

Immunostimulatory Sequence CpG Elicits Th1-Type Immune Responses in Inflammatory Skin Lesions in an Atopic Dermatitis Murine Model

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Key Words

Immunostimulatory sequence CpG • Atopic dermatitis •
Cytokines • Chemokines • Animal model

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease, for which no fundamental therapy exists. Immunostimulatory sequence CpG (ISS CpG) has potential in reducing susceptibility to allergic diseases and reversing established allergic reactions. **Objective:** To investigate the effects of ISS CpG in the prevention and treatment of AD in an AD murine model. **Methods:** BALB/c mice were epicutaneously exposed to ovalbumin (OVA) for 3 or 4 weeks with a 2-week resting period between each exposure week. ISS i.d. injection was given either on the 1st day of each exposure week (in the prevention experiment) or 3 days before and on the 1st, 4th and 7th day of the last exposure week (in the treatment experiment). Skin biopsy and blood were obtained at the end of the experiments. **Results:** ISS CpG treatment increased drastically mRNA expression of proinflammatory and Th1-type cytokines and chemokines in OVA-treated skin both in the prevention and treatment experiments. The suppressing effect of ISS CpG on Th2-type

cytokines and chemokines was weak and limited to IL-13 and CCL24 in the treatment experiment. No significant reduction in OVA-elicited infiltration of eosinophils and T cells in the skin was seen after ISS administration but infiltration of plasmacytoid dendritic cells was absent in ISS CpG-treated skin. In contrast, ISS injection elicited dramatic infiltration of F4/80+ and CCR5+ cells into the dermis and subcutaneous tissue. **Conclusion:** Due to unwanted side effects and minor beneficial effects in our model, administration of ISS CpG may not be suitable for the treatment of AD in humans.

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Introduction

Atopic dermatitis (AD) is a chronic pruritic inflammatory disease of the skin that affects about 10–20% of the general population. Prevalence of this disease has increased two- to threefold during the past three decades in industrialized countries [1]. So far there have been no effective therapies which can cure the disease fundamen-

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tally. Topical corticosteroids have been the mainstay of treatment in AD [2]. However, long-term application of corticosteroids may lead to loss of efficacy, local skin atrophy [3] and decreased immune cell response [4]. These observations underscore the need for developing safer and more effective therapies to prevent and treat the disease.

AD is characterized by a Th2-type dominated response – tissue eosinophilia, elevated serum IgE and significant production of Th2-type cytokines. The two types of T cells (Th1 and Th2 cells) in allergic inflammation interact in a counter-regulatory fashion. On one hand, IL-4 promotes Th2 cell maturation from naïve Th0 cells, and suppresses Th1 cells and their cytokine production. On the other hand, IFN- γ inhibits the proliferation of Th2 cells and promotes Th1 cells [5]. Thus, agents that selectively elicit Th1 immunity and finely tune the balance between the production of Th1- and Th2-type cytokines might be useful in controlling allergic inflammation in AD.

Studies have shown that bacterial DNA and synthetic oligodeoxynucleotides (ODNs) that contain immunostimulatory CpG motifs trigger an immunomodulatory cascade which skews the immune milieu of the host in favor of Th-1 type responses [6]. The therapeutic potentials of CpG ODNs in reducing susceptibility to allergic diseases and reversing established allergic reactions have been extensively investigated in asthma murine models. The results demonstrated that CpG ameliorates airway hyper-responsiveness, decreases serum IgE level and reduces the number of eosinophils both in bronchoalveolar lavage (BAL) and lung tissue [7–13]. Since both allergic asthma and atopic dermatitis are atopic disorders, we were interested whether CpG would have similar down-regulatory effects in AD inflammation as well. We explored preventive and treatment effects of immunostimulatory sequence CpG (ISS CpG) using a previously developed murine model of AD.

Materials and Methods

CpG

ISS CpG and control ODN [14] were obtained from Dynavax Technologies, Corporation, USA.

Mice and Sensitization

6- to 8-week-old female BALB/CJBOM mice were purchased from Taconic M & B (Ry, Denmark) and maintained under pathogen-free conditions. All procedures performed on the mice were approved by the Animal Care and Use Committee of Uusimaa District, Finland.

Epicutaneous (EC) exposure of mice was performed as described previously [15]. Briefly, mice were anesthetized with inhalation of Isoflurane (Abbott Laboratories Ltd., Queenborough, UK). Mice backs were shaved with an electric razor and tape-stripped four times by transparent adhesive tape (Tegaderm, 3M Health Care, St Paul, Minn., USA) to introduce a standardized skin injury. A patch of sterile gauze (1 $\times$ 1 cm²) soaked with 100 μ l of 0.1% OVA or 0.9% saline was put on the back skin secured with Tegaderm and kept there for 1 week. Each mouse had a total of 3 (in prevention experiment) or 4 (in treatment experiment) exposure weeks with a 2-week resting period between each exposure week. Intradermal (i.d.) injection of 5 μ g (in the prevention experiment) or 10 μ g (in the treatment experiment) of ISS CpG or control ODN in 100 μ l of saline was given on each side of the patch on the 1st day of each exposure week in the prevention experiment, or 3 days before and on the 1st, 4th and 7th days of the last exposure week in the treatment experiment. Mice were sacrificed 1 or 2 days after the last exposure week. Blood samples were then collected for antibody analysis. Skin biopsies from exposure areas were used for RNA isolation, histology and immunohistochemistry examination.

Antibodies

Rat mAbs against mouse CD3 (clone 17A2), CD4 (clone RM4-5) and CD8a (clone 53-6.7) in immunohistochemical analysis, and rat anti-mouse IgE (clone 35-72) and biotin-conjugated rat anti-mouse IgG2a (clone 19-15) in ELISA were purchased from BD PharMingen. Biotinylated anti-rat IgG (H+L) for immunohistochemistry was obtained from Vector Laboratories, Inc. Burlingame, Calif., USA.

Table 1. Sequence of mouse primer pairs and fluorogenic probes for real-time quantitative PCR

	Sense primer (5'-3')	Antisense primer (5'-3')
CCL17	5'-CAG GAA GTT GGT GAG CTG GTA TAA G-3'	5'-TGG CCT TCT TCA CAT GTT TGT CT-3'
CCL11	5'-ATG CAC CCT GAA AGC CAT AGT C-3'	5'-CAG GTG CTT TGT GGC ATC CT-3'
CCL24	5'-CGG CCT CCT TCT CCT GGT A-3'	5'-TGG CCA ACT GGT AGC TAA CCA-3'
CCR2	5'-CCT TGG GAA TGA GTA ACT GTG TGA-3'	5'-TGT GTC ATT CCA AGA GTC TCT GTC A-3'
CCR3	5'-TTG CAG GAC TGG CAG CAT T-3'	5'-TTC ATT CTT AGA GCA TGG AAA CGT T-3'
CCR4	5'-TCA TGA CTT CCG TGA CGC TTT-3'	5'-GTT TTC TTC CTC AGA GCC CTG TT-3'
CCR5	5'-TTG CAA ACG GTG TTC AAT TTT-3'	5'-TCT CCT GTG GAT CGG GTA TAG AC-3'
CCR8	5'-CTT CTG GGT CCC ATT CAA CGT-3'	5'-CAG TGG GTA AAA GAG ATG ACC TCT GT-3'
IFN- γ	5'-AGC GCC AAG CAT TCA ATG A-3'	5'-CGC TTC CTG AGG CTG GAT T-3'

ELISA

OVA-specific IgG2a and IgE were measured as described by Spergel et al. [15]. Briefly, 96-well microtiter plates were coated with 50 µl/well of OVA (for IgG2a) or rat anti-mouse IgE (for IgE) (2 µg/ml in 0.05 M NaHCO₃) at 4°C overnight. The plates were washed and blocked with PBS-3% bovine serum albumin for 2 h at room temperature. 100 µl of diluted serum (1:400 for IgG2a, 1:40 for IgE) was added and incubated in duplicate at 4°C overnight. After washing with PBS-Tween 20, biotin-conjugated rat anti-mouse IgG2a (2 µg/ml) or biotin-labeled OVA was added and incubated for 2 h. Streptavidin-horseradish peroxidase (BD PharMingen) and peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) were used to develop color. The absorption at 405 nm was read with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Histological Analysis

Skin biopsies were obtained from the patched area after the mice were sacrificed. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut 4 µm thick and stained with hematoxylin and eosin (HE) for inflammatory cell counting or with o-toluidine blue for mast cell counting. Inflammatory cells were counted in 15–30 high-power fields (HPF) at ×400–1,000 magnification under light microscope and expressed as cells per HPF, with mean and SEM calculated. The thickness of epidermis and dermis was measured by using a scaled ocular lens on the light microscope. 10–15 measurements were obtained for each mouse. The measured values were adjusted for the magnification optics and expressed in micrometers with mean and SEM calculated.

Immunohistologic Analysis

Skin samples were embedded in Tissue-Tek O.C.T compound (Sakura Finetek Europe B.V., the Netherlands) on dry ice. Sections of 4 µm were prepared and stained by avidin-biotin complex method using ChemMate™ Detection Kit (DakoCytomation, Denmark). Briefly, the sections were fixed in cold acetone for 2 min, air-dried, and incubated with purified rat anti-mouse mAbs (anti-CD3, anti-CD4, anti-CD8a, anti-F4/80, anti-PDCA-1, anti-IL10, anti-CCR5), followed by incubation with biotinylated anti-rat IgG. The dendritic cell marker CD11c is a hamster antibody and a biotinylated anti-hamster antibody was used as a secondary antibody. Endogenous peroxidase activity was blocked

by incubation with peroxidase-blocking solution. Sections were then incubated with peroxidase-conjugated streptavidin and stained in a substrate solution containing hydrogen peroxide and 3-amino-9-ethylcarbazole, followed by 1 min incubation in hematoxylin. The tissue sections were mounted with aquamount (BDH, Gurr, Pole, UK). The positively stained cells were counted in 10–15 HPFs at ×1,000 magnification and expressed as cells per HPF, with mean and SEM calculated.

RNA Isolation, cDNA Synthesis and Quantitative Real Time PCR Analysis

Total RNA was isolated from skin specimens using Trizol (Gibco BRL, New York, N.Y., USA) according to the manufacturer's instructions. cDNA was synthesized using 1 µg of total RNA in a 50-µl reaction mixture which contained MultiScribe reverse transcriptase, random hexamers, RNase inhibitor, dNTP, MgCL and reverse transfer buffer (PE Applied Biosystems, Foster City, Calif., USA) in a thermal cycler (Mastercycler gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 48°C for 90 min, and stopped at 95°C for 5 min. The mRNA expression levels of cytokines, chemokines/receptors were determined with real-time quantitative PCR using the ABI 7700 sequence detector system (PE Applied Biosystems). PCR primers and probes for TNF-α, IL-4, IL-5, IL-13, IL-10, CCL1, CCL2, CCL5, CXCL9, CXCL10, CXCR3, TLR9 and 18S rRNA were purchased from PE Applied Biosystems as pre-developed reagents. Primers and probes for IFN-γ, CCL11, CCL17, CCL24, CCR2, CCR3, CCR4, CCR5 and CCR8 were designed by Primer Express version v1.5 software (PE Applied Biosystems) (table 1). Taqman® universal PCR master mix was purchased from PE Applied Biosystems. One microliter of sample cDNA was added to the master mix during amplification. The thermal cycling conditions were set to 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min for each cycle. PCR amplification of the endogenous 18S rRNA was performed for each sample to control sample loading and to allow normalization between samples. The results were expressed as relative units (RU), which was calculated by the comparative C_T method according to the manufacturer's instructions. First, the C_T for the target amplicon and the C_T for the internal control (18S rRNA) was determined for each sample. Differences between the C_T of the target and the internal control, termed ΔC_T, were calculated to normalize the differences in the amounts of cDNA added to each reaction mixture. The ΔC_T for each experimental sample was then subtracted from the ΔC_T of the calibrator. This difference is termed ΔΔC_T. The amount of target, normalized to the internal (18S rRNA) and relative to the calibrator, was then calculated by the equation 2^{-ΔΔC_T}. A FAM C_T value 40, reflecting the situation in which the gene is not expressed at the mRNA level, was used as the calibrator.

Statistical Analysis

The data were analyzed with the GraphPad Prism Software. t test or nonparametric Mann-Whitney U test was used to compare the difference between different groups. p < 0.05 was considered statistically significant.

Probe (5'-3')

5'-TGT CCA GGG CAA GCT CAT CTG TGC-3'
5'-AGC ACA GAT CTC TTT GCC CAA CCT GGT-3'
5'-CCC TCA TCT TGC TGC ACG TCC TTTV ATT TC-3'
5'-TGA CAA GCA CTT AGA CCA GGC CAT GCA-3'
5'-TGG AGA GTT TTC CTG CAG TCC TCG CTA TC-3'
5'-TCG CCT TGT TTC AGT CAG GGT GCC-3'
5'-AGC AAG ACA ATC CTG ATC GTG CAA GCTC-3'
5'-CGT CCC TGC ACG ACC TGC ACA TC-3'
5'-ATC CGA GTG GTC CAC CAG CTG TTG C-3'

Results

Subcutaneous Infiltration of F4/80+ Macrophages Is Increased and Dermal Accumulation of Plasmacytoid Dendritic Cells Decreased after ISS CpG Treatment

After repeated EC exposures of mice to OVA, a significant thickening of epidermis and dermis, as well as a dermal cell infiltration were induced, which are in consistence with our previous study [16] (fig. 1a). All results shown below are focused on OVA sensitized mice only to reduce the excess of information. Local injection of ISS CpG showed a very slight tendency to lower the total dermal infiltration of inflammatory cells and mast cells (fig. 1b), and reduce the thickness of epidermis and dermis (fig. 1c) in the prevention experiment compared with control ODN and OVA groups. The inhibiting effects were statistically significant regarding to mast cells and the thickness of dermis compared with control groups ($p < 0.05$). In the treatment experiment, the total dermal infiltration of inflammatory cells and eosinophils also showed a decreasing tendency after ISS CpG treatment compared with the control and OVA groups, yet the differences were not statistically significant (fig. 1b).

Local ISS CpG injection induced vigorous mononuclear cell infiltration in the lower subcutaneous tissue on both sides of panniculus carnosus in both prevention and treatment experiments (fig. 1e, g). This effect was ISS CpG specific because this local cell infiltration was not found in control ODN groups (fig. 1d, f). Immunohistochemical studies using a marker for plasmacytoid dendritic cells (PDCA+ cells; fig. 2c) or dendritic cells (CD11c+ cells; data not shown) failed to stain the infiltrate. In contrast, the macrophage specific marker F4/80 strongly stained this infiltrate (fig. 2g). Interestingly, OVA exposure alone increased dermal staining for plasmacytoid dendritic cells, this staining was strongly reduced by ISS CpG (fig. 2b, c). Additional immunohistochemical studies showed that the numbers of CD3-, CD4- and CD8-positive cells in OVA exposed mice were not significantly affected by ISS CpG administration compared with control ODN groups in the treatment experiment (data not shown).

ISS CpG Increases Gene Expression of Proinflammatory, Th1-Type and Regulatory Cytokines in the Inflammatory Skin

To study the effects of ISS CpG on the Th1/Th2 balance in AD, cytokine mRNA expression in OVA-sensitized skin areas was analyzed. Compared with control ODN and OVA groups, ISS CpG increased significantly the mRNA expression of TNF- α and IFN- γ in both pre-

vention and treatment experiments (fig. 3a, b). No significant changes in the expression of IL-13 mRNA was found by ISS CpG treatment in the prevention experiment but IL-13 mRNA expression was weakly suppressed by ISS CpG in the treatment experiment (fig. 3c). IL-4 was significantly expressed in OVA treated mice in both prevention and treatment experiments. The level was not significantly decreased by ISS CpG treatment in either the prevention or treatment experiments (data not shown). The expression of regulatory cytokine IL-10 mRNA was dramatically increased by ISS CpG treatment in both experiments (fig. 3d). Immunohistochemistry revealed that IL-10 protein was expressed in the subcutaneous macrophage infiltrate induced by CpG ISS administration (fig. 2i). TLR9 mRNA expression was also significantly increased in ISS CpG-treated mice compared with control ODN and OVA groups in both experiments (fig. 3e).

ISS CpG Increases Proinflammatory and Th1-Type Chemokines in Inflammatory Skin

Chemokines are critical molecules for recruiting leukocytes into inflammatory sites. ISS CpG administration increased significantly the mRNA expression of Th1-type chemokines (CCL2, CXCL9 and CXCL10) in the prevention experiment (fig. 4a, e, f). Accordingly, Th1-type receptors (CCR2, CCR5 and CXCR3) were also significantly increased compared with control ODN and OVA groups (fig. 4g–i). In the treatment experiment, ISS CpG administration significantly increased Th1-type chemokines (CCL2 and CXCL9) (fig. 4a, e), as well as receptors (CCR2 and CCR5) (fig. 4g–h). Immunohistochemistry revealed that CCR5+ cells (i.e. Th1 cells and macrophages) were heavily accumulated in the subcutaneous tissue after ISS CpG treatment (fig. 2l). The effects of ISS CpG on Th2-type chemokines were heterogeneous. On one hand, ISS CpG significantly induced CCL11 expression in both the prevention and the treatment experiment (fig. 4c). On the other hand, ISS CpG reduced CCL24 in the treatment experiment (fig. 4d). The expression of CCL1, CCL17 and Th2-type receptors CCR3, CCR4 and CCR8 were not significantly affected by ISS CpG in either experiment (data not shown). Finally, CCL5, an unpolarized chemokine, was upregulated significantly in both the prevention and the treatment experiment (fig. 4b).

CpG Treatment Enhances Allergen-Specific IgG2a Levels but Does Not Affect IgE Levels

Epicutaneous exposures of mice to OVA induced significant induction of OVA-specific IgE antibodies (fig. 5).

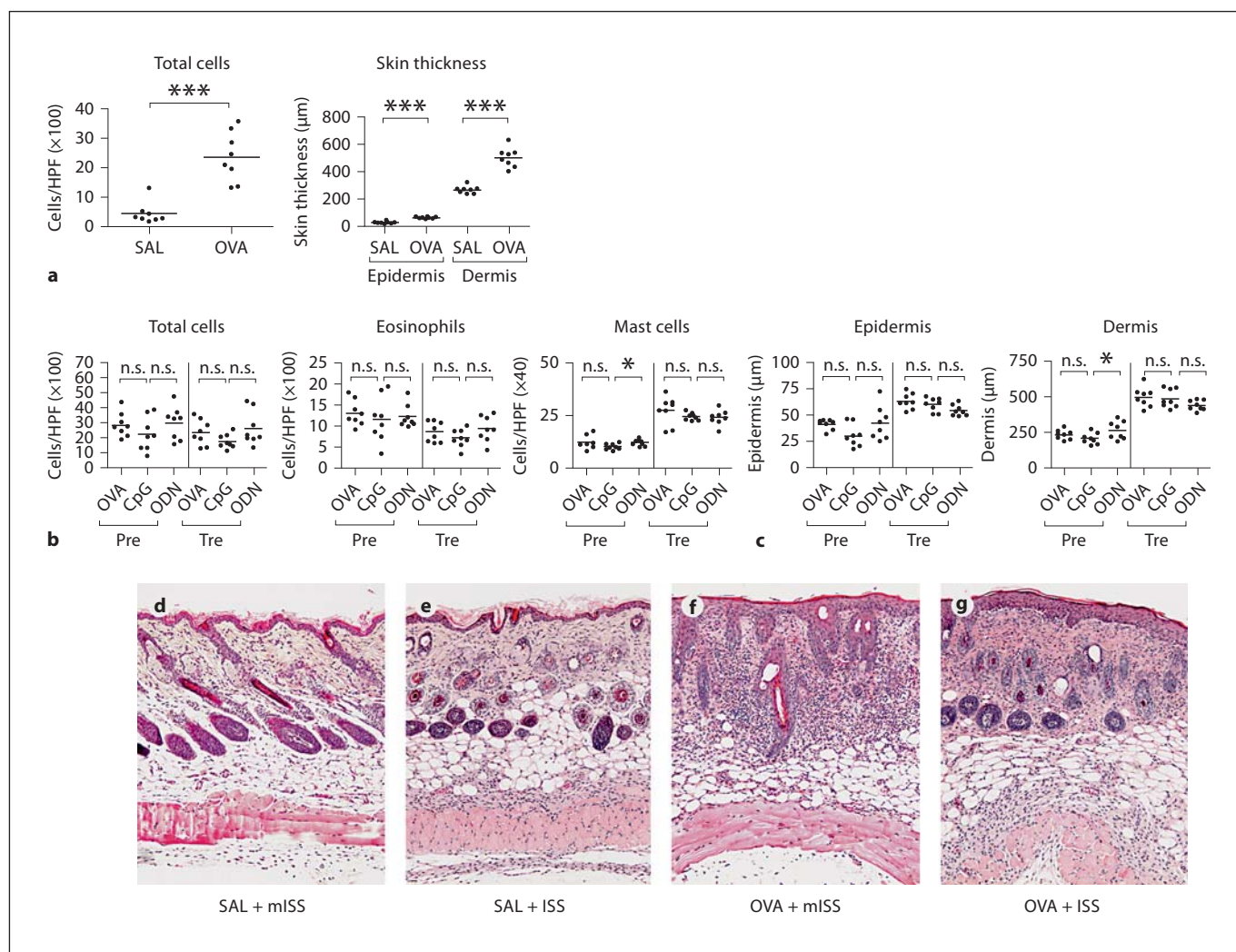


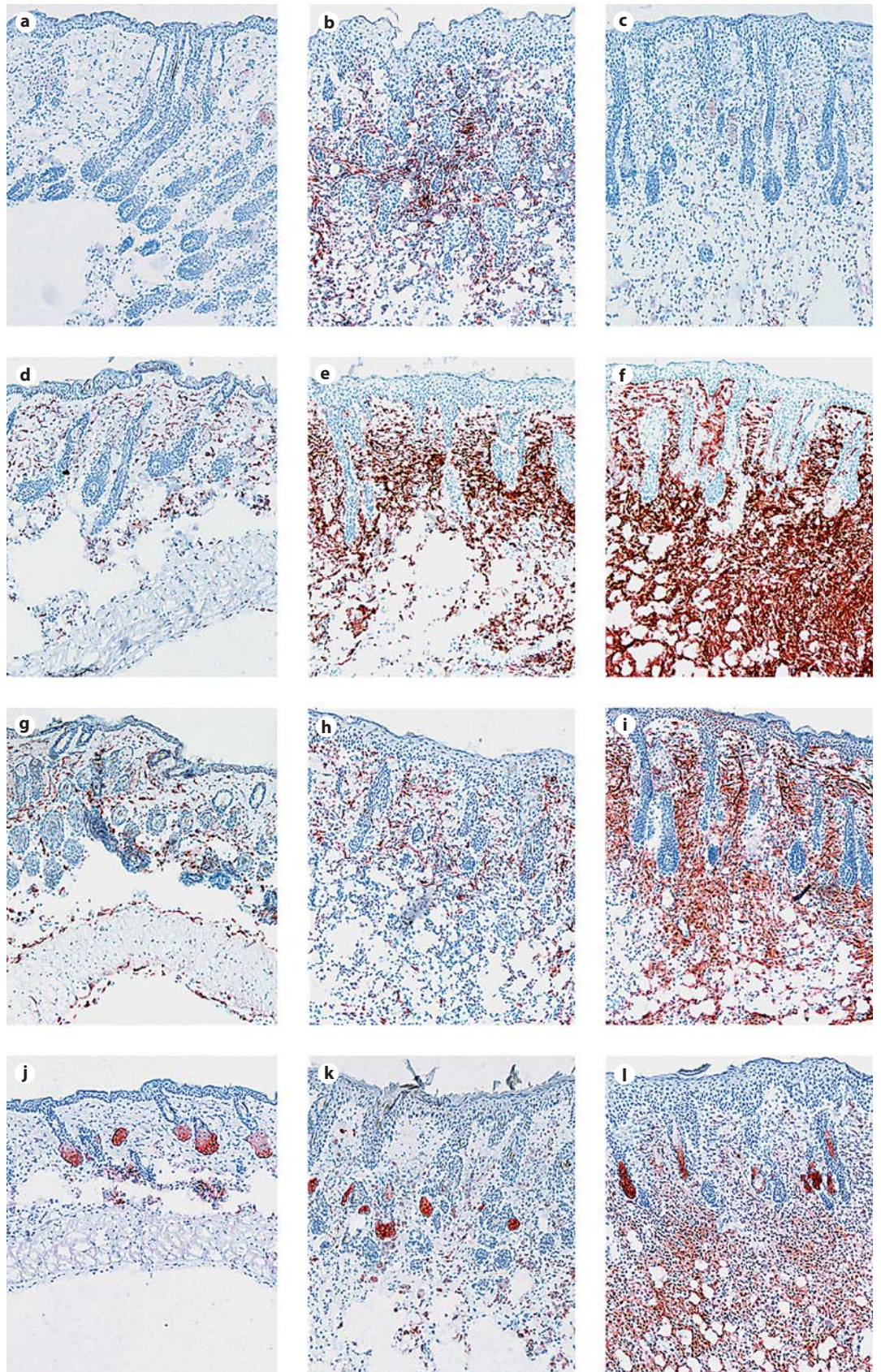
Fig. 1. Histological changes of the mouse skin after OVA and ISS CpG treatment. Treated skin of the backs of the mice was collected and processed for histologic examination. The number of inflammatory cells was counted under light microscope in 15–30 HPFs ($\times 400$ – $1,000$) per mouse and expressed as cells per HPF. The thickness of epidermis and dermis was measured 15–20 times per skin sample and expressed in micrometers. * $p < 0.05$, *** $p < 0.001$, n.s. = not statistically significant; Pre = prevention experiment; Tre = treatment experiment. **a** Total inflammatory cells and

thickness of epidermis and dermis of mice exposed to saline and OVA without ISS CpG treatment. **b** Total inflammatory cells, eosinophils and mast cells in OVA-exposed mice after treatment with ISS CpG and control ODN. **c** The thickness of epidermis and dermis in OVA-exposed mice after treatment with ISS CpG and control ODN. **d–g** Subcutaneous tissue infiltration with mononuclear cell in ISS CpG-treated mice (**e, g**), but absent in control ODN-treated mice (**d, f**). The represented HE sections are shown at $\times 10$ magnification.

ISS CpG did not affect significantly the level of OVA-specific IgE in either the prevention (fig. 5a) or the treatment experiments (fig. 5b). However, ISS CpG administration elicited significant induction of OVA specific IgG2a levels in the prevention experiments (fig. 5a) but not in the treatment experiments (fig. 5b).

Discussion

In this study, we investigated the effects of intradermally injected ISS CpG in the prevention and treatment of atopic dermatitis-like skin lesions in a well-established AD murine model [15, 16]. The results showed that OVA-elicited inflammatory cell infiltration in the skin was re-



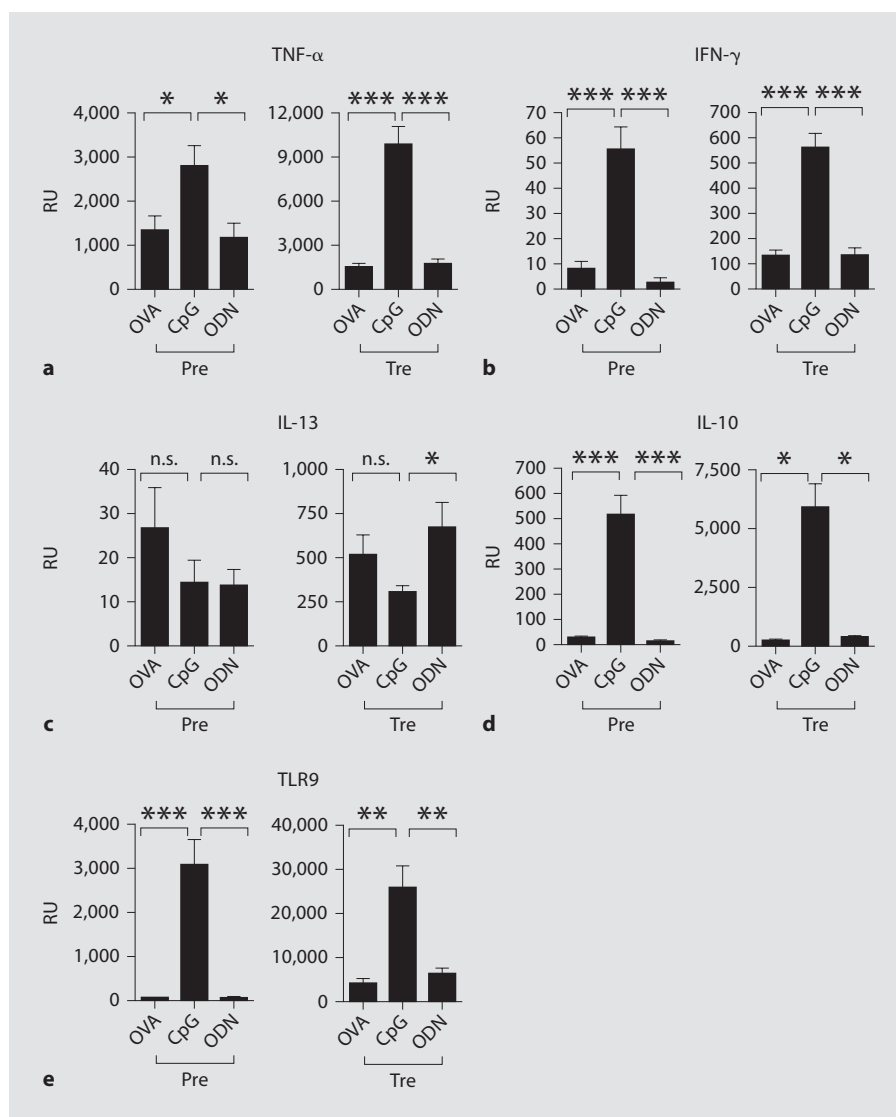
duced only partially and very weakly after ISS CpG administration. In contrast, ISS CpG administration induced vigorous infiltration of F4/80+ CCR5+ macrophages in the dermis and subcutaneous tissue as well as drastic expression of proinflammatory and Th1-type cytokines and chemokines in the skin.

CpG can initiate innate immune response by activating macrophages and dendritic cells to secrete cytokines such as IL-12, IL-18, IFN- α , β or natural killer cells to secrete IFN- γ [17–19]. Since some of these cytokines are involved in inhibiting the generation of Th2-type cytokines and biasing the immune response toward development of Th1-type immune response, CpG thus promises

a therapeutic potential for Th2 dominated allergic diseases. Studies have shown that CpG exerts inhibitory effects on pulmonary eosinophilia in asthma model as early as 24 h after CpG administration [13]. The effects sustained for at least 6 weeks after CpG administration [10]. The early onset of the effect is due to the immediate activation of innate immunity. In the prevention experiment of our study, mice were sacrificed 7 days after CpG injection. The significant increase in gene expressions of IFN- γ , Th1-type chemokines and chemokine receptors in the skin reflects an ongoing innate immune response initiated by CpG treatment. In the treatment experiment, owing to several administrations of CpG within 10 days and

Fig. 2. Immunohistochemical staining of mouse skin after OVA and ISS CpG treatment. Skin samples were embedded in Tissue-Tek O.C.T compound. 4- μ m sections were cut and stained with immunohistochemistry method. Skin of (a, d, g, j) saline, (b, e, h, k) OVA-treated, and (c, f, i, l) OVA+ISS-treated mice. Representative figures of mouse skin after (a–c) PDCA-1, (d–f) F4/80, (g–i) IL-10 or (j–l) CCR5 staining. The brown dots are positively stained cells. Magnification in immunohistological figures is $\times 100$.

Fig. 3. ISS CpG increases gene expression of proinflammatory, Th1-type and regulatory cytokines in inflammatory skin. Real-time quantitative PCR was used to analyze the mRNA expression levels. RU is expressed as fold differences relative to the calibrator. Bars and errors represent mean $\pm$ SEM. n = 8. * p < 0.05, ** p < 0.01, *** p < 0.001. n.s. = Not statistically significant; Pre = prevention experiment; Tre = treatment experiment.



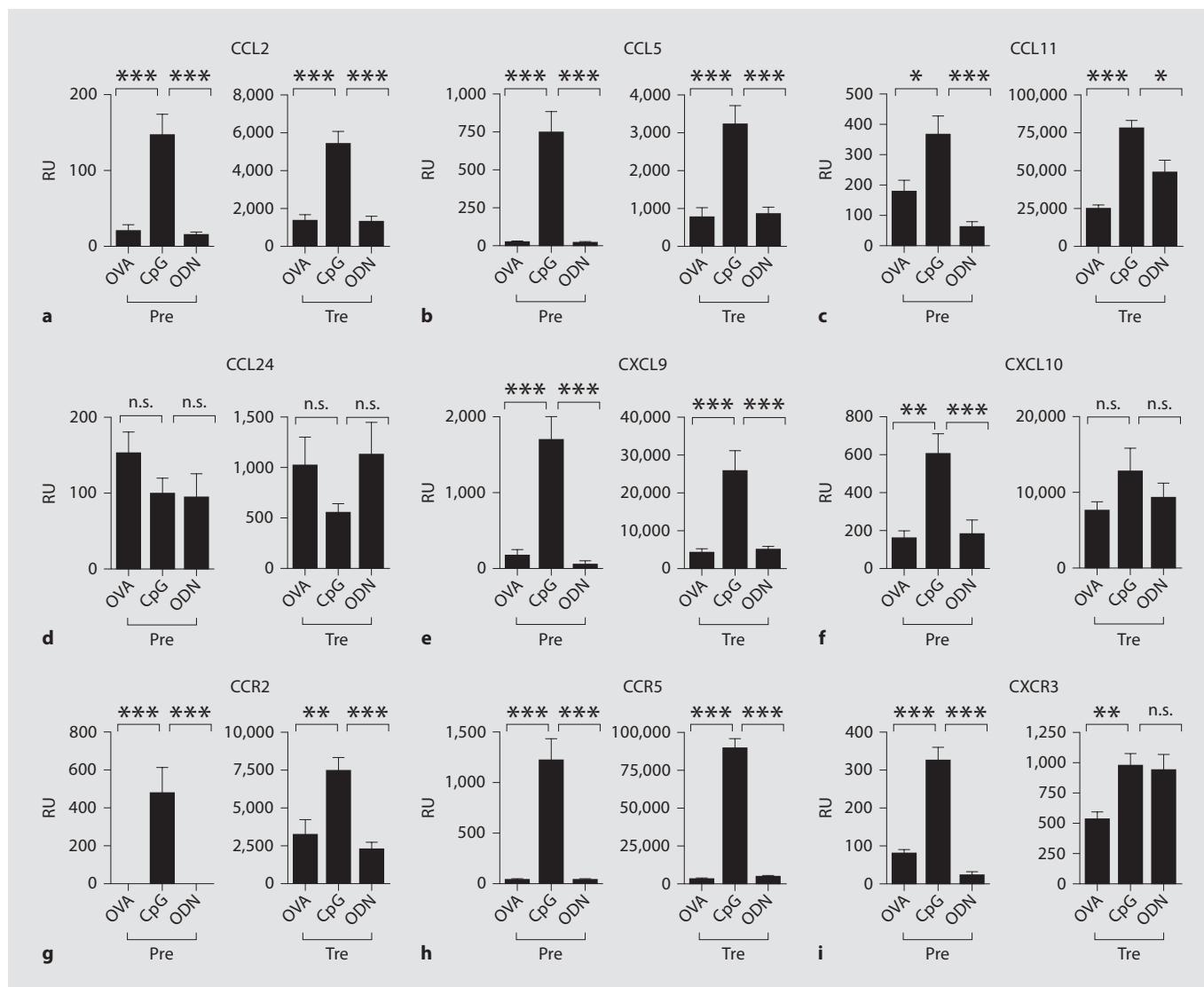
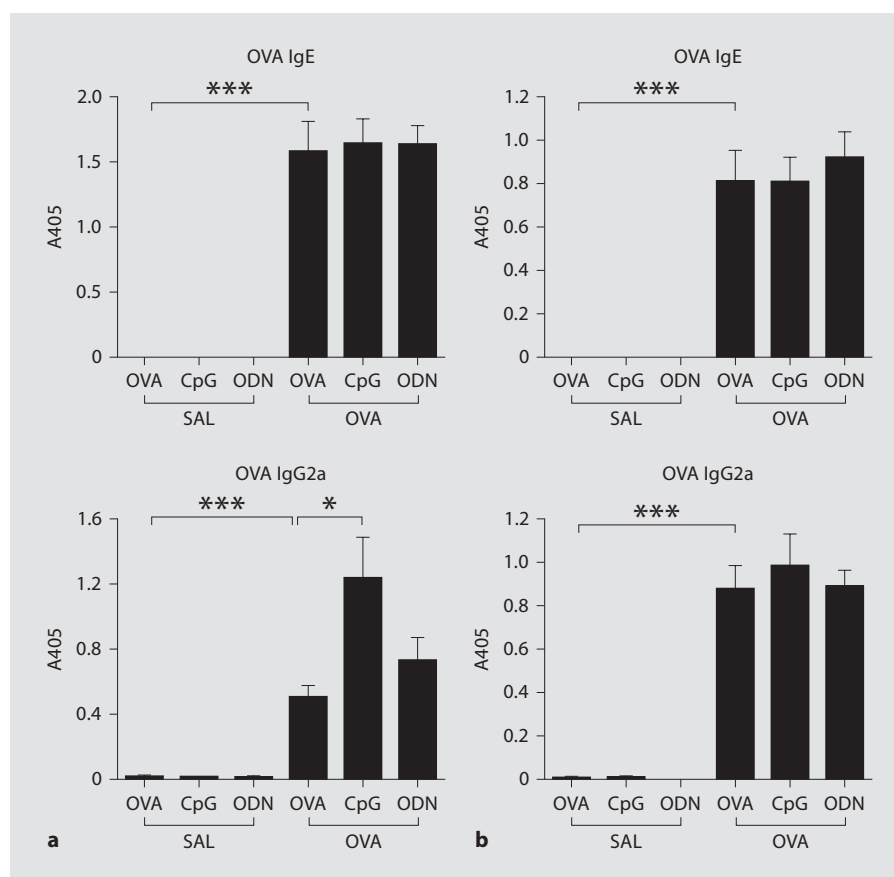


Fig. 4. ISS CpG increases gene expression of proinflammatory and Th1-type chemokines/receptors in inflammatory skin. Real-time quantitative PCR was used to analyze the mRNA expression levels. RU is expressed as fold differences relative to the calibrator. Bars and errors represent mean $\pm$ SEM. $n = 8$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = Not statistically significant; Pre = prevention experiment; Tre = treatment experiment.

a shorter time between the last CpG treatment and sacrifice of the mice, the relative cytokine and chemokine gene expressions were significantly higher compared to the prevention experiment. The effects of CpG on Th2 cytokine production have varied significantly between different investigations. Some studies have shown that CpG downregulates IL-4 and IL-5 along with the induction of Th1-type cytokines, while others have revealed different results. Santeliz et al. [9] have shown that the

suppression of eosinophilic inflammation and airway hyper-reactivity in an asthma model is associated with an increase in CpG induced IFN- γ and IgG2a, but in the absence of suppression of IL-5 and IgG1. In the study by Sur et al. [10], the therapeutic effect of CpG seems to result from an enhanced IFN- γ /IL-4 ratio. The absolute number of cells secreting IL-4 in spleen or lung tissue was, however, not significantly decreased [10].

Fig. 5. CpG treatment enhances allergen-specific IgG2a levels but does not affect IgE levels. **a** OVA-specific IgG2a and IgE levels in the preventive experiments. **b** OVA-specific IgG2a and IgE levels in the treatment experiment. Bars and errors represent mean $\pm$ SEM. $n = 8$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. = Not statistically significant; SAL = saline-treated control mice; OVA = ovalbumin-sensitized mice.



In the present study, the suppressing effect of ISS CpG on Th2-type cytokines and chemokines was very weak and limited only to IL-13 and CCL24 in the treatment experiment. The present study also showed a very minor and mostly insignificant suppression in the number of inflammatory cells and skin thickness in the skin lesions treated by ISS CpG. We speculate that one reason for this incomplete amelioration of skin lesions may be related to the formulation of ISS CpG used in our study. Shirota et al. [20] examined the intratracheal administration of allergen (OVA)-ISS-CpG conjugation (AIC) on cytokine production and clinical parameters in a mouse model of asthma, and found that AIC were 100-fold more efficient than a mix of OVA and ISS CpG in inducing IFN- γ production, and decreasing IL-4 and IL-5 secretion. AIC was also 100-fold more efficient in reducing AHR and lung eosinophilia. Tighe et al. [21] reported that AIC induced much higher IgG2a production and inhibited more efficiently IgE production compared with Ag and ISS CpG mixture. In the present study, ISS CpG and OVA were given separately with ISS CpG injected alone into the

subcutaneous tissue and OVA applied epicutaneously. ISS CpG administration, however, elicited elevated levels of allergen-specific IgG2a (Th1 surrogate marker) antibodies and induction of a major Th1 cytokine IFN- γ and also IFN- γ -regulated chemokines CXCL9 and CXCL10. In contrast to the study by Tighe et al. [21], allergen-specific IgE levels remained unchanged and only a very modest inhibition of Th2 cytokine IL-13 in the skin was seen after CpG administration. Thus, CpG administration induces induction of Th1 cells but is not able to significantly inhibit the Th2 response in the present study settings.

Our study revealed a massive accumulation of F4/80+ cells (F4/80 antigen is present on major subpopulations of resident tissue macrophages and Langerhans cells [22]) in the dermis and upper subcutis after ISS CpG administration. The number of CD11c+ cells was, however, not notably increased in the skin after CpG treatment, excluding the possibility that the F4/80+ cells would correspond to Langerhans cells. In line with results collected from atopic dermatitis patients [23], plasmacytoid dendritic cells were numerous in OVA-induced AD lesions.

However, plasmacytoid dendritic cells were absent in ISS CpG-treated skin, suggesting that ISS CpG administration suppresses their survival or recruitment to the skin. Absence of these regulatory type of dendritic cells may in turn additionally amplify CpG induced skin inflammation and cytokine response. CCR5+ cells, which correspond to Th1 cells and macrophages, were heavily accumulated in CpG-treated skin, confirming the predominance of a Th1-type immune reaction. It is of note that no significant changes in the infiltration of CD3-, CD4- and CD8-positive cells in OVA-exposed mice after CpG treatment were seen. Thus, the major responders to CpG administration are rather F4/80+CCR5+ macrophages than T cells. It is, however, likely that changes in the T cell phenotype (e.g. polarization into Th1 cells) exist although total numbers of T cells in the skin do not change significantly in response to CpG administration. Finally, the expression of regulatory cytokine IL-10 mRNA and protein was dramatically increased in the skin by ISS CpG administration. IL-10 was likely derived from macrophages and monocytes, but not from plasmacytoid dendritic cells or T cells on the basis of the immunohistochemistry results. Local inflammatory reactions induced

by CpG injections have been reported in several studies including in human skin [24–26]. The presence of localized cell recruitment is reported to be necessary for the immunoprotective effects of CpG against viral and bacterial infection since they are activated to produce IFN- α and IFN- γ [24, 25]. However, such cell infiltration is an unwanted side effect in the practice of immunotherapy for allergic diseases.

In conclusion, our results demonstrate that intradermally introduced ISS CpG stimulates innate immune activities in the local skin in an AD murine model, which only weakly reduced OVA-induced atopic dermatitis-like skin inflammation. However, intradermal injection of CpG causes massive local mononuclear cell recruitment. Due to unwanted side effects and at most minor beneficial effects in our model, administration of ISS may not be suitable for the treatment of AD in humans.

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