免疫組織染色により、PNA で染色される胚中心の形成が確認された（未発表データ）。

D．考察

カルシポトリオール誘導アトピー性皮膚炎モデルは、カルシポトリオール刺激をうけた表皮角化細胞の TSLP 過剰産生によってアトピー性皮膚炎様の皮膚炎を発症することが報告されている。今回、本モデルにおける IgE 誘導も TSLP 依存性であることが明らかとなった。また、本モデルでは、抗原反復塗布やダニ寄生によるアトピー性皮膚炎モデルと異なり外来抗原による感作を伴わない。すなわち、表皮角化細胞の TSLP 過剰産生のみで IgE 産生が誘導されることが示唆された。こうした IgE 産生も、外来抗原に対する抗原特異的 IgE 誘導と同様に T 細胞依存性であり、所属リンパ節における胚中心形成と胚中心 B 細胞分化を伴っていることが本研究により明らかとなった。

E．結論

カルシポトリオール誘導アトピー性皮膚炎マウスモデルにおける IgE 誘導は、T 細胞依存性であり、所属リンパ節における胚中心形成と胚中心 B 細胞分化を伴う。

F．健康危険情報

なし

G．研究発表

1. 論文発表

なし

2. 学会発表

Kitoh A., Shibuya R., Kabashima K. Underlying mechanism of IgE induction by type 2 skin inflammation in mice. Cell symposia: The Multifaceted Roles of Type 2 Immunity, 2014, Bruges, Belgium

H. 知的財産権の出願・登録状況

なし

厚生労働科学研究委託費（難治性疾患等実用化研究事業）
委託業務成果報告（業務項目）

表皮を標的としたアトピー性皮膚炎の治療の最適化を目指す新規薬剤の開発に関する研究
化合物の探索とバリア機能評価に関する研究

担当責任者：谷崎英昭、大日輝記、宮地良樹（京都大学）

研究要旨

体表のプロテアーゼとそのインヒビターのバランスは、化学的バリアとして病原体の定着や侵入を防いでいる。申請者らは、表皮における化学的バリアの障害はアトピー様皮膚炎の重要な発症因子であり、また治療の標的となり得ると考えた。バリア障害による皮膚炎の発症に関与する分子を探索する目的で、４種類のアトピー性皮膚炎動物モデルから採取した皮膚検体より、転写産物の発現を網羅的に解析した。その結果、最終的に共通して発現が上昇したのは、既知の３つを含む、ただ４つの遺伝子のみであった。第４の新規遺伝子の発現は、アトピー性皮膚炎を対象とした既存の３つの網羅的解析試験でも発現が上昇していた。さらに、システインプロテアーゼを感作したマウスの表皮で確認したところ、感作の 2 日目までに、定常状態の 1,000 倍に発現が上昇していた。以上より、この新規遺伝子の転写産物を含む 4 つの分子が、プロテアーゼを含む化学的バリアの異常を背景とした皮膚炎の発症に関与しており、新規薬剤の標的となる可能性が示唆された。

A．研究目的

アトピー性皮膚炎において皮膚のバリアの障害がTh2型の炎症に関与すると考えられている。体表のプロテアーゼとそのインヒビターのバランスは、化学的バリアとして病原体の定着や侵入を防いでいる。我々は化学的バリアに注目し、その異常が生体でTh2型免疫を誘導する機序を、探索型研究と、分子指向型研究の双方向から明らかにする。

B．研究方法

バリアの以上から湿疹型の皮膚炎を生じるアトピー性皮膚炎の動物モデルとして、ダニが寄生したNC/Nga マウス、突然変異でバリア障害を生じた Flaky tail マウス、フィラグリン欠損マウス、卵白アルブミン反復感作モデルの４つの動物モデルから、計 40 の皮膚検体を採取し、転写産物の発現を網羅的に解析した。4,000 以上の non-coding RNA を含む35,240の遺伝子発現について検討を行った。

C．研究結果

最終的に共通して発現が上昇したのは、既知の３つを含む、ただ４つの遺伝子のみであった。第４の新規遺伝子の発現を、アトピー性皮膚炎を対象とした既存の４つの網羅的解析の試験結果に照会したところ、３つの試験で、オルソログ遺伝子の発現上昇を確認できた。さらに、パパインを感作したマウスの表皮で確認したところ、感作の 2 日目までに、定常状態の 1,000 倍に発現が上昇していた。

D．考察

以上の研究結果より、この新規遺伝子

“X” が、システインプロテアーゼの間接的な標的であり、即時性の転写を通じてTh2 型の免疫応答を誘導する可能性が示唆された。

E．結論

表皮を対照としたアトピー性皮膚炎の新たな治療の標的となり得る、新規の分子を含む４つの分子を決定した。

F．健康危険情報

なし

G．研究発表

1. 論文発表
2. 学会発表

H．知的財産権の出願・登録状況

（予定を含む）

様式第１９

学 会 等 発 表 実 績

委託業務題目「表皮を標的としたアトピー性皮膚炎の治療の最適化を目指す新規薬剤の開発」
機関名 京都大学

１．学会等における口頭・ポスター発表

発表した成果 （発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所 （学会等名）	発表した 時期	国内・ 外の別
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The histamine release from epidermal keratinocytes is involved in ?-melanocyte-stimulating hormone-induced itching in mice.	Shimizu K, Andoh T, Yoshihisa Y, Shimizu T.	The 39th Annual meeting of the Japanese Society for Invetigative Dermatology; Osaka.	2014 Dec 12-14;	国内

2. 学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Possible new therapeutic strategy to regulate atopic dermatitis through upregulating filaggrin expression.	Otsuka A1, Doi H2, Egawa G2, Maekawa A3, Fujita T3, Nakamizo S2, Nakashima C2, Nakajima S2, Watanabe T4, Miyachi Y2, Narumiya S3, Kabashima K5.	J Allergy Clin Immunol 133: 139-46	2014 Jan	国外
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Possible new therapeutic strategy to regulate atopic dermatitis through upregulating filaggrin expression

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Background: Nonsense mutations in filaggrin *(FLG)* represent a significant genetic factor in the cause of atopic dermatitis (AD).
Objective: It is of great importance to find drug candidates that upregulate FLG expression and to determine whether increased FLG expression controls the development of AD.
Methods: We screened a library of bioactives by using an *FLG* reporter assay to find candidates that promoted *FLG* mRNA expression using a human immortalized keratinocyte cell line (HaCaT). We studied the effect of the compound on keratinocytes using the human skin equivalent model. We examined the effect of the compound on AD-like skin inflammation in NC/Nga mice.
Results: JTC801 promoted *FLG* mRNA and protein expression in both HaCaT and normal human epidermal keratinocytes. Intriguingly, JTC801 promoted the mRNA and protein expression levels of FLG but not the mRNA levels of other makers for keratinocyte differentiation, including loricrin, keratin 10, and transglutaminase 1, in a human skin equivalent model. In addition, oral administration of JTC801 promoted the protein level of Flg and suppressed the development of AD-like skin inflammation in NC/Nga mice.
Conclusion: This is the first observation that the compound, which increased FLG expression in human and murine keratinocytes, attenuated the development of AD-like skin inflammation in mice. Our findings provide evidence that modulation of FLG expression can be a novel therapeutic target for AD. (J Allergy Clin Immunol 2014;133:139-46.)

Key words: Atopic dermatitis, filaggrin, JTC801, keratinocyte differentiation

Abbreviations used
AD: Atopic dermatitis
AP-1: Activator protein 1
BMDC: Bone marrow–derived dendritic cell
C_T: Cycle threshold
FLG: Filaggrin
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
K10: Keratin 10
NHEK: Normal human epidermal keratinocyte
ORL1: Opioid receptor-like 1
SC: Stratum corneum
TCR: T-cell receptor
TEWL: Transepidermal water loss
TGM1: Transglutaminase 1

The main function of the skin is to form a protective barrier against external stimuli, such as irritants and allergens. In the epidermis terminal differentiation of keratinocytes leads to the formation of the stratum corneum (SC), an impenetrable physical barrier consisting of flat anucleated corneocytes imbedded in an intercellular matrix rich in nonpolar lipids.[1] The cross-linking of filaggrin (FLG) monomers derived from profilaggrin produced in the keratinocytes with keratin filaments induces an aggregation of the keratin filaments into tight bundles.[2] In the upper layers of the SC, FLG monomers are further processed to hydroscopic amino acids and their derivatives by proteases[3] called natural moisturizing factors, which play a major role in SC hydration.[4]

Barrier disruption and continuous percutaneous exposure to allergens presumably initiate and drive the development of atopic dermatitis (AD).[5-7] AD is a common skin condition characterized by a complex and heterogeneous pathogenesis.[6-9] Direct evidence of a link between the incidence of AD and nonsense mutations in the gene encoding *FLG* has been discovered.[5] *FLG*-null mutations are seen in approximately 20% to 30% of patients with AD.[5,10-12] Additionally, irrespective of mutations in *FLG*, FLG expression is downregulated in almost all cases of moderate-to-severe AD.[13] This finding suggests that barrier dysfunction is a primary cause of AD.

We and others have previously demonstrated that flaky tail mice, namely those deficient in the *Flg* gene, elicited a severe contact hypersensitivity reaction compared with wild-type mice and exhibited spontaneous AD-like skin lesions.[14-18] Consistently, mice with mutations in *Flg* allowed the penetration of both haptens and protein antigens through the SC, which resulted in exaggerated immune responses.[19] Moreover, a large-scale study proved that intragenic copy number variation within *FLG*, with alleles encoding 10, 11, or 12 repeats of FLG monomers, contributes to the risk of AD.[20] Moreover, a recent highlight in the allergy field is that the mutations in the *FLG* gene are associated with not only skin but also other allergic

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diseases. The biology of this molecule in its altered function offers new insights into a range of conditions not previously thought to be related.[21] Therefore a therapeutic strategy through modulation of FLG expression is promising not only to control AD but also to prevent the development of other allergic diseases.

In this study we screened a library of bioactives using an FLG reporter assay and found some candidates that promoted *FLG* mRNA expression in a human immortalized keratinocyte cell line (HaCaT). Among the candidates, JTC801 promoted *FLG* expression with normal human epidermal keratinocytes (NHEKs) independent of opioid receptor-like 1 (ORL1) receptor signaling. The human skin equivalent model revealed that JTC801 promotes the expression level of FLG but has no marginal affect on other differentiation markers of keratinocytes. Furthermore, oral administration of JTC801 promoted the protein level of Flg and suppressed the development of AD-like skin inflammation in NC/Nga mice.

METHODS

Cell culture and reagents

Second-passage neonatal foreskin NHEKs were purchased from Kurabo Industries (Osaka, Japan) and cultured in the serum-free keratinocyte growth medium HuMedia-KG2 (Kurabo Industries) containing human epidermal growth factor (0.1 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), and bovine brain pituitary extract (0.4% vol/vol) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The Ca^{2+} concentration in HuMedia-KG2 was 0.15 mmol/L. Cells were passaged at 60% to 70% confluence to avoid differentiation, and the experiments were conducted with subconfluent cells at passage 3 or 4 in the proliferative phase at 60% to 80% confluence. Induction of keratinocyte differentiation was achieved by culturing the keratinocytes for 48 hours in a culture medium containing 1.35 mmol/L Ca^{2+}. Keratinocyte differentiation was confirmed based on morphologic changes determined by using microscopy. The wild-type and activator protein 1 (AP-1)–deleted profilaggrin constructs were generated as in previous reports.[22] NHEKs were transfected with pGL4.17 plasmid by using TransIT-LT1 reagent (Panvera, Madison, Wis), according to the manufacturer's protocol.

JTC801 was purchased from Tocris Bioscience (Bristol, United Kingdom). Recombinant human IL-4 and IL-13 were purchased from R&D Systems (Minneapolis, Minn).

Mice

C57BL/6NCrSlc (B6) mice were purchased from SLC (Shizuoka, Japan). Pure flaky tail mice were backcrossed to B6 mice for 10 generations to have the single homozygous Flg mutation from flaky tail mice. Female mice were used in all experiments, unless otherwise stated. They were maintained on a 12-hour light/dark cycle at a temperature of 24°C and a humidity of 50% ± 10% under specific pathogen-free conditions at Kyoto University Graduate School of Medicine. NC/Nga mice were purchased from SLC, maintained under conventional conditions, and orally administrated JTC801 (30 mg/kg in 0.5% methyl cellulose) or vehicle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Human skin equivalent model

A 3-dimensional human dermal model kit (TESTSKIN LSE-high; Toyobo, Osaka, Japan) derived from human normal keratinocytes and fibroblasts was cultured in the presence of JTC801 for 5 days, according to the manufacturer's protocol.

Immunohistochemistry

Deparaffinized sections were immersed in 0.3% H_2O_2 to abolish endogenous peroxidase activity and incubated with a monoclonal mouse anti-human FLG (clone, AKH-1; Santa Cruz Biotechnology, Dallas, Tex) overnight. The secondary antibody and 3,39-diaminobenzidine staining were added according to the manufacturer's protocol (R&D Systems). The skin sections were counterstained with hematoxylin.

Transepidermal water loss and SC conductance

Measurements of transepidermal water loss (TEWL) and SC conductance were performed at room temperature (22°C-26°C) and 40% to 60% humidity. TEWL was measured on the lesional skin by using a Vapo Scan AS-VT100RS machine (Asahi Biomed, Tokyo, Japan). SC hydration was evaluated by analyzing skin electrical impedance with a Corneometer SKICON-200 (IBS, Hamamatsu, Japan). All data are presented as the median of 3 repeated recordings.

Quantitative PCR analysis

Quantitative PCR analysis was performed, as reported previously.[23] Briefly, total RNAs were isolated from NHEKs with Trizol (Life Technologies, Gaithersburg, Md). cDNA was reverse transcribed with a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR with a Light Cycler real-time PCR apparatus was performed (Roche Diagnostics, Foster City, Calif) by using SYBR Green I (Roche, Basel, Switzerland). The primer sequences are described in the Methods section in this article's Online Repository at www.jacionline.org. Expression of mRNA (relative) was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA by the change in the Δ cycle threshold (ΔC_T) method and calculated based on $2^{-\Delta CT}$.

Western blotting

Proteins were obtained from ear skin, as reported previously.[24] These samples were used for immunoblotting with polyclonal antibodies against FLG, loricrin, and keratin 10 (K10; COVANCE, Berkeley, Calif). Horseradish peroxidase–conjugated secondary anti-mouse IgG antibody (GE Healthcare, Piscataway, NJ) was used for detection with ECL (GE Healthcare).

Clinical observation

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria that were used for the NC/Nga mouse.[25] In brief, the total clinical score for skin lesions was designated as the sum of individual scores of the right ear, left ear, dorsum, and face graded as 0 (none), 1 (mild), and 2 (severe) for the symptoms of erythema/hemorrhage, edema, crust, excoriation/erosion, and scaling/dryness.

Statistical analysis

Unless otherwise indicated, data are presented as means ± SDs and are representative of 3 independent experiments. *P* values were calculated with the Wilcoxon signed-rank test. *P* values of less than .05 are considered significantly different.

RESULTS

JTC801 promotes the transcription level of profilaggrin

To explore candidate compounds that promote the *FLG* mRNA, we screened the 1120-compound library of bioactives (Tocriscreen Mini) using HaCaT cells by means of real-time PCR.[26] This screening revealed that several compounds, such as a histone deacetylase inhibitor (Scriptaid, Tocris Bioscience), actinomycin D, and JTC801, potently promoted the *FLG* mRNA expression (see Table E1 in this article's Online Repository at www.jacionline.org). Because HaCaT is an immortalized keratinocyte cell line, we further examined whether these compounds increase *FLG* mRNA expression with NHEKs

using real-time PCR. Contrary to the observations in HaCaT cells, actinomycin D, CP 55,940, and MG132 did not increase *FLG* expression (data not shown). We chose Scriptaid and JTC801 for further analysis because they most highly promoted *FLG* mRNA expression among these compounds and upregulated *FLG* mRNA expression in both HaCaT cells (see Fig E1 in this article's Online Repository at www.jacionline.org) and NHEKs (Fig 1, *A*).

For further examination to verify that Scriptaid and JTC801 promoted *FLG* expression and to identify the responsible motif for regulating *FLG* expression, we prepared reporter constructs in which the expression of a luciferase gene is under the control of a wild-type promoter sequence and transfected them into NHEKs. A significant increase in the relative activity of *FLG* was found after a 1-day incubation with Scriptaid but not with JTC801 or high Ca^{2+} levels (Fig 1, *B*). After a 3-day incubation, JTC801 and a high Ca^{2+} condition significantly increased the relative activity of *FLG*. The relative activity of *FLG* under both high Ca^{2+} levels and JTC801 was increased compared with that under the condition of high Ca^{2+} or JTC801. Scriptaid decreased the *FLG* expression after a 3-day incubation, and trypan blue staining showed that all NHEKs were dead with Scriptaid after a 5-day incubation (see Fig E1). In addition, JTC801 promoted pro-FLG expression by means of Western blotting (see Fig E2 in this article's Online Repository at www.jacionline.org). Therefore we chose JTC801 for further analysis.

Previous reports have demonstrated that differentiation of keratinocytes in the presence of IL-4 and IL-13, which are the T_H2 cytokines overexpressed in acute AD skin, significantly reduced FLG protein expression.[13,27] Consistently, the relative activity of *FLG* was significantly decreased 3 days after the incubation of NHEKs in the presence of IL-4 and IL-13 (Fig 1, *C*). Intriguingly, JTC801 increased the relative activity of *FLG*, even with IL-4 and IL-13, which indicated that JTC801 potentially increased FLG expression in the skin lesions of patients with acute AD.

Keratinocyte differentiation can be identified by examining morphologic changes with microscopy. Although NHEKs changed to a differentiated morphology after a 3-day incubation under the condition of high Ca^{2+} levels, NHEKs with JTC801 had the same morphology as those under control conditions (Fig 1, *D*). The extent of the upregulation of *FLG* expression elicited by JTC801 was higher than that elicited by Ca^{2+} 3 days after incubation (Fig 1, *B*). These results suggest that JTC801 increases *FLG* expression but does not promote the differentiation of keratinocytes in general.

AP-1 region is a main sequence of JTC801-dependent *FLG* expression

To identify which motif regulates the JTC801-induced *FLG* expression, we prepared several lengths of *FLG* promoter constructs (−2204, −1504, −980, and −246) and transfected them into NHEKs. All constructs were sufficient to maintain JTC801-induced *FLG* expression (Fig 2, *A*). To further clarify the primary control of *FLG* production at the transcription level, we generated an AP-1–deleted construct and transfected it into NHEKs to examine the relative activity of *FLG* because AP-1–regulated transcription factors control genes expressed in terminally differentiating keratinocytes, including *FLG*.[22] Consistent with this, AP-1–deleted constructs abolished the induction of the relative activity of *FLG* by JTC801 (Fig 2, *B*).

Next, we examined whether the effect of JTC801 on the activity of the *FLG* promoter is limited to keratinocytes. A transfected *FLG* promoter construct in a cervical cancer cell line of HeLa cells did not increase the promoter activity of *FLG* in the presence of JTC801, Ca^{2+}, or JTC801 plus Ca^{2+} in combination (Fig 2, *C*). These results suggest that the effect of JTC801 on *FLG* expression was selective in cell types.

To assess the affect of JTC801 on the development of tight junctions, we evaluated mRNA expression of claudin-1, which is an essential element of tight junctions,[28] in NHEKs in the presence or absence of JTC801. There was no difference in mRNA expression of claudin-1 between them (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org).

Next, to assess the affect of JTC801 on immune cell function, we performed a proliferative assay using T cells from NC/Nga mice. The numbers of total T cells, $CD4^+$ T cells, $CD8^+$ T cells, IFN-γ^+ cells, and IL-4^+ cells were comparable, irrespective of the presence of JTC801 (see Fig E3, *B*).

Moreover, we generated bone marrow–derived dendritic cells (BMDCs) and evaluated the effect of JTC801 on the expression of maturation markers. The results showed no effect of JTC801 on dendritic cell maturation at any concentrations tested (see Fig E3, *C*). Taken together, these results suggest that the effects of JTC801 on the development of tight junctions and on immune cell function are limited.

JTC801 promotes the protein expression level of FLG monomers in the human skin equivalent model

The human skin equivalent model closely simulates the morphologic and biochemical features of differentiated epidermis.[29] We studied the effect of JTC801 on keratinocytes by using this model. The mRNA level of *Ki67*, which is associated with cell proliferation, was comparable, irrespective of the presence or absence of JTC801. On the other hand, *FLG* mRNA was significantly increased by the addition of JTC801 (Fig 3, *A*). Loricrin, K10, and transglutaminase 1 (TGM1) were other makers for keratinocyte differentiation. The mRNA levels of loricrin, *K10*, and *TGM1* were not increased by the addition of JTC801 (Fig 3, *A*). *FLG* is expressed in the terminal differentiation stage of keratinocytes, which is different from other markers, such as *K10* and *TGM1*. Therefore the effect of JTC801 seems to be limited to the terminal differentiation of keratinocytes. Profilaggrin is dephosphorylated and proteolyzed into FLG monomers in the stratum granulosum during the later stages of epidermal terminal differentiation. FLG monomers bind to and assemble keratin intermediate filaments and are postulated to contribute to the mechanical strength and integrity of the epidermis. Therefore it is of great importance to examine whether JTC801 promotes FLG monomer expression. Intriguingly, its protein expression level was upregulated by the presence of JTC801 (Fig 3, *B*). Additionally, JTC801 promoted FLG protein expression with human skin organ culture by means of immunohistochemistry (Fig 3, *C*). Of note, the positively staining cells below the stratum granulosum (around the basal layer) stain nonspecifically.

JTC801 promotes the expression level of mouse Flg by using flaky tail mice

Flaky tail mice, which are essentially deficient in Flg, have been used to investigate the role of Flg in AD. We used flaky tail

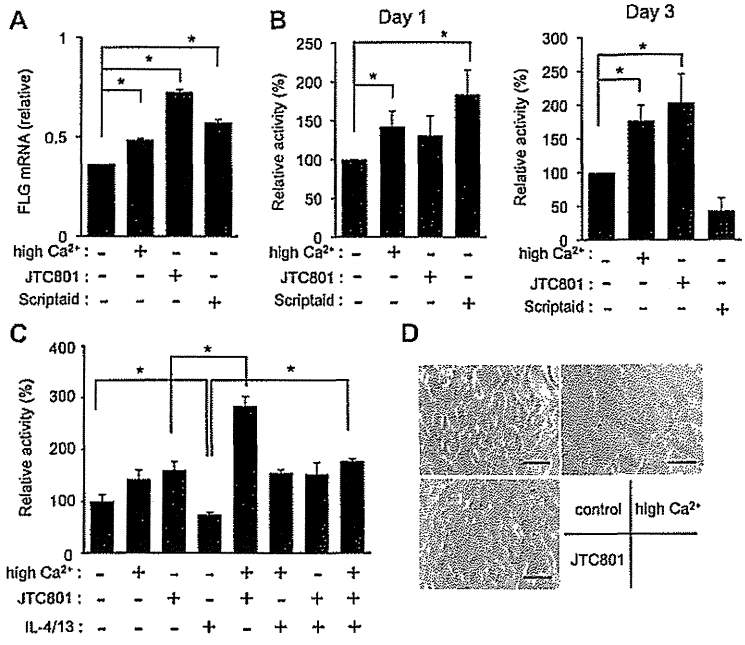

FIG 1. JTC801 promotes the transcription level of *FLG*. A, mRNA levels of *FLG* using NHEKs after 1 day. B, Relative activity of *FLG* was examined by using NHEKs after 1- and 3-day incubations with high Ca^{2+} levels, Scriptaid, or JTC801. C, Relative activity of *FLG* was evaluated in the presence of IL-4 and IL-13 after a 3-day incubation. D, Morphology of NHEKs after a 3-day incubation under conditions of high Ca^{2+} or JTC801. *$P < .05$.

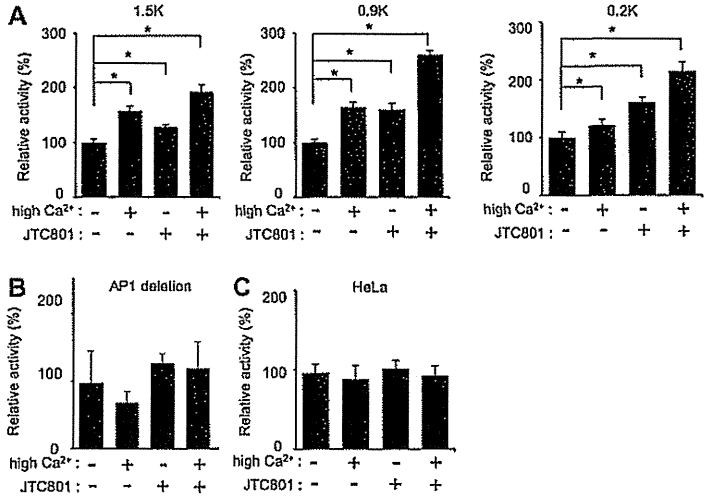

FIG 2. The AP-1 lesion is a main sequence of JTC801-dependent *FLG* expression. A and B, Relative activity of *FLG* was examined by using transfected NHEKs (1.5 K [−1504bp], 0.9 K [−980bp], 0.2 K [−246bp], and AP-1 deleted). C, Relative activity of *FLG* was examined by using transfected HeLa cells after a 3-day incubation. *$P < .05$.

mice to examine the effect of JTC801 on the expression of Flg. Homozygous mutant flaky tail mice did not express Flg protein, and the subcutaneous injection of JTC801 did not induce Flg expression by means of Western blotting (Fig 4, *A*). On the other hand, the expression levels of both profilaggrin and the Flg monomer were increased in heterozygous deficient flaky tail mice by subcutaneous JTC801 injection (Fig 4, *A*).

Next, we sought to evaluate the effect of JTC801 on dryness and barrier functions of the skin. It is generally agreed that TEWL correlates with the amount of water that evaporates from the body surface and that increased TEWL is indicative of SC barrier dysfunction. TEWL is thus used as a surrogate marker to assess skin dryness and SC barrier function in patients with AD.[30] In our model the TEWL value was significantly decreased by subcutaneous injection with JTC801 (Fig 4, *B*). SC conductance is another widely used method to evaluate SC hydration and skin barrier function. SC conductance, which is an indicator of SC hydration, was significantly increased by subcutaneous injection with JTC801 (Fig 4, *B*). These results suggest that the upregulation of Flg expression by JTC801 might contribute to the promotion of skin barrier functions.

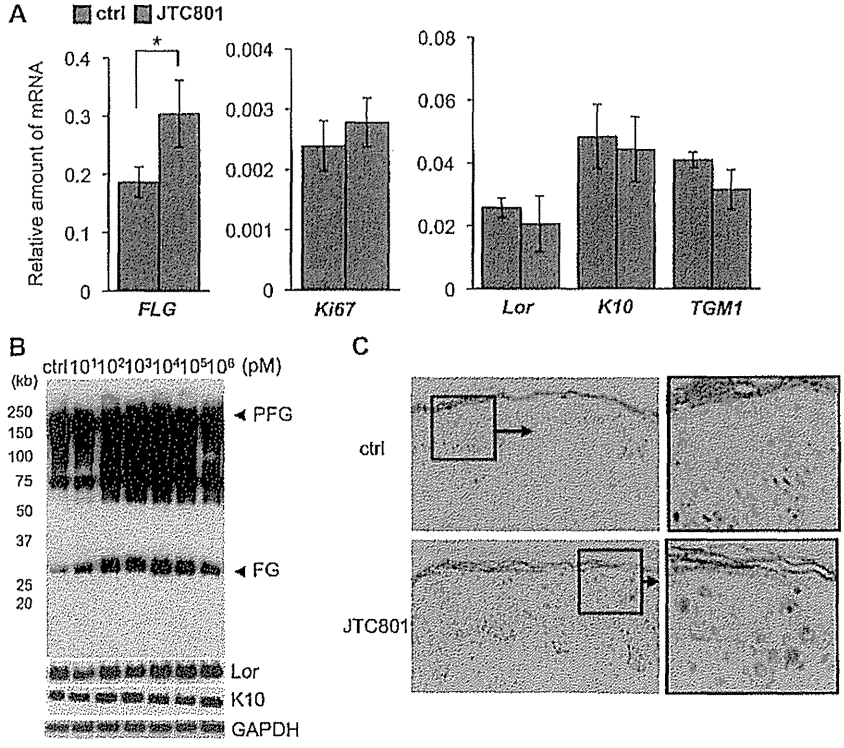

FIG 3. JTC801 promotes expression levels of the FLG monomer in the human skin equivalent model. A, mRNA levels of *FLG*, *Ki67*, loricrin *(Lor)*, *K10*, and *TGM1* in the human skin equivalent model after a 5-day incubation in the presence of JTC801. B, Western blot analysis for FLG expression at several concentrations of JTC801 (from *left*: 0, 0.01 nmol/L, 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 μmol/L). These results are representative of 2 independent experiments. *FG*, Filaggrin; *Lor*, loricrin; *PFG*, profilaggrin. C, FLG expression with human skin organ culture by means of immunohistochemistry under JTC801 conditions or not. $*P < .05$.

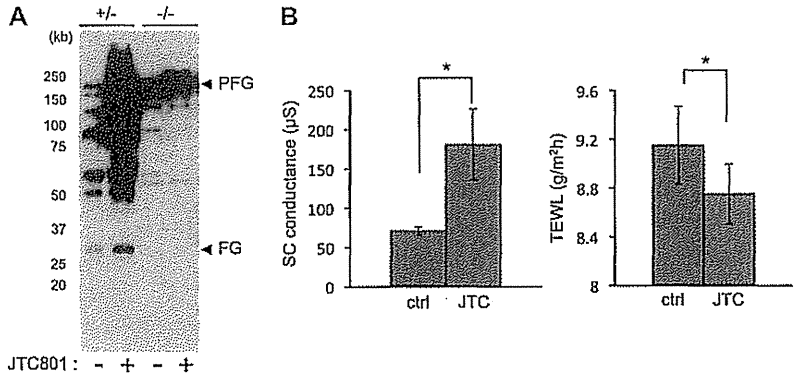

FIG 4. JTC801 promotes the expression level of Flg by using flaky tail mice. A, Western blot analysis of Flg expression in skin injected with JTC801 subcutaneously. +/−, Heterozygous deficient flaky tail mouse; −/−, homozygous deficient flaky tail mouse. B, TEWL and skin conductance were measured in this model. $*P < .05$. *FG*, Filaggrin; *PFG*, profilaggrin.

Increased Flg expression by JTC801 led to the attenuation of AD-like skin inflammation in NC/Nga mice

We used NC/Nga mice as an animal model of AD. When NC/Nga mice are raised under conventional conditions but not under specific pathogen-free conditions, the skin lesions are clinically and histologically very similar to those of human AD.[31] Consistent with previous reports,[32] the Flg expression was determined by means of Western blotting to be decreased in the lesional skin compared with the nonlesional skin of NC/Nga mice (Fig 5, *A*). We next examined the effect of JTC801 on AD-like skin inflammation. TEWL and skin conductance were improved after 2 weeks of oral administration of JTC801 (Fig 5, *B*). In addition, the clinical scores of

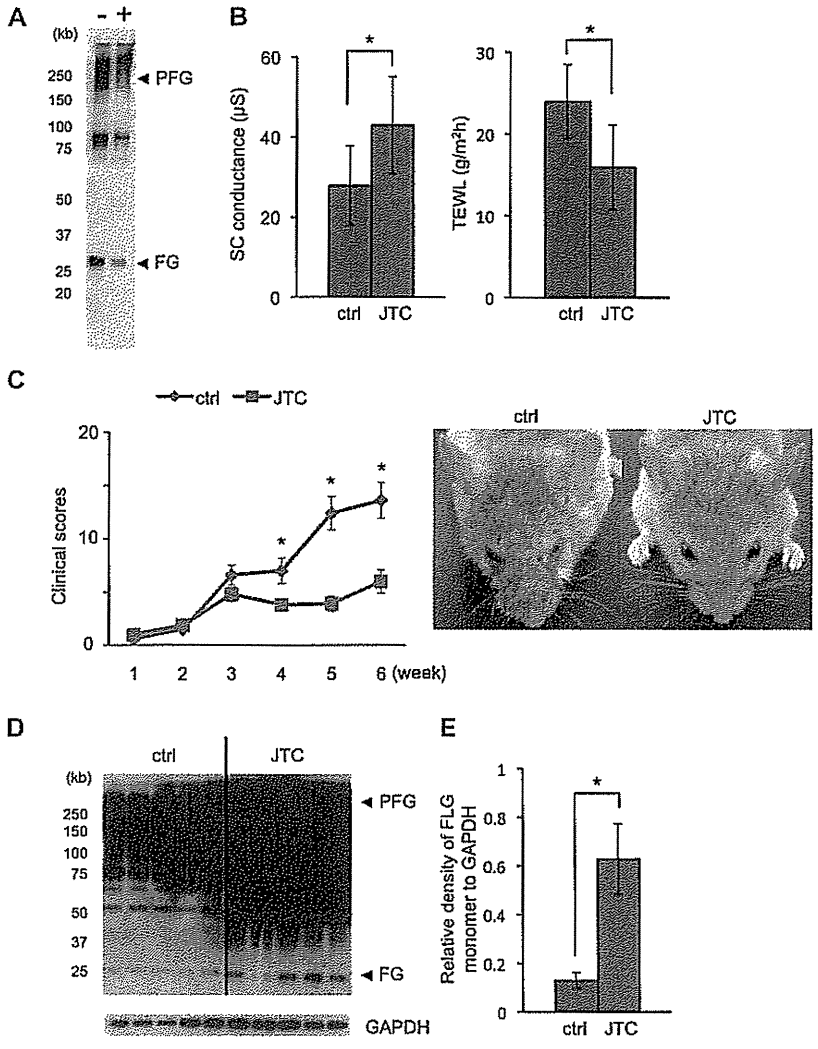

FIG 5. JTC801 attenuated atopic skin inflammation with NC/Nga mice. A, Western blot analysis of Flg expression in the lesional *(+)* or control *(−)* skin of NC/Nga mice. B, TEWL and skin conductance were measured after administration of JTC801 for 2 weeks. C, Time course of total clinical severity scores *(left panel)* and clinical photographs of NC/Nga mice after 6 weeks of administration of JTC801 *(right panel)*. D, Western blot analysis of Flg expression with these mice. E, Relative density of the FLG monomer to GAPDH. Data in Fig 5, *B*, are presented as means ± SDs, and those in Fig 5, *C* and *E*, are presented as means ± SEMs. *FG*, Filaggrin; *PFG*, profilaggrin. **P* < .05.

JTC801-administered mice were significantly decreased compared with those of control mice in terms of the symptoms of erythema/hemorrhage, edema, crust, excoriation/erosion, and scaling/dryness (Fig 5, *C*, and see Table E2 in this article's Online Repository at www.jacionline.org). We evaluated the number of T-cell receptor $(TCR)^+$ T cells, $CD45R^+$ B cells, and MHC class II^+ cells using immunohistochemistry (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Consistent with clinical observations, the numbers of these immune cells were significantly decreased in lesions of JTC801-treated NC/Nga mice compared with those of control-treated NC/Nga mice (see Fig E4, *B*).

When mice were orally administrated 10 mg/kg JTC801, the concentration of JTC801 in the serum remained greater than 1 ng/mL for 8 hours (see Fig E5 in this article's Online Repository at www.jacionline.org). According to *in vitro* findings (Fig 3, *B*), this concentration (>1 ng/mL) is an effective value to increase FLG expression. Consistently, the Flg expression was significantly increased in the mouse skin after administration of JTC801 (Fig 5, *D* and *E*). On the other hand, the histology of nonlesional skin was not modulated by treatment with JTC801 (see Fig E6 in this article's Online Repository at www.jacionline.org), which suggests that JTC801 might not affect keratinocyte differentiation in the steady state. In addition, treatment with JTC801 did not affect scratching behavior in NC/Nga mice (see Fig E7 in this article's Online Repository at www.jacionline.org), which suggests that treatment with JTC801 did not directly attenuate the pruritic sensation. Taken together, treatment with JTC801 increased Flg expression, which might lead to attenuation of atopic skin inflammation.

DISCUSSION

In this study we screened more than 1000 compounds in a bioactive chemical library to find candidates that promote *FLG* mRNA expression using the human immortalized keratinocyte cell line HaCaT. We found that JTC801 promoted the *FLG* mRNA expression in HaCaT cells, as well as in NHEKs. In addition, the human skin equivalent model revealed that JTC801 promotes the expression level of *FLG* but not that of other markers for keratinocyte differentiation, including loricrin, *K10*, and *TGM1*. Consistent with the *in vitro* findings, the oral administration of JTC801 promoted the protein level of Flg and suppressed the development of AD-like skin inflammation in NC/Nga mice *in vivo*. This is the first observation that the compound, which increased Flg expression, attenuated the development of AD-like skin inflammation in mice. Our findings provide evidence that modulation of FLG expression can be a novel therapeutic target for AD.

AD is a relapsing chronic inflammatory skin disease. It has been suggested that barrier dysfunction is a primary cause of AD.[5,8] Intriguingly, irrespective of mutations in *FLG*, FLG expression is downregulated in almost all cases of moderate-to-severe AD,[33] making it an appropriate therapeutic target for the disease. The filaggrin index effect, in which a modest 20% increase in filaggrin copy number leads to a 40% reduction in AD susceptibility,[20] is highly suggestive that filaggrin upregulation therapies will be of a potential utility in patients with AD.

In this study, for the first time, we demonstrated that JTC801 promoted FLG protein and mRNA expression *in vitro* and *in vivo*. It is of note that this compound did not affect other differentiation markers, including *K10*, *TGM1*, and loricrin, in NHEKs *in vitro*. In addition, the histology of murine epidermis in the steady state was not affected by this compound (see Fig E3). These findings suggest that this compound only enhances the terminal differentiation of keratinocytes through directly inducing FLG in keratinocytes. Importantly, we observe no obvious side effects in other organs after use of this compound (data not shown). In line with induction of FLG expression, this compound successfully suppressed AD-like skin inflammation, including the symptoms of erythema/hemorrhage, edema, crust, excoriation/erosion, and scaling/dryness.

AP-1 factors are key regulators of cell survival and death through the control of gene transcription in response to extracellular stimuli, such as growth factors and cytokines.[34] In the epidermis, among the AP-1 family,[35] c-Jun/c-Fos heterodimers have been reported to be activators of the transcription of the genes encoding profilaggrin,[22] loricrin, involucrin, and *TGM1*,[36] all of which are components of terminal differentiation of keratinocytes. We demonstrated here that JTC801 promotes the expression level of *FLG* but not that of other makers for keratinocyte differentiation, including loricrin, *K10*, and *TGM1*. Although AP-1 transactivation seems to be a key downstream event that results from JTC801 stimulation in keratinocytes, our results indicated that the *FLG* expression might be controlled by specific pathways different from other terminal differentiation markers. One alternative might be that JTC801 has an effect downstream rather than upstream of AP-1.

Comparatively little is known about the regulation of *FLG* gene expression, although this is now an area of interest to academia and the pharmaceutical industry. Because FLG expression is shown to be regulated by environmental humidity,[37] there are signaling pathways in the skin that actively control FLG expression and skin barrier homeostasis.

JTC801 is considered a nonpeptidergic ORL1 receptor antagonist. The ORL1 receptor is a G protein–coupled receptor structurally related to the opioid receptors, the endogenous ligand of which is the heptadecapeptide nociceptin/orphanin FQ.[38] Nociceptin, the endogenous peptide ligand for ORL1 receptor, has been implicated in inflammation and pain in the skin. Nociceptin also acts on the ORL1 receptor on keratinocytes to produce leukotriene B, which induces itch-associated responses in mice.[39] JTC801 is a 4-aminoquinoline derivative, which is considered a nonpeptidergic ORL1 receptor antagonist. JTC801 is known to attenuate allodynia, hyperalgesia, and aggravation of formalin-evoked pain behaviors,[40] but the effect of JTC801 remains unclear. Intriguingly, another ORL1 antagonist, J113397, did not increase the relative activity of *FLG* unexpectedly (data not shown). At present, it remains unclear how JTC801 regulates FLG expression in detail. We are currently working to understand the underlying mechanism of how FLG expression is controlled and to identify the action site of this compound.

Therefore the precise underlying mechanism remains unclear, but our current findings provide 2 important insights: (1) there seems to be a specific signaling pathway that selectively modulates the terminal differentiation of keratinocytes or controls FLG expression, and (2) the compound that induces Flg expression attenuates the development of AD-like skin lesions in mice. Therefore although further studies are required, this compound has potential as a new therapeutic strategy to control AD.

We thank Ms Kaori Tomari for technical assistance.

Clinical implications: We screened a library of bioactives using a reporter assay and found that JTC801 increased FLG expression in human and murine keratinocytes. JTC801 attenuated the development of AD-like skin inflammation in mice.

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METHODS

Quantitative PCR analysis for screening the compounds

HaCaT cells in the condition of each compound of Tocriscreen Mini were cultured in 96-well plates (IWAKI, Tokyo, Japan) for 24 hours. Total RNA was extracted with RNeasy (Qiagen, Hilden, Germany). cDNA was synthesized from 2 mg of RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). Quantitative RT-PCR with a Light Cycler real-time PCR apparatus was performed (Roche Diagnostics) with SYBR Green I (Roche). Expression of mRNA (relative) was normalized to the expression of *GAPDH* mRNA by the change in the ΔC_T method and calculated based on $2^{-\Delta CT}$.

Primer sequences

The primer sequences for *GAPDH* were as follows: 5′- CAT GAG AAG TAT GAC AAC AGC CT -3′ (forward) and 5′- AGT CCT TCC ACG ATA CCA AAG T -3′ (reverse). The primer sequences for *FLG* were as follows: 5′- TCG GCA AAT CCT GAA GAA TCC AGA -3′ (forward) and 5′- GCT TGA GCC AAC TTG AAT ACC ATC AG -3′ (reverse). The primer sequences for *K10* were as follows: 5′- TTG CTG AAC AAA ACC GCA AAG -3′ (forward) and 5′- GCC AGT TGG GAC TGT AGT TCT -3′ (reverse). Other primer sequences were obtained from PrimerBank.[E1] Details of the primers used in these experiments are available on request.

Effect of JTC801 on T cells and BMDCs

T cells were sorted from splenocytes and lymph nodes with NC/Nga mice by using the autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was confirmed to be greater than 95% through flow cytometry by using the FACSFortessa system (BD) and analyzed with FlowJo software (Tree Star, Ashland, Ore). In a plate coated with anti-CD3/CD28 antibodies, cells (1×10^5/well) were cultured with or without JTC801 (1, 10, and 100 nmol/L) for 3 days. The samples were stained with anti-mouse CD4 and CD8 antibodies (eBioscience, San Diego, Calif) and analyzed with flow cytometry. For intracellular cytokine staining, cells were incubated during the last 4 hours of 3 days in the presence of GolgiStop (BD Biosciences). After washing, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), according to the manufacturer's instruction. Anti-mouse IFN-γ and IL-4 antibodies were purchased from BD Biosciences.

For BMDC induction, 5×10^6 bone marrow cells were cultured in complete RPMI supplemented with 10% FCS in the presence of 10 ng/mL recombinant GM-CSF (PeproTech, Rocky Hills, NJ) for 5 days. The purity was confirmed to be greater than 95% through flow cytometry. After generation of BMDCs, cells were incubated with or without JTC801 (1, 10, and 100 nmol/L) for 2 days and analyzed by using flow cytometry. Anti-mouse CD11c, MHC class II, CD80, and CD86 antibodies were purchased from eBioscience.

Immunohistochemistry

Immunohistochemistry was performed, as reported previously.[E2,E3] Anti-mouse TCR^+ (ab91494), $CD45R^+$ (ab964100), and MHC class II^+ (ab139365) antibodies were purchased from Abcam (Cambridge, United Kingdom). The number of positively staining cells reflects the average number in 5 different fields.

Scratching behavior

Scratching behavior was measured in detail with the SCLABA-Real system (Noveltec, Kobe, Japan). Mice were put into the machine 20 minutes before measurement to allow them to adapt to the new environment. JTC801 (30 mg/kg in 0.5% methyl cellulose) or vehicle was then orally administered to 9-week-old NC/Nga mice, and the numbers of scratching sessions were counted for 22 hours, according to the manufacturer's protocol.

NHEK Western blotting

NHEKs were seeded at 3×10^5 cells per well on a 6-well plate and cultured overnight. The next day, vehicle (0.001% dimethyl sulfoxide) or 10 or 1000 nmol/L JTC801 was added and incubated for 3 days at 37°C in a humidified atmosphere with 5% CO_2. The cells were harvested with ice-cold 10% tricarboxylic acid and incubated on ice for 30 minutes. The cells were centrifuged, and the pellets were lysed and sonicated in 150 μL of 50 mmol/L Tris-HCl (pH 8.0) buffer containing 9 mol/L urea, 2% TritonX-100, and a protease inhibitor cocktail (Roche). Forty microliters of Laemmli SDS-PAGE sample buffer with 1 mmol/L dithiothreitol was added to the lysate, adjusted to pH 7.5, and boiled for 5 minutes. The samples were resolved by using SDS-PAGE on an e-PAGEL 5-20% Gel (ATTO) with SDS running buffer (25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS) and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with either 1:500 dilution of anti-filaggrin antibody (sc-66192, Santa Cruz Biotechnology) or 1:1000 dilution of anti-tubulin antibody (clone DM1A; Sigma, St Louis, Mo) and horseradish peroxidase–conjugated anti-mouse IgG (GE Healthcare) and developed by means of chemiluminescence (GE Healthcare), according to the manufacturer's instructions.

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E3. Otsuka A, Ozaki M, Horiguchi Y, Murata Y, Kumano K, Nogami R, et al. Basophils infiltrate the skin lesions in lepromatous leprosy. Acta Derm Venereol 2013;93:88-9.

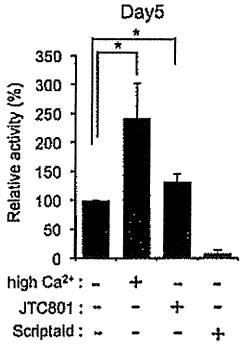

FIG E1. Relative activity of *FLG* was examined by using NHEKs after 5-day incubation with high Ca^{2+} levels, Scriptaid, or JTC801. $*P < .05$.

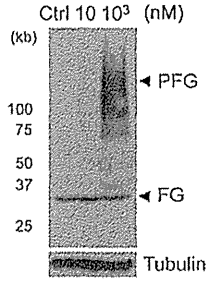

FIG E2. Western blot analysis for FLG with NHEKs. Western blot analysis for FLG expression under several concentrations of JTC801 (from *left*: 0, 10, and 1000 nmol/L) is shown. These results are representative of 3 independent experiments. *FG*, Filaggrin; *PFG*, profilaggrin.

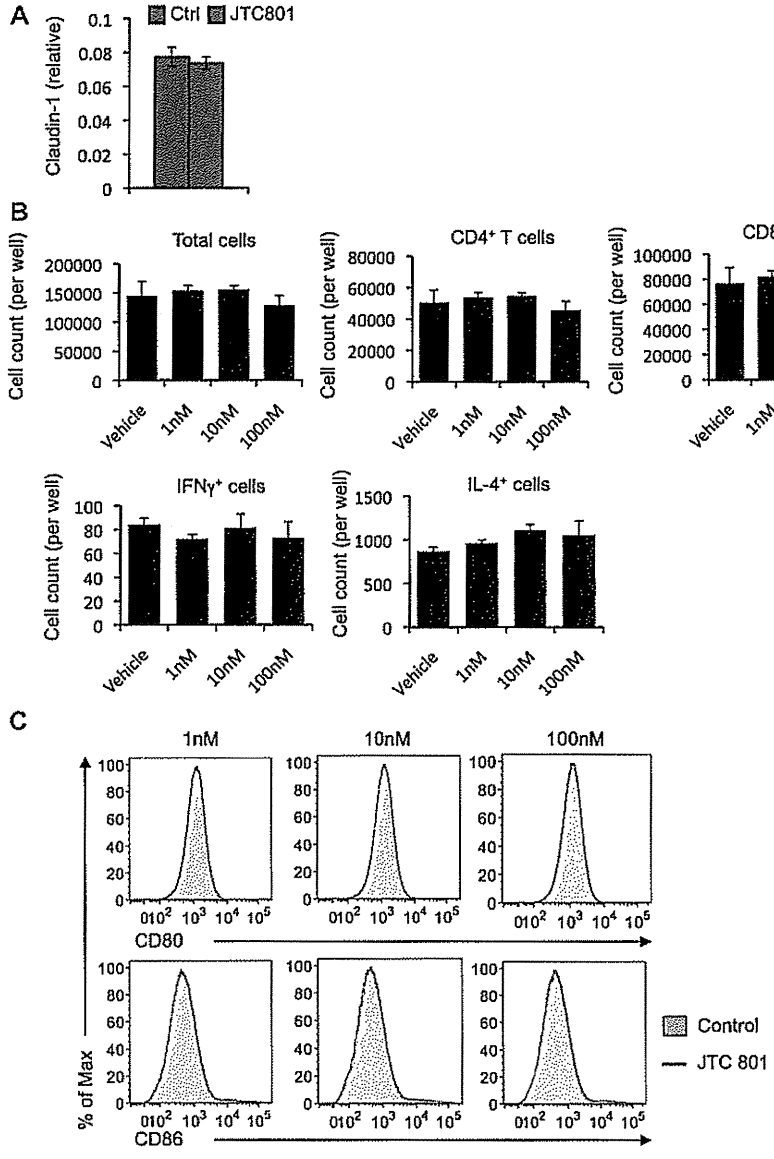

FIG E3. Effects of JTC801 on development of tight junction and immune cell functions. **A**, mRNA levels of claudin-1 of NHEKs for a 3-day incubation with or without JTC801. **B**, Numbers of total T cells, $CD4^+$ T cells, $CD8^+$ T cells, IFN-γ^+ cells, and IL-4^+ cells with or without JTC801 (1, 10, and 100 nmol/L). **C**, Flow cytometric histogram of CD80 and CD86 on BMDCs in the presence or absence of JTC801. These results are a representative of 2 independent experiments.

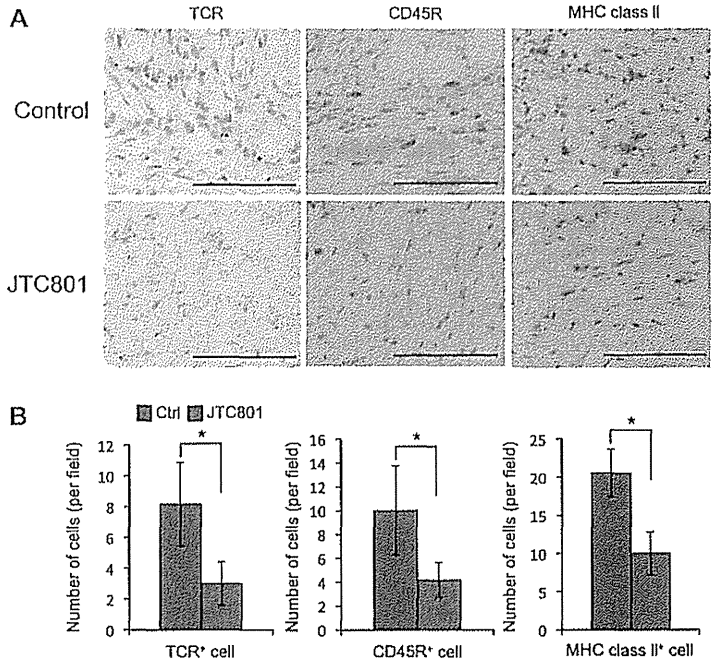

FIG E4. Immunohistochemistry of TCR^+, $CD45R^+$, and MHC class II^+ cells. *A*, Immunohistochemistry of TCR^+, $CD45R^+$, and MHC class II^+ cells with or without the treatment of JTC801 in the lesional skin of NC/Nga mice. *Scale bar* = 50 μm. *B*, Numbers of TCR^+, $CD45R^+$, and MHC class II^+ cells per field (n = 5). All data are presented as the mean ± SD and are representative of 2 experiments. $*P < .05$.

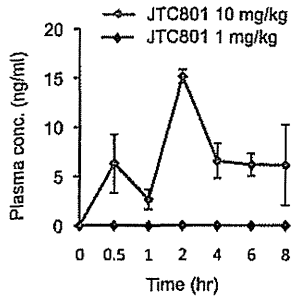

FIG E5. Concentrations of JTC801 in serum. Serum concentrations of JTC801 were measured 0.5, 1, 2, 4, 6, and 8 hours after the oral administration of 1 or 10 mg/mL JTC801.

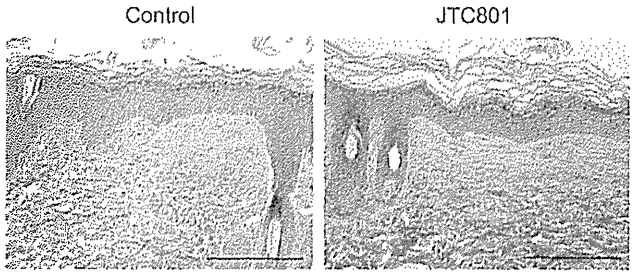

FIG E6. Histology of nonlesional skin in mice treated with or without JTC801. *Scale bar* = 100 μm.