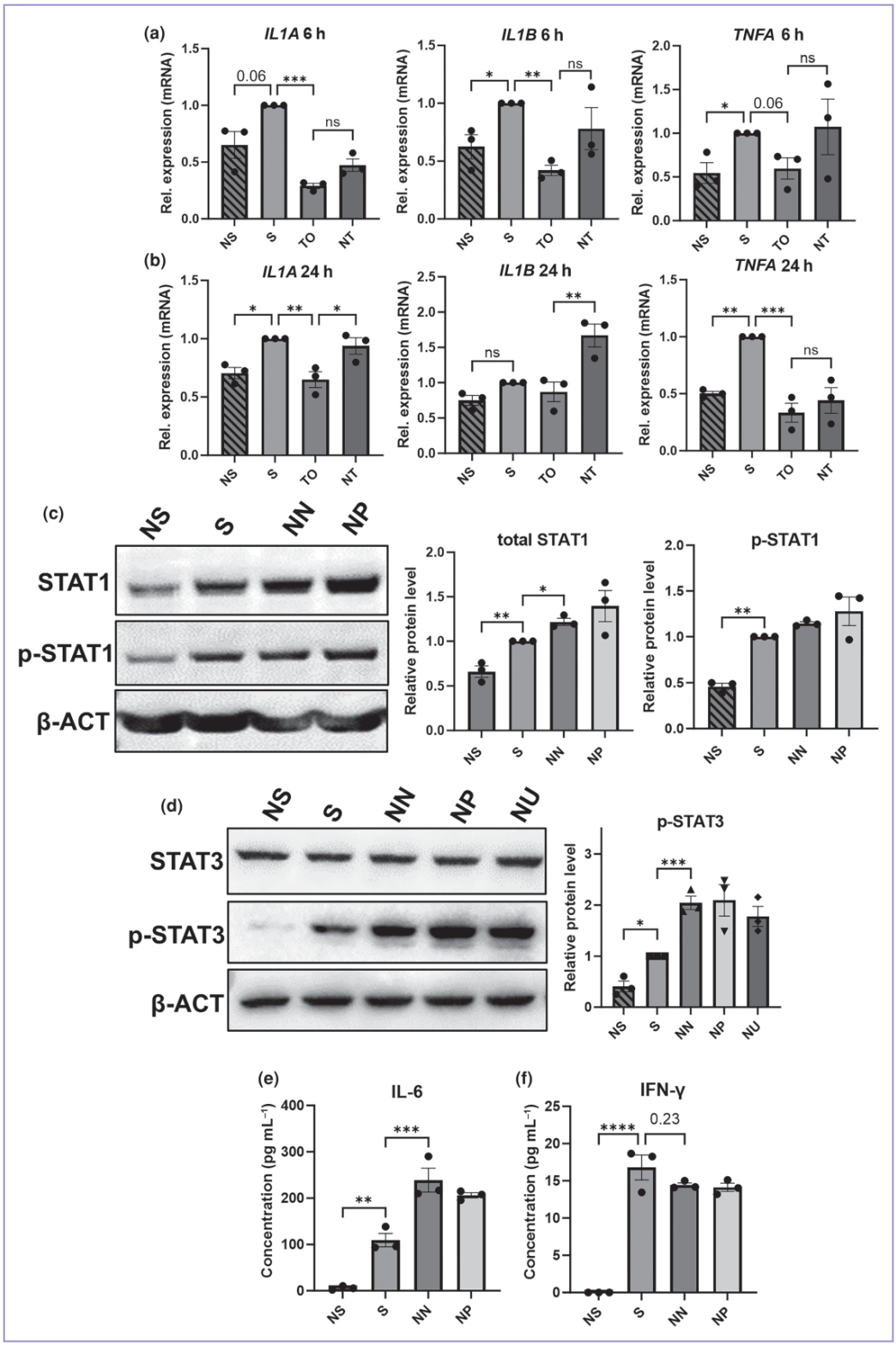


Figure 3 Arginase 1 (ARG1) activity can affect Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling. (a, b) Quantitative polymerase chain reaction (qPCR) mRNA level analysis of proinflammatory *IL1A*, *IL1B*, *IL36G* and *TNFA* in scratch-wounded N/TERT-1 keratinocytes treated with the JAK inhibitor tofacitinib (TO) or a combined treatment between the ARG1 inhibitor nor-NOHA (NN) and TO (NT) for (a) 6 h or (b) 24 h postwounding. (c) Representative Western blot images illustrating total STAT1 and phosphorylated (p)-STAT1 expression and quantification 24 h postwounding in scratch-wounded N/TERT-1 cells treated with the ARG1 inhibitor NN and the polyamine putrescine (NP). (d) Representative Western blot images illustrating total STAT3 and p-STAT3 expression and quantification 6 h postwounding in scratched N/TERT-1 cells treated with NN, NP and urea (NU). β-actin (β-ACT) was used as a loading control. (e, f) Cytokine array analysis of (e) interleukin (IL)-6 and (f) interferon (IFN)-γ secretion at 24 h postwounding in scratched N/TERT-1 cell monolayers treated with NN and NP. For all experiments, results represent the mean (SEM) of at least three independent biological replicates. ns, not significant (i.e. $P > 0.05$); NS, not scratched; S, scratched. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$.

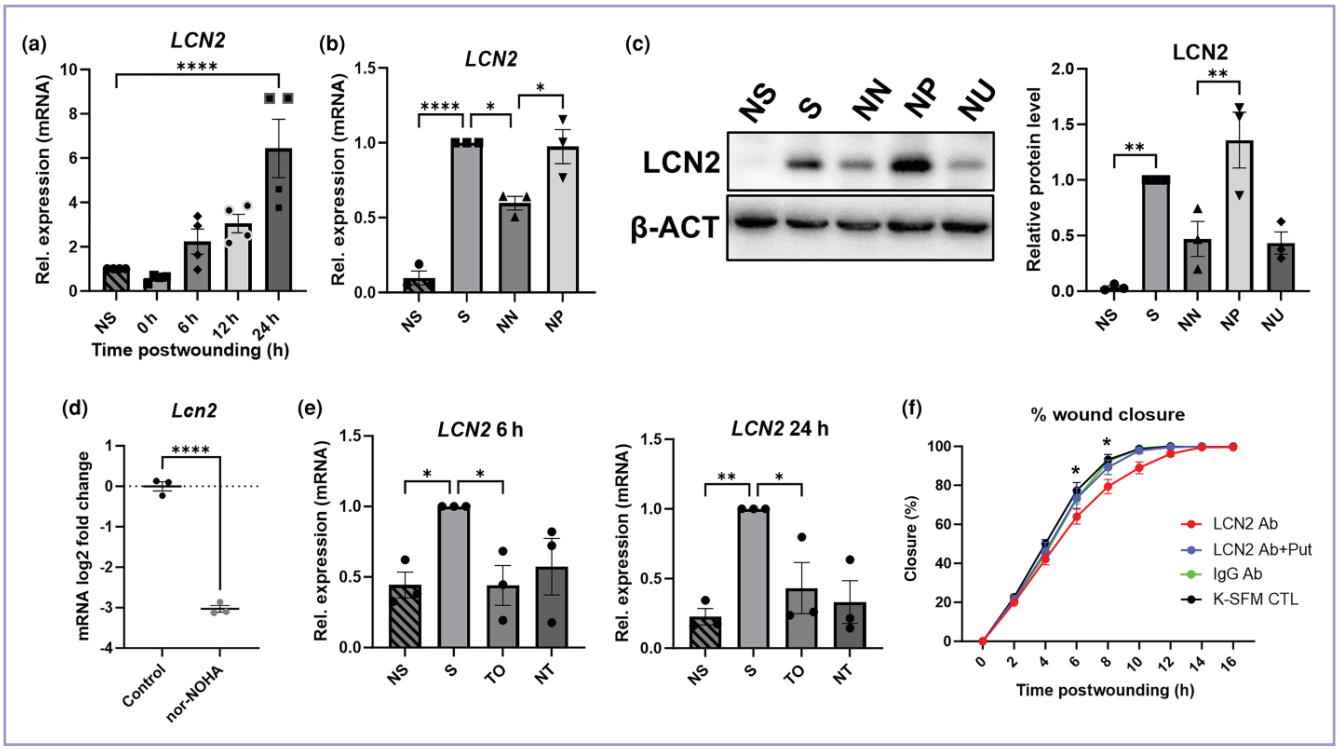


Figure 4 Arginase 1 (ARG1) modulates the expression of lipocalin 2 (LCN2), which can contribute to keratinocyte scratch closure. (a, b) Quantitative polymerase chain reaction (qPCR) mRNA-level analysis of *LCN2* in (a) a time course of *in vitro* scratch-wounded N/TERT-1 keratinocytes and (b) scratched N/TERT-1 keratinocytes treated with the ARG1 inhibitor nor-NOHA (NN) and the polyamine putrescine (NP) for 24 h after wounding; mRNA expression was normalized to the housekeeping gene *RPLP0*. (c) Representative Western blot images illustrating LCN2 expression and quantification in scratch-wounded N/TERT-1 cell monolayers treated with NN, NP or rescued with urea (NU); β-actin (β-ACT) was used as a loading control. (d) qPCR analysis of *Lcn2* expression in 3-day cutaneous excisional wounds subcutaneously injected with NN. (e) qPCR mRNA-level analysis of *LCN2* in scratch-wounded N/TERT-1 keratinocytes treated with the Janus kinase inhibitor tofacitinib (TO) or a combination of the ARG1 inhibitor NN and TO (NT) for 6 h or 24 h postwounding. (f) Percentage wound closure of *in vitro* scratched N/TERT-1 keratinocyte treated with LCN2-specific antibodies (Ab) and putrescine (Put); nonspecific IgG antibody isotypes (IgG) were used as control (CTL); data were quantified using the Incucyte® Live-Cell Analysis Systems v2019B and normalized to initial wound width measured at time 0 h. For all experiments, results represent the mean (SEM) of at least three independent biological replicates. K-SFM, keratinocyte serum-free medium; ns, not significant ($P > 0.05$); NS, not scratched; S, scratched. *$P < 0.05$; **$P < 0.01$; ****$P < 0.001$.

downstream arginase metabolite putrescine on LCN2 inhibition-related scratch closure delay was determined (Figure 4f). Exogenous putrescine rescued the impaired scratch closure caused by LCN2 inhibition. Collectively, these data suggest that, on wounding, ARG1 promotes LCN2 expression in epidermal keratinocytes, contributing to keratinocyte migration via putrescine metabolism.

Arginase 1-sensitive lipocalin 2 responds to microbial ligand stimulation on wounding

To promote inflammation, scratched keratinocytes were challenged with LPS or live bacteria. *IL1A, IL1B*, *TNFA* and *RELA* mRNA levels were upregulated on LPS stimulation in scratched keratinocytes. LPS-induced *IL1A* expression decreased upon ARG1 inhibition via NN (Figure S5; see Supporting Information), suggesting that IL-1α induction by LPS is dependent on ARG1. *LCN2* mRNA and LCN2 protein significantly increased upon LPS stimulation in wounded keratinocytes, relative to scratch controls (Figure 5a, b). Similarly, *LCN2* mRNA expression increased on challenge with *Pseudomonas aeruginosa* (Figure 5c). ARG1 inhibition via NN led to an abrogation of the increases in *LCN2* mRNA and LCN2 protein, despite bacterial challenge (Figure 5c). Taken together, these data suggest that ARG1 is required – at least in part – for mounting keratinocyte-specific inflammatory responses on wounding.

Discussion

We found that ARG1 is increased in the epidermis of human chronic wounds and scratched keratinocytes. Our results corroborate previous work, which showed that a lack of arginase impairs re-epithelialization in an epidermal-specific arginase knockdown murine model and in NN-treated human wound explants that could be rescued by the arginase metabolite putrescine.[4] Putrescine is likely converted into spermidine and spermine to drive increased motility, corroborating previous findings showing that spermine can enhance cell migration.[11] Similarly to putrescine, the addition of exogenous urea led to scratch closure rescue upon ARG1 inhibition. Urea is an important regulator of keratinocyte proliferation, which prolongs the generation time of postmitotic cells by decreasing DNA synthesis in epidermal basal cells.[32] It is likely that, on wounding NN-treated cells, urea

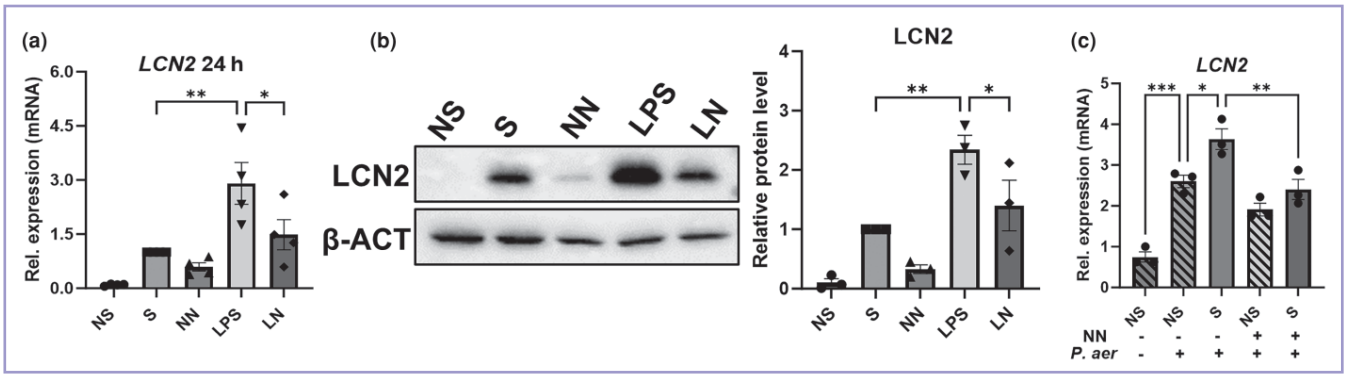


Figure 5 Lipocalin 2 (LCN2) shows arginase 1 (ARG1) dependence and responds to bacterial challenges on wounding. (a) Quantitative polymerase chain reaction (qPCR) mRNA-level analysis of *LCN2* in *in vitro* scratched N/TERT-1 keratinocytes treated with the ARG1 inhibitor nor-NOHA (NN), the bacterial ligand lipopolysaccharide (LPS) or a combination of the two (LN). (b) Representative Western blot images illustrating LCN2 expression and protein quantification in scratch-wounded N/TERT-1 cells treated with NN, LPS and LN; protein expression was normalized to β-actin (β-ACT), which was used as a loading control. (c) qPCR mRNA-level analysis of *LCN2* in *in vitro* scratched N/TERT-1 keratinocytes treated with NN and challenged with live *Pseudomonas aeruginosa* for 6 h postwounding; mRNA expression was normalized to the housekeeping gene *RPLP0*. For all experiments, the results represent the means (SEM) of at least three independent biological replicates. ns, not significant (i.e. $P > 0.05$); NS, not scratched; S, scratched. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

helped rescue scratch closure by promoting migration at the expense of cell proliferation. Further studies are needed to confirm this.

Cell–cell communication via soluble factors represents an important regulator of proper re-epithelialization.[33] Members of the IL-1 cytokine family, including IL-1β, IL-18 and IL-36γ, have been associated with wound inflammation and re-epithelialization.[26,34,35] Human keratinocytes are an important source of IL-36γ, which contributes to the initiation and amplification of an inflamed state that can hinder skin healing.[34] Expression of pro-IL-1β, IL-1α, IL-36γ and the proinflammatory cytokine TNF-α were upregulated on ARG1 inhibition in scratched keratinocytes. An *in vivo* epidermal-specific inhibition of ARG1 also showed similar trends with IL-1β and TNF-α; however, this was not statistically significant, perhaps due to alternative sources of ARG1 in the tissue from cells such as macrophages or the NN not penetrating the entire tissue. This suggests that keratinocyte proinflammatory responses are prolonged upon ARG1 downregulation, providing a putative link between chronic inflammation and decreased ARG1 expression, both of which are features of ageing-related delayed healing.[36,37]

Our data suggest that IL-1α and IL-1β are upregulated on wounding in a p-STAT1/3-related manner and that ARG1 regulates this response, at least partially, through inhibition of p-STAT3 levels. IL-1β, IL-36γ and TNF-α can all activate proinflammatory responses in keratinocytes via NF-κB,[38] forming a positive feedback loop that stimulates their reciprocal production.[39] Wound-driven upregulation of keratinocyte IL-1β, IL-36γ and TNF-α corresponded with increased NF-κB subunit p65 nuclear localization, which was further augmented upon ARG1 inhibition. The increase in these cytokines may have a synergistic effect, converging on keratinocyte NF-κB activation that may be dampened by ARG1. This could account for the increased nuclear localization of the NF-κB subunit p65 in ARG1-inhibited keratinocytes upon scratch. However, NF-κB can shuttle in and out of the nucleus,[40] and a single snapshot of NF-κB nuclear translocation at 24 h postwounding cannot accurately highlight whether ARG1 modulates NF-κB rhythmicity: further studies are needed to evaluate this.

LCN2 has well-established roles in anti-inflammatory responses and keratinocyte migration.[41] LCN2 secretion in scratched cultured cells, as well as *in vivo* murine skin wounds, increased healing rates by driving re-epithelialization.[42] A new aspect of LCN2 biology is highlighted by the finding that upregulation of *LCN2* on wounding is arginase dependent. Putrescine rescued the delay in scratch closure caused by the LCN2-neutralizing antibodies. A potential explanation is that putrescine supplementation and conversion to spermine promoted higher LCN2 expression and secretion to rescue the phenotype.[11]

Owing to the disruption of skin architecture, wounding facilitates bacterial colonization, which – in turn – promotes inflammation.[2] We showed that *IL1A, IL1B* and *TNFA* were upregulated upon microbial ligand (LPS) stimulation in scratched keratinocytes and that NN treatment partially abrogated the increased expression of *IL1A*. This suggests that ARG1 might play a dual role in IL-1α-related responses, as ARG1 inhibition resulted in increased IL-1α under sterile conditions but inhibited IL-1α upon microbial ligand treatment. LPS or live *P. aeruginosa* stimulation in scratched keratinocytes significantly increased LCN2. The upregulation of LCN2 was abrogated on ARG1 inhibition via NN in the presence of LPS. These results highlight that LCN2 could play a dual role in driving scratch closure and keratinocyte inflammatory processes, and that this role is ARG1-dependent upon wounding. LCN2, as an iron sequester, also has an antimicrobial function; future studies could explore the arginase dependence of keratinocyte antimicrobial responses. Furthermore, future studies evaluating the impacts of arginase in infection or in response to a broader range of bacterial ligands would be informative.

Clinically, conflicting reports of the implications of ARG1 in healing responses are reported. Downregulation of ARG1 expression is linked to delayed healing in ageing patients,[37] whereas increased arginase levels and activity have been reported in diabetic wounds.[23,43] In line with previous studies,[43,44] we demonstrated that ARG1 expression was altered

in perilesional regions of a skin pressure ulcer where there was stronger ARG1 expression in the upper parakeratotic epidermis. One explanation is that the wound is trying to close by upregulating prohealing mechanisms, but protracted inflammation via the IL-6–STAT3 axis delays wound closure. Alternatively, arginase malfunction may only be seen in certain chronic wounds. This warrants future investigation. Our finding that ARG1 inhibition could be rescued *in vitro* by the addition of downstream metabolites suggests that manipulation of arginase or its metabolic products has potential in wound repair treatment. Dietary or intravenous supplementation with arginase pathway metabolites, including the amino acids L-arginine and L-ornithine, has already been trialled in the treatment of nonhealing wounds.[45] Urea, the direct byproduct of L-arginine breakdown via ARG1, stimulates healing in chronic purulent wounds.[46] Considering the results of this study, putrescine or urea supplementation might help promote re-epithelialization, especially in conditions where only the epithelium is lost, such as superficial burn injury or skin graft donor sites. Given the propensity of chronic lesions to develop infections,[2] harnessing these metabolites might also help drive antimicrobial responses, as suggested by the ARG1-sensitive antimicrobial highlighted in this study, LCN2.

In summary, our data demonstrate that, on wounding, the ARG1 pathway plays an important role in anti-inflammatory responses and timely re-epithelialization via LCN2, highlighting the importance of tightly controlled ARG1 levels and metabolic activity for the healing outcome of skin lesions.

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Conflicts of interest

The authors declare no conflicts of interests.

Data availability

The data underlying this article are available in the article and in its supplementary material. Datasets related to this article can be found at https://www.ebi.ac.uk/arrayexpress, hosted at ArrayExpress (accession number: E-MTAB-10213).

Ethics statement

Clinical investigation was conducted according to the principles of the Declaration of Helsinki. Human skin specimens were obtained from six adult patients following surgery (Manchester Skin Health Biobank, Research Ethics Committee under 07/Q1406/14 or 13/SC/0499).

Patient consent

Written patient consent for publication was obtained.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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ACR 50, ≥50% response in the American College of Rheumatology criteria; **AS**, ankylosing spondylitis; **CRP**, C-reactive protein; **DMARD**, disease-modifying antirheumatic drug; **HS**, hidradenitis suppurativa; **IGA**, Investigator's Global Assessment; **(m)NRI**, (modified) non-responder imputation; **MRI**, magnetic resonance imaging; **nr-axSpA**, non-radiographic axial spondyloarthritis; **NSAID**, non-steroidal anti-inflammatory drug; **PASI 75/90/100**, ≥75/90/100% improvement from baseline in Psoriasis Area and Severity Index; **PsA**, psoriatic arthritis; **PsD**, psoriatic disease; **PsO**, psoriasis; **TNFi-IR**, tumour necrosis factor-α inhibitor – inadequate responder; **TRAE**, treatment-related adverse event.

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