

Overexpression of Cathepsin S Induces Chronic Atopic Dermatitis in Mice

Nari Kim^{1,5}, Ki Beom Bae^{1,5}, Myoung Ok Kim¹, Dong Hoon Yu¹, Hei Jung Kim¹, Hyung Soo Yuh¹, Young Rae Ji¹, Si Jun Park¹, Sol Kim², Kyu-Hee Son², Sang-Joon Park², Duhak Yoon³, Dong-Seok Lee¹, Sanggyu Lee¹, Hyun-Shik Lee^{1,6}, Tae-Yoon Kim^{4,6} and Zae Young Ryoo^{1,6}

Atopic dermatitis (AD) is a chronically relapsing, noncontagious pruritic skin disease with two phases: acute and chronic. Previous studies have shown that the cysteine protease cathepsin S (CTSS) is linked to inflammatory processes, including atherosclerosis and asthma. The possibility that this or other cysteine proteases might cause itching or be part of a classical ligand–receptor signaling cascade has not been previously considered. Recently, CTSS was shown to be a ligand for proteinase-activated receptor-2 (PAR-2), which is associated with itching. In this study, we show that CTSS-overexpressing transgenic (TG) mice spontaneously develop a skin disorder similar to chronic AD. The results of this study suggest that CTSS overexpression triggers PAR-2 expression in dendritic cells (DCs), resulting in the promotion of CD4⁺ differentiation, which is involved in major histocompatibility complex (MHC) class II expression. In addition, we investigated mast cells and macrophages and found significantly higher mean levels of T helper type 1 (Th1) cell-associated cytokines than T helper type 2 (Th2) cell-associated cytokines in CTSS-overexpressing TG mice. These results suggest that increased PAR-2 expression in DCs as a result of CTSS overexpression induces scratching behavior and Th1 cell-associated cytokine expression, and can trigger chronic AD symptoms.

Journal of Investigative Dermatology advance online publication, 15 December 2011; doi:10.1038/jid.2011.404

INTRODUCTION

Atopic dermatitis (AD) has long been a problem for immunologists because it is associated with a wide range of immunological abnormalities (Coombs, 1963). The course of AD comprises two phases: acute AD with T helper type 2

(Th2) predominance and chronic AD with T helper type 1 (Th1) cell-associated cytokine expression. Several other observations suggest that Th1 cells, rather than Th2 cells, are important in the pathogenesis of AD (Grewé *et al.*, 1998).

Cathepsin S (CTSS) is a lysosomal cysteine protease that can function as an elastase over a broad pH range in alveolar macrophages and may participate in the degradation of antigenic proteins to peptides for presentation on major histocompatibility complex (MHC) class II molecules. Recently, human CTSS was shown to activate human protease-activated receptor-2 (PAR-2), consistent with a possible pruritic role in inflammatory skin diseases such as AD (Reddy *et al.*, 2010).

PAR-2 is member of the G-protein-coupled receptor family (Vu *et al.*, 1991). Serine proteases cleave receptors within their N termini. They thereby expose a tethered ligand domain that binds and activates the cleaved receptor. The only endogenous proteases known to activate PAR-2 are serine proteases, i.e., proteases with serine residues in their active sites, which include trypsin, tryptase, and several kallikrein-related peptidases (Steinhoff *et al.*, 1999; Oikonomopoulou *et al.*, 2006; Stefansson *et al.*, 2008). Tryptase and PAR-2 are involved in serine protease-mediated receptor activation and itching (Steinhoff *et al.*, 2000, 2003).

Mucinain is an inducer of histamine-independent itch (Arthur and Shelley, 1955; Shelley and Arthur, 1955). The recent findings that mucunain, which is an exogenous cysteine protease and the active component of cowhage

¹School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Buk-ku, Daegu, Republic of Korea;

²Department of Histology, College of Veterinary Medicine, Kyungpook National University, Buk-ku, Daegu, Republic of Korea; ³Department of Animal Science, Kyungpook National University, Sangju, Kyungpook, Republic of Korea and ⁴Department of Dermatology, Kangnam St Mary's Hospital, College of Medicine, The Catholic University, Seocho-gu, Seoul, Republic of Korea

⁵These authors contributed equally to this work.

⁶These corresponding authors contributed equally to this work.

The work was done in Daegu, Republic of Korea.

Correspondence: Zae Young Ryoo or Hyun-Shik Lee, School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, 1370 Sankyuk-dong, Buk-ku, Daegu 702-701, Republic of Korea. E-mail: jaewoong64@hanmail.net or leeh@knu.ac.kr or Tae-Yoon Kim, Department of Dermatology, Kangnam St Mary's Hospital, College of Medicine, The Catholic University, 505, Banpo-dong, Seocho-gu, Seoul 137-701, Republic of Korea. E-mail: tykimderma@catholic.ac.kr

Abbreviations: AD, atopic dermatitis; cDNA, complementary DNA; CTSS, cathepsin S; DC, dendritic cell; MHC, major histocompatibility complex; PAR-2, proteinase-activated receptor-2; RT-PCR, reverse transcription-PCR; TG, transgenic; Th1, T helper type 1; Th2, T helper type 2; WT, wild type

Received 22 February 2011; revised 5 August 2011; accepted 19 September 2011

(or cowitch; *Mucuna pruriens*), and CTSS evoke itching and activate PAR-2 extended these observations (Reddy *et al.*, 2008, 2010). CTSS shares active site sequence homology with cowhage and binds to PAR-2 (Johanek *et al.*, 2007), which leads to the stimulation of C fibers (Namer *et al.*, 2008). This pathway is distinct from the neuronal circuits transmitting histamine itch (Davidson *et al.*, 2007). The cleavage site for cysteine protease activation of PAR-2 remains to be identified. In addition, the presence of PAR-2 on free nerve endings in the skin, keratinocytes, dendritic cells (DCs), and dorsal root ganglia suggests a link between this receptor and both itching and pain (Steinhoff *et al.*, 2000, 2003).

Because of the overall implications of these findings with respect to the physiological role of CTSS, we postulated that CTSS overexpression may be responsible for boosting itching and scratching. Therefore, we focused on the role of CTSS in the induction of chronic AD *in vivo*.

RESULTS

CTSS induces PAR-2 overexpression in CTSS-overexpressing mice

The human CTSS gene (GenBank NM_004079) was amplified from human placental RNA. To induce overexpression in mice, a *Bam*HI–*Xho*I fragment containing full-length human CTSS complementary DNA (cDNA) was cloned into the pEGFP-N1 vector, which contains the cytomegalovirus promoter (Figure 1a). CTSS overexpression was examined in various tissues and compared with wild-type (WT) expression by reverse transcription–PCR (RT–PCR). Transgenic (TG) mice showed high overall expression of CTSS in the skin, as well as in other tissues (Figure 1b–d).

AD symptoms in CTSS-overexpressing mice

We observed significant morphological changes in TG mice. These changes included reddening and scaling of the dorsal skin and neck region, frequent face rubbing, and scratching (Figure 2a). Because pruritus is one of the major symptoms of AD, we examined scratching behaviors in the mice. Total scratching counts in TG mice were significantly higher than those in WT mice. In addition, TG mice exhibited increased scratching behavior with increased AD severity (Figure 2b).

Aggravation of inflammation and mast cell accumulation

The skin histology in hematoxylin and eosin and toluidine blue staining showed a progressive increase in inflammatory cell infiltration in the epidermis and dermis of 16-week-old TG mice as the disease progressed. TG samples were distinguished from WT ones by a substantial increase in epidermal hyperproliferation, epidermis rupture, dermis thickness, and infiltration of mast cells, which is a histological marker of chronic inflammation in AD (Figure 3a). Notably, defects in keratinocyte proliferation are an important component in the pathogenesis of AD. TG mice showed increased keratinocyte proliferation and differentiation in the basal and suprabasal layers of the epidermis (Figure 3d). Proliferating cell nuclear antigen- and Ki67-positive cells indicated keratinocyte proliferation in the epidermis (visua-

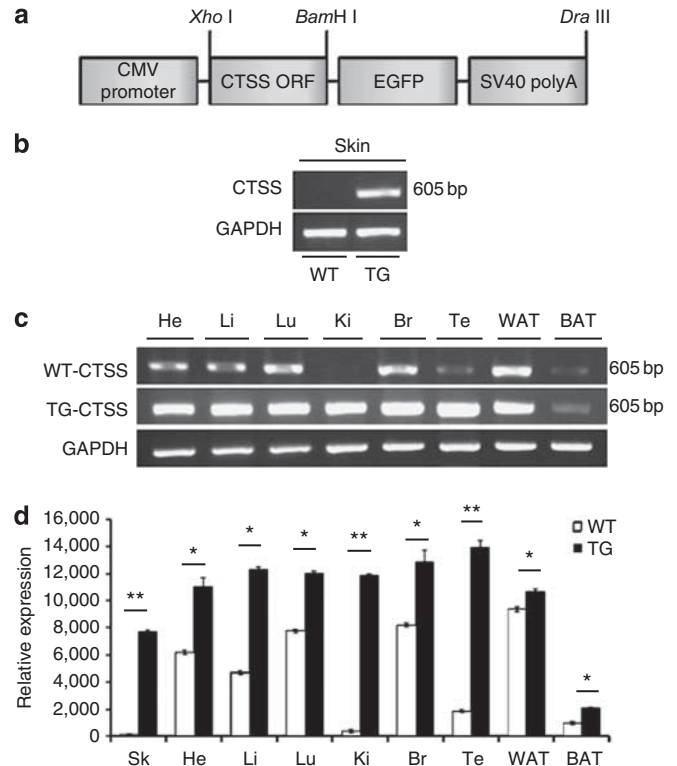


Figure 1. Cathepsin S (CTSS)-overexpression vector construction and transgene expression in various tissues. (a) We used the cytomegalovirus (CMV) promoter to overexpress CTSS in the whole body. EGFP, enhanced green fluorescent protein; ORF, open reading frame. (b, c) The primers used for PCR in various tissues are indicated; they yielded 605 bp bands, indicating expression of the CTSS transgene in 6-week-old mice. (d) Relative CTSS mRNA expression in 6-week-old mice. Filled columns, TG; open columns, WT. BAT, brown adipose tissue; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sk, skin; Te, testis; TG, CTSS-overexpressing transgenic mice; WAT, white adipose tissue; WT, C57BL/6 wild-type mice. The columns and error bars represent the means $\pm$ SEM of three independent experiments. Statistics: Student's *t*-test. **P* < 0.01, ***P* < 0.001 compared with WT controls.

lized by K10 staining). Moreover, the dermis in the TG samples was densely infiltrated by mast cells (Figure 3b). The numbers of mast cells were higher in TG samples than in those from WT mice (Figure 3c).

Increased PAR-2 expression is caused by CTSS overexpression

We were interested in the effect of CTSS overexpression on PAR-2 expression. We performed real-time PCR analysis of dorsal skin tissue from WT mice and TG mice with AD of different severity. TG mice exhibited increasing CTSS and PAR-2 expression with increasing AD severity compared with WT mice (Figure 4a and b). We performed western blotting to further quantify CTSS and PAR-2 protein expression in 16-week-old mice. Expression of CTSS and PAR-2 was significantly increased (Figure 4c), supporting the utility of CTSS-overexpressing TG mice in investigating the role of the interaction between CTSS and PAR-2 in the pathogenesis of AD.

We performed immunohistochemical staining in 16-week-old mice. We examined CTSS overexpression, which was

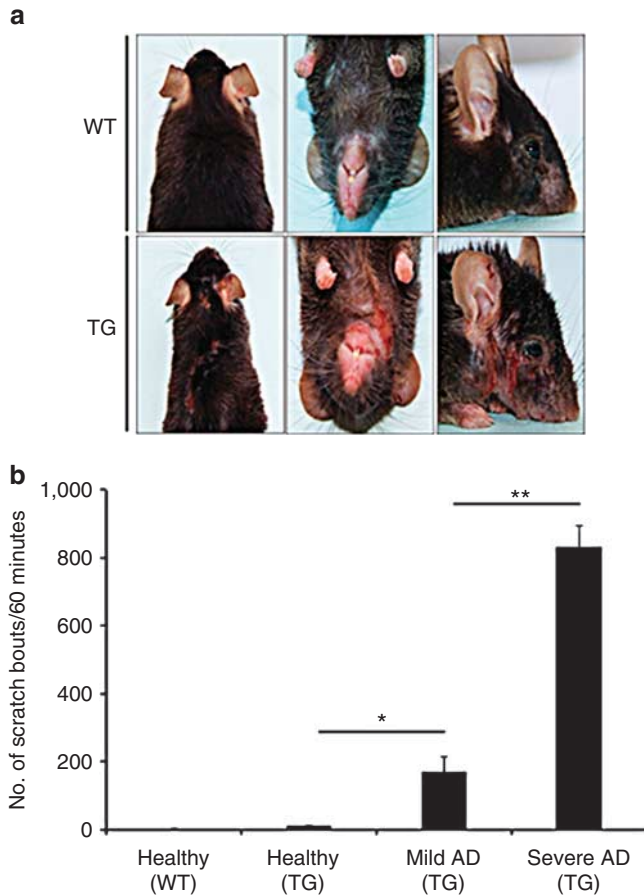


Figure 2. Atopic dermatitis phenotype in cathepsin S (CTSS)-overexpressing mice. (a) Appearance of 16-week-old WT and TG mice. (b) Scratch bouts were counted for 60 minutes ($n=3$). TG, CTSS-overexpressing transgenic mice; WT, C57BL/6 wild-type mice. The columns and error bars represent the means $\pm$ SEM of three independent experiments. Statistics: Student's *t*-test. * $P<0.05$, ** $P<0.01$ compared with WT controls.

found to result in marked induction of PAR-2 expression in the epidermis and dermis of TG mice compared with WT controls (Figure 4d). Notably, expression of both CTSS and PAR-2 was strongly increased in the thickened epidermis, indicating that overexpression of the two genes was correlated with hyperkeratosis (see also Figure 3d).

A recent study indicated that PAR-2 is expressed in DCs and may have a significant role in priming the Th cell response by inducing DC maturation (Lim *et al.*, 2006). A fully mature DC displays CD11c and MHC class II on its membrane. We found high levels of CD11c expression in the epidermis and dermis of TG mice (Figure 4d). In addition, we found high levels of molecules typically expressed by DCs, such as CD4 and MHC class II (Figure 4e). Therefore, our results suggest that there is a positive relationship between CTSS and PAR-2, supporting the role of DC maturation in CD4⁺ T-cell stimulation. We next examined the presence and degree of inflammatory cell infiltration in the skin. Our results indicated significant infiltration of cells expressing CD68, a marker for macrophages, in the skin of TG mice (Figure 4e).

Increased production of cytokines, chemokines, and IgE

As cytokines and chemokines orchestrate atopic skin inflammation, their mRNA and protein levels were examined in skin from 16-week-old WT and TG mice by RT-PCR and ELISA. In TG mice, we found increased levels of transcripts for Th2-type cytokines, including IL-4 and IL-10, which were not detected in WT controls. Interestingly, transcripts for the Th1-type cytokines tumor necrosis factor- α , IFN- γ , IL-2, and IL-1 β were also upregulated in TG mice (Figure 5a). In addition, increased transcript levels of the chemokines monocyte chemoattractant protein-1 and granulocyte macrophage-colony stimulating factor were found in TG mice (Figure 5a). These chemokines attract monocytes to inflammatory lesions and induce proliferation and differentiation.

Next, we performed ELISA to further examine cytokine expression. The results were similar to those of RT-PCR, but we observed greater increases in Th1-type cytokines, especially IFN- γ , as compared with Th2-type cytokines in TG mice compared with WT (Figure 5b). The Th2-mediated response showed a relatively small effect as compared with the Th1-mediated response in TG mice. Therefore, we concluded that the Th1-mediated response is the main factor responsible for the rapid development of chronic inflammation in TG mice.

We also found that serum IgE levels were an average of 2-fold higher in TG mice as compared with WT controls (Figure 5c).

DISCUSSION

AD is a type of eczema or skin inflammation that arises from an allergic reaction. It causes an itchy rash that becomes crusty and scaly with scratching, and is often associated with elevated serum IgE levels (Hanifin and Rajka, 1980; Rudikoff and Lebowitz, 1998; Leung and Bieber, 2003). Although the precise causes of AD are not entirely understood, most evidence suggests that complex immune dysregulation and environmental allergenic susceptibility are both involved in the underlying etiology (Kang *et al.*, 2010).

Cysteine proteases belong to the papain family, and 11 isoforms have been identified to date. The majority of cysteine proteases are localized in acidic vesicles, lysosomes, and endosomes, where they possess highly specific and directed proteolytic activity (Chapman *et al.*, 1997; Turk *et al.*, 2000). Their participation in antigen processing and proteolytic proprotein activation (Riese and Chapman, 2000; Brix *et al.*, 2001; Rudensky and Beers, 2006) indicates their pivotal roles in numerous cellular events, and secreted cysteine proteases have the capacity to promote remodeling of the extracellular matrix during wound healing and tumor cell invasion (Baici, 1998; Brix, 2005; Mohamed and Sloane, 2006). In marked contrast to other isoforms, CTSS can function both inside and outside of the lysosomes (Wiederanders *et al.*, 1991). In the extracellular compartment, CTSS exhibits a large portion of its proteolytic activity at a neutral pH, which is a specific feature of this isoform (Bromme *et al.*, 1993).

In this study, CTSS-overexpressing TG mice were generated using the cytomegalovirus promoter, which induced

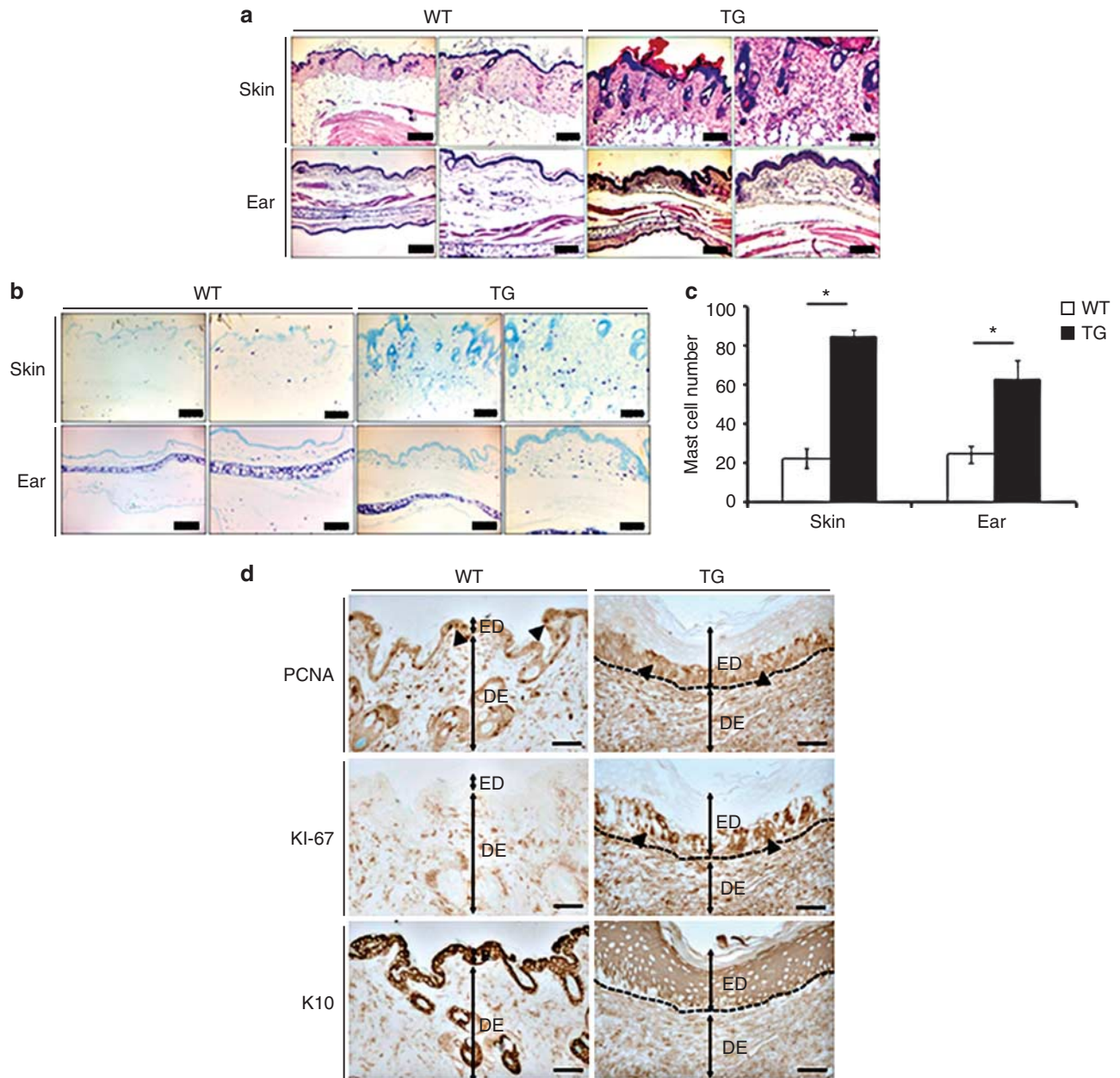


Figure 3. Cathepsin S (CTSS)-overexpressing mice develop chronic skin inflammation. (a) Hematoxylin and eosin (H&E) staining. (b) Toluidine blue staining. Mast cells show purple staining. Scale bar = 50 μ m. (c) Mast cell numbers were counted. (d) Analysis of keratinocyte proliferation in the epidermis. Arrowheads identify proliferating keratinocytes, and dotted lines identify the boundaries between the epidermis (ED) and dermis (DE). Positive cells show brown staining. Bar = 200 μ m. Filled columns, TG; open columns, WT. PCNA, proliferating cell nuclear antigen; TG, CTSS-overexpressing transgenic mice; WT, C57BL/6 wild-type mice. The columns and error bars represent the means $\pm$ SEM of three independent experiments. Statistics: Student's *t*-test. **P* < 0.05 compared with WT controls.

CTSS expression in various tissues. However, this study focused on skin tissue because marked skin defects were observed in TG mice. In future studies, we will access abnormalities and changes caused by CTSS overexpression in other organs. Protein alignment revealed that human CTSS is very similar to its mouse counterpart with which it shares 74% overall homology (data not shown). However, there is a small possibility that the observed results could have resulted from overexpression of a foreign protease. In this TG mouse

model, the onset of AD occurred spontaneously at the age of 14–16 weeks, and the disease progressed rapidly to the extent that the lesions covered ~40% of the body. TG mice showed vigorous scratching at varying times, which triggered even more skin irritation than did the initial lesions (Figure 2).

NC/Nga mice were derived from Japanese fancy mice (Nishiki-Nezumi) and were established as an inbred strain in 1955. Some Japanese researchers noticed the spontaneous development of dermatitis just before or after weaning (Suto

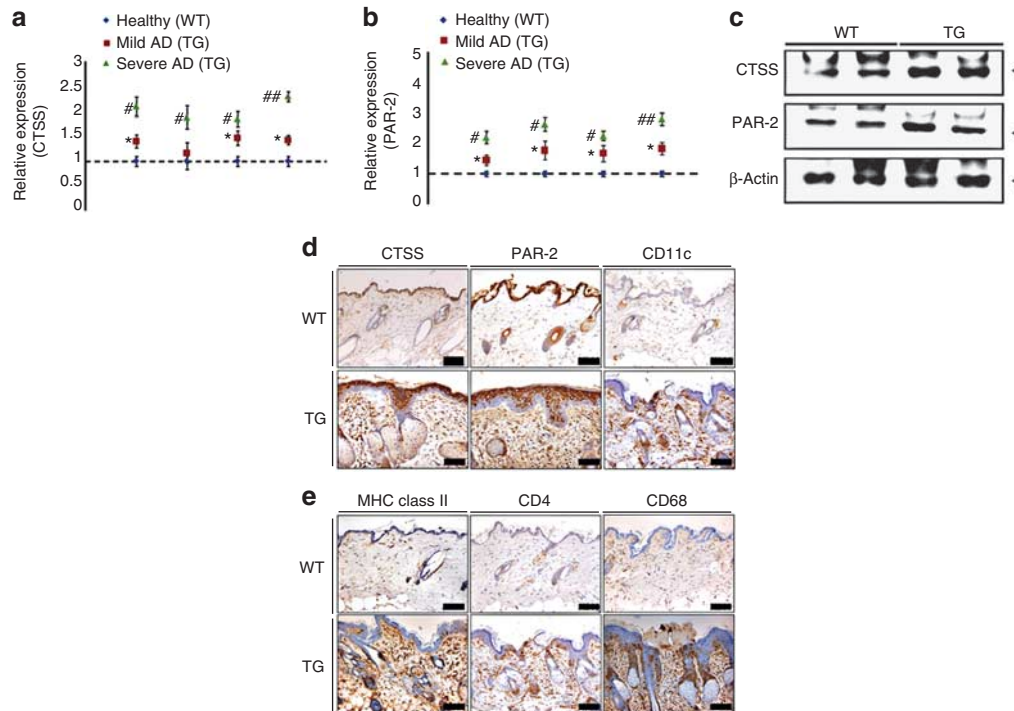


Figure 4. Proteinase-activated receptor-2 (PAR-2) expression and inflammatory cell infiltration in dorsal skin. Real-time PCR analysis of (a) cathepsin S (CTSS) and (b) PAR-2 expression. Diamonds, C57BL/6 WT mice; squares and triangles, CTSS-overexpressing TG mice ($n = 4$). The average value for WT mice was set to 1. * $P < 0.05$, diamonds and squares; # $P < 0.05$, ## $P < 0.01$, squares and triangles. (c) Western blot analysis of dorsal skin tissue homogenates with antibodies specific for CTSS, PAR-2, and β -actin (arrow). Immunostaining of dorsal skin with antibodies specific for (d) CTSS, PAR-2, and CD11c, as well as (e) major histocompatibility complex (MHC) class II, CD4 (CD4⁺ T-cell marker), and CD68 (macrophage marker). Markers of mature dendritic cells: CD11c and MHC class II. Bar = 50 μ m. Positive cells show brown staining. TG, CTSS-overexpressing transgenic mice; WT, C57BL/6 wild-type mice.

et al., 1999). NC/Nga mice display clinical and histological features that are very similar to those that characterize AD (Suto *et al.*, 1999). Furthermore, NC/Nga mice with skin lesions frequently scratch their faces, ears, and the rostral parts of their backs using their hind paws (Tohda *et al.*, 1997). Features similar to these were seen in CTSS-overexpressing TG mice.

Similar to NC/Nga mice, CTSS-overexpressing TG mice showed expression of Th2-type cytokines from allergen-specific Th2 cells in AD. IL-4 and IL-10 act on B cells to induce IgE synthesis (Farrar *et al.*, 2002). IL-6 and IL-10 block the generation of Th1 cells from naive T cells (Murphy *et al.*, 2000; Feizy and Ghobadi, 2006). Interestingly, we showed marked increases in the levels of the Th1-type cytokines tumor necrosis factor- α , IFN- γ , IL-2, and IL-1 β , which have important roles during the chronic phase of AD. In AD, Th1 cytokines such as tumor necrosis factor- α and IL-1 β from resident cells (keratinocytes, mast cells, and DCs) bind to receptors on the vascular endothelium, activating cellular signaling pathways. In addition, they induce expression of vascular endothelial cell adhesion molecules and IL-2, which are important for T-cell growth and activation (Ahn *et al.*, 2010). In previous studies, the marked expression of IFN- γ was shown to be involved in chronic eczematous AD skin lesions and was suggested to be related to the activation of tissue macrophages (Grewe *et al.*, 1994; Tsiopoulos *et al.*, 1994), which have an

important role in skin hypertrophy during delayed hypersensitivity reactions. These findings were consistent with our observation that TG mice showed relatively strong expression of IFN- γ , resulting in thickening of the epidermal and dermal layers.

The key questions with respect to the pathophysiology of chronic AD are how CTSS is initiated and maintained and whether CTSS itself may have a role. We found that epidermal hyperproliferation accompanies an increase in itch and scratching. PAR-2 is expressed on endothelial and immune cells, and has been implicated in DC differentiation. DCs are strong candidates to explain how PAR-2 modulates the immune response during inflammation (Allshurafa *et al.*, 2004). PAR-2 expression on the surface of DCs induces stimulation of CD4⁺ T cells, which produce IFN- γ , consistent with a Th1 phenotype (Csernok *et al.*, 2006). The results of this study indicate that CTSS induces PAR-2 expression, resulting in DC maturation, as indicated by the expression of CD11c and MHC class II. These CTSS-matured DCs stimulated CD4⁺ T cells to increase the expression of Th1-type cytokines.

In conclusion, increased PAR-2 activation caused by CTSS contributed to the development of AD. CTSS overexpression induced the expression of PAR-2 on DCs, and PAR-2-matured DCs stimulated CD4⁺ T cells, which produced Th1-type cytokines in CTSS-overexpressing TG mice. These findings may improve our understanding of the

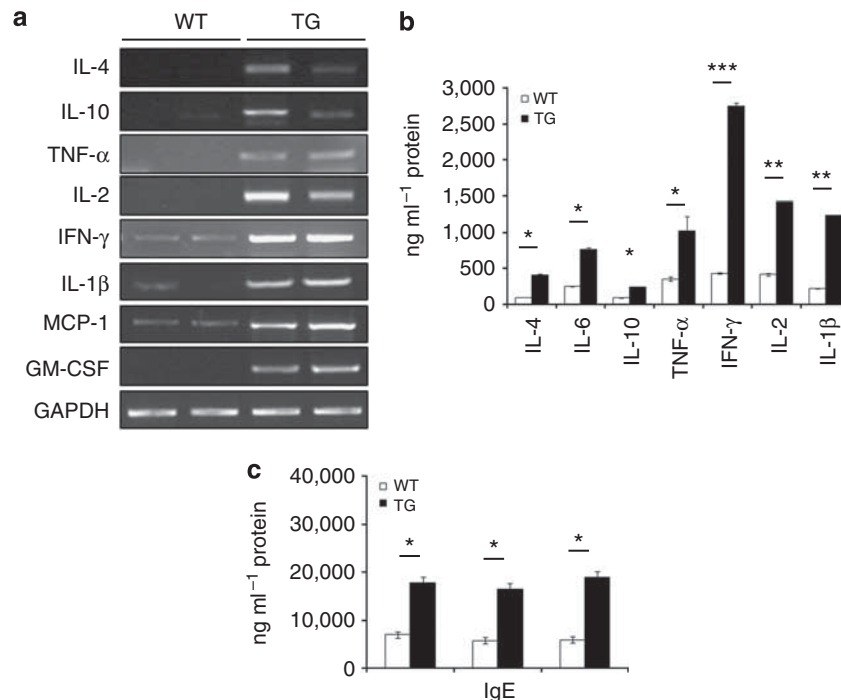


Figure 5. Increased tissue production of cytokines, chemokines, and serum IgE expression in TG mice. (a) mRNA levels of cytokines and chemokines in dorsal skin from 16-week-old WT and TG mice. (b) Tissue cytokine and chemokine levels in dorsal skin homogenates were measured by ELISA. (c) Serum IgE levels in 16-week-old mice were measured by ELISA ($n = 3$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage-colony stimulating factor; MCP-1, monocyte chemoattractant protein-1; TG, CTSS-overexpressing transgenic mice; TNF- α , tumor necrosis factor- α ; WT, C57BL/6 wild-type mice. Filled columns, TG; open columns, WT. The columns and error bars represent the means $\pm$ SEM of three independent experiments. Statistics: Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with WT controls.

development of AD with enhancement of both Th1- and Th2-mediated immunological responses, especially how Th1 cytokines contribute to chronic inflammation in AD. In addition, CTSS-overexpressing TG mice showed increased mast cell infiltration, hyperproduction of IgE, and keratinocyte hyperproliferation. Our findings suggest that CTSS-overexpressing TG mice may be useful as a model of chronic AD. This model will provide insights into the pathogenesis of this condition as well as facilitate the development of treatment regimens.

MATERIALS AND METHODS

Plasmid construction

A *Bam*HI-*Xho*I fragment containing full-length human CTSS cDNA was cloned into the pEGFP-N1 vector driven by the cytomegalovirus promoter. This expression cassette was prepared for microinjection by digesting the recombinant vector with restriction enzymes.

Generation and screening of transgenic mice

The transgene construct was microinjected into embryos obtained from female C57BL/6J mice. Microinjected embryos were transferred into the oviducts of pseudopregnant female ICR mice.

To isolate TG mice, total genomic DNA was extracted from the biopsied tails of founder candidates, and PCR screening was performed. The following primers were used: pEGFP-N1 forward (5'-CGTGTACGGTGGGAGGTCTA-3') and pEGFP-N1 reverse (5'-TGGTGCAGATGAACCTCAGG-3'). PCR was performed as 30 cycles

of 94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 40 seconds.

Two transgenic founder mice were identified based on the results of this screening. Each founder mouse was mated with WT C57BL/6J mice. The offspring produced as a result of this mating were genotyped through PCR analysis of tail lysates. Offspring that did not inherit the transgene were used as controls.

All mice were generated by a standard procedure (Hogan *et al.*, 1986). They were raised and maintained under conventional conditions in a room with a 12-hour light/dark cycle, and a controlled temperature (23 °C), and were given free access to food and water.

All animal experiments were carried out in accordance with the guidelines for animal experimentation and under permission from the Institutional Animal Care and Use Committee. At 6 weeks of age, pups were raised individually (one per cage) under the same conditions. At the beginning of the experiment, 20 WT and 20 TG male mice aged 16 weeks were used.

RT-PCR analysis

Patterns of human CTSS mRNA expression in various tissues were assessed by RT-PCR.

Total RNA was isolated from various tissues from 6-week-old WT and TG mice using TRIzol reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. Total RNA was reverse transcribed for 1 hour at 42 °C using AMV reverse transcriptase (Promega, Madison, WI). The synthesized cDNAs were amplified using the following primers: CTSS forward (5'-TGGATCACCCTG

GCATCTCT-3') and CTSS reverse (5'-TGAACATGTGGCAGCAC GAT-3'). After denaturation for 5 minutes at 94 °C, PCR was performed as 30 cycles of 94 °C for 30 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds. The *GAPDH* gene was used as an internal control. Additional RT-PCR analyses were conducted to confirm the levels of various cytokines and chemokines (see Supplementary Table S1 online).

Quantitative real-time PCR analysis

cDNAs were synthesized from dorsal skin tissue from 13-week-old WT and TG mice.

Real-time PCR was performed using a Step One Plus PCR system (Applied Biosystems, Foster City, CA), which exploited the ability of SYBR Green to fluoresce after hybridization with double-stranded DNA. The following primers were used: CTSS forward (5'-CGGCTG GTTGTGTGCTCTT-3'), CTSS reverse (5'-AGAGATGCCAGTGGTG ATCCA-3'), PAR-2 forward (5'-CGATGAGTTCTCTGCGTCCAT-3'), and PAR-2 reverse (5'-GCCATTACTGGGCAAACCA-3'). After an initial denaturation step at 95 °C for 30 seconds, PCR was performed as 50 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C for 30 seconds, and elongation at 72 °C for 10 seconds. The *GAPDH* gene was used as an internal control for quantification. Levels of CTSS, PAR-2, and *GAPDH* gene expression in all cDNA samples were determined based on SYBR Green fluorescence using Step One software, version 2.1 (Applied Biosystems).

Behavioral experiments

We observed individual animals for 60 minutes, during which time we counted bouts of scratching. A bout of scratching was defined as the animal lifting a hind paw from the cage floor, scratching with the paw, and returning the paw to the floor. Animals were tested in the cages in which they were housed.

Antibodies

The following antibodies were used: anti-mouse cytokeratin 10 (K10) monoclonal antibody (Covance, Princeton, NJ), anti-rabbit Ki67 monoclonal antibody (Thermo, Hudson, CA), anti-mouse proliferating cell nuclear antigen monoclonal antibody, anti-goat CTSS polyclonal antibody, anti-rabbit PAR-2 polyclonal antibody, anti-mouse MHC class II monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-hamster CD11c monoclonal antibody, anti-rabbit CD4 polyclonal antibody (Novus Biologicals, Littleton, CO), and anti-rat macrophage monoclonal antibody (Abcam, Cambridge, UK).

Histology and immunohistochemistry

Skin sections were excised from dorsal skin from 16-week-old WT and TG mice. Tissues were fixed through incubation in Bouin's solution for 1 day and embedded in paraffin. The paraffin-embedded tissues were sectioned to a thickness of 3–5 µm. Tissue sections were mounted on glass slides. The slides were deparaffinized before staining.

For histological assessment, tissue sections were stained with hematoxylin and eosin and toluidine blue. In some experiments, numbers of mast cells in 10 randomly selected low-power fields were counted.

For immunohistochemistry, tissue sections were fixed in Bouin's solution, subjected to antigen retrieval (cooking for 3 minutes in 0.01 M citrate buffer, pH 6.0), and incubated with 3% normal

serum. They were then washed with phosphate-buffered saline and immunostained with primary antibody and the corresponding secondary antibody. Representative images were captured using LSM 510 Meta confocal microscope (Carl Zeiss, Hertfordshire, UK).

Western blot analysis

CTSS and PAR-2 protein levels were determined by western blotting using specific antibodies. Dorsal skin tissues were washed twice with phosphate-buffered saline, and tissue lysates were then prepared in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail (Sigma, St Louis, MO). Proteins were resolved by SDS-PAGE. As a loading control, tissue lysates were blotted with anti-β-actin antibody. C57BL/6 mice were used as a control.

Cytokine and IgE ELISA

Cytokine levels in dorsal skin tissue extracts were measured by a sandwich ELISA (R&D Systems, Minneapolis, MN). Briefly, a 96-well plate was coated with monoclonal capture antibody. After washing and blocking steps, standards and samples were then added. The plate was incubated for 2 hours, and biotinylated detection antibody was added. The color reaction was generated using a substrate solution containing tetramethylbenzidine and hydrogen peroxide. The reaction was terminated by the addition of 1 M sulfuric acid, and the optical density at 450 nm was determined using a microplate reader with wavelength correction at 550 nm. Three washes with blocking buffer were performed between the different steps.

For measurement of total IgE, blood samples (5 µl) were collected from the tail vein, and serum IgE levels were determined using an ELISA kit (BioLegend, San Diego, CA).

Statistical analyses

The results are expressed as means ± SEM of at least three independent experiments. Groups were compared using Student's *t*-test. In all analyses, *P* < 0.05 was taken to indicate statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by the SRC program (Center for Food & Nutritional Genomics: grant 2010-0001886) of the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology, a grant from the BioGreen 21 Program (PJ0071812009), Rural Development Administration, Republic of Korea, and a grant from the Korean Ministry of Education, Science and Technology (the Regional Core Research Program/Anti-Aging and Well-Being Research Center).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Ahn JY, Choi SE, Jeong MS *et al.* (2010) Effect of taxifolin glycoside on atopic dermatitis-like skin lesions in NC/Nga mice. *Phytother Res* 24:1071–7
- Allshurafa HN, Stenton GR, Wallace J *et al.* (2004) Protease receptor-2 (PAR-2) activating peptide, tc-LIGRLO-NH₂, induces protease release from mast cells: role of TNF degradation. *BMC Pharmacol* 4:1–9
- Arthur RP, Shelley WB (1955) The role of proteolytic enzymes in the production of pruritus in man. *J Invest Dermatol* 25:341–6

- Baici A (1998) Inhibition of extracellular matrix-degrading endopeptidases: problems, comments, and hypotheses. *Biol Chem* 379:1007-18
- Brix K (2005) Chapter 5: Lysosomal proteases: revival of the sleeping beauty. In: Saftig P (ed) *Lysosomes*, vol. 1. Springer Science, Landes Bioscience: New York, pp 259-64
- Brix K, Linke M, Tepel C et al. (2001) Cysteine proteinases mediate extracellular prohormone processing in the thyroid. *Biol Chem* 382: 717-25
- Bromme D, Bonneau PR, Lachance P et al. (1993) Functional expression of human cathepsin S in *Saccharomyces cerevisiae*. Purification and characterization of the recombinant enzyme. *J Biol Chem* 268:4832-8
- Chapman HA, Riese RJ, Shi GP (1997) Emerging roles for cysteine proteases in human biology. *Annu Rev Physiol* 59:63-88
- Coombs WH (1963) Twenty centuries of public health in Britain. *Radiography* 29:275-82
- Csernok E, Ai M, Gross WL et al. (2006) Wegener autoantigen induces maturation of dendritic cells and licenses them for Th1 priming via the protease-activated receptor-2 pathway. *Blood* 107:4440-8
- Davidson S, Zhang X, Yoon CH et al. (2007) The itch-producing agents histamine and cowhage activate separate populations of primate spinothalamic tract neurons. *J Neurosci* 27:10007-14
- Farrar JD, Asnagli H, Murphy KM (2002) T helper subset development: roles of instruction, selection, and transcription. *J Clin Invest* 109:431-5
- Feizy V, Ghobadi A (2006) Atopic dermatitis and systemic autoimmune diseases: a descriptive cross sectional study. *Dermatol Online J* 12:3
- Grewe M, Bruijnzeel-Koomen CA, Schopf E et al. (1998) A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 19:359-61
- Grewe M, Gyufko K, Schopf E et al. (1994) Lesional expression of interferon-gamma in atopic eczema. *Lancet* 343:25-6
- Hanifin JM, Rajka G (1980) Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 92:44-7
- Hogan B, Costantini F, Lacy E (1986) Manipulating the mouse embryo. In: Hogan B (ed) *A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp 1-332
- Johanek LM, Meyer RA, Hartke T et al. (2007) Psychophysical and physiological evidence for parallel afferent pathways mediating the sensation of itch. *J Neurosci* 27:7490-7
- Kang MJ, Eum JY, Park SH et al. (2010) Pep-1 peptide-conjugated elastic liposomal formulation of taxifolin glycoside for the treatment of atopic dermatitis in NC/Nga mice. *Int J Pharm* 402:198-204
- Leung DY, Bieber T (2003) Atopic dermatitis. *Lancet* 361:151-60
- Lim SY, Tennant GM, Kennedy S et al. (2006) Activation of mouse protease-activated receptor-2 induces lymphocyte adhesion and generation of reactive oxygen species. *Br J Pharmacol* 149:591-9
- Mohamed MM, Sloane BF (2006) Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer* 6:764-75
- Murphy KM, Ouyang W, Farrar JD et al. (2000) Signaling and transcription in T helper development. *Annu Rev Immunol* 18:451-94
- Namer B, Carr R, Johanek LM et al. (2008) Separate peripheral pathways for pruritus in man. *J Neurophysiol* 100:2062-9
- Oikonomopoulou K, Hansen KK, Saifeddine M et al. (2006) Proteinase-activated receptors, targets for kallikrein signaling. *J Biol Chem* 281:32095-112
- Reddy VB, Iuga AO, Shimada SG et al. (2008) Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. *J Neurosci* 28:4331-5
- Reddy VB, Shimada SG, Sikand P et al. (2010) Cathepsin S elicits itch and signals via protease-activated receptors. *J Invest Dermatol* 130:1468-70
- Riese RJ, Chapman HA (2000) Cathepsins and compartmentalization in antigen presentation. *Curr Opin Immunol* 12:107-13
- Rudensky A, Beers C (2006) Lysosomal cysteine proteases and antigen presentation. *Ernst Schering Res Found Workshop* 56:81-95
- Rudikoff D, Leibold M (1998) Atopic dermatitis. *Lancet* 351:1715-21
- Shelley WB, Arthur RP (1955) Mucinase, the active pruritogenic proteinase of cowhage. *Science* 122:469-70
- Stefansson K, Brattsand M, Roosterman D et al. (2008) Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases. *J Invest Dermatol* 128:18-25
- Steinhoff M, Corvera CU, Thoma MS et al. (1999) Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Exp Dermatol* 8:282-94
- Steinhoff M, Neisius U, Ikoma A et al. (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *J Neurosci* 23:6176-80
- Steinhoff M, Vergnolle N, Young SH et al. (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* 6:151-8
- Suto H, Matsuda H, Mitsuishi K et al. (1999) NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* 120(Suppl 1):70-5
- Tohda C, Yamaguchi T, Kuraishi Y (1997) Increased expression of mRNA for myocyte-specific enhancer binding factor (MEF) 2C in the cerebral cortex of the itching mouse. *Neurosci Res* 29:209-15
- Tsicopoulos A, Hamid Q, Haczku A et al. (1994) Kinetics of cell infiltration and cytokine messenger RNA expression after intradermal challenge with allergen and tuberculin in the same atopic individuals. *J Allergy Clin Immunol* 94:764-72
- Turk B, Turk D, Turk V (2000) Lysosomal cysteine proteases: more than scavengers. *Biochim Biophys Acta* 1477:98-111
- Vu TK, Hung DT, Wheaton VI et al. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64:1057-68
- Wiederanders B, Broemme D, Kirschke H et al. (1991) Primary structure of bovine cathepsin S. Comparison to cathepsins L, H, B and papain. *FEBS Lett* 286:189-92

Table 1**Primer sequence for cytokines and chemokines**

Cytokines/ Chemokines	Forward primer	Reverse primer	NO. of PCR cycles
IL-4	5'-AGCTAGTTGTCATCCTGCT-3'	5'-AGGAAGTCTTTCAGTGATGT-3'	30
IL-10	5'-AGCCTTGCAGAAAAGAGAGC-3'	5'-TGGAGTCCAGCAGACTCAAT-3'	30
TNF- α	5'-AACTGGCAGAAGAGGCACTC-3'	5'-CACAGAGCAATGACTCCAAAAGT-3'	30
IL-2	5'-CCTCACAGTGACCTCAAGTCC-3'	5'-GCCTTATGTGTTGTAAGCAGGA-3'	30
IFN- γ	5'-ATAGCTGCCATCGGCTGAC-3'	5'-TGGACCTGTGGGTTGTTGA-3'	30
IL-1 β	5'-CTAAAGTATGGGCTGGACTG-3'	5'-AGCTTCAATGAAAGACCTCA-3'	30
MCP-1	5'-AGCCAACTCTCACTGAACCC-3'	5'-CTAACAGAAGTGCTTGAGGTGGT-3'	30
GM-CSF	5'-ATGTGGCTGCAGAATTTACT-3'	5'-ATGAAATCCGCATAGGTGGT-3'	30
GAPDH	5'-AATGCATCCTGCACCACCAA-3'	5'-GTAGCCATATTCATTGTCAT-3'	30

Table 1. Primer sequences for cytokines and chemokines.