

CCL27-transgenic mice show enhanced contact hypersensitivity to Th2, but not Th1 stimuli

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CCL27 is one of the CC chemokines produced by epidermal keratinocytes and is suggested to be involved in the pathogenesis of inflammatory skin diseases. To clarify the contribution of CCL27 in skin inflammation, we created transgenic C57BL/6 mice that constitutively produce CCL27 in epidermal keratinocytes. These mice had high serum CCL27 levels and did not show any phenotypical change. Thus we stimulated these mice with various reagents by single and repeated application. Interestingly, only contact hypersensitivity to repeated application with fluorescein isothiocyanate was significantly enhanced in transgenic mice compared to non-transgenic mice. Under this condition, the numbers of inflammatory cells, CCR10-positive cells, CCR4-positive cells and cutaneous lymphocyte-associated antigen-positive cells were increased, and IL-4 mRNA expression was higher in the lesional skin of transgenic mice. Increased number of mast cells and higher serum IgE levels, which were similar to atopic dermatitis, were also observed. These results indicated that CCL27 modified inflammation by attracting CCR10-positive and CCR4-positive cells into the lesional skin, and may participate in the pathogenesis of Th2-shifted skin diseases such as atopic dermatitis.

Received 20/7/07

Revised 18/11/07

Accepted 15/1/08

[DOI 10.1002/eji.200737685]

Key words:

Allergy · Chemokines
· Inflammation · Skin
· Transgenic/
knockout mice

Introduction

Chemokines are a family of polypeptides that govern the chemotaxis and activation of different subsets of leukocytes during immune and inflammatory responses. They are divided into four subgroups: CC, CXC, CX3C, and C. The receptors for chemokines are subdivided in the same manner, as CCR1–10, CXCR1–6, CX3CR1, and XCR1 [1, 2]. CCR4 is predominantly expressed on Th2 lymphocytes, basophils, and mature dendritic cells; CXCR3 is predominantly expressed on Th1 lymphocytes and B cells [1].

Cutaneous T cell-attracting chemokine/CCL27 is a ligand for CCR10. It is selectively and constitutively produced in skin by epidermal keratinocytes [3] and displayed on the surface of dermal endothelial cells [4]. Its mRNA is detected only in keratinocytes [5]. CCR10 is expressed in melanocytes, dermal fibroblasts, dermal endothelial cells, cutaneous lymphocyte-associated antigen (CLA)-positive, memory T cells, Langerhans cells and plasma cells [5, 6]. In a previous study we have shown that lesional keratinocytes of patients with atopic dermatitis and psoriasis vulgaris were immunohistochemically positive for CCL27 and that serum CCL27 levels were elevated in both diseases [7]. It has been reported that CCL27-CCR10 interactions may be involved in toxic epidermal necrolysis, Stevens–Johnson syndrome [8], and allergic contact dermatitis [9]. Furthermore, intracutaneous mouse CCL27 (mCCL27) injection into BALB/c mice attracted lymphocytes expressing CCR10 and, conversely, neutralization of CCL27-CCR10 interactions impaired lymphocyte re-

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Abbreviations: **CHS:** contact hypersensitivity · **CLA:** cutaneous lymphocyte-associated antigen · **hGH:** human growth hormone · **hK14:** human keratin 14 · **mCCL27:** mouse CCL27

cruitment to the skin, leading to the suppression of allergen-induced skin inflammation [4].

To clarify the contribution of CCL27 produced by keratinocytes in skin diseases such as atopic dermatitis and psoriasis vulgaris, we have created transgenic (Tg) mice that constitutively produce mCCL27 in the epidermis under the control of the human keratin 14 (hK14) promoter. Interestingly, these mice did not show any macroscopic or microscopic phenotypical changes. Thus, analysis of the contact hypersensitivity (CHS) reaction were performed and compared between Tg and non-Tg mice.

Results

Generation and screening for CCL27-Tg mice

Four founders were identified by PCR analysis of tail DNA from 50 pups born after embryonic transfer. Among them, only one founder was successfully bred to yield a line. Mice were screened for transgene presence by PCR analysis of tail genomic DNA with the forward primer located in the hK14 gene and the reverse primer located in the mCCL27 gene. A fragment of transgene was detected only in Tg mice (Fig. 1A). CCL27 mRNA in Tg mice was approximately fourfold higher than in non-Tg mice (Fig. 1B).

Increased production and secretion of CCL27 protein in keratinocytes of Tg mice

To establish that CCL27 mRNA was being translated into protein, immunohistochemistry and ELISA were performed. (Fig. 2A shows that epidermis of Tg mice was strongly positive with the anti-CCL27 antibody while that of non-Tg mice was virtually negative (Fig. 2B). The concentration in the supernatants of Tg keratinocytes (3400.6 ± 36.5 pg/mL) was markedly higher than that of non-Tg keratinocytes (209.5 ± 137.8 pg/mL) ($p < 0.05$; Fig. 2C). Serum CCL27 levels were significantly higher in Tg mice (2891.6 ± 1023.4 pg/mL) than in non-Tg mice (269.5 ± 72.7 pg/mL) ($p < 0.005$; Fig. 2D). We also measured serum levels of mouse CCL17 (Fig. 2E) and CCL22 (Fig. 2F), but there was no significant difference between non-Tg and Tg mice.

CCL27 produced by Tg keratinocytes has biologic and functional activity

The biologic function of CCL27 produced by Tg keratinocytes was assayed with chemotaxis assay. Supernatant of Tg keratinocytes and recombinant mCCL27 displayed a potent chemoattractant activity for CCR10⁺ L1.2 cells that acts in a characteristic dose-

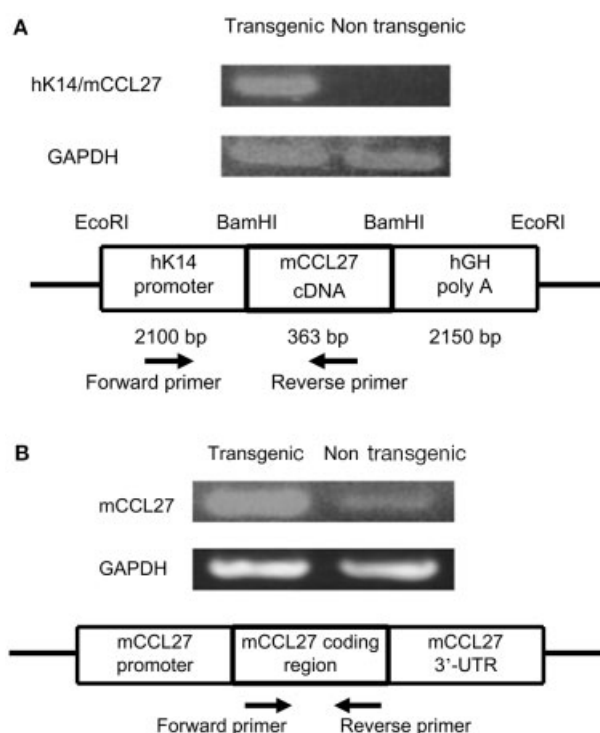


Figure 1. Tg construct was used to produce hK14-mCCL27-Tg mice, and expression of CCL27 mRNA was increased in Tg mice. (A) The 4613-bp segment of DNA isolated after EcoRI digestion and used for microinjection is illustrated. A fragment of transgene was detected only in Tg mice. (B) CCL27 mRNA expression was analyzed. The fragment within mCCL27 coding region which was included in both the transgene and genomic DNA was amplified. One representative of three experiments is shown. UTR means untranslated region.

dependent fashion, but showed no chemoattractant activity for vector L1.2 cells (Fig. 3A). Medium alone and supernatant of non-Tg keratinocytes chemoattracted neither of these cells. These chemoattractant activities were blocked by anti-mCCL27 antibody. We also performed Western blotting and detected that the molecular weight of CCL27 produced by keratinocytes was the same as that of recombinant CCL27 (Fig. 3B).

Chronic CHS in response to FITC challenge is enhanced in CCL27-Tg mice

The CHS reaction after a single challenge and that following repeated challenges were designated as “acute CHS” and “chronic CHS”, respectively [10]. In acute CHS following FITC challenge (“FITC acute CHS”), there was no significant difference in ear swelling between non-Tg mice and Tg mice (Fig. 4A). In FITC chronic CHS, compared to non-Tg mice, Tg mice showed significantly increased ear swelling after day 5 (Fig. 4B). In oxazolone acute or chronic CHS, after irritation by croton oil and repeated tape stripping, there was no

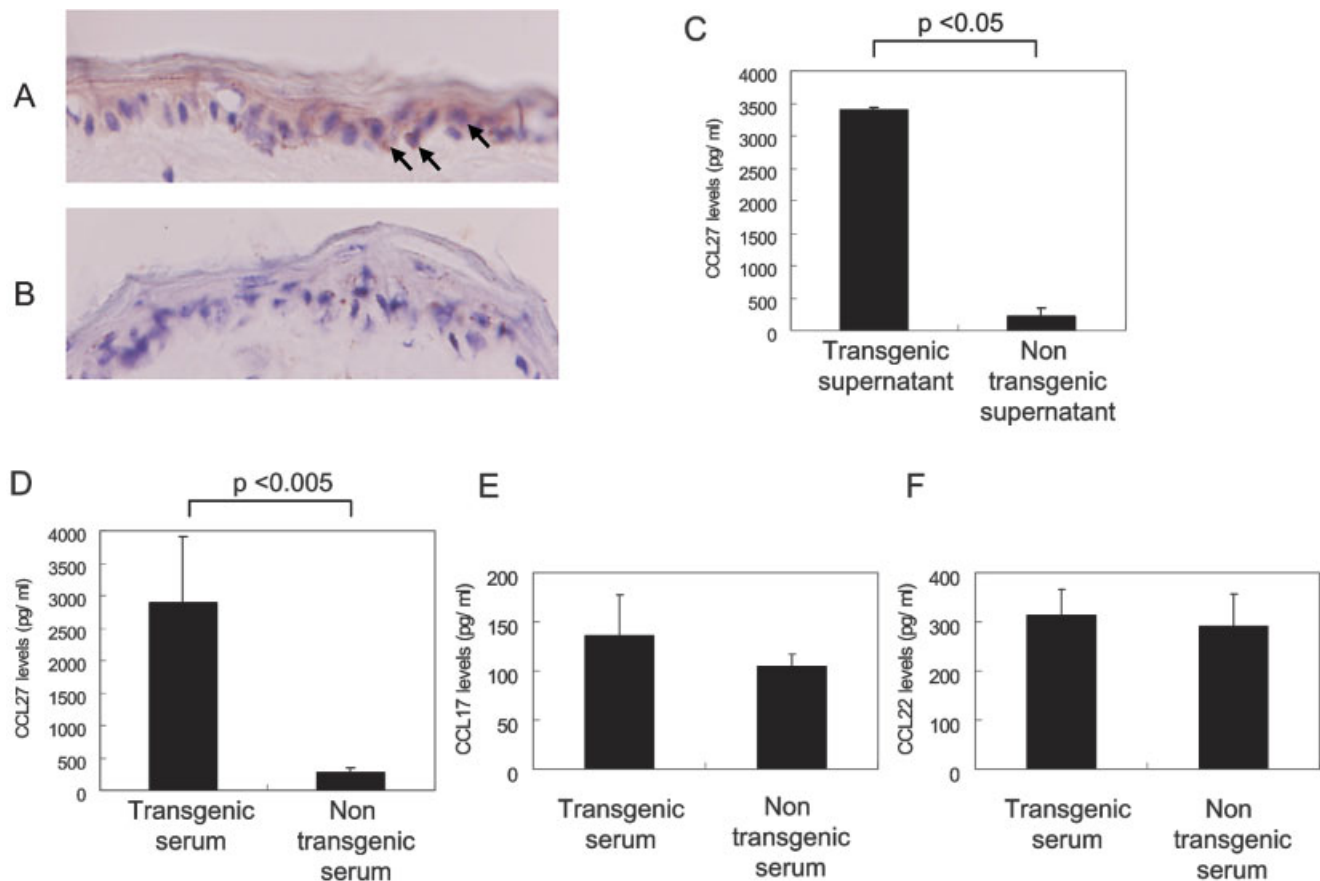


Figure 2. Increased CCL27 expression in Tg mice. (A, B) Frozen sections of ear skin from Tg (A) and non-Tg (B) mice were stained with monoclonal rat anti-mCCL27 antibody. Arrows indicate positive staining. (C, D) ELISA was used to quantitate the amount of CCL27 in supernatants of keratinocytes ($n=4$) (C) and sera ($n=17$) (D) from non-Tg and Tg mice. (E, F) Serum CCL17 (E) and CCL22 (F) levels were also measured.

significant difference between non-Tg mice and Tg mice (data not shown).

We also performed intravenous injection of anti-mCCL27 antibody to Tg mice in normal condition or FITC acute CHS. In both conditions, the injection did not cause any difference in phenotype or skin leukocyte infiltration (data not shown).

Furthermore, we also examined serum CCL27 levels in FITC acute or chronic CHS. In both conditions, these levels of Tg mice were significantly higher than those of non-Tg mice ($p<0.05$). In Tg mice, there was no significant difference between non stimulated condition and FITC acute or chronic CHS (Fig. 4C).

The number of inflammatory cells in FITC chronic CHS is higher in CCL27-Tg mice

Pathological findings on the skin were compared between non-Tg and Tg mice. With no treatment, there were few inflammatory cells in either group and there was no significant difference (Fig. 5A, B). In oxazolone acute or chronic CHS, and FITC acute CHS, the number

of inflammatory cells was increased compared to non-stimulated mice, but there was no significant difference between Tg and non-Tg mice (Fig. 5C–H). In FITC chronic CHS, at day 22, the number of inflammatory cells was increased compared to non-stimulated mice. Furthermore, a significantly higher number of inflammatory cells were found in Tg mice than in non-Tg mice ($295.3 \pm 70.4/\text{field}$ vs. $210.0 \pm 17.0/\text{field}$, $p<0.05$) (Fig. 5I–K).

The number of mast cells, CCR10⁺, CLA⁺, and CCR4⁺ cells is increased in FITC chronic CHS in CCL27-Tg mice

To further examine the pathological change of FITC chronic CHS, we performed further staining. The number of mast cells, CCR10⁺ cells, CLA⁺ cells, and CCR4⁺ cells in FITC chronic CHS were significantly increased in Tg mice compared to non-Tg mice (mast cells: $30.8 \pm 7.1/\text{field}$ vs. $17.1 \pm 5.7/\text{field}$; CCR10⁺: $92.6 \pm 53.4/\text{field}$ vs. $19.0 \pm 1.7/\text{field}$; CLA⁺: $56.3 \pm 18.4/\text{field}$ vs. $8.1 \pm 2.0/\text{field}$; CCR4⁺: $37.3 \pm 7.6/\text{field}$ vs. 17.5

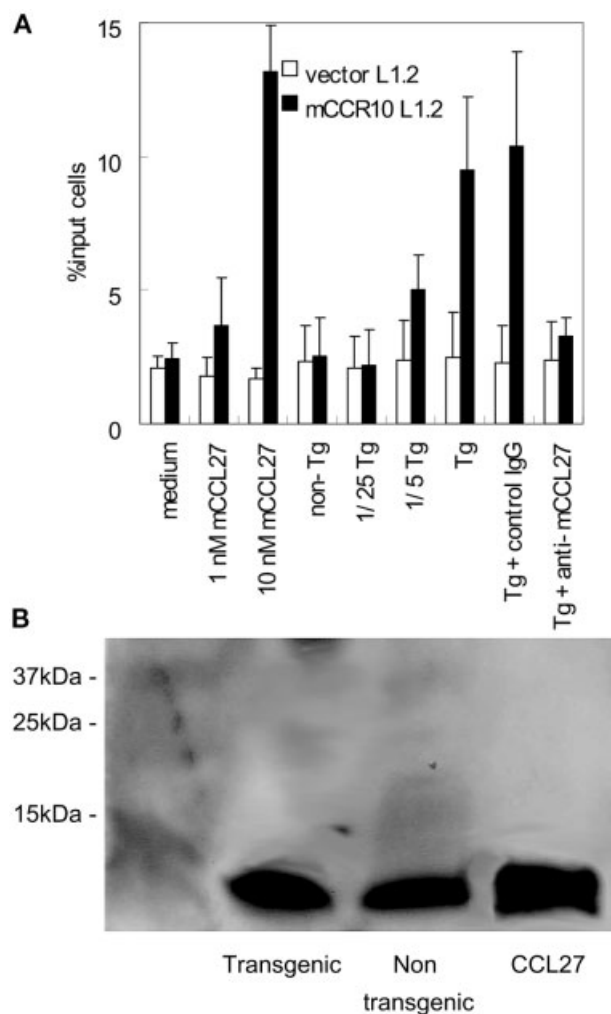


Figure 3. CCL27 produced by Tg keratinocytes had functional activity and exact molecular weight. (A) Supernatants of keratinocytes from non-Tg and Tg mice, and recombinant mCCL27 were serially diluted. CCR10-expressing L1.2 cells and vector L1.2 cells were used in 96-well disposable chemotaxis chambers fitted with an 8- μ m polycarbonate filter. The number of migrated cells was expressed as a percentage of the number of cells loaded in the upper chamber ($n=6$). (B) Western blotting to measure molecular weight of CCL27 produced by keratinocytes from non-Tg and Tg mice.

$\pm 3.8/\text{field}$) ($p<0.05$; (Fig. 6A–H, K). However, there was no significant difference in the number of CXCR3⁺ cells between non-Tg and Tg mice (Fig. 6I, K). Without treatment and in other CHS, there was no significant difference between Tg and non-Tg mice (data not shown).

IL-4 mRNA expression is increased in FITC chronic CHS in CCL27-Tg mice

In order to examine cytokine profile in FITC chronic CHS, IL-4 and IFN- γ expression were examined. RT-PCR was performed using mRNA extracted from the ear

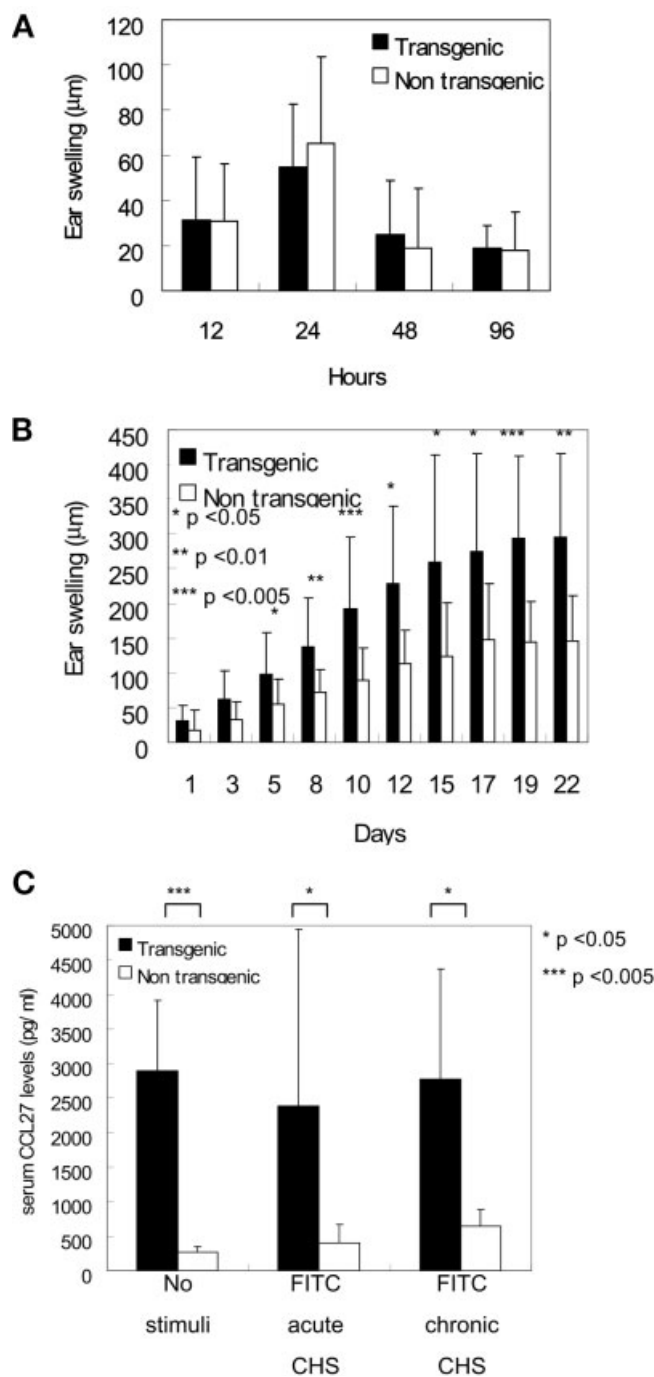


Figure 4. Enhanced FITC chronic CHS in Tg mice. CHS to FITC was assayed in non-Tg mice and Tg mice. (A) FITC acute CHS ($n=10$). (B) FITC chronic CHS ($n=14$). (C) Serum CCL27 levels in FITC acute and chronic CHS ($n=8$).

skin at 24 h after the last challenge in FITC chronic CHS. Without treatment, neither IL-4 mRNA nor IFN- γ mRNA was detected in non-Tg and Tg mice (data not shown). In FITC chronic CHS, IFN- γ mRNA was not detected in either group, but IL-4 mRNA in Tg mice was approximately 470-fold higher than in non-Tg mice (Fig. 7).

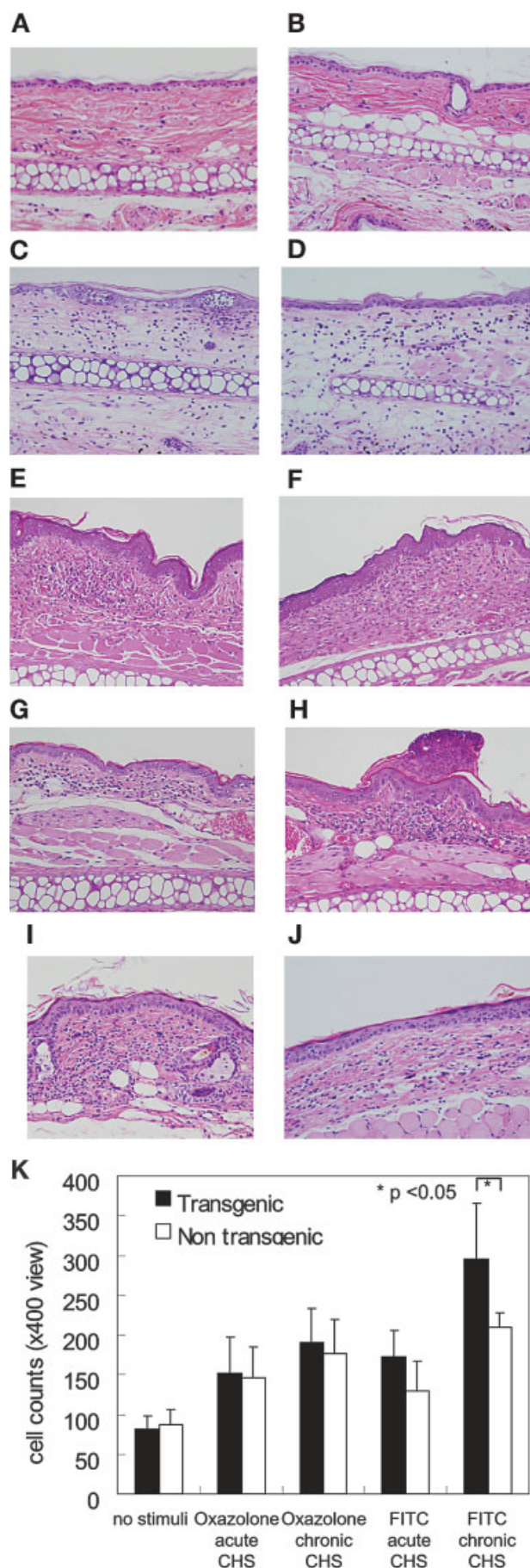


Figure 5. Increased inflammatory cell infiltration in the ear of Tg mice in FITC chronic CHS at day 22. H&E staining from untreated Tg (A) and non-Tg mice (B), after oxazolone acute CHS from Tg (C) and non-Tg mice (D), after oxazolone chronic CHS from Tg (E) and non-Tg mice (F), after FITC acute CHS from Tg (G) and non-Tg mice (H); and after FITC chronic CHS from Tg (I) and non-Tg mice (J). Number of inflammatory cells was presented as mean $\pm$ SD (K).

Increased serum IgE levels after FITC chronic CHS in CCL27-Tg mice

Serum IgE levels of non-Tg and Tg mice were measured at 24 h after the end of challenge (Fig. 8). Without treatment, the serum IgE concentration of Tg mice was not significantly higher than that of non-Tg mice. After FITC chronic CHS to ears and abdomen, Tg mice showed significantly higher levels of serum IgE than non-Tg mice ($p < 0.01$, $p < 0.05$, respectively). We also measured serum levels of mouse IL-4, IFN- γ , CCL17, CCL22, and CXCL9 after FITC chronic CHS, but there was no significant difference between non-Tg and Tg mice (data not shown).

Discussion

We have created a line of Tg mice where CCL27 expression is increased in epidermal keratinocytes under the control of hK14 promoter. It was confirmed that transgene was integrated, CCL27 mRNA was transcribed from transgene, a large amount of mCCL27 protein was produced and secreted by keratinocytes, and the produced CCL27 protein is bioactive for CCR10 $^{+}$ cells.

However, no spontaneous infiltration of inflammatory cells into the skin could be identified. CCR10 desensitization was not observed, because in normal condition and FITC acute CHS, intravenous injection of anti-mCCL27 antibody to Tg mice did not cause any macroscopic or microscopic phenotypical change. The mechanism of lymphocyte invasion into the tissues is a multistep process in which adhesion molecules participate [11]. Stimuli by contactants induce pro-inflammatory cytokines from keratinocytes, which in turn induce endothelial activation and expression of adhesion molecules [12], enabling various cells to infiltrate into the inflammation sites. The lack of spontaneous inflammation in Tg mice would be due to absence of the endothelial activation and expression of adhesion molecules. CLA is thought to be important in leading skin-associated T cells to inflammatory skin sites by interacting with the endothelial cell ligand E-selectin which is pronounced in inflamed skin [13]. It would be possible that CCR10 $^{+}$ lymphocytes cannot infiltrate

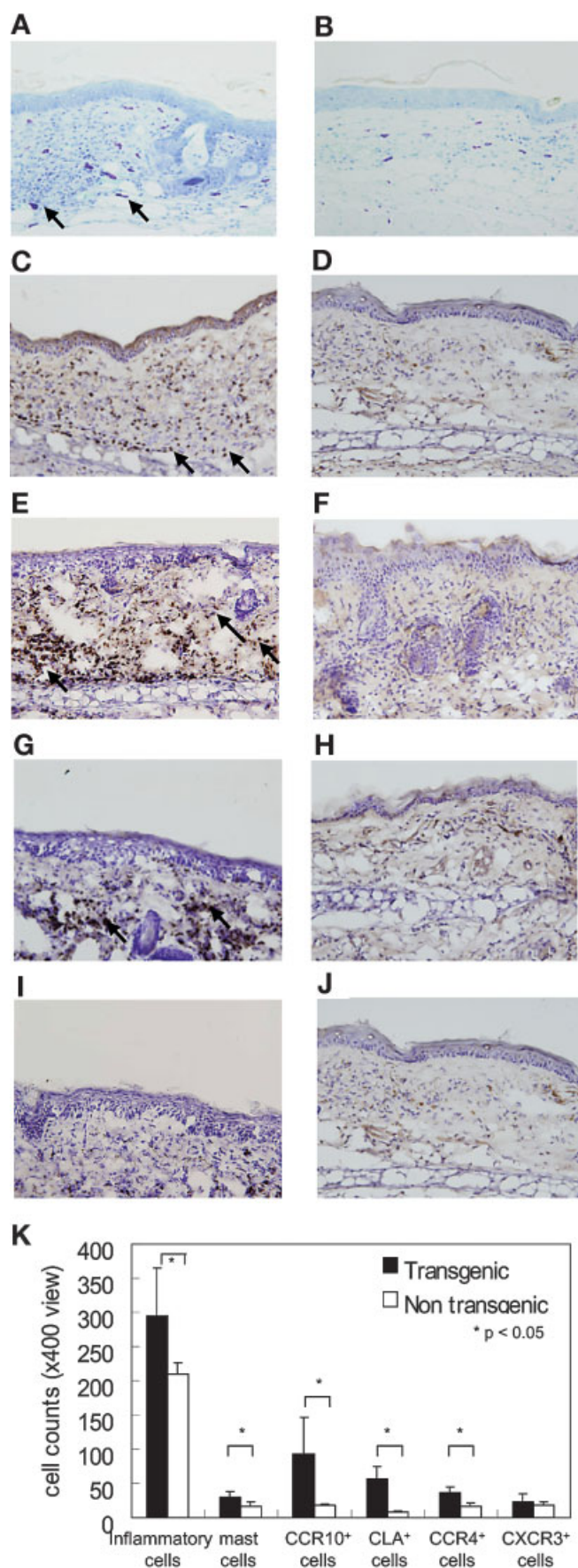


Figure 6. Increased mast cell, CCR10⁺, CLA⁺, CCR4⁺ cell infiltration in the ear of Tg mice in FITC chronic CHS in day 22. Ear skin from non-Tg and Tg mice in FITC chronic CHS was biopsied and stained. Toluidine blue staining from Tg (A) and non-Tg mice (B). CCR10 staining from Tg (C) and non-Tg mice (D). CLA staining from Tg (E) and non-Tg mice (F). CCR4 staining from Tg (G) and non-Tg mice (H). CXCR3 staining from Tg (I) and non-Tg mice (J). The number of infiltrated cells was presented as mean $\pm$ SD (K). Arrows indicate positive staining.

when E-selectin is not expressed even if CCL27 concentration is sufficient for migration.

Homey *et al.* [4] reported that intracutaneous CCL27 injection into BALB/c mice attracted lymphocytes. This is in conflict with our result where no spontaneous inflammation was observed in Tg mice that constitutively produce CCL27 in the epidermis. One possible explanation is that the stimulation caused by injection induced pro-inflammatory cytokines and mechanical stress, which up-regulate the expression of adhesion molecules on endothelial cells. In addition, they used BALB/c mice, which are a Th2-dominant strain, while we used C57BL/6 mice, which are a Th1-dominant strain. The minimum CCL27 concentration, which can induce inflammation, might be different between these mice. Similar phenomenon was observed in mice overexpressing CCL17 in the epidermal keratinocytes [10]. These CCL17-Tg mice created in C57BL/6 mice did not show spontaneous inflammation. Interestingly, it has been reported that intracutaneous recombinant CCL17 injection into BALB/c mice attracted lymphocytes exhibiting Th2-shifted inflammation [14].

In this study, CHS reactions were examined, since these mice did not show any macroscopic or microscopic phenotypical change. Oxazolone and FITC were employed as sensitizers, and two ways of challenge, namely acute CHS and chronic CHS, were performed. Oxazolone induces a Th1-dominant response. In cytokine levels, initial challenge with antigens such as oxazolone and trinitrochlorobenzene leads to predominant production of Th1 cytokines such as IFN- γ and IL-2, and minimal production of Th2 cytokines such as IL-4 and IL-10 in the lesional skin [15, 16]. Continued exposure to antigens induces a down-regulation of Th1 cytokine and up-regulation of Th2 cytokine production in the lesional skin [15, 16]. On the other hand, FITC induces a Th2-dominant response in C57BL/6 mice and increment of serum IgE level was observed [17].

In FITC chronic CHS, Tg mice showed stronger ear swelling compared to non-Tg mice. On the other hand, Tg mice did not show significant difference in FITC acute CHS, or after other stimuli. These results indicate that CCL27 production and secretion by keratinocytes is not sufficient for the induction of local inflammatory responses, but enhances the inflammation caused by

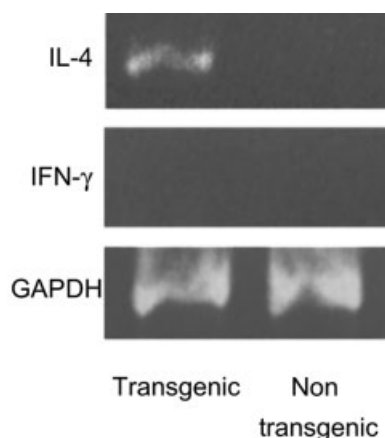


Figure 7. IL-4 mRNA expression was increased in Tg mice with FITC chronic CHS. RT-PCR was performed with RNA extracted from the ear skin at 24 h after the last challenge in FITC chronic CHS. One representative of three experiments is shown.

Th2-directed stimuli. A single challenge of FITC was not sufficient to clarify the difference between Tg and non-Tg mice; however, repeated challenges of FITC enhanced CHS reaction in Tg mice. Repeated application might evoke stronger effect of Th2-directed stimuli.

The results of immunohistological analysis and RT-PCR of the ear indicate that CCL27 produced by the epidermal keratinocytes in Tg mice attracts mast cells, CLA⁺, CCR10⁺ and CCR4⁺ cells, and these cells secrete IL-4, resulting in a Th2-dominated condition in Tg mice. Furthermore, recent evidence suggests that mast cells can contribute to CHS or delayed-type hypersensitivity reactions through several distinct mechanisms [18, 19].

As Th2 cells produce IL-4 and IL-13, which is closely related to the promotion of IgE production, IgE is a reliable marker of Th2 activity [17]. Without treatment, the difference was not significant between non-Tg and Tg mice. However, increased and significantly higher

serum IgE levels were observed in Tg mice after FITC chronic CHS. This is consistent with FITC being a Th2-dominant sensitizer and chronic CHS inducing Th2-type inflammation [17]. These results suggest that CCL27 induces Th2 cytokines, such as IL-4 and IL-13 produced by CCR10⁺ and CCR4⁺ Th2 cells, which enhances IgE production by plasma cells [20]. Bryce *et al.* [21] speculated that IgE is required for optimal immune sensitization in certain models of CHS. In such models, IgE can mediate antigen-independent, but high-affinity IgE receptor-dependent, priming effects on dermal mast cells that render these cells better able to produce cytokines and chemokines, which can in turn enhance CHS [21].

Chen *et al.* [22] reported that CCL27-CCR10 interaction is important for the development of skin inflammation in the hK14-IL-4-Tg mice. Reiss *et al.* [23] reported that both CCL27 and CCR4 can support homing of T cells to skin, and that either one or the other is required for lymphocyte recruitment in cutaneous delayed-type hypersensitivity. Furthermore, Vestergaard *et al.* [24] reported that a part of CCR10⁺ cells in atopic dermatitis expressed CCR4, whereas the CCR10⁺ cells in psoriasis did not express CCR4. Therefore, both CCL27-CCR10 and CCL17-CCR4 interactions are important for skin inflammation. Reduced Th1-type reaction, and enhanced Th2-type reaction and irritation were observed in CCL17 Tg mice [25]; however, only FITC chronic CHS was enhanced in CCL27 Tg mice, and CCR10⁺ cells and CCR4⁺ cells were increased in Tg mice in FITC chronic CHS. These results suggest that CCL27-CCR10 interactions do not have a direct effect on skin inflammation, but CCL27 attracts CCR10⁺ CCR4⁺ lymphocytes into the skin and these cells enhance skin inflammation under the stronger Th2-shifted condition, although we failed to detect CCR10 and CCR4 double staining in this study.

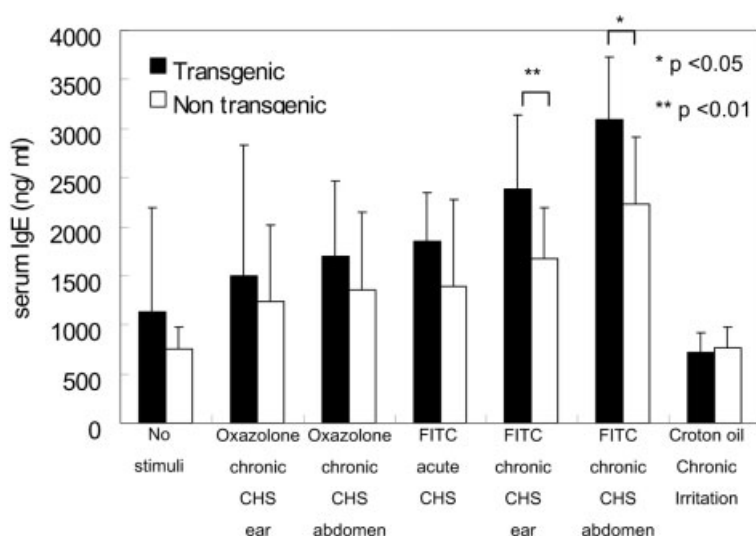


Figure 8. Serum IgE levels before and after the challenge. Serum levels of IgE from non-Tg and Tg mice were measured at 24 h after the end of challenge.

In summary, we studied the effect of CCL27 produced by epidermal keratinocytes in CCL27-Tg mice. Although CCL27 itself is not sufficient for the induction of inflammation, CCL27-CCR10 interactions enhance skin inflammation if inflammation exhibit stronger Th2-shifted response, probably by attracting CCR4-expressing Th2 cells into the skin. In addition, an atopic dermatitis-like condition such as increment of the number of mast cells and high levels of serum IgE was observed in chronically challenged Tg mice. CCL27 may participate in the pathogenesis of skin diseases like atopic dermatitis by regulating chronic allergic inflammation.

Materials and methods

Mice

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). All mice used in the experiments were 6–8 wk old and mice of the same sex were used in each experiment. They were maintained under specific pathogen-free conditions and were kept under standard conditions in a 12-h day/night rhythm with access to food and water *ad libitum*. All the mice received humane care and the studies were approved by the internal ethics committee.

Generation of CCL27-Tg mice

The pTTT3D-Pac vector (Pharmacia, Kalamazoo, MI) containing a 402-bp full-length cDNA for mCCL27 (GenBank accession No. BC028511) was used [26]. DNA construct was generated as previously described [27]. To introduce *Bam*HI linkers to the CCL27 cDNA, PCR was performed using a set of primers including *Bam*HI linkers at both ends: 5'-GACGGA-TCCATGATGGAGGGGCTCTCCCC-3' (forward primer) and 5'-GACGGATCCTTAGTTTTGCTGTTGGGGGT-3' (reverse primer) (the underlined parts indicate the *Bam*HI linkers, the *italic* in the forward primer and in the reverse primer indicate the ATG start codon and the TAA stop codon, respectively). The PCR product was subcloned into pT7 BlueT vector (Takara Bio Inc., Shiga, Japan) and digested by endonuclease *Bam*HI. The resulting fragment was inserted into *Bam*HI site, between hK14 and human growth hormone (hGH) of the hK14/hGH expression vector [a pGEM-3Z vector (Promega) including hK14 promoter/enhancer and a portion of the hGH gene with poly(A) signal] [28]. The 4613-bp segment of hK14 promoter/enhancer-mCCL27-hGH fusion products was released with *Eco*RI and this fragment was used for microinjection (Fig. 1A). To generate Tg mice, hK14 promoter/enhancer-mCCL27 hGH inserts were microinjected into fertilized eggs of C57BL/6 mice followed by re-implantation of injected eggs into pseudopregnant C57BL/6 females [27].

Genotyping of CCL27-Tg mice

Mice were screened for transgene by PCR amplification of tail skin DNA using forward primer 5'-ACACCTCCCCCTGTGAA-TCA-3' located in the hK14 gene and reverse primer

5'-AGTTTTGCTGTTGGGGGTTT-3' located in the mCCL27 gene (Fig. 1A). Mouse GAPDH gene was employed as internal control and primers for mouse GAPDH were as follows: 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (forward primer) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (reverse primer). Tail genomic DNA was extracted with QIAGEN DNeasy Tissue Kit (Qiagen, Hilden, Germany). PCR products (594 bp) were subjected to electrophoresis and the presence of transgene was confirmed.

Keratinocyte cell culture

Dorsal and ventral portions of split ear skin were floated dermal-side-down in 0.5% trypsin (Sigma, St. Louis, MO) in PBS for 40 min at 37°C. The epidermis was removed from the dermis as a sheet and further mechanically agitated with 0.05% DNase I (Sigma) in PBS containing 3% FBS (Sigma) by aspirating and expiring cells with a syringe. The resulting single-cell suspension was filtered through nylon filter to remove clumps. The cells were spun down and washed with 5% FBS in PBS. They were cultured and maintained in collagen type I-coated tissue culture flask (Iwaki, Chiba, Japan) using Keratinocyte-SFM supplemented with 5 ng/mL of epidermal growth factor and 50 µg/mL bovine pituitary extract (Invitrogen, CA) in humidified 5% CO₂, 95% air at 37°C. For ELISA, these keratinocytes were cultured in collagen type I-coated six-well plates (Iwaki) using the same medium. Supernatant was collected from keratinocyte cultures grown for 48 h at 80–90% confluence without media exchange.

RT-PCR

Tissues were pulverized and homogenized with Ultra-turrax T8 (IKA, Staufen, Germany). Total RNA was extracted using a QuickPrep micro mRNA purification kit (Amersham Biosciences, Buckinghamshire, England). The RNA was treated with Amplification Grade DNase I (Invitrogen) to eliminate any residual genomic DNA. One microgram of RNA was reverse-transcribed with oligo(dT)_{12–18} primers to synthesize cDNA using a SuperScript First-Strand Synthesis System (Invitrogen). The primers used in the experiments with keratinocytes were as follows: 5'-AGCCTCCCGCTGTTACT-GT-3' (mCCL27, forward primer), 5'-AGTTTTGCTGTTGGG-GTTTT-3' (mCCL27, reverse primer), 5'-GAGGAGCGAGACCC-CACTAA-3' (mouse GAPDH, forward primer), 5'-GGCATCG-GGCATCGAAGGTGGAAGAGT-3' (mouse GAPDH, reverse primer). Primer sets for mouse IFN-γ and IL-4 were described previously [15]. The mixture was predenatured for 10 min at 95°C and then subjected to 40 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C, then 7 min at 72°C. Amplified DNA fragments were judged from 2% agarose gel electrophoresis.

Relative real-time mRNA quantitation

We used ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). A 50-µl reaction mixture containing 500 ng cDNA diluted in RNase-free water and 2.5 µl of TaqMan Gene Expression Assays Inventoried was mixed with 25 µl of TaqMan Universal PCR Master Mix, No AmpErase UNG (2×, Applied Biosystems). TaqMan Gene

Expression Assays Inventoried consists of a 20× mix of unlabeled PCR primers and TaqMan MGB probes (FAM dye-labeled). Assay ID: Mm00445259_m1, Mm00801778_m1, Mm00441257_g1, and Mm99999915_g1 were used for IL-4, IFN- γ , CCL27, and GAPDH, respectively. The reaction conditions were designed as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles with 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. The threshold cycle, the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined.

ELISA

The concentration of mCCL27 was measured by Quantikine mouse CTACK/CCL27 (R&D Systems). The serum levels of mouse IgE were determined using Mouse IgE EIA kit Yamasa (Yamasa Corporation, Tokyo, Japan). The serum was collected 24 h after the challenge in acute CHS and 48 h after the last challenge of chronic CHS.

Chemotaxis assay

CCL27 chemoattractant activity was determined by chemotaxis assay using L1.2 cells [29] (a kind gift from E. Butcher, Stanford University School of Medicine, Stanford, CA) stably transfected with mouse CCR10 cDNA [30] (CCR10⁺ L1.2 cells). L1.2 cells were cultured in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine and 5 μ M 2-mercaptoethanol (Sigma). Chemotaxis assay was performed in 96-well disposable chemotaxis chambers fitted with an 8- μ m polycarbonate filter (PVP free, ChemoTX, Neuroprobe, Cabin John, MA). Briefly, 28 μ L of medium plus serially diluted mCCL27 [supernatants of keratinocytes or recombinant mCCL27 (R&D Systems)] was added to the lower compartment of each well. The framed filter was aligned with the holes in the corner of the filter frame and placed over the wells. L1.2 cells (2×10^5) in 25 μ L of RPMI 1640 medium were added to the upper compartment. The chamber was then incubated at 37°C in a humidified atmosphere of 5% CO₂ for 4 h.

After incubation, cells in the lower compartment of each well were collected and counted by Coulter counter Z1 (Beckman Coulter, Fullerton, CA). Values are expressed as the percentage of input cells that migrate through the filter. Untransfected L1.2 cells (vector L1.2 cells) were used as a control. Monoclonal rat anti-mCCL27 antibody (MAB725, R&D Systems) was used for antibody blocking study. Various dilutions of mCCL27 (supernatants of keratinocytes or recombinant mCCL27) were incubated with 20 μ g/mL of the antibody for 20 min at room temperature and used for the chemotaxis assay described above.

Western blot

Fresh keratinocytes were disrupted in lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM PMSF (Boehringer Mannheim, Indianapolis, IN), 1 mg/mL leupeptin and 1 mM sodium orthovanadate (Sigma)]. The concentrations of extracted proteins were measured using a BCA Protein Assay

Kit (Pierce, Rockford, IL). The samples were boiled in sample buffer (50 mM Tris pH 7.4, 0.14% sodium dodecylsulfate, 1% v/v β -mercaptoethanol) for 5 min and separated by 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (10 μ g of protein per lane).

After transfer to an Immobilon-P® transfer membrane (Millipore Corp., Bedford, MA), the membrane was incubated in 0.2 μ g/mL monoclonal rat anti-mouse CCL27 antibody (MAB725, R&D Systems) overnight at 4°C. The membrane was washed and incubated with 40 ng/mL horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rat IgG HRP, sc-2065; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature and visualized using a chemiluminescence method (Phototope-HRP Western Blot Detection System, LumiGLO Reagent and Peroxidase; New England Biolabs Inc., Beverly, MA). The membranes were exposed to X-ray film, which was developed and printed. The molecular mass of mCCL27 is 10.9 kDa [31].

Contact hypersensitivity

CHS to oxazolone and FITC was assayed as described previously [10]. Briefly, in oxazolone CHS, mice were sensitized on the shaved abdominal skin with 100 μ L of 10% oxazolone (Sigma) in 4:1 acetone/olive oil. Six days after sensitization (day 0), the mice were challenged on both sides of one ear with 10 μ L of 1% oxazolone each [25]. In FITC CHS, mice were sensitized on the shaved abdominal skin with 400 μ L of 0.5% FITC (Sigma) in 1:1 acetone/dibutylphthalate three times at an interval of 1 wk. Six days after the last sensitization (day 0), the mice were challenged on both sides of one ear with 10 μ L of 0.5% FITC each [32].

Ear thickness was measured using a dial thickness gauge (Adachi, Tokyo, Japan). The CHS reaction by a single challenge was designated as “acute CHS”. Changes in ear thickness (ear swelling) were calculated as [(ear thickness at each time point) minus (ear thickness just before the challenge)]. In the experiment of CHS reaction to repeated challenges (designated as “chronic CHS”), mice were sensitized in the same way as described for acute CHS; each reagent was repeatedly applied to the same ear three times a week. The ear swelling was expressed as [(ear thickness just before each elicitation) minus (ear thickness on day 0)]. To examine the serum levels of IgE, single or repeated application of 100 μ L of oxazolone or FITC to the abdomen was also performed. Irritation by croton oil or tape stripping was performed as described previously [10].

Anti-CCL27 treatment

Monoclonal rat anti-mCCL27 antibody (125 ng, MAB725, R&D Systems) in 100 μ L of PBS was intravenously injected into the tail of Tg mice in normal condition and with FITC CHS. In FITC acute CHS, we injected three times just before the sensitization and once just before the challenge. The same amount of normal rat IgG (sc-2026, Santa Cruz Biotechnology) was used as a control. Due to the high costs of the antibodies, six mice were included in each treatment group.

Histological and immunohistochemical analysis

Tissue biopsies were fixed in 10% buffered formalin and embedded in paraffin, or were embedded in Tissue-Tek OCT compound (Sakura Finetech, Tokyo, Japan) and frozen in liquid nitrogen. H&E stain and toluidine blue stain were performed on formalin-fixed and paraffin-embedded sections.

For mCCL27 immunostain, 6- μ m cryostat sections were fixed in cold acetone (-20°C) for 15 min. The slides were air-dried and endogenous peroxidase activity was blocked by treating the slides with 1% hydrogen peroxide for 10 min, followed by incubation with normal goat serum. Then they were incubated with monoclonal rat anti-mCCL27 (5 $\mu\text{g}/\text{mL}$, MAB7251, R&D Systems) overnight at 4°C , and were incubated with biotin-conjugated goat anti-rat IgG (sc-2041, Santa Cruz Biotechnology) for 30 min at room temperature, followed by incubation with ABC complex using the VECTASTAIN ABC KIT (Vector Laboratories, Burlingame, CA). Stain was developed by adding the diaminobenzidine solution until brown color was visible, and counterstained with hematoxylin.

For mouse CCR10, CCR4, CLA, and CXCR3 immunostain, frozen sections were stained with polyclonal goat anti-mouse CCR10 (ab1661; Abcam, Cambridge, UK) in 1:500 dilution, goat anti-mouse CCR4 (CI0122; Capralogics, MA) in 1:500 dilution, rat anti-human CLA (HECA-452, which is also reactive to mouse CLA; BD Biosciences, San Jose, CA) in 1:250 dilution [33], and goat anti-mouse CXCR3(Y-16, Santa Cruz Biotechnology) in 1:250 dilution as described above, respectively. The number of cells was counted in three lesions. Six visual fields of $\times 400$ magnification were selected at random under a light microscopy (Labophot-2; Nikon, Tokyo, Japan) [10].

Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical analysis was performed using Mann–Whitney's U test; $p < 0.05$ was considered significant.

Acknowledgements: This work was supported by Health Science Research Grants from the Ministry of Health, Welfare and Labor of Japan, and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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