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CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14 IL-4 transgenic mouse model

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Abstract

The keratin-14 IL-4 transgenic (Tg) mouse model of atopic dermatitis (AD) is characterized by skin infiltration of T cells, early up-regulation of T_h2 cytokines and late surge of T_h1 cytokines. In the present study, we investigated the role of CCL27, a T cell skin-homing chemokine known to be elevated in sera of human AD patients, in disease development in our animal model of AD. The results showed that the mRNA and protein levels of CCL27 in the skin and serum were significantly increased in IL-4 Tg mice. The percentage of T cells expressing CCR10 in skin draining lymph nodes of IL-4 Tg mice was increased, consistent with the findings of >80% of skin-infiltrating T cells in Tg mice expressing CCR10. Chemotaxis transmigration assay demonstrated that CCL27 promotes a greater degree of migration of T cells in diseased Tg mice. Subcutaneous injection of neutralizing anti-CCL27 to IL-4 Tg mice with early skin lesions resulted in reduced clinical progression of inflammation, accompanied with decreased T cell and mast cell infiltration in the skin, and down-regulation of inflammatory cytokines. In conclusion, CCL27 and CCR10 interaction is important for the development of skin inflammation in our AD model.

Introduction

Atopic dermatitis (AD) is a common chronic pruritic inflammatory skin disease characterized by skin-infiltrating T cells, mast cells and eosinophils (1, 2). AD is a typical example of a T_h2-mediated inflammatory skin disease at the acute stage followed by a biphasic T_h1 and T_h2 immunophenotypes at the chronic stage of the disease (3–5). We have generated an animal model of AD by expressing a T_h2 cytokine IL-4 in the basal epidermis using a basal keratinocyte-specific keratin-14 promoter/enhancer (6). The transgenic (Tg) mice spontaneously developed a pruritic, chronic, inflammatory skin disease (6). The inflammatory skin disease in Tg mice fulfills the clinical and histological diagnostic criteria for human AD (7). Immunologically, we found that T cells including CD4+ and CD8+ cells were highly activated and were the major infiltrates in both the epidermis and dermis of the inflammatory skin lesions (8). We also demonstrated that there was an early up-regulation of T_h2 cytokines followed by a late surge of T_h1 cytokines (9) and that total serum levels of IgE and IgG1 significantly increased in the early stage of the disease (10).

Chemokines, a superfamily of structurally related small proteins, contribute to leukocyte extravasations and localization within peripheral sites (11, 12). Chemokines such as CXCL9 [monokine induced by interferon-γ, (MIG)], CXCL10 [IFN-inducible protein 10, (IP-10)] and CXCL11 [interferon-inducible T cell α chemoattractant, (I-TAC)] recruit lymphocytes mainly to T_h1-type inflammatory sites, while chemokines such as CCL11 (eotaxin), CCL17 [thymus and activation-regulated chemokine, (TARC)] and CCL22 [macrophage-derived chemokine, (MDC)] lead to a T_h2-dominated pattern of cell recruitment (13). One of the CC chemokines, CCL27 (cutaneous T cell-attracting chemokine, CTACK) (14), has recently been described in humans and mice as exclusively expressed by

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keratinocytes (14). CCL27 is an essential chemokine for T cell skin homing (14). The most abundant expression of CCL27 is in the basal keratinocytes (15). In normal skin suprabasal keratinocytes express minimum amount of CCL27; yet in the lesions of human AD, contact dermatitis and psoriasis, they demonstrate stronger expression of CCL27 (15). In addition, CCL27 is found in the dermal extracellular matrix (ECM), fibroblasts and endothelial cells of the superficial plexus especially in inflamed skin (15). However, fibroblasts and endothelial cells do not express CCL27 mRNA (16). It is suggested that CCL27 is secreted into the papillary dermis, immobilized on the ECM and displayed on the surface of endothelial cells (15). It is not clear how the secreted CCL27 localizes on the surface of endothelial cells, and where is it involved in the activation of integrins. The presentation of CCL27 on endothelial cells probably occurs through transcytosis, a mechanism known to localize chemokines such as CXCL8 and CCL19 on endothelial cells (17, 18). CCR10, the receptor for CCL27, is expressed by T cells, primary dermal microvascular endothelial cells, dermal fibroblasts and melanocytes, but not keratinocytes (16). In humans, CCL27 selectively attracts cutaneous lymphocyte-associated antigen (CLA)+ memory T cells by interacting with CCR10 expressed in these lymphocytes (14–16). The expression of CCR10 in circulating cells is restricted to a specific subset of CLA+CD45RO+ T lymphocytes derived from normal human donors (19). Intra-dermal injection of recombinant CCL27 induces the recruitment of CD3+ lymphocytes to the injection site (15). Additionally, the neutralization of CCL27 impairs lymphocyte recruitment in the mouse skin of 2,4-dinitro-1-fluorobenzene, (DNFB)-induced contact dermatitis and ovalbumin-induced AD (15). Neutralization of CCL27 also results in suppression of inflammation-induced skin thickening (15), indicating that CCL27–CCR10 interaction directly regulates T cell recruitment to inflamed skin.

Although the role of CCL27 in human AD has not been delineated, recent studies showed that the serum level of CCL27 was significantly elevated in patients with AD (20–22). This elevation correlated with disease severity (21, 22), which highly suggested that CCL27 might play a role in the inflammatory process of AD. Using our mouse model of AD, we proceeded to evaluate the dynamic changes of CCL27 in the skin and CCR10 on the lymphocytes as the disease progressed and the effect of anti-CCL27 treatment. Our results demonstrated that CCL27 was highly up-regulated in the skin and serum of Tg mice and that CCR10 is expressed on T cells in both skin lesions and skin draining lymph nodes (LNs). Locally administered anti-CCL27-neutralizing antibody abrogated the severity of the skin inflammation, which may provide a novel potential therapeutic strategy for human AD.

Methods

Mice

The establishment and systematic studies of the K-14 IL-4 epidermal Tg mouse line were published previously (6, 8–10). The Tg mice and their non-Tg littermates were housed in special pathogen-free cages. We have previously determined that all Tg mice and none of non-Tg mice developed a chronic inflammatory skin disease under these conditions (8–10). The study complied with the Animal Care Policies and Procedures of the University of Illinois at Chicago.

Disease phenotype classification

Skin lesions from IL-4 Tg mice that developed for 1 week or shorter duration were defined as early skin lesion (EL). Skin lesions that developed for 3 weeks or longer were defined as late skin lesion (LL). Tg mice before disease onset were defined as BO as described in our previous publications (8–10).

Anti-CCL27 treatment

Anti-mouse CCL27-neutralizing antibody (200 μg) (R&D Systems, Minneapolis, MN, USA) was intra-dermally injected into the base of the ears of Tg mice at EL stage. The same amount of isotype rat IgG2b (R&D Systems) was used in age-matched Tg mice with the same extent of skin lesions at the EL stage. The injection was repeated every 3 days for 24 days. Due to the high costs of the antibodies, three mice were included in each treatment group.

Cell culture

Mouse keratinocyte cell line PAM212 provided by Jonathan C. R. Jones was grown in 24-well flat-bottom plastic culture plates in MEM with 10% fetal bovine serum (FBS). To examine the effects of cytokines on keratinocyte CCL27 expression, IL-1β (20 ng ml^{-1}), tumor necrosis factor (TNF)-α (20 ng ml^{-1}), IL-1β and TNF-α (20 ng ml^{-1} for each), IL-4 (30 ng ml^{-1}), IL-5 (30 ng ml^{-1}), IL-6 (3 ng ml^{-1}), IL-13 (30 ng ml^{-1}) and IFN-γ (20 ng ml^{-1}) were added to the medium when the cells became 90% confluent. The culture media were collected 24 h later and used for a CCL27 ELISA as described below. For primary keratinocyte cell culture, neonatal mouse pups of both IL-4 Tg mice and non-Tg mice, between 3 and 4 days post-partum were used. After the mice were sacrificed, the pelts from the pups were placed dermis-side down into a 60-mm tissue culture dish containing 3 ml of dispase (Roche, Indianapolis, IN, USA, 10 mg ml^{-1} in keratinocyte culture medium) and incubated overnight at 4°C. The epidermis was separated from the dermis and transferred to another tissue culture dish containing 2 ml trypsin versene and incubated for 15 min at 37°C. The trypsinized epidermal sheets were then transferred to a prescratched dish (to provide rougher surface optimal for cell release) containing 2 ml FBS and rubbed back and forth for 30 s. The released keratinocytes were then collected and washed with PBS. Finally, the cells were re-suspended in KGM-2 keratinocyte media (Cambrex Bio Science, Walkersville, MD, USA) with 50 mM $CaCl_2$ and placed onto rat tail collagen I-coated 75-cm^2 flasks. When cells were 70–80% confluent, they were subcultured into 24-well collagen I-coated plates. When cells had again reached 70–80% confluence, cytokines, including IL1-β (20 ng ml^{-1}) and TNF-α (20 ng ml^{-1}) or both (20 ng ml^{-1} for each) were added. Control

samples contain culture medium only. Supernatants were collected at 24 h post-cytokine stimulation for CCL27 ELISA. The tails from pups were genotyped as described in previous publications (6, 8–10).

ELISA

Blood was collected from non-Tg mice and Tg mice at BO, EL, and LL stages. Concentrations of CCL27 in the mice sera and culture media of PAM212 cells, and primary keratinocytes from IL-4 Tg mice and non-Tg mice, were determined by a sandwich ELISA. Briefly, a 96-well Nunc-Immuno plate (Nalge Nunc Int., Rochester, NY, USA) was coated with 100 μl per well of rat anti-mouse CCL27 capture antibody (2 μg ml^{-1}, R&D Systems) and incubated overnight. After the washes, the plate was blocked with PBS containing 1% BSA, 5% sucrose, then recombinant CCL27 standards (PeproTech, Rocky Hill, NJ, USA), diluted mice sera, and culture media were added and incubated for 2 h. The biotinylated goat anti-mouse CCL27 antibody (200 ng ml^{-1}, R&D Systems) was added and incubated for 2 additional hours followed by incubating the plate with streptavidin–peroxidase polymer (Sigma, St Louis, MO, USA) for 1 h and then 3,3=B4,5,5=B4-tetrametylbenzidine substrate (Sigma) for 15 min. H_2SO_4 (0.5 M) was added to stop the reaction. Optical densities (ODs) were read at 450 nm in a μQuant microplate reader (Bio-TEK, Inc., Winooski, VT, USA). The concentrations were determined by the standard curve generated from recombinant mouse CCL27 standards. The assay detected as low as 62 pg ml^{-1} of CCL27. To determine the concentrations of IL-1β and TNF-α in the skin extracts, mouse ears from non-Tg, Tg-BO, Tg-EL and Tg-LL were collected. Ears were then cut into small pieces in tissue lysis buffer containing 20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA and 0.1% SDS (pH 7.5) with protease inhibitors cocktail (Sigma). Tissue was then homogenized and centrifuged for 20 min at 13 000 rpm at 4°C. Supernatants were collected and the amount of protein was quantified using protein microassay (Bio-Rad, Hercules, CA, USA). The protein concentrations in all samples were diluted to 5 mg ml^{-1}. IL-1β and TNF-α concentrations were then determined by commercially available ELISA kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. One hundred microliters (500 μg proteins) of each diluted sample was used in the assay. These ELISA kits detected as low as 4 pg ml^{-1} of IL-1β and TNF-α.

Flow cytometric analysis

The single-cell suspensions from LNs of non-Tg, Tg-BO, Tg-EL and Tg-LL mice and skin lesions from Tg-EL and Tg-LL mice prepared as described in references (8, 9) were stained with FITC–rat anti-mouse CD4/CD8 (eBioscience) and goat anti-mouse CCR10 (Abcam, Cambridge, MA, USA) followed by PE–swine anti-goat IgG (Caltag Laboratories, Burlingame, CA, USA) staining, then the cells were analyzed in Calibur FACS system (BD Bioscience). Live cells were gated based on 7-aminoactinomycin D (BD Bioscience) uptake. Percentages of CCR10 expressing CD4+ or CD8+ cells were calculated. All antibodies were used at a final concentration of 0.5 μg per 1×10^6 cells.

Chemotaxis assay

Standard transmigration assay using 5-μm pore Costar transwell inserts (Corning Incorporated, Corning, NY, USA) was performed as previously described for T cell migration (23). T cells were purified from skin draining LNs of non-Tg mice and Tg mice in BO, EL and LL stages using anti-Thy1.2 antibody beads (Miltenyi Biotec Inc., Auburn, CA, USA). The purity of T cells was >97% as confirmed by flow cytometry using PE–anti-CD3 (BD Bioscience). Purified T cells (1×10^6) suspended in 100 μl RPMI 1640 with 10% FBS were added into transwell inserts (upper chamber), which were then placed in contact with lower chambers containing 900 nM recombinant mouse CCL27 or CXCL-2 macrophage-inflammatory protein-2, (MIP-2) (PeproTech) in 600 μl medium and cultured for 2 h. Replicate wells were used. The cells that migrated into the lower chambers were collected and counted by hemocytometer and the percentages of migrated cells were then calculated based on the number of input and migrated cells.

Histology

Twenty-four days after the antibody treatment, mice were sacrificed and lesional skins were processed for haematoxylin and eosin and Giemsa staining. The number of purple-stained mast cells in Giemsa stained sections were counted per high power field (HPF, ×40 objective lens). Ten fields of each section were counted.

Immunofluorescence microscopy

Examination of CD4+ and CD8+ cells in the lesional skin 24 days after the antibody treatment by immunofluorescence microscopy was performed as previously described (8). The numbers of CD4+ and CD8+ T cells per HPF (×40 objective lens) were counted.

Real-time reverse transcription–PCR

The detailed protocol of real-time PCR and the primers for cytokines were published previously (9, 24). Briefly, total RNAs were extracted from the skin samples (ears) of non-Tg, Tg-BO, Tg-EL and Tg-LL mice and from lesional skins of Tg mice treated with anti-CCL27 or isotype using TRIzol (Invitrogen, Carlsbad, CA, USA). The RNAs were reverse transcribed to cDNAs by Retroscript Kit (Ambion, Austin, TX, USA).

Table 1. Chemokine and chemokine receptor primers

	Forward primer (5′–3′)	Reverse primer (5′–3′)
CXCL-2 (MIP-2)	cctgccaagggttgacttca	ttctgtctgggcgcagtg
CXCL9 (MIG)	ttctcggacttcactcca	cactgctccaggaagatg
CXCL10 (IP-10)	gtcctaattgcccttggt	tcttgcttcggcagttac
CXCL11 (I-TAC)	aacatgtgacatcctggg	cctggtaatacgtggctg
CCL5 (RANTES)	cagcagcaagtgctccaatctt	ttcttgaacccacttcttctctgg
CCL11 (eotaxin)	tgagaggctgagatccaa	ctgggaggtgaaggaagt
CCL22 (MDC)	attctgggagtttcaggc	attctgagcctgctcctt
CCL17 (TARC)	agcagaagtccctgttcc	aggctttattccgttgct
CCL27 (CTACK)	ccgctgttactgttgctt	cttgggagtggctgtcta
CCR10	tgctcctactgagaccca	ccctgggattgtttcttt

Real-time PCR was performed to determine the cDNA copy number of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-γ and TNF-α) using control plasmid standards (9, 24). The relative quantities of CXCL-2 (MIP-2), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CCL-5 [regulated on activation normal T cell expressed and secreted, (RANTES)], CCL11 (eotaxin), CCL17 (TARC), CCL22 (MDC), CCL27 (CTACK), CCR10, IL-8 and RANTES were also determined. The primer sequences for these chemokines or receptor are listed in Table 1. Real-time PCR was performed in Mx3000p real-time PCR machine (Stratagene, La Jolla, CA, USA).

T cell proliferation

LN cells (2×10^5) from Tg mice treated with anti-CCL27 or isotype in triplicate were incubated in the presence or absence of anti-CD3 (1 μg ml^{-1}, eBioscience), for 72 h, and then the cell proliferation rate was examined using a Cell Counting Kit-8 (Kumamoto, Japan) as described in our previous publication (8). OD_{450} was read in a μQuant microplate reader (Bio-TEK, Inc.) with a reference wavelength at 600 nm.

Statistics analysis

All experimental data were expressed as mean ± SD. Significant differences between two groups were determined by Student's *t*-test. All analyses were performed by GraphPad Instat Software (San Diego, CA, USA). A *P*-value ⩽0.05 was considered significantly different.

Results

CCL27 is significantly elevated in the skin and sera of IL-4 Tg mice

We first used real-time PCR to examine mRNA expression of chemokines including CXCL9, CXCL10, CXCL11, CCL11, CCL17, CCL22 and CCL27 in the skin of non-Tg and Tg mice at different stages of the disease. As demonstrated in Fig. 1(A), there was an increase of mRNA expression in the late stage of skin lesions of Tg mice (Tg-LL) for almost all the chemokines examined. However, distinguished from other chemokines, CCL27 was the only one significantly elevated in the skin of BO in Tg mice (Tg-BO), which has a 4.36-fold increase compared with the level of non-Tg mice ($P < 0.001$). As the disease progressed in the Tg early lesion (Tg-EL) mice, the level of CCL27 mRNA somewhat diminished, but was still significantly higher than that of non-Tg mice (2.7-fold increase, $P < 0.001$) (Fig. 1A). However, the CCL27 mRNA expression was nearly normalized in Tg-LL mice skin, close to the level of non-Tg mice ($P > 0.05$) (Fig. 1A). We next examined the soluble CCL27 in the serum by ELISA. As shown in Fig. 1(B), CCL27 was detectable in non-Tg mice serum. But in the Tg mice, this molecule in all three groups of Tg mice was markedly increased compared with that of non-Tg mice ($P < 0.001$). The peak level was observed in Tg-EL stage, which may simply reflect the fact that the synthesis (translation) and secretion of CCL27 occur subsequent to the mRNA transcription. Based on above findings and reported roles of CCL27 in skin inflammation in the literature (14, 15), it seemed that CCL27 would be a more important factor for the initiation of skin lesions than other chemokines. Therefore, in the following experiments we primarily focused on function analyses of CCL27 and CCR10 in the IL-4 Tg mice.

Inflammatory cytokines up-regulate the production of CCL27 by keratinocytes

Having demonstrated that the skin mRNA expression and serum level of CCL27 were elevated in the Tg mice, we investigated the possible activators responsible for this elevation. Previously, we showed that cytokines including IL-1β, IL-4, IL-5, IL-6, IL-13, IFN-γ and TNF-α were significantly increased in the skin of Tg-BO mice, suggesting that these cytokines may play an inductive role in the disease (9). To investigate whether these cytokines modulate the keratinocyte expression of CCL27, we cultured mouse keratinocyte cell line PAM212 in the presence or absence of different cytokines and examined the CCL27 production in the culture media. The results showed that the cells treated with TNF-α secreted significantly more CCL27 protein than untreated cells ($P = 0.05$). IL-1β increased the production of CCL27, but no statistical significance was observed (Fig. 2A). However, the combination of IL-1β and TNF-α had the strongest effect on CCL27 production ($P < 0.05$), a finding consistent with other studies (15, 25). In addition, IL-5, IL-13 and IFN-γ had a similar but less significant effect as IL-1β (Fig. 2A). IL-4 or IL-6 did not stimulate CCL27 production (Fig. 2A). When primary keratinocytes isolated from IL-4 Tg mice were used, similar results were obtained (Fig. 2B) compared with that of PAM212 cells (Fig. 2A). However, keratinocytes from IL-4 Tg mice produced

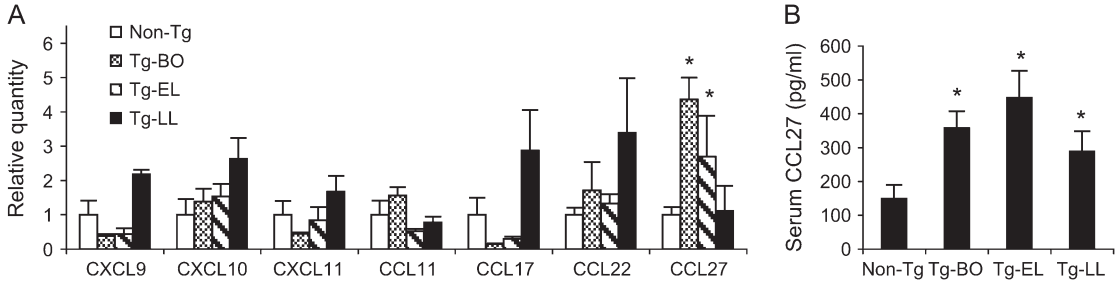

Fig. 1. Chemokine profile in the skin and serum of the IL-4 Tg mice. CXCL9, CXCL10, CXCL11, CCL11, CCL17, CCL22 and CCL27 mRNA expression in the skin of non-Tg, Tg-BO, Tg-EL and Tg-LL mice was examined by semi-quantitative real-time PCR. Relative quantities of chemokines compared with non-Tg mice were shown in (A). Serum levels of CCL27 at different stages of the disease were examined by ELISA (B). $n = 15$ for each group, $^*P < 0.01$ compared with non-Tg mice.

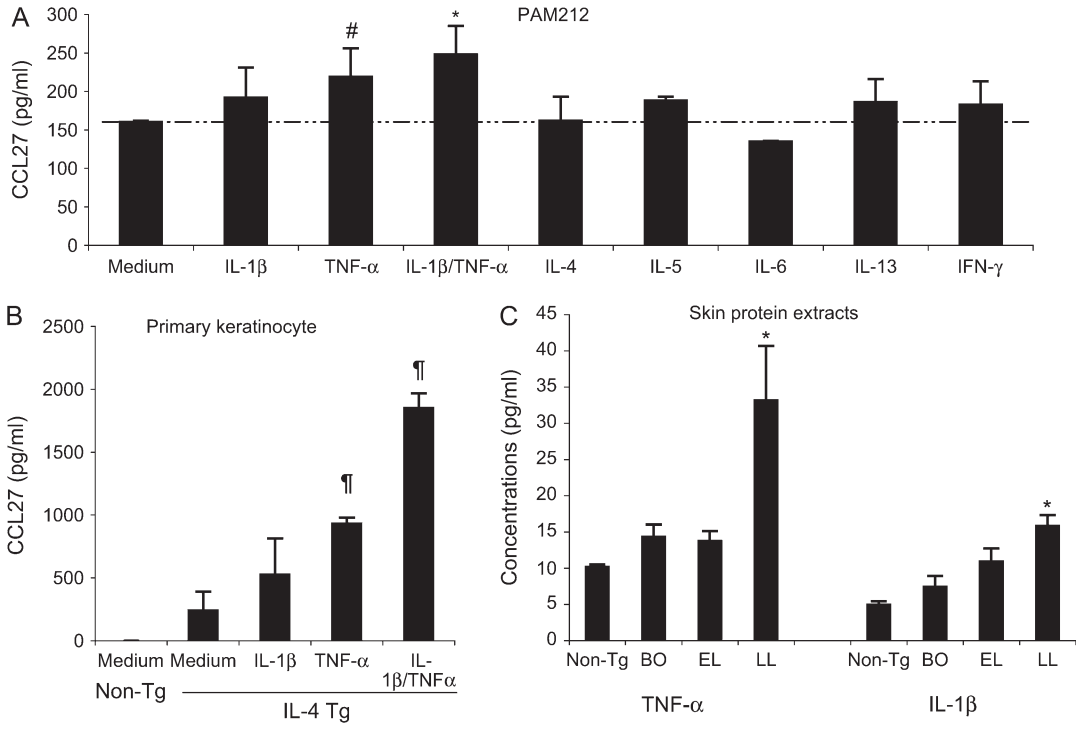

Fig. 2. Cytokines up-regulate the production of CCL27 by keratinocytes. (A) PAM212 cells were incubated with recombinant IL-1β (20 ng ml^{-1}), TNF-α (20 ng ml^{-1}), IL-1β and TNF-α (20 ng ml^{-1} for each), IL-4 (30 ng ml^{-1}), IL-5 (30 ng ml^{-1}), IL-6 (3 ng ml^{-1}), IL-13 (30 ng ml^{-1}) and IFN-γ (20 ng ml^{-1}) for 24 h. #$P = 0.05$, *$P < 0.05$ compared with medium control. (B) Primary keratinocytes isolated from neonatal mice were incubated with IL-1β (20 ng ml^{-1}), TNF-α (20 ng ml^{-1}) and IL-1β and TNF-α (20 ng ml^{-1} for each) for 24 h. ¶$P < 0.001$ compared with medium control. The concentrations of CCL27 in supernatants were determined by ELISA. Similar results for (A) and (B) were obtained in another experiment. (C) TNF-α and IL-1β in the skin extracts. The concentrations of TNF-α and IL-1β in the skin extracts of non-Tg, Tg-BO, Tg-EL, and Tg-LL mice were determined by ELISA. $n = 4$ for each group. *$P < 0.01$ compared with non-Tg mice.

much higher level of CCL27 than those of PAM212 cells under the same cultural conditions, including basal condition (medium only, without cytokine stimulation) (Fig. 2B). Furthermore, keratinocytes from IL-4 Tg mice produced a higher basal level of CCL27 compared with non-Tg mouse keratinocytes, which do not secrete a detectable level of CCL27 protein (Fig. 2B). This experiment suggests that the cytokine milieu created by the epidermal IL-4 transgene and the subsequent elevation of IL-1β, IL-5, IL-13, TNF-α and IFN-γ in the skin of Tg mice at BO stage may play a pivotal role in the elevation of CCL27 by epidermal cells, thus may change the skin-associated homeostatic chemokine CCL27 into an inflammatory one (15). Given the findings that IL-1β and TNF-α were the two most important cytokines shown to up-regulate CCL27 in keratinocytes by the above experiments and that mRNAs of IL-1β and TNF-α were significantly elevated in the skin of IL-4 Tg mice including Tg-BO, Tg-EL and Tg-LL mice in our previous report (9), we took one step further to examine the protein levels of IL-1β and TNF-α in the skin extracts from different stages of the disease by quantitative ELISA. Results showed that protein concentrations of IL-1β and TNF-α started to increase from Tg-BO stage and reached peaks in Tg-LL stage, paralleling the findings of skin mRNA increases of these two cytokines (9). The evidence of elevated protein levels of IL-1β and TNF-α in the skin of Tg-BO mice further suggests that these two cytokines may play an important role in the disease induction by up-regulating CCL27 *in vivo*, which thereby enhances the recruitment of extra-cutaneous T cells into the skin.

CCR10 is expressed on the majority of CD4+ and CD8+ cells in the skin lesions

Having demonstrated that CCL27 expression is elevated in the Tg mice, we further asked the question of what is the *in vivo* outcome of this CCL27 up-regulation. Using flow cytometric analysis, we found that 91.6 ± 3.5% and 97.8 ± 3.8% of CD4+ cells in the lesional skin of Tg-EL and Tg-LL mice expressed CCR10, respectively, while there were 84.2 ± 5.4% and 95.6 ± 4.0% of CD8+ cells expressing CCR10 (Fig. 3A). This result strongly suggests that CCR10+ T cells are preferentially concentrated in the skin lesions, a finding that indirectly points to the role of CCL27 in recruiting the skin-infiltrating T cells. In the LNs, we found that as the disease progressed, the frequencies of CCR10 expressing CD4+ cells gradually increased from 1.3 ± 0.4% in non-Tg mice to 1.5 ± 0.5% in Tg-BO, 3.1 ± 0.6% in Tg-EL and 3.4 ± 0.6% in Tg-LL (Fig. 3B). The expressions in EL and LL groups were significantly higher than that of non-Tg mice ($P < 0.05$). The frequencies of CCR10 on LN CD8+ cells also increased in Tg mice with a peak at the Tg-EL stage (Fig. 3B), however, no significant difference was observed among these groups.

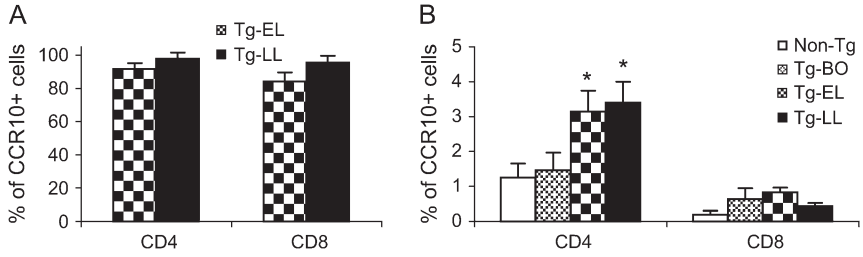

Fig. 3. CD4+ and CD8+ cells in the LNs and skin express CCR10. (A) Majority of CD4+ and CD8+ cells in the skin lesions express CCR10. (B) CCR10 expressing CD4+ and CD8+ cells in LNs increase in IL-4 Tg mice as the disease progresses. Data were the mean percentages of CCR10 expressing CD4 or CD8 cells pooled from three experiments. $*P < 0.05$ compared with non-Tg mice.

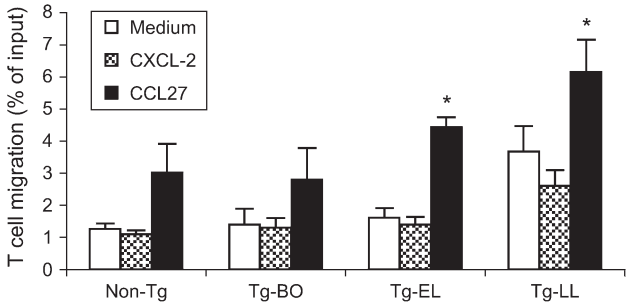

Fig. 4. CCL27 promotes T cell migration. Transmigration assay was performed. Purified T cells (1×10^6) from non-Tg g-BO, Tg-EL and Tg-LL mice and 900 nM recombinant mouse CCL27 or CXCL-2 were used. The percentages of migrated cells were calculated based on the number of input and migrated cells. Data were pooled from four experiments. $*P < 0.05$ compared with medium control or CXCL-2-treated cells.

CCL27 promotes migration of T cells from diseased IL-4 Tg mice

To further confirm the role of CCL27 in T cell migration as suggested by the indirect evidence of high percent CCR10+ T cells in the skin, we assessed the effect of CCL27 on chemotrafficking of T cells isolated from LNs by a transwell chemotaxis method. The results showed that more T cells migrated to CCL27 in all groups of mice including non-Tg mice than those that migrated to the medium control (Fig. 4). As the disease progressed, T cell migration rates in response to CCL27 were increased from 2.80 ± 0.98% (Tg-BO mice) to 4.43 ± 0.31% (Tg-EL mice) and to 6.16 ± 1.00% (Tg-LL mice) (Fig. 4), with the number of T cells from Tg-EL and Tg-LL mice migrating in the presence of CCL27 significantly being higher than those from non-Tg or Tg-BO mice ($P < 0.05$, Fig. 4). This is likely due to the greater number of CCR10-expressing T cells in the diseased mice (Fig. 3B). Interestingly, in the absence of CCL27, more T cells from Tg-LL mice migrated compared with those from other groups (Fig. 4). The mechanism of this increased migration is not clear at this point. A higher level of activation in these T cells demonstrated by their significantly up-regulated activation molecules (CD44 and CD69) and/or stronger proliferation (8) cannot adequately explain this accelerated migration, since T cells from Tg-EL mice also have similar features but nevertheless did not have an increased migration rate. The migration of T cells treated with MIP-2, a chemokine serving as a negative control, showed a similar rate to that of medium control, indicating the specific chemoattractive activity of CCL27 to T cells.

Anti-CCL27-neutralizing antibody treatment reduces skin inflammation and the expressions of skin inflammatory cytokines

Based on the above findings, the interaction of CCL27 and CCR10 is most likely involved in the T cell homing to the skin in our animal model, leading us to further explore the effect of CCL27 neutralization. Injection of anti-CCL27-neutralizing antibody into the bases of the diseased ears of Tg mice with early lesions clearly decreased the clinical progression of the inflammatory lesions including the size, thickness and scaling compared with the animals that received isotype control at 8, 14 and 24 days after treatments were initiated (Fig. 5A). The clinical effect of anti-CCL27 treatment was confirmed by histopathology, showing skin lesions of anti-CCL27-treated mice having less acanthosis, parakeratosis, and leukocyte infiltrate (Fig. 5B), a finding similar to what Homey *et al.* (15) reported using a similar treatment in a contact dermatitis model induced by DNFB and an AD model induced by epicutaneous application of ovalbumin. Furthermore, less numbers of CD4+ and CD8+ cells in both the epidermis and dermis (Fig. 5C, $P < 0.01$–0.001) and less number of mast cells in the dermis (Fig. 5D, $P < 0.05$) were present in treated mice than that of control mice. In addition, the reduction of skin inflammatory cell infiltrates by anti-CCL27 treatment correlates with the reduction of skin inflammatory cytokines. PCR analysis showed that the treatment substantially reduced the mRNA expression of IL-1β, IL-2, IL-4, IL-6 and IL-10, IL-12p40, IFN-γ and TNF-α compared with that of control mice (Fig. 5F). The treatment also resulted in less expression of CCR10 in the skin lesions, corresponding to the reduction of T cell infiltrate as illustrated by the immunopathology finding (Fig. 5C). Given the specificity of CCL27 for T cell recruitment, the reduction of mast cell infiltration in the skin lesions was unlikely to be directly related to CCL27 but rather a secondary effect of down-regulated chemoattractants for mast cells such as IL-8 and RANTES (26, 27). Mice do not express IL-8. However, MIP-2 is functionally equivalent to human IL-8 (28–31). Therefore, we examined MIP-2 mRNA expression in the skin lesions. Results showed that the skin lesions in CCL27-treated group had less MIP-2 and RANTES mRNA expression compared with IgG-treated control (Fig. 5F), suggesting this reduction of MIP-2/RANTES expression may play a role in reducing the mast cells in the CCL27-treated skin lesions.

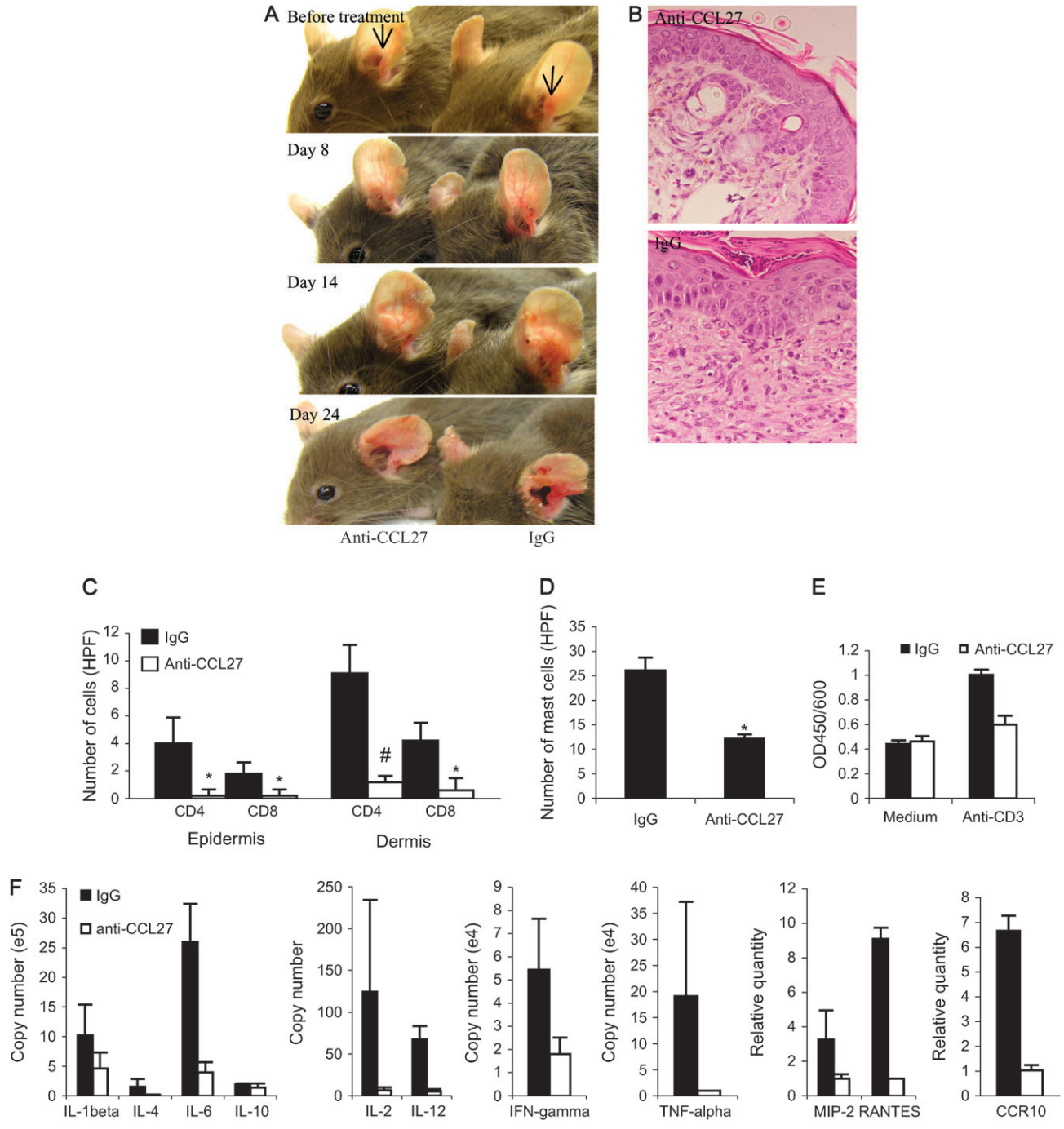

Fig. 5. Anti-CCL27 treatment reduces the skin inflammation in IL-4 Tg mice. (A) Photos of skin lesions before the treatment and 8, 14 and 24 days after the treatment. Arrows indicate lesions before the treatment. (B) Reduced acanthosis, parakeratosis and leukocyte infiltrates in the skin lesions after anti-CCL27 treatment. Haematoxylin and eosin staining, magnification: ×40. (C) Impaired numbers of CD4+ and CD8+ cell infiltrates in the epidermis and dermis after anti-CCL27 neutralization. $*P < 0.01$, $\#P < 0.001$. (D) Impaired numbers of mast cells in the skin lesions after anti-CCL27 neutralization. $*P < 0.01$. (E) Down-regulated T cell proliferation after anti-CCL27 neutralization. Results are expressed as the mean ± SD of triplicate. Data are representative of one of the three mice in each group. (F) Decreased mRNA expression of inflammatory cytokines and CCR10 in lesional skin after anti-CCL27 neutralization. Data were obtained from three mice in each group.

Anti-CCL27-neutralizing antibody treatment down-regulates T cell proliferation

Having shown that anti-CCL27 partially blocked T cell homing to the skin in our AD model, we asked the question of whether this local inhibition of T cell migration might also influence the activation status of T cells in LNs. *In vitro* T cell proliferation in response to anti-CD3- in anti-CCL27-treated mice was significantly lower than that of control mice (Fig. 5E), suggesting

that T cells in the anti-CCL27-treated mice were less activated or less reactive to stimulation compared with those in control group.

Discussion

With the knowledge that T cells are the major infiltrates in the lesional skin of our AD mouse model (8), we logically move to identify the mechanism that regulates lymphocyte recruitment to the skin by investigating CCL27's role in T cell homing to the skin. In the present study, we found that the mRNA and protein levels of CCL27 in the skin and serum were significantly increased in IL-4 Tg mice. The frequency of T cells expressing CCR10 in LNs of Tg mice was increased, consistent with findings of the majority of skin-infiltrating T cells in Tg mice expressing CCR10. Chemotaxis transmigration assay demonstrated that CCL27 promotes a greater degree of migration of T cells in diseased Tg mice. Subcutaneous injection of neutralizing anti-CCL27 resulted in reduced clinical progression of inflammation, accompanied with decreased T cell and mast cell infiltration in the skin, down-regulation of inflammatory cytokines and the T cell proliferation.

Based on available literature and the data from our present and previous studies (8–10), we propose the role of CCL27 in the lymphocyte recruitment to the skin in the AD model as the following. Epidermal expression of IL-4 changes the homeostasis in the skin, which may induce the production of IL-1β and TNF-α by keratinocytes (and perhaps also by other cell types in the skin) by a yet-to-be-determined mechanism. IL-1β and TNF-α could enhance the production of constitutively expressed CCL27 by keratinocytes. CCL27 then could be displayed on endothelial cells by a transcytosis process (17, 18) and serve as a binding ligand for CCR10-bearing T cells. The signal transduction generated from the interaction of CCL27 and CCR10 then activates leukocyte function-associated antigen (LFA)-1 ($\alpha_L\beta_2$ integrin) and very late antigen-4, (VLA-4) ($\alpha_4\beta_1$ integrin) on lymphocytes (13). Upon activation, LFA-1 and VLA-4 then interact with intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 up-regulated by IL-4 (13, 32) and mediate firm adhesion of lymphocytes on endothelial cells (13) prior to lymphocyte extravasation. Upon infiltrating the skin, lymphocytes could then produce IL-5, IL-13 and IFN-γ, as documented in the skin of Tg mice at BO and EL stages (9), which provides additional stimulation to keratinocytes for producing CCL27 that recruit more lymphocytes into the skin. Unless a sufficient negative feedback mechanism is in place to terminate this cycle of inflammatory cell recruitment, this process will go on indefinitely.

It is intriguing to determine the mechanism by which anti-CCL27 treatment diminishes the skin inflammation in our mouse model of AD. We propose that this neutralizing antibody binds to CCL27 on endothelial cells, which leads to the blockade of CCL27-induced activation of adhesion molecules (LFA-1 and VLA-4) on CCR10+ T cells. Thus, the following events including lymphocyte's firm adhesion on endothelial cells and subsequent transendothelial migration of lymphocyte into the skin are impaired. The decreased T cell infiltrate then results in reduction of cytokines (IL-2, IL-4, IL-6, IFN-γ and TNF-α) as shown in Fig. 5(F). This reduction could then further dampen the release of other inflammatory factors produced by T cells or other skin cells including keratinocytes, Langerhans' cells and fibroblasts. Therefore, anti-CCL27 treatment eventually decreases the skin inflammation. In addition, the antibody may also bind to CCL27 in the ECM and cytoplasm of keratinocytes, which may then reduce the supply of CCL27 for endothelial cells.

Since anti-CCL27 treatment does not completely block T cell infiltrate in the skin, other chemokines, especially another T cell skin-homing chemokine, CCL17, may also be involved in the recruitment pathway of T cells in this AD model. CCL17 is constitutively expressed and inducible on cutaneous venules and some other systems' venules (23). The receptor for CCL17 is CCR4 (3, 33), which is mainly expressed on skin-homing CLA+ cells (23). Studies have shown that CCL17 selectively recruits T_h2 lymphocytes (34, 35), and serum CCL17 is significantly increased in patients with AD (21, 36). Up-regulated CCL17 may cooperate with CCL27 to mediate adhesion of T cells to endothelial cells. In our preliminary study, we have found that the CCL17 mRNAs in skin and the CCR4-bearing CD4+ T cells in the LNs of Tg-LL mice were increased by an average 2.9-fold (Fig. 1A) and 3.9-fold (data not shown) compared with that of non-Tg mice, respectively (unpublished data). Both CCL27 and CCL17 support homing of T cells to the skin, and that either one or the other is required for lymphocyte recruitment in mouse cutaneous delayed-type hypersensitivity (37). In the skin lesions of human allergic delayed-type hypersensitivity, most skin-infiltrating lymphocytes express both CCR4 and CLA, but only ~10% express CCR10, suggesting that CCR10+ T cells have no advantage over other CLA+/CCR4+ T cells in homing to the cutaneous sites (38). Our finding of the majority of T cells in the skin lesions being CCR10+ (Fig. 3A) suggests a more prominent role of CCL27 in our model, but did not rule out the roles of CCL17 and CCR4. Further study needs to be done to elucidate the roles of CCL17/CCR4 in this model.

In humans, CLA+ memory T cells constitute a skin-homing population of memory T cells that preferentially migrate into normal (39) and inflamed skin (40). The majority (80–90%) of the T cells in the inflammatory skin express CLA. However, only 5–10% of T cells in the peripheral blood and <5% of lymphocytes in non-cutaneous inflamed tissues express CLA (40). CLA+ T cells migrate into the skin through E-selectin on endothelial cells (41). However, the CLA/E-selectin binding cannot fully explain the skin-homing CLA+ memory T cells because neutrophils also express CLA and they do not selectively migrate to the skin (42). In addition, E-selectin is also induced in non-cutaneous sites (41). The discovery of CCL27 and CCR10 allowed a more in-depth study of the migration mechanism of skin-homing CLA+ memory T cells into the skin. CCR10+/CD4+ T cells have been identified entirely to be included in the CD45RA-/CD45RO+ memory population and most CCR10+ cells are found within the CLA+ memory CD4+ T cell subset, and about one-third of CLA+ memory CD4 T cells express CCR10 (19). Unfortunately, an equivalent CLA molecule in mice has not yet been found. Therefore, we could not verify how T cells equivalent to human CLA+ memory T cells are involved in the skin inflammation in the mouse model of AD at the present time.

Although serum level of CCL27 was found significantly increased in Tg mice, the role of CCL27 released into circulation

is not clear in the present study. Some reports show that chemokines are expected to activate leukocyte integrins systemically and transiently (43, 44). The activation of integrins by serum CCL27 at locations remote from the skin and the events following the activation needs to be identified. However, remote activation of integrins by serum CCL27 is unlikely to control lymphocyte trafficking into the skin and this process is instead determined by cutaneous CCL27 as suggested in (13). Nevertheless, the serum elevation of CCL27 seems to be a good disease marker in human patients as well as in our mouse model.

In conclusion, CCL27 in the skin/serum and CCR10 on T cells were found significantly elevated in the K-14 IL-4 Tg mice. Neutralizing CCL27 impaired the skin inflammation development. Thus, the interaction of CCL27 and CCR10 plays an important role in the pathogenesis of AD in this animal model.

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Abbreviations

AD	atopic dermatitis
BO	before disease onset
CLA	cutaneous lymphocyte-associated antigen
CTACK	cutaneous T cell-attracting chemokine
DNFB	2,4-dinitro-1-fluorobenzene
ECM	extracellular matrix
EL	early skin lesion
FBS	fetal bovine serum
HPF	high power field
IP-10	IFN-inducible protein 10
I-TAC	interferon-inducible T cell α chemoattractant
LFA	leukocyte function-associated antigen
LL	late skin lesion
MDC	macrophage-derived chemokine
MIG	monokine induced by interferon-γ
MIP-2	macrophage-inflammatory protein-2
OD	optical density
RANTES	regulated on activation normal T cell expressed and secreted
TARC	thymus and activation-regulated chemokine
Tg	transgenic
TNF	tumor necrosis factor
VLA-4	very late antigen-4

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