

Histamine induces proliferation in keratinocytes from patients with atopic dermatitis through the histamine 4 receptor

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Background: Epidermal hyperproliferation resulting in acanthosis is an important clinical observation in patients with atopic dermatitis, and its underlying mechanisms are not completely understood.

Objective: Because increased levels of histamine are present in lesional skin, we investigated the effect of histamine, especially with regard to histamine 4 receptor (H4R) activation, on the proliferation of human and murine keratinocytes.

Methods: The expression of H4R on human and murine keratinocytes was detected by using real-time PCR.

Keratinocyte proliferation was evaluated by using different *in vitro* cell proliferation assays, scratch assays, and measurement of the epidermal thickness of murine skin.

Results: We detected H4R mRNA on foreskin keratinocytes and on outer root sheath keratinocytes; H4R mRNA was more abundant in keratinocytes from patients with atopic dermatitis compared with those from nonatopic donors. Stimulation of

foreskin keratinocytes, atopic dermatitis outer root sheath keratinocytes, and H4R-transfected HaCaT cells with histamine and H4R agonist resulted in an increase in proliferation, which was blocked with the H4R-specific antagonist JNJ7777120.

Abdominal epidermis of H4R-deficient mice was significantly thinner, and the *in vitro* proliferation of keratinocytes derived from H4R-deficient mice was lower compared with that seen in control mice. Interestingly, we only detected H4R expression on murine keratinocytes after stimulation with LPS and peptidoglycan.

Conclusion: H4R is highly expressed on keratinocytes from patients with atopic dermatitis, and its stimulation induces keratinocyte proliferation. This might represent a mechanism that contributes to the epidermal hyperplasia observed in patients with atopic dermatitis. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Histamine, keratinocyte, proliferation, histamine 4 receptor, human, mouse, atopic dermatitis

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Atopic dermatitis affects up to 20% of the population, and its incidence is steadily increasing.¹ Moreover, the therapeutic interventions for atopic dermatitis are limited because the disease is not fully understood. Atopic dermatitis is a multifactorial disease which can be caused by numerous trigger factors and involves the interaction of different cell types: skin-resident keratinocytes or immune cells present in the skin or infiltrating the skin on inflammation.² Keratinocytes have been shown to release a set of mediators, among them chemokines and cytokines, which are important for the attraction of immune cells to atopic dermatitis lesions.³ In turn, the expression of cell-surface receptors is upregulated on keratinocytes in response to signals from the surrounding immune cells.⁴ Apart from the involvement in the immunologic response, keratinocytes contribute to the pathology of atopic dermatitis through a change in their growth characteristics. Thickening of the epidermis is evident in chronic lesions of eczema, as indicated clinically by lichenification and histologically by acanthosis. It has been shown previously that this epidermal hyperproliferation might in part be due to the inflammatory mediators TGF- α and GM-CSF.^{5,6}

Histamine is another important inflammatory mediator that is present at high levels in the skin of patients with atopic dermatitis⁷ and plays an important role in disease pathology.⁸ Keratinocytes were already shown to express histamine 1 receptor (H1R) and histamine 2 receptor (H2R), and histamine stimulation modulates the expression of inflammatory mediators, such as IL-6, IL-8, and CCL5, as well as the cell-surface molecules MHC class II and intercellular adhesion molecule 1.^{4,9} The previously performed

Abbreviations used

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
 H1R: Histamine 1 receptor
 H2R: Histamine 2 receptor
 H3R: Histamine 3 receptor
 H4R: Histamine 4 receptor
 4-MH: 4-Methylhistamine
 nKC: Neonatal keratinocyte
 orsKC: Outer root sheath keratinocyte

work with regard to histamine and keratinocytes did not include the most recently described histamine 4 receptor (H4R).^{10,11} H4R was shown to be involved in the pathogenesis of atopic dermatitis by modulating the function of various immune cells present in the skin (ie, T cells¹² and dendritic cells¹³); however, the expression and function of this receptor on keratinocytes had not yet been identified.

Here we investigated the function of H4R on human and murine keratinocytes with regard to proliferation. Importantly, we found higher expression of H4R on and higher proliferation in response to its stimulation in keratinocytes derived from patients with atopic dermatitis. Accordingly, we found that H4R in murine neonatal keratinocytes (nKCs) is expressed after the mimicry of inflammatory conditions by treatment with LPS and peptidoglycan.

METHODS**Animals**

Male and female wild-type and H4R knockout mice 20 to 22 weeks of age were used for this study. All animals were healthy and housed in groups of 6 mice per cage at 22°C with a 12-hour light/dark cycle. Water and a standard diet (Altromin, Lage/Lippe, Germany) were available *ad libitum*. H4R knockout mice were kindly provided by Johnson and Johnson Pharmaceutical Research and Development (New Brunswick, NJ). The H4R knockout mice were generated by Lexicon Genetics (Woodlands, Tex), as described previously,¹⁴ and were cross-bred with BALB/c mice from Charles River (Sulzfeld, Germany) to obtain heterozygote mice for H4R. These mice were used to obtain homozygote H4R knockout mice and their respective wild-type mice.

Reagents

Various histamine receptor ligands were used in this study: histamine (agonist for all histamine receptors; ALK-Scherax, Wedel, Germany), 4-methylhistamine (4-MH; H4R agonist), 2-pyridylethylamine (H1R agonist), amthamine (H2R agonist; all agonists from Tocris Bioscience, Bristol, United Kingdom), JNJ7777120 (JNJ, H4R antagonist, Tocris Bioscience), levocetirizine (H1R antagonist, UCB, Anderlecht/Brussels, Belgium), and ranitidine (H2R antagonist, Biomol, Hamburg, Germany).

LPS (0111:B4) and peptidoglycan (both from Sigma-Aldrich, Munich, Germany) were used for stimulation of mouse keratinocytes.

Keratinocyte isolation and culture

Human primary nKC cultures were prepared from foreskin, as described previously.¹⁵ The foreskin was cut into pieces and incubated overnight at 4°C in 2.4 U of Dispase II (Roche, Mannheim, Germany). The next day, the epidermis was separated from the dermis and placed for 20 minutes at 37°C in EDTA (0.02%)–trypsin (0.05%) solution (PAN-Biotech, Aidenbach, Germany). After stopping the trypsin reaction by addition of FCS (PromoCell, Heidelberg, Germany), the cell suspension was filtered through a sterile gauze

(40 μ m) and washed 2 times in PBS. The obtained single-cell suspension of nKCs was incubated in the serum-free growth medium Keratinocyte Growth Medium 2 Kit (PromoCell) at 37°C in a humidified atmosphere containing 5% CO₂.

Adult human keratinocytes were isolated from the outer root sheath from plucked hair (outer root sheath keratinocytes [orsKCs]), as described previously.¹⁶ Briefly, the hairs were placed in dishes with a feeder layer of 3T3 fibroblasts that had been treated with mitomycin C (Roche), the medium was changed every 2 to 3 days, and when sufficient orsKCs had outgrown, they were selectively trypsinized and passaged further. Keratinocytes in passages 3 to 6 cultured in hydrocortisone and epidermal growth factor-free medium were used for experiments.

Murine primary nKC cultures were prepared according to the method of Caldelari and Muller.¹⁷ Skin samples of neonatal wild-type (BALB/c) and histamine H4R knockout mice were collected and incubated overnight in Dispase II (5 mg/mL, Sigma-Aldrich) at 4°C. Skins of 2 to 5 mice were pooled for 1 experiment. The next day, the epidermis was peeled off the dermis and incubated in trypsin/EDTA 0.125%/0.05% (Biochrom) for 30 minutes at room temperature. Trypsin/EDTA was inactivated with CnT-07 (CELLnTEC, Bern, Switzerland) plus 10% FCS (PAA, Pasching, Austria), and the epidermis was washed 3 times in CnT-07 medium. A single-cell suspension was obtained by means of gentle rubbing and pipetting. Keratinocytes were seeded at a density of 1×10^6 cells in rat-tail collagen-coated (Roche) 25-cm² flasks (Nunc; Thermo Fisher Scientific, Braunschweig, Germany) and cultured at 37°C in a 5% CO₂ atmosphere. Cell passages 1 to 3 were used for experiments.

Transfection of the HaCaT keratinocyte cell line

To generate HaCaT cells¹⁸ stably expressing H4R, we used a retroviral gene transfer and expression System (Clontech, Mountain View, Calif) based on the retroviral transduction method described by Ory et al.¹⁹ In brief, human H4R was amplified from pcDNA3-HR4 by using the following primers: aaaaaccgggtgccaccatgccagataactaatagcaca (sense primer) and aaaaagatcct-taagaagatactgaccgactg (antisense primer). The amplicon was digested with *Age*I and *Bam*HI and cloned into the retroviral pQCXIN transfer vector (Clontech) that allows bicistronic expression of the gene of interest in combination with a neomycin resistance gene. The generated pQCXIN-H4R construct was confirmed by sequencing of the open reading frame and used for transfection of the 293gpg retrovirus packaging cell line. Retrovirus-containing supernatants of the transfected 293gpg cells were used to transduce HaCaT cells, according to the manufacturer's protocol (Clontech). H4R-expressing, neomycin-resistant cells were selected with 800 mg/L G-418 from Calbiochem (Merck4Biosciences, Darmstadt, Germany).

mRNA isolation, reverse transcription, and quantitative RT-PCR

Murine keratinocytes were stimulated for 24 hours with 50 μ g/mL LPS 0111:B4 and 50 μ g/mL peptidoglycan when they reached confluence. Murine and human keratinocytes were washed in PBS and lysed for RNA isolation by using the Mini RNA Isolation II Kit (Zymo Research, Orange, Calif), and reverse transcription was performed with the First Strand cDNA Synthesis Kit (MBI Fermentas, St Leon-Rot, Germany). Real-time quantitative PCR was performed on a LightCycler (Roche Molecular Biochemicals) using SYBR Green with Quantitect primer assays for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; QT01192646), human H1R (QT00199857), human H2R (QT00210378), human histamine 3 receptor (H3R; QT00210861), and human H4R (QT00032326), as well as murine GAPDH (QT00199388) and murine H4R (QT00135884), according to the manufacturer's instructions (Qiagen, Hilden, Germany). The following PCR settings were used. An initial activation step of 15 minutes at 95°C with a ramp of 20°C/s was followed by 3-step cycling (45 cycles): denaturation for 15 seconds at 94°C, annealing for 20 seconds at 55°C, and extension for 20 seconds at 72°C (all 3 with a ramp of 2°C/s). Melting curve analysis was performed from 60°C to 90°C with a ramp of 20°C/s. The amount of target genes relative to the reference gene GAPDH

was quantified with the Relative Quantification Software (Roche Molecular Biochemicals). Agrose gel electrophoresis was performed with 2% agarose (Roth, Karlsruhe, Germany) in 1× Tris-Borat-EDTA buffer (Roth) to visualize the amplification products after completion of the PCR run.

Cell proliferation assays

Human keratinocytes were seeded in 96-well plates at a density of 1.5×10^4 cells per well and treated with 10 $\mu\text{mol/L}$ histamine or 10 $\mu\text{mol/L}$ agonist (2-pyridylethylamine, amthamine, or 4-MH). For blocking experiments, the cells were incubated with 10 $\mu\text{mol/L}$ specific antagonist (levocetirizine, ranitidine, or JNJ7777120) 30 minutes before addition of the agonists. The proliferation of human keratinocytes was measured at different time points by using the CellTiter96 nonradioactive proliferation assay (MTT assay; Promega, Madison, Wis), according to the manufacturer's recommendations, or by using the thymidine incorporation test. After 1 day in culture, keratinocytes were pulsed with tritiated thymidine (0.5 μCi per well) for approximately 16 hours. Decay as a measure of thymidine incorporation during cell division was counted in a liquid scintillation counter (Wallac System1409). The proliferation index was defined by the ratio of mean counts per minute of stimulated to unstimulated cultures.

Murine wild-type and H4R knockout keratinocytes were seeded in collagen-coated 96-well plates (Greiner, Frickenhausen, Germany) at a density of 2×10^4 cells per well and cultured until 70% confluency. Cells were grown for 12, 24, and 48 hours, and cell proliferation was measured by using BrdU incorporation (BrdU colorimetric assay, Roche), according to the manufacturer's recommendations.

Scratch assay

Scratch assays are regularly used to investigate wound closure mediated by cell migration and proliferation, and they have been used before to evaluate the proliferative capacity of keratinocytes.²⁰ Keratinocytes or HaCaT cells were grown in 6-well plates until they reached a confluence of approximately 90% to 100%. A scratch was set in the middle of the well with a standard 1000- μL pipette tip, and a photograph was taken. After 24 hours, the cells were stained with carboxyfluorescein succinimidyl ester, photographs were taken, and the scratch thickness was evaluated by a group of blinded observers and ranked from 0 (no closure of the scratch) to 6 (complete closure of the scratch).

Evaluation of murine epidermal thickness

The abdominal hair of mice was removed with depilatory cream (Veet, Zurich, Switzerland). Mice were killed 24 hours later, and the abdominal skin of the mice was separated. Skin specimens were fixed in Bouin solution, and paraffin sections (6 μm) were prepared and stained with hematoxylin and eosin. The sections were examined histologically by a blinded investigator. Epidermal thickness (distance from the basal membrane to the stratum corneum) of 10 randomly selected areas was measured with Axiovision 4.8 software (Carl Zeiss, Jena, Germany). The stratum corneum itself could not be measured because it was partially or completely torn off during sectioning.

Statistical analysis

For statistical evaluation of normal distributed data, paired *t* test was used, and for nonnormally distributed data, the Mann-Whitney test (unpaired, nonparametric) or the Wilcoxon matched-pairs signed-rank test (paired, nonparametric) was used; a *P* value of less than .05 was regarded as significant. The program GraphPad Prism (version 3.02; GraphPad Software, San Diego, Calif) was used for statistical analysis.

Ethics

Animal experiments were approved by the LAVES (Oldenburg, Germany; AZ.G33.9-42502-04-10/0252). The investigation of the role of histamine

receptors in human allergic skin inflammation was approved by the local ethics committee of the Hannover Medical School (Vote No. 4253) and was conducted according to the principles of the Declaration of Helsinki.

RESULTS

nKCs express H4R and its stimulation induces proliferation

LightCycler real-time PCR experiments showed that human primary nKCs isolated from foreskin express H4R on the mRNA level (Fig 1, A). We also detected expression of H1R mRNA but not H2R or H3R mRNA (Fig 1, B). Stimulation with histamine or the H4R agonist 4-MH induced proliferation of keratinocytes (1.5- to 2-fold increase), as shown with 2 different cell proliferation assays (MTT and thymidine incorporation). The induction of cell proliferation was most pronounced after 48 hours of treatment (Fig 1, D) and was induced by histamine at concentrations between 10^{-4} and 10^{-8} mol/L (Fig 1, C). Because 4-MH is known to bind not only H4R but also H2R,²¹ we performed experiments in the presence of the selective H4R antagonist JNJ7777120 to show that keratinocyte proliferation was induced by selective H4R stimulation (Fig 1, E). To investigate the role of H1R or H2R, we incubated the keratinocytes with specific H1R and H2R agonists and antagonists and showed no significant involvement of these receptors in inducing proliferation (Fig 1, F).

H4R-transfected HaCaT keratinocytes respond to histamine stimulation with proliferation

The keratinocyte cell line HaCaT (a spontaneously immortalized keratinocyte cell line¹⁸) expresses low to undetectable levels of H4R mRNA and was therefore stably transfected with a plasmid expressing H4R or a control vector, respectively. H4R-transfected HaCaT cells showed a marked increase in H4R expression (Fig 2, A). Furthermore, HaCaT cells expressed H1R mRNA but not H2R or H3R mRNA (Fig 2, B). H4R-transfected HaCaT cells responded to histamine treatment with proliferation, whereas nontransfected and control-transfected HaCaT cells did not show induction of proliferation (Fig 2, D). Histamine dose-dependently induced the increase in proliferation at concentrations between 10^{-4} and 10^{-6} mol/L (Fig 1, C). The H4R antagonist JNJ7777120 blocked the 4-MH-induced proliferation in H4R-transfected HaCaT cells (Fig 2, E). Incubation of H4R-transfected HaCaT cells with specific H1R and H2R agonists and antagonists showed no influence of these receptors on proliferation (Fig 2, F).

H4R is highly expressed on and triggers proliferation of keratinocytes derived from patients with atopic dermatitis

We investigated the expression level of H4R on orsKCs from healthy subjects and patients with atopic dermatitis or psoriasis. The patients with atopic dermatitis showed higher H4R mRNA expression on orsKCs compared with healthy control subjects; for patients with psoriasis, no significant difference was detected (Fig 3, A). Keratinocytes from patients with atopic dermatitis but not those from patients with psoriasis also expressed significantly more H1R mRNA compared with that seen in healthy donors (Fig 3, A). In the proliferation assay orsKCs derived from patients

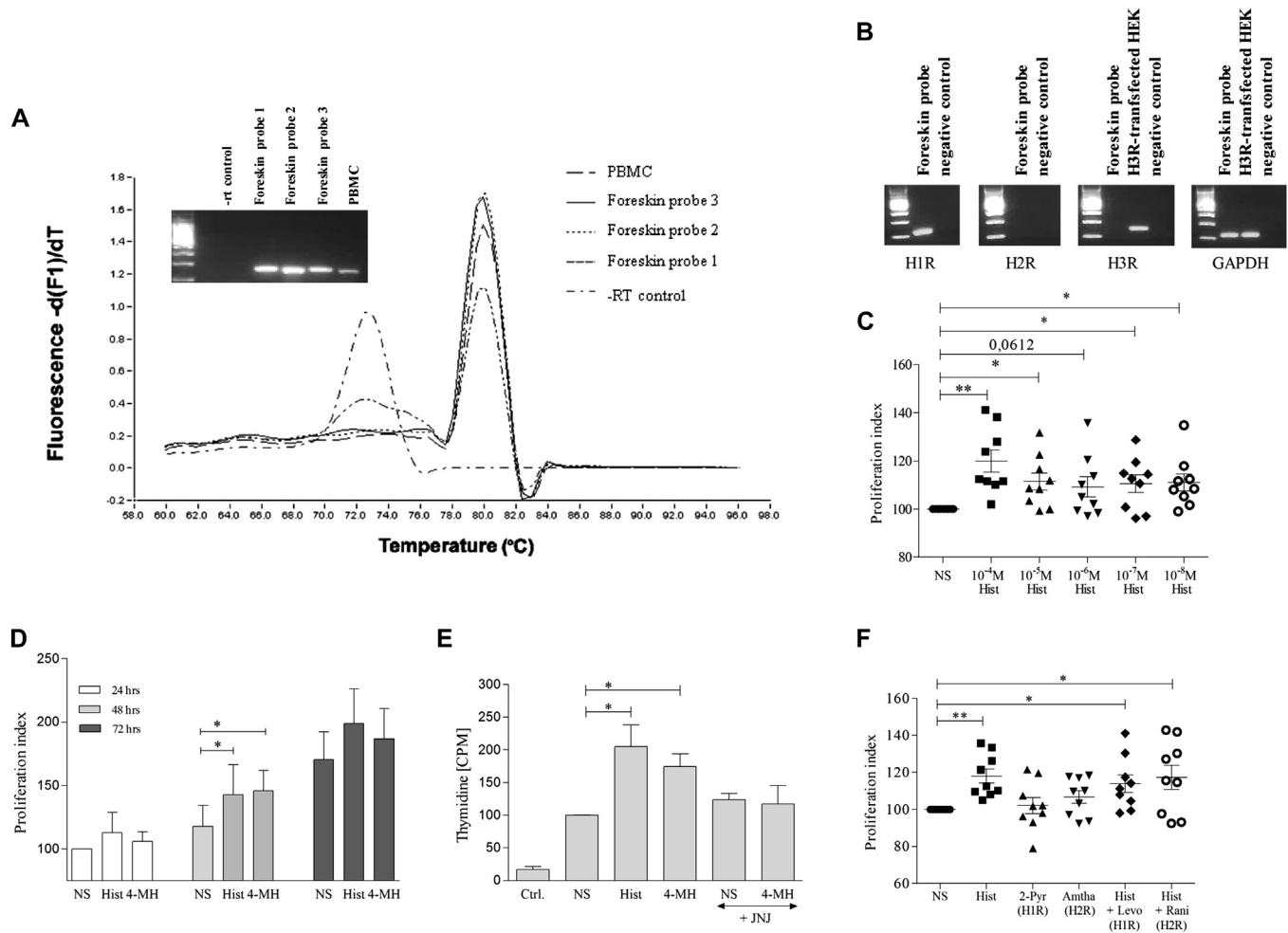


FIG 1. Histamine induces proliferation in human foreskin keratinocytes. **A** and **B**, Representative amplification products and melting peaks of 3 independent real-time PCR experiments are shown. **C** and **D**, Foreskin keratinocytes were treated with different histamine concentrations (Fig 1, **C**) and stimulated with histamine or H4R agonist (4-MH) for different time points (Fig 1, **D**). **E**, The H4R antagonist JNJ777120 (*JNJ*) abolished the H4R agonist-induced effect. **F**, H1R and H2R agonists and antagonists had no influence on proliferation. Means and SEMs of 4 to 8 experiments are depicted. 2-Pyr, 2-pyridylethylamine; Antha, amthamine; Hist, histamine; Levo, levocetirizine; NS, no stimulation; Rani, ranitidine. * $P < .05$ and ** $P < .005$, paired t test.

with psoriasis showed higher basal proliferation, but their proliferative capacity was not changed by histamine. orsKCs from patients with atopic dermatitis responded to stimulation with histamine or an H4R agonist with approximately 1.5-fold higher proliferation compared with that seen in nonstimulated control cells (Fig 3, **B**). The H4R antagonist JNJ777120 blocked histamine-induced proliferation in orsKCs derived from patients with atopic dermatitis (Fig 3, **C**).

Closure of scratching wounds is accelerated on histamine stimulation in H4R-expressing keratinocytes

Stimulation of keratinocyte monolayer cultures of cells that express high H4R levels (ie, H4R-transfected HaCaT cells and orsKCs derived from patients with atopic dermatitis) with histamine or the H4R agonist 4-MH resulted in faster closure of scratching wounds (Fig 4). In untransfected or enhanced green fluorescent protein-transfected HaCaT cells and orsKCs

from healthy subjects, histamine did not influence the rate of wound closure (Fig 4, **B** and **C**). The H4R antagonist JNJ777120 blocked the effect of histamine and 4-MH (Fig 4, **A**).

H4R knockout mice show reduced epidermal thickness and decreased *in vitro* proliferation of keratinocytes

Histologic analyses revealed that the epidermis of healthy abdominal skin of H4R knockout mice is significantly thinner than the epidermis of wild-type mice. Wild-type epidermis was $21 \pm 4.6 \mu\text{m}$ (mean $\pm$ SD) thick, whereas the epidermis of H4R knockout mice was only $15.9 \pm 2 \mu\text{m}$ thick (Fig 5, **A**). Proliferation of neonatal murine keratinocytes was analyzed by using a colorimetric BrdU assay after 12, 24, and 48 hours of culture. H4R knockout keratinocytes showed a trend toward reduced basal proliferation compared with that seen in wild-type keratinocytes at each time point (Fig 5, **B**). Stimulation

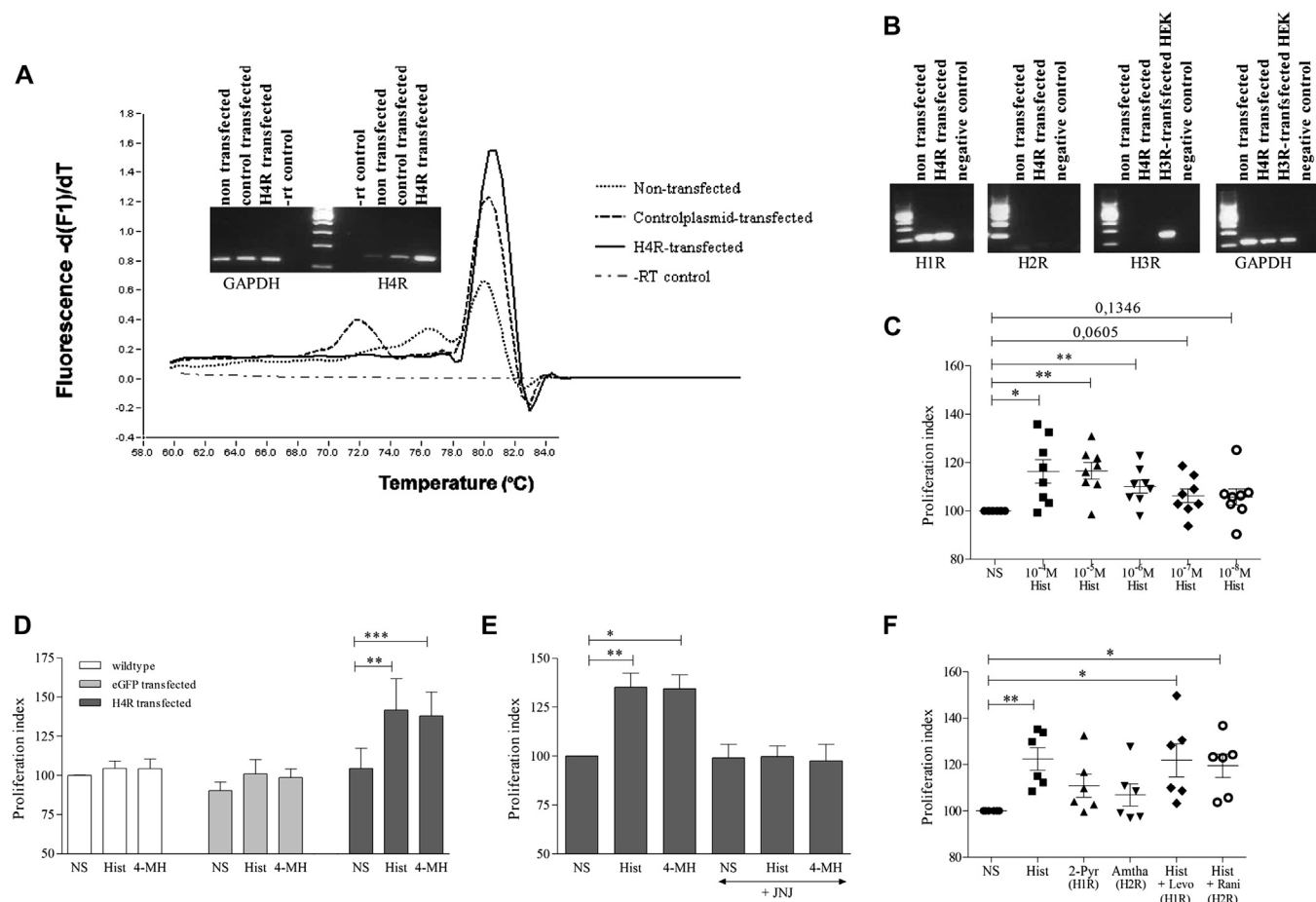


FIG 2. Histamine induces proliferation in H4R-transfected HaCaT keratinocytes. **A** and **B**, Representative amplification products and melting peaks of 5 independent real-time PCR experiments are shown. **C**, H4R-transfected HaCaT cells were treated with different histamine concentrations. **D**, Forty-eight hours of stimulation with histamine and H4R agonist (4-MH) induced proliferation of H4R-transfected HaCaT keratinocytes, as determined by using the MTT assay. **E** and **F**, The H4R antagonist JNJ777120 abolished the H4R agonist-induced effect (Fig 2, **E**), whereas H1R and H2R agonists and antagonists had no influence (Fig 2, **F**). Means and SEMs of 6 to 12 experiments are depicted. 2-Pyr, 2-pyridylethylamine; Amtha, amthamine; Hist, histamine; Levo, levocetirizine; NS, no stimulation; Rani, ranitidine. * $P < .05$, ** $P < .005$, and *** $P < .001$, paired t test.

with histamine did not modulate the proliferation of wild-type and H4R knockout keratinocytes (data not shown).

Murine keratinocytes express H4R under stimulation with LPS and peptidoglycan

Expression of H4R mRNA could not be detected in untreated murine nKCs isolated from healthy wild-type mice. Stimulation of murine keratinocytes with the inflammatory molecules LPS and peptidoglycan for 24 hours induced H4R expression (Fig 6). In contrast, in human keratinocytes stimulation with peptidoglycan, polyinosinic-polycytidylic acid, LPS, or a combination of peptidoglycan and LPS did not result in increased expression of H4R (data not shown).

DISCUSSION

Changes in epidermal proliferation and differentiation are observed in patients with inflammatory skin diseases, such as atopic dermatitis and psoriasis, and represent an important

disease phenotype.^{22,23} Jensen et al²⁴ demonstrated that in lesional skin of patients with atopic dermatitis, the proliferation is 5-fold increased, and even in nonlesional skin keratinocytes show a 2-fold increase of proliferation compared with that seen in healthy subjects. Accordingly, other studies report an enhanced expression of the proliferation-associated keratins 6 and 16 in the skin of patients with atopic dermatitis and those with psoriasis.²⁵ The mechanisms underlying the keratinocyte hyperproliferation are not fully elucidated yet, although several local skin mediators have been shown to be involved. Especially growth factors and cytokines, such as TNF- α , IFN- γ , and IL-1, are involved in the growth of keratinocytes.²⁶ The cytokines IL-21 and IL-23 were recently shown to induce the keratinocyte proliferation and epidermal hyperplasia associated with psoriasis.^{27,28}

In the present study we analyzed the influence of histamine on keratinocyte proliferation because this mediator is present at increased levels in the skin of patients with atopic dermatitis or psoriasis.^{7,29} Moreover, previous studies showed histamine-induced proliferation of different cell types, among them hepatoma cells through H3R stimulation,³⁰ hematopoietic stem

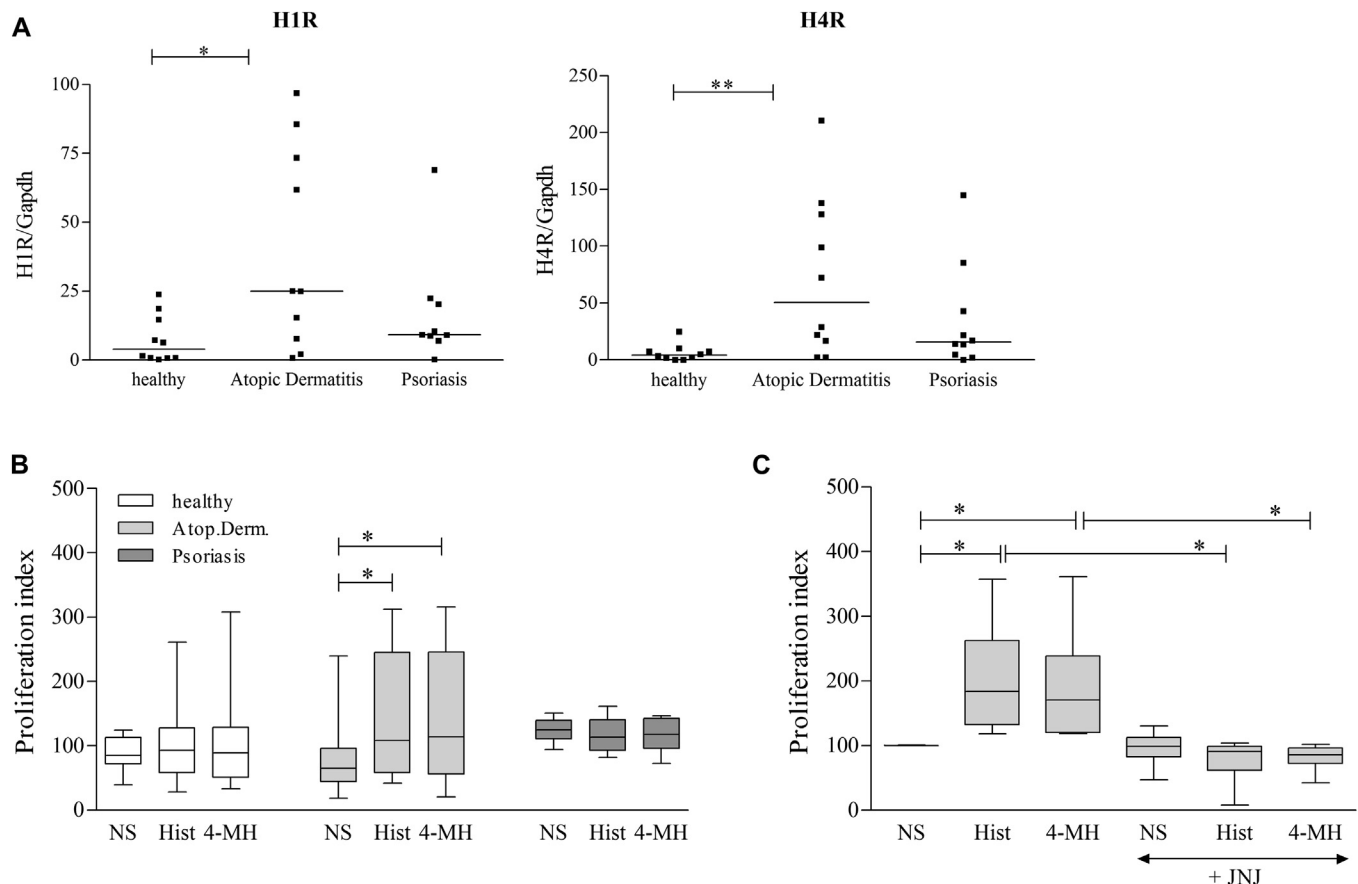


FIG 3. Keratinocytes derived from patients with atopic dermatitis express high levels of H4R and respond with proliferation. **A**, orsKCs derived from patients with atopic dermatitis showed higher H1R and H4R levels than healthy control subjects and patients with psoriasis. **B**, Forty-eight hours of stimulation with histamine and H4R agonist (4-MH) induced proliferation of keratinocytes from patients with atopic dermatitis. **C**, The H4R antagonist JNJ777120 (*JNJ*) abolished the H4R agonist-induced effect in keratinocytes from patients with atopic dermatitis. Medians and quartiles of 6 to 8 experiments are depicted. *Hist*, Histamine; *NS*, no stimulation. The Mann-Whitney test was used for statistical analysis in Fig 3, *A*, and the Wilcoxon matched-pairs signed-rank test was used in Fig 3, *B* and *C*. * $P < .05$ and ** $P < .005$.

cells through H2R stimulation,³¹ and airway smooth muscle cells.³² There is also evidence that histamine influences the proliferation of different cell types in human skin: histamine inhibits the proliferation of skin fibroblasts through activation of H1R and protein kinase C.³³

In the present study we show that histamine can increase the proliferation of human keratinocytes from patients with atopic dermatitis through H4R, which might be relevant in the epidermal hyperplasia seen in patients with atopic dermatitis. We found increased proliferation of nKCs and H4R-transfected HaCaT cells in response to histamine or an H4R agonist.

Furthermore, we observed, using histologic analysis, that H4R knockout mice have a thinner epidermis compared with the corresponding wild-type mice. Macroscopically, no skin abnormalities could be detected. The *in vitro* proliferation potential of keratinocytes derived from H4R-deficient mice was lower compared with that seen in keratinocytes from wild-type control animals, thus correlating with the epidermal phenotypes observed. Surprisingly, H4R expression on murine nKCs was only detectable after stimulation with LPS and peptidoglycan but not on untreated cells. In contrast, we did not observe upregulation of H4R mRNA in human

keratinocytes after stimulation of Toll-like receptors. The lack of H4R mRNA expression in normal murine nKCs complicates the interpretation of the differences that we observed in H4R knockout mice with regard to epidermal thickness and basal proliferative capacity. Moreover, in contrast to other studies, we did not observe proliferation of murine keratinocytes in response to histamine. Maurer et al³⁴ showed that histamine stimulates the proliferation of keratinocytes in epidermal sheets obtained from 6- to 8-week-old C57BL/6 mice. Explanations for the differences could be the fact that Maurer et al used telogen skin of adult mice, which displays the lowest rate of basal keratinocyte proliferation under organ culture conditions in the murine hair cycle and is therefore most sensitive for the detection of even discrete stimulatory effects. The proliferative activity of murine nKCs is high at birth,³⁵ and therefore these cells might not be sensitive for the detection of stimulatory effects. In addition to cellular activation of keratinocytes (ie, anagen vs telogen skin), the local microenvironment most likely influences the proliferative responses of epidermal cells as well.³⁶ The influence of proliferation-modulatory extracellular signals like the local cytokine network cannot be studied in isolated keratinocyte populations. This might explain

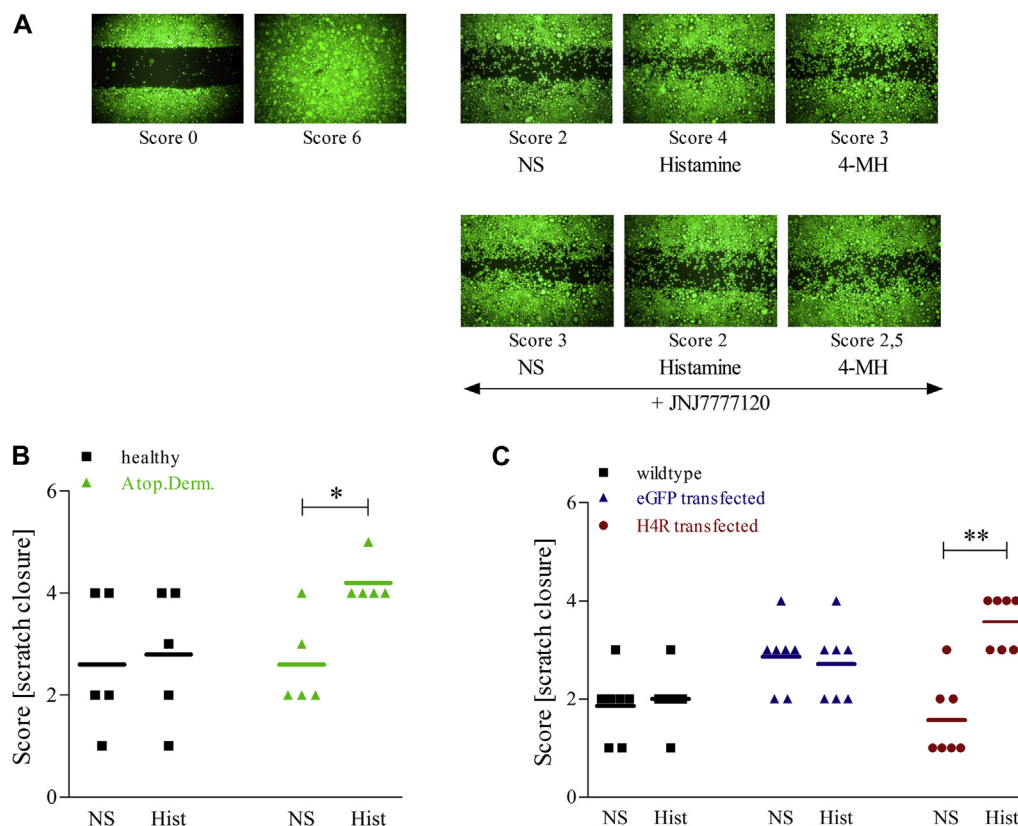


FIG 4. Histamine induces fast scratch wound healing in high H4R-expressing keratinocytes and H4R-transfected HaCaT cells. **A** and **B**, In keratinocytes from patients with atopic dermatitis, the scratch wound was better closed after 24 hours stimulation with histamine or the H4R agonist (4-MH) compared with nontreated control subjects. In contrast, histamine did not modify wound healing in healthy keratinocytes (Fig 4, **B**). **C**, Histamine induced faster scratch wound healing in H4R-transfected HaCaT keratinocytes but not in nontransfected or control-transfected cells. Individual values and medians of 5 to 7 experiments are depicted. eGFP, Enhanced green fluorescent protein; Hist, histamine; NS, no stimulation. * $P < .05$ and ** $P < .005$, paired t test.

the differences in findings by Maurer et al,³⁴ as well as our *ex vivo* data (ie, decreased thickness of epidermis in H4R knockout mice) and the fact that histamine stimulation did not modulate the proliferation of nKCs *in vitro*. Moreover, in the experimental protocol used in Maurer et al, inflammatory conditions upregulating H4R expression might have been induced, such as the reaction to hair removal. However, why H4R knockout keratinocytes showed a lower basal proliferation in the absence of any external stimulus compared with wild-type keratinocytes still needs to be investigated. Autocrine secretion of factors that modulate keratinocyte proliferation, such as TGF- α and GM-CSF, might be decreased in H4R knockout keratinocytes.^{5,6} Also, in another study Lin et al³⁷ showed that other histamine receptors are involved in murine keratinocyte proliferation; they observed that H1R and H2R antihistamines stimulate *in vivo* murine epidermal proliferation in adult albino hairless (Skh1) mice. This further complicates the area because both histamines and antihistamines can modulate epidermal proliferation, probably depending on the experimental system. From our studies, we have the impression that the H4R knockout mouse does not represent a good model for further *in vivo* investigation of the findings from human keratinocytes, and it is unclear whether H4R represents a functional receptor on murine keratinocytes.

An important observation that we made in *in vitro* experiments with human keratinocytes is that H4R stimulation induced proliferation in keratinocytes derived from patients with psoriasis but not in cells from patients with atopic dermatitis or donors without skin disease. The reason for this difference in the response to histamine might be explained by the observation that keratinocytes from patients with atopic dermatitis express higher levels of H4R, whereas the receptor is expressed at relatively low levels on keratinocytes from healthy control and patients with psoriasis. A study describing differences in H4R expression in Chinese patients with atopic dermatitis compared with healthy control subjects because of polymorphisms in the HRH4 gene³⁸ provides a hint that regulation on the genetic level might be involved. However, the reason for the higher expression of H4R on keratinocytes from patients with atopic dermatitis still needs to be elucidated and will be subject to future studies.

It was noted already that the epidermal hyperplasia in patients with atopic dermatitis and those with psoriasis might be triggered by different mechanisms. For example, IL-21 and IL-23 were shown to induce epidermal hyperplasia only in patients with psoriasis.^{26,27} Similarly, the inducer of keratinocyte proliferation peroxisome proliferator-activated receptor δ is upregulated in lesional skin of patients with psoriasis but not in skin from patients with atopic dermatitis.³⁹ Here we identified histamine

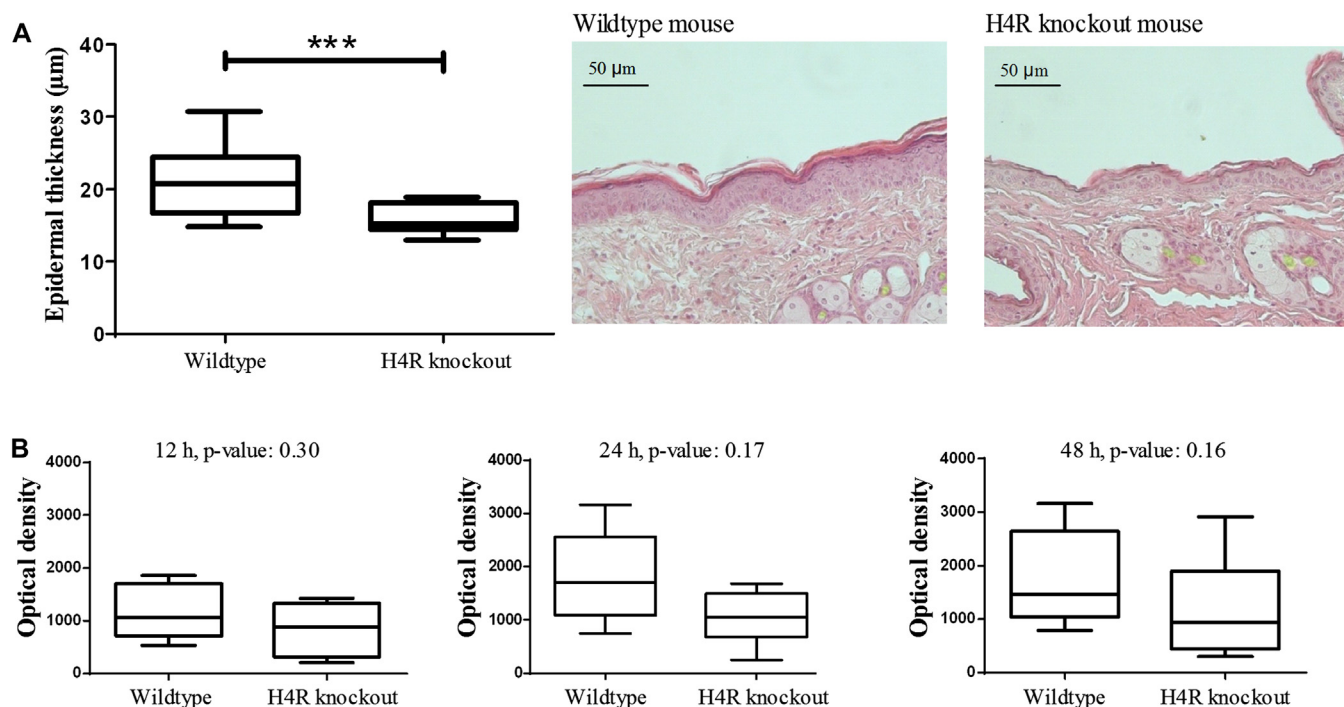


FIG 5. H4R-deficient mice show decreased epidermal thickness, and keratinocytes derived from H4R-deficient mice have lower *in vitro* proliferative capacity. **A**, Biopsy specimens were taken from abdominal skin of wild-type and H4R-deficient mice, and epidermal thickness was measured. **B**, nKCs from H4R knockout and wild-type mice were cultured for 12, 24, and 48 hours, and cell proliferation was determined by using BrdU incorporation. Means and SEMs of 5 (12 hours), 6 (24 hours), and 7 (48 hours) experiments are shown. The Mann-Whitney test was used for statistical analysis. *** $P < .001$.

as an inducer of orsKC proliferation selectively in patients with atopic dermatitis but not in those with psoriasis.

Interestingly, we found a difference in the response to histamine depending on the keratinocyte source. In contrast to healthy nKCs, healthy orsKCs derived from the hair root did not show increased proliferation on stimulation with histamine. Because both cell types express H4R, the variation should be in response to histamine stimulation or in the proliferative capacity of the keratinocytes themselves. However, at the moment, we can only speculate on an explanation for the difference in histamine response of nKCs and orsKCs, both of healthy origin.

Keratinocyte proliferation can be induced in a paracrine or autocrine manner by various mediators. For example, fibroblast growth factor 7 and insulin-like growth factor I secreted by fibroblasts and melanocytes act as paracrine proliferation inducers.^{33,40} On the other hand, TGF- α and GM-CSF are synthesized by keratinocytes themselves and promote their proliferation in an autocrine manner.^{5,6} Because histamine is mainly secreted by mast cells, it regulates keratinocyte proliferation in a paracrine manner, if we assume that histamine directly affects keratinocyte proliferation. However, it cannot be excluded that it is an indirect effect through the upregulation of other mediators, such as GM-CSF, which is known to be upregulated in keratinocytes on histamine stimulation⁴¹ and is by itself an inducer of keratinocyte proliferation.⁶ Therefore further studies are required to investigate the level of endogenous histamine and other factors (eg, GM-CSF, TGF- α , and endothelin) produced in human and murine keratinocytes.

Recent studies have investigated the effect of histamine on keratinocyte differentiation, a process closely linked to keratinocyte proliferation. In a murine model it was shown that H1R and H2R antihistamines enhance epidermal differentiation and epidermal proliferation; the involvement of H4R was not investigated because it was not detected in mouse keratinocytes.³⁷ In human *in vitro* keratinocyte cultures and skin equivalents, it was described that histamine reduces the keratinocyte differentiation dependent on H1R stimulation, whereas experiments with histamine receptor agonists and antagonists showed no effect of H4R on keratinocyte differentiation.⁴² This study, together with our results, indicates that histamine mediates keratinocyte growth by 2 different receptors: proliferation through H4R and differentiation through H1R. However, it might still be worth investigating the differentiation capacity of keratinocytes derived from patients with atopic dermatitis in response to histamine and H4R stimulation because the previous data on differentiation was obtained from healthy keratinocytes.⁴²

In summary, we demonstrate that H4R is expressed on human keratinocytes. Interestingly, receptor expression is significantly increased in patients with atopic dermatitis compared with that seen in healthy subjects or patients with psoriasis. Stimulation of H4R resulted in induction of proliferation of human keratinocytes. The hyperproliferation of keratinocytes in response to histamine in patients with atopic dermatitis might play an important pathophysiologic role, and based on our results, H4R antagonists could represent a new strategy for the therapy of atopic dermatitis.

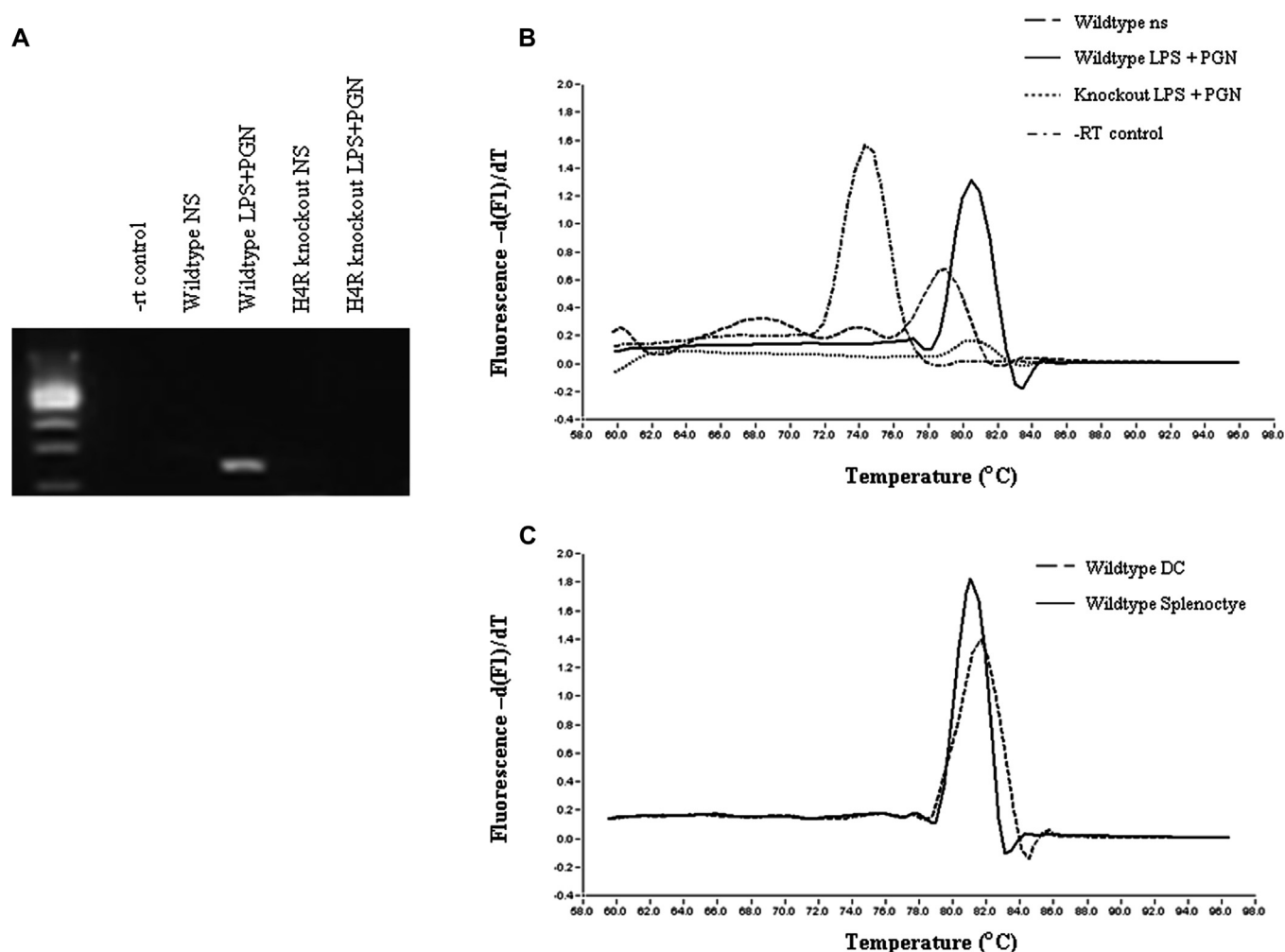


FIG 6. LPS and peptidoglycan induce expression of H4R in murine nKCs. **A** and **B**, Representative amplification products and melting peaks of 3 independent experiments are shown. **C**, As a control, H4R was amplified from wild-type mice spleen and dendritic cell (DC) mRNA samples, showing the same melting peak as the treated keratinocytes. PGN, Peptidoglycan; NS, no stimulation.

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Key messages

- H4R is highly expressed on human keratinocytes isolated from patients with atopic dermatitis and on murine keratinocytes cultured under inflammatory conditions.
- Stimulation of H4R induces proliferation of human keratinocytes isolated from patients with atopic dermatitis.
- H4R stimulation might play a role in the epidermal acanthosis observed in patients with chronic eczema.
- H4R knockout mice have thinner abdominal epidermis compared with wild-type mice.

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