Suppression of Inflammation by Recombinant *Salmonella typhimurium* Harboring *CCL22* MicroRNA

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Atopic dermatitis (AD) is an inflammatory, chronically relapsing, pruritic skin disorder. These syndromes result from multifactorial inheritance, with interaction between genetic and environmental factors. In particular, the macrophage-derived chemokine CCL22 is directly implicated in skin inflammatory reactions and its levels are significantly elevated in serum and correlated with disease severity in AD. We tested the suppression of the *CCL22* gene by microRNA (miRNA) and observed the effects in mice with inflammation similar to AD. We used *Salmonella* as a vector to deliver miRNA. The recombinant strain of *Salmonella typhimurium* expressing *CCL22* miRNA (ST-miRCCL22) was prepared for *in vivo* knockdown of *CCL22*. ST-miRCCL22 was orally inoculated into mice and the *CCL22* gene suppressed with *CCL22* miRNA in the activated lymphocytes. IgE and interleukin-4 were inhibited and interferon- γ was induced after treatments with ST-miRCCL22 and *CCL22* was suppressed. Further, Th17 cells were suppressed in the atopic mice treated with ST-miRCCL22. These results suggested that suppression of the *CCL22* gene using *Salmonella* induced anti-inflammatory effects.

Introduction

ATOPIC DERMATITIS (AD) is a chronic and persistent inflammatory skin disease that results from a combination of genetic predisposition, imbalanced immune responses, epidermal barrier abnormalities, and severe pruritus (Jesena and Banovcin, 2006). A Th1/Th2 imbalance is known to be the key factor in the pathogenesis of AD. The Th2-cytokine interleukin-4 (IL-4) is necessary for IgE synthesis, IL-4 suppression, and generation of Th1 cells. The marker cytokine of Th1 cells, interferon gamma (IFN- γ), inhibits IgE synthesis and Th2 expansion, but induces Th1-cell growth (Bohm and Bauer, 1997). Thus, in allergic diseases such as AD, Th2 responses are considered to play a primary role in inducing disease (Yanai *et al.*, 2007). Macrophage-derived chemokine (MDC/CCL22) is a recently described CC chemokine known to chemoattract the Th2 cytokine, producing cells that express the receptor CCR4. The intestinal mucosa produced a chemokine and regulated the trafficking of T cells that produce anti-inflammatory cytokines (Berin *et al.*, 2001). Additionally, CCL22 was significantly elevated and correlated with the severity of disease in atopic patients (Nakazato *et al.*, 2008). The imbalance in serum CCL22 concentrations contributes to AD development (Narbutt *et al.*, 2009).

To suppress the *CCL22* gene, we used RNA interference (RNAi) technology. Despite the great potential of RNAi, clinical trials with RNAi are difficult because of their transient nature and instability as well as the lack of appropriate delivery methods (Yang *et al.*, 2008). To overcome these limitations of siRNA, we used live *Salmonella* as a vector enabling the expression of engineered microRNA (miRNA).

miRNAs are expressed as small single-stranded RNA (ssRNA) sequences that naturally direct gene silencing through components shared with the RNAi pathway (Bartel, 2004). *Salmonella typhimurium* was used to invade and transfer the *CCL22* miRNA expression vector (miRCCL22) into mucosal epithelial cells to solve the problems associated with delivery of the RNAi. Additionally, *Salmonella* has been exploited to transfer eukaryotic expression plasmids to mammalian cells *in vitro* and *in vivo* (Weiss and Chakraborty, 2001). To this end, oral administration of attenuated *S. typhimurium* carrying an expression vector has been shown to restore the production of IFN- γ in the macrophages of IFN- γ -deficient mice (Paglia *et al.*, 2000). When delivered orally to mice, *S. typhimurium* strains engineered to express IL-12 or granulocyte/macrophage colony-stimulating factor mediate cytokine gene expression and exert genetic effects (Yuhua *et al.*, 2001). Therefore, it is possible to utilize *Salmonella* to deliver expression vectors encoding various effector genes to

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cells, with the aim of enhancing the endogenous genetic modulation.

In this study, we tested the efficacy of live, attenuated *S. typhimurium* as a vector for oral gene delivery and the anti-inflammatory efficacy of CCL22 gene suppression in mice with inflammation.

Materials and Methods

Mice and allergic mouse model

Four-week-old CD-1(ICR) female mice were purchased from Daehan Biolink. The mice were housed in an animal room maintained at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$, with a 12-h light/dark cycle. They were given CRF-1 standard laboratory rodent feed (Oriental Yeast) and water ad libitum. Mice were treated according to institutional animal care and use guidelines.

To make allergic mouse model, mice were acclimatized to the animal facility for 1 week before experimentation. The hair covering specific body regions of each mouse was removed by hair clippers and hair remover, and 48 h later, 2,4-dinitro-1-chlorobenzene (DNCB; Sigma) was used as the contact allergen. Mice were sensitized with 100 μL of 1% DNCB (Olive oil:Acetone, 1:3) for 3 days. After 48 h, the mice were treated with 120 μL of 2% DNCB daily for 3 days. The elicited scratching behavior was measured (Shah *et al.*, 2010; Yamashita *et al.*, 2010).

Construction of miRCCL22

To design two single-stranded DNA oligonucleotides encoding the CCL22 target pre-miRNA (GenBank accession no. NM_009137), we used an RNAi design program (Invitrogen). The top and bottom single-strand oligos (top strand: 5' TGCTGTTATGGAGTAGCTTCTTCACCGTTTTGGCCACT GACTGACGGTGAAGACTACTCCATAA 3'; bottom strand: 5' CCTGTTATGGAGTAGTCTTCACCGTCAGTCAGTGGC CAAAACGGTGAAGAAGCTACTCCATAAC 3') were annealed to generate a double-stranded oligonucleotide for cloning into the miRNA expression vector. A 22-nucleotide miRNA double-stranded oligonucleotide was inserted into the pcDNATM6.2-GW/EmGFP-miR expression vector. Once the double-stranded oligos were generated, they were cloned into the miRNA expression vector by T4 DNA Ligase. This new plasmid was named miRCCL22, and the scrambled miRNA-expressing plasmid was named miRCV. miRNA expression in the cell was also detected using fluorescence microscopy ($\times 200$) after transfection for 48 h.

Development of CCL22 miRNA-expressing Salmonella strains and growth conditions

Competent *Escherichia coli* DH5 α cells were transformed with the resultant plasmid vector, and the plasmid DNA was isolated from the DH5 α cells using a plasmid mini-prep kit (GeneAll). Then, the *S. typhimurium* SF586 strain (SF586) was transformed with the plasmid DNA by electroporation (Bang *et al.*, 2000). The plasmid from the transformed SF586 was used to transform *S. typhimurium* BRD 509 (BRD 509) cells, which are a mutant *aroA/aroD* variant of SL1344 (Strugnelli *et al.*, 1992). These *Salmonella* strains were contributed by I.S. Lee (Hannam University, Korea). The engineered *S. typhimurium* expressing CCL22 miRNA was designated as ST-miRCCL22. *S. typhimurium* containing the scrambled

miRNA-expressing plasmid and untransformed *S. typhimurium* that was used as the control were termed ST-miRCV and ST-control, respectively. Each bacterial strain was grown in Luria-Bertani (LB) broth, consisting of 1% tryptone, 0.5% NaCl, and 0.5% yeast with spectinomycin at 50 $\mu\text{g}/\text{mL}$ for *Escherichia coli*, 250 $\mu\text{g}/\text{mL}$ for the SF586 strain, and 500 $\mu\text{g}/\text{mL}$ for the BRD 509 strain.

Transfection of CCL22 miRNA

RAW 264.7 cells were purchased from the Korean Cell Line Bank and cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/mL penicillin and gentamycin (Welgene), 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS) (Welgene). One day before transfection, the cells were plated at a density of 2×10^4 cells in eight-well culture slides and incubated overnight at 37°C in a 5% CO_2 incubator so that the cells reached 90%–95% confluence at the transfection time. After incubation, 0.5 μg of plasmid DNA was transfected with lipofectamine 2000 (Invitrogen) or with transformed *S. typhimurium*, and the cells were incubated at 37°C in a 5% CO_2 incubator for 48 h.

Splenocyte culture and infection of Salmonella strains

Four-week-old CD-1(ICR) female mice were sacrificed by dislocation of the cervical vertebrae, and the spleen was dissociated from the mice under aseptic conditions. The extracted spleen was transferred to 10 mL of RPMI 1640. Connective tissues and fat were removed. The spleen was then homogenized using a homogenizer, and red blood cells were lysed in red blood cell lysing buffer (Sigma). The cells were cultured in RPMI 1640 medium supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% FBS (Welgene). The cells were plated at a density of 5.0×10^7 cells in six-well culture plates and treated with lectin (10 $\mu\text{g}/\text{mL}$; Sigma) and IL-4 (20 ng/mL; Invitrogen) to overexpress CCL22 for 24 h. The whole mouse splenocytes were infected with ST-miRCCL22 (6×10^7 cfu) and gentamycin was added (50 $\mu\text{g}/\text{mL}$; Welgene) for 90 min to kill any remaining extracellular bacteria. Transfected mouse splenocytes were incubated for 48 h to express the eukaryotic plasmid vector. Total RNA was isolated and cDNA was synthesized from mouse splenocytes after treatment with ST-miRCCL22.

RNA isolation and RT-PCR

Cultured cells were collected by centrifugation at 1500 g, and total RNA was isolated by TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using random primers and the M-MLV-RT enzyme for cDNA synthesis (Promega). The resulting cDNA was used in PCRs (final volume of 20 μL) with *Taq* polymerase and its reaction mix (Han-taq), 0.2 mM dNTP (Takara), and 0.3 μM forward and reverse primers. Primers used for PCR were as follows: CCL22: 5' ATGAGGTCACCTCAGATGCT 3' and 5' AGGTCACG GCCTTGGGTTTT 3' IFN- γ , IL-1 α , TNF- α , IL-18, IL-4, and β -actin: premade primers (Bioneer Co, Korea). CCL22 was amplified from 1 μL (~ 20 ng) cDNA in a 35-cycle PCR program with cycling conditions as follows: 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. IL-4 and IFN- γ were amplified from 1 μL (~ 20 ng) cDNA in a 35-cycle PCR program with cycling conditions as follows: 95°C for 1 min, 50°C for 1 min,

and 72°C for 1 min. Normalized template volumes were subjected to 31–35 PCR cycles using mouse β -actin primers. Additionally, miRNA RT was performed with 500 ng of total RNA using the Applied Biosystems cDNA kit. qPCR was performed with the Sybr Green or TaqMan Gene Expression Assay using the ABI Prism 7900 Sequence Detection System instrument (Applied Biosystems) according to the company's manual (Genolution).

Invasion assay

RAW cells were infected with *Salmonella* strains at an MOI of 100 for 1 h. After infection, the cells were washed three times with phosphate-buffered saline (PBS) and then treated with 100 μ g/mL of gentamycin (Invitrogen Corporation) for 1 h at 37°C. After antibiotic treatment, the cells were washed again with PBS and then incubated with 1% Triton X-100 for 5 min at 37°C for detection of *Salmonella*. The number of internalized bacteria was determined by plating 10-fold serial dilutions of the cell lysates on LB plates. Invasion rate (%) was determined using the following formula: Invasion rate (%) = (Number of internalized recombinant bacteria / Number of internalized wild-type *Salmonella*) $\times$ 100.

Bacteria replication in normal tissues

Bacteria were grown to mid-log phase, harvested by centrifugation (3000 g) and resuspended in a 10% sodium bicarbonate buffer. ICR mice were inoculated with $\sim$ 100 μ L of the bacterial suspension (10^8 cfu/mouse). Each group of mice received the corresponding strain of recombinant *Salmonella*. Control mice received subcutaneously the buffer alone.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot assay

The bacterial culture supernatant was run on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and electrophoretically transferred to nitrocellulose membranes. The membranes were pre-equilibrated with TBS-T solution containing 5% skim milk overnight at 4°C and were incubated with mouse anti-GFP antibody for 1 h at room temperature. After three washes with TBS-T, the membranes were incubated with goat anti-mouse IgG HRP conjugate for 1 h at room temperature. The immunoreactive protein bands were visualized using the BM Chemiluminescence Blotting Substrate (Roche).

Cytokine analysis

Mice with AD were orally inoculated with 1.6×10^8 cfu ST-miRCCL22, ST-miRCV, and PBS. One week after inoculation, total serum was collected from each mouse for the detection of IL-4 by ELISA. Serum samples of the control and test groups were collected by eye bleeding from mice at 1 week after bacteria delivery. Additionally, 96-well plates were coated with 100 μ L/well of IL-4, IFN- γ , or IgE capture antibody and incubated overnight at 4°C. The wells were aspirated and washed three times with PBS-T (PBS with 0.05% Tween 20), and the wells were blocked with dilution buffer (PBS with 10% FBS) for 2 h at room temperature. After three washes, 100 μ L of each sample was pipetted into the wells and incubated for 2 h at room temperature. The wells were aspirated and washed five times. Then, 100 μ L of

each detection antibody and SAV-HRP conjugate reagent were added and incubated for 1 h at room temperature. The wells were aspirated by washing seven times, and then 100 μ L of substrate solution (R&D system) was added to each well and incubated for 30 min at room temperature in the dark. Subsequently, 50 μ L of stop solution (2N H₂SO₄) was added. The absorbance was read at 450 nm by a microplate ELISA reader within 30 min of stopping the reaction.

Cytokine serum levels were also measured by a multiplexed assay combined with flow cytometry using commercially available kits BD™ Mouse TH1/TH2 Cytokine cytometric bead array (CBA) according to the kit procedure (BD Biosciences Immunocytometry Systems and BD Bioscience Pharmingen).

Scratching behavior test

Induced hypersensitiveness of pruritus mice were observed for 7 days. The total number of scratching bouts was scored over a 15-min period. A scratching bout was defined as one or more rapid hind paw movements directed toward the region in direct contact with the DNCB agent. The end point of scratching was when the mouse bit or licked its toes or placed the hind paw on the floor. Grooming or other movements directed toward areas other than the DNCB-hypersensitive site were not counted. Observers were always blinded to the treatment group and genotype.

Flow cytometry

Single-cell suspension of splenic cells was prepared by pressing the organs through a nylon mesh and removing red cells with lysis buffer. A part of splenic cell suspension was subjected to flow cytometry for the expression of Th17 cells, as described by the manufacturer, using the mouse T-cell staining kit (R&D system). Cell samples were subjected to FACScan flow cytometry and data were collected and analyzed using CellQuest software (BD Biosciences).

Results

Suppression of CCL22 by plasmid expressing CCL22 miRNA

To construct the CCL22 miRNA plasmid for RNAi against CCL22, we designed two single-stranded DNA oligonucleotides encoding the CCL22 target pre-miRNA using an RNAi design program. We observed that successful transfer of the CCL22 miRNA expression into mammalian cells occurred. RAW 264.7 cells were transfected with miRCCL22. miRNA was detected in the RAW 264.7 cells transfected with miRCCL22 but not with the negative control vector. These data show that the miRNA expression vector in *S. typhimurium* can be expressed in mammalian cells (Fig. 1A). *E. coli* DH5 α cells were transformed with miRCCL22, and the plasmid was isolated and used to transform *S. typhimurium* SF586. The plasmid from the transformed SF586 cells was used to further transform *S. typhimurium* BRD509, and this was used for further experiments. To evaluate the expression of miRCCL22 in bacteria, we also observed green fluorescence proteins in ST-miRCCL22 and ST-miRCV. Cells were infected with recombinant *Salmonella*, and GFP expression by ST-miRCCL22 and ST-miRCV was observed (Fig. 1B). The invasive ratio of ST-miRCCL22 was similar to ST-miRCV (Fig. 1C).

FIG. 1. Expression of *CCL22* miRNA by recombinant *Salmonella typhimurium* in RAW cells. To examine expression of miRNA vector and *S. typhimurium* expressing *CCL22* miRNA, RAW 264.7 cells were infected with ST-miRCCL22, ST-miRCV, or EmGFP negative control. Recombinant *Salmonella* invaded RAW cells (A) and expression of the miRNA was observed in cells with ST-miRCCL22 (B). Invasion rates of recombinant *Salmonella* were examined (C). ST-miRCCL22, *Salmonella typhimurium* expressing *CCL22* miRNA; miRNA, microRNA. Color images available online at www.liebertonline.com/dna

Suppression of the *CCL22* gene by ST-miRCCL22

To determine the *CCL22* gene silencing effects of engineered *Salmonella*, we treated *CCL22*-expressing immune cells with recombinant bacteria. In mice orally inoculated with recombinant bacteria, *Salmonella* invaded cells and were distributed in spleen, kidney, and liver at 1 day (Fig. 2A). Mice orally inoculated with ST-miRCCL22 showed GFP expression in spleen cells at 1 day (Fig. 2B); this result suggested that ST-miRCCL22 would be transferred and miRNA gene would be expressed in spleen.

To examine whether the ST-miRCCL22 silenced the *CCL22* gene in spleen cells, whole mouse splenocytes were ex-

tracted. Splenocytes were treated with lectin and IL-4 to induce the overexpression of *CCL22*. The expression of *CCL22* was only silenced in mouse splenocytes treated with ST-miRCCL22 (Fig. 2C).

Modulation of *CCL22*, IgE, and cytokines after treatment with ST-miRCCL22 in mice with cutaneous disease

To examine the effects of ST-miRCCL22 in mice with cutaneous diseases, *CCL22*, cytokines, and IgE levels were analyzed after oral treatments. Mouse blood samples were collected to analyze changes in inflammatory cytokine levels

FIG. 2. Gene silencing of *CCL22* and alteration of inflammatory cytokine levels. Distribution of ST-miRCCL22 in mice was observed in spleen, kidney, and liver (A). Expression of ST-miRCCL22 was detected in spleen cells at 1 day after treatments by fluorescence microscopy (B). Expression of *CCL22* was silenced in splenocytes after treatment with ST-miRCCL22; ST-miRCV did not affect *CCL22* expression (C). One experiment, representative of three independent analyses with similar results, is shown. Color images available online at www.liebertonline.com/dna

FIG. 3. Changes in CCL22 and cytokine levels in serum from atopic dermatitis mice treated with ST-miRCCL22. Mice were orally infected with 1.6×10^8 cfu ST-miRCCL22 or ST-miRCV. Total CCL22 (**A**), IgE (**B**), IL-4 (**C**), and IFN- γ (**D**) levels of ST-miRCCL22-treated mice were examined by ELISA. Other inflammatory cytokines levels were examined by cytometric bead array assay (**E**). These experiments were repeated at least three times with similar results. IL, interleukin; IFN- γ , interferon gamma. Color images available online at www.liebertonline.com/dna

in vivo. At 1 week after oral treatment with ST-miRCCL22, mice demonstrated suppression of CCL22 protein in blood (Fig. 3A). The total IgE level of ST-miRCCL22-treated mice was significantly suppressed compared with mice treated with PBS or ST-miRCV (Fig. 3B). IL-4 levels were suppressed in cells treated with ST-miRCCL22 (Fig. 3C). The ST-miRCCL22 treatment groups also showed induction of IFN- γ production than the ST-miRCV-treated cells (Fig. 3D). These results suggested that ST-miRCCL22 altered inflammatory cytokine levels. Other inflammatory cytokines (IL-6, IL-10, MCP-1, IFN- γ , TNF- α , IL-12p70) were also analyzed by CBA method (Fig. 3E). The results showed that IFN- γ and TNF- α were more induced in blood than other cytokines.

Anti-inflammatory effects of ST-miRCCL22 mice with cutaneous disease

To examine the scratching behavior of mice, ST-miRCCL22 were orally inoculated in mice with cutaneous disease. The total scratching counts in the ST-miRCCL22-treated group were significantly lower than that in the mice treated with PBS or ST-miRCV (Fig. 4A). In histological analysis, ST-miRCCL22-treated mice showed reduced skin inflammations. These data showed that specific gene silencing against CCL22 relieved pruritus (Fig. 4B). Additionally,

cytokines and Th17 cells were also examined in skin tissue. IL-4 and IL-1 α levels were slightly reduced in mice inoculated with ST-miRCV or ST-miRCCL22 (Fig. 4C). The ST-miRCCL22-treated mice showed slightly reduced Th17 cells compared to control groups (Fig. 4D).

Discussion

Both Th2 and Th1 chemokines (TARC/CCL17, MDC/CCL22, and Mig/CXCL9) were elevated in sera from patients with AD (Shimada *et al.*, 2004), but these chemokines' role in cutaneous diseases was not clear. This study suggested that CCL22 was related to inflammation, and *S. typhimurium* could be used as a vector for miRNA delivery in mice with AD.

In particular, *S. typhimurium*-harboring plasmids that express CCL22 miRNA could reduce *in vitro* and *in vivo* CCL22 gene expression in mouse models of AD. The intestinal mucosa contains a subset of lymphocytes that produce Th2 cytokines; MDC/CCL22 is a recently described CC chemokine known to chemoattract the Th2 cytokine-producing cells that express the receptor CCR4. CCL22 modulated and regulated the production by intestinal epithelial cells (Berin *et al.*, 2001). *Salmonella* is a reasonable tool for intestinal cell delivery because of its intracellular invasive abilities.

FIG. 4. Improvement in symptoms of atopic dermatitis by oral administration of ST-miRCCL22 in mice. Total scratch counts of ST-miRCCL22-treated mice were dramatically lower when compared with PBS, ST-control, or ST-miRCV group (A). * $p < 0.05$, comparing ST-miRCCL22 with ST-miRCV-control groups ($n = 7$). The skin tissue of ST-miRCCL22-treated mice showed more improved inflammation compared with PBS or ST-miRCV groups (B). TNF- α , IFN- γ , IL-18, IL-4, and IL-1 α were examined by RT-PCR in skin after treatments. Expression of IL-4 was slightly suppressed in the ST-miRCCL22-treated mice (C). Th17 cells were analyzed by Th17 FACS kit. The arrows indicate gene expression. (D). These experiments were repeated at least three times with similar results. PBS, phosphate-buffered saline. Color images available online at www.liebertonline.com/dna

To suppress the *CCL22* gene, we engineered bacteria with vector-based miRNA for easy and stable delivery. Previous research showed that *S. typhimurium* could transfer eukaryotic vector-based RNAi-expressing plasmids *in vitro* or *in vivo* by oral administration (Yang *et al.*, 2008). Oral administration of ST-miRCCL22 reduced *CCL22* gene expression in mouse spleen and induced therapeutic effects.

Our plasmid DNA, under the control of the CMV promoter, can be expressed in a eukaryotic system as well as a prokaryotic system. This plasmid was partially generated with bacterial promoters placed in the CMV promoter sequence (Goussard *et al.*, 2003; Gahan *et al.*, 2009).

Macrophages are normal targets for *Salmonella* during natural infections, and it has been demonstrated that attenuated bacteria can deliver nucleic acid vaccine constructs (Paglia *et al.*, 2000). Therefore, we examined whether *Salmo-*

nella can be used for the *in vivo* delivery of miRNA to their natural cellular target, in an attempt to suppress target gene associated with inflammation. Our results showed miRNA expression by recombinant *Salmonella* (Fig. 1). Using a reporter gene (GFP), we demonstrated that recombinant *Salmonella* could be used as an effective *in vivo* delivery system to transfer miRNA into immune cells such as murine macrophage. *In vivo*, the oral administration of attenuated *Salmonella* allows targeted delivery of transgenes to spleen cell and subsequent expression of miRNA at a systemic level (Fig. 2). These results are interesting but difficult to understand. *CCL22* principally attracts CD4⁺ T cells, which express the skin-homing receptor cutaneous lymphocyte-associated antigen (Campbell and Kemp, 1999; Ghia *et al.*, 2002). They are also directly implicated in Th2-associated skin inflammatory responses and is significantly elevated

and correlated with disease severity in AD (Gilet *et al.*, 2009). In this study, ST-miRCCL22 reduced IL-4, whereas IFN- γ inhibits IgE secretion through the antagonistic effect of the Th2 cytokines (Herberth *et al.*, 2010). These data demonstrated that CCL22 gene suppression induced the Th1 shift and balance in AD. In the AD model, the IgE response was elevated, as evidenced by the increased Th2 cytokines' responses and the simultaneous decrease in IFN- γ production in patients with AD (Yoshizawa *et al.*, 2002).

Itching is an important symptom in AD (Takaoka *et al.*, 2006). This causes histamines to be secreted through the activation of Th2 and IgE/mast-cell-driven responses (Thurmond *et al.*, 2008). The treatment of ST-miRCCL22 induced reduced scratching behavior and inflammation of skin in mice with AD (Fig. 4).

In addition to suppressive effects on the CCL22 gene, orally inoculated *Salmonella* induced not only Th1 immune responses but also suppression of Th17 cells (Fig. 4D). *Salmonella* contributed adjuvant effects and induced Th1 cytokines and cell-mediated immunity (Capozzo *et al.*, 2004). Th17 cells were probably responsible for chronic tissue inflammation. Skewing of response away from Treg cells may lead to the onset and/or progression of autoimmune diseases or acute transplant rejection in humans (Fietta and Delsante, 2009). The *Salmonella* vector induced anti-inflammatory effects with suppression of the CCL22 gene. In conclusion, recombinant *S. typhimurium* that suppresses the CCL22 gene reduced CCL22 gene expression in cells and induced anti-inflammatory effects. These results suggest that *Salmonella*-based gene therapy would be an alternative for gene therapy against AD.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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