



Therapeutic effects of streptococcal preparation OK-432 on atopic dermatitis-like lesions in NC/Nga mice: possible shift from a Th2- to Th1-predominance

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KEYWORDS

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Th1/Th2

Summary

Background: The inducement of Th1 cell-mediated immune response, possibly brought about through bacterial stimulation, may serve to control atopic disorders such as atopic dermatitis (AD). The streptococcal preparation, OK-432, has been shown a potent Th1 inducer through the action of IL-12. NC/Nga mice under ordinary conditions have been found to contract dermatitis similar to human AD.

Objective: Examination was made of the therapeutic effects of OK-432 local intra- and/or subcutaneous injections on AD-like lesions in NC/Nga mice.

Methods: Immunohistochemical staining with IL-4/IL-12p40 and CD80/86 and phosphorylated STAT4/p-STAT6 and RT-PCR for IL-4/IL-12p40 and STAT6/STAT4 mRNA was conducted for the evaluation of OK-432 treatment of spontaneous AD-like lesions in NC/Nga mice.

Results: At 5 weeks following injection of OK-432, for treating head and back lesions in NC/Nga mice, 10 of 12 OK-432 treated NC mice were found to have clinically improved quite considerably. On the head and back skin of OK-432-treated mice, IL-12p40/CD80 positive cellular infiltration was conspicuous, in contrast to non-treated mice. IL-4/CD86 positive cellular infiltrates in OK-432-treated mice had decreased significantly more than in non-treated mice and IL-4 mRNA expression was virtually absent in OK-432-treated mice. P-STAT4 positive cells could be seen abundantly present in OK-432-treated mice, and p-STAT6 positive cells were much fewer than in non-treated mice.

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Conclusions: OK-432-treatment appears to induce Th1 cellular response and to down-regulate that of the Th2 pathway in AD-like lesions of NC/Nga mice. The present results demonstrate bacterial components from such *Streptococcus* to likely constitute an effective new therapeutic approach in the treatment of AD.

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1. Introduction

Atopic dermatitis (AD) is clinically characterised by acute and chronic repeated eczematous skin lesions with history of hereditary atopic diathesis and frequently high IgE antibody production. For functionally distinct subsets [1] of CD4 T cells, Th1 and Th2, Th2 predominance in AD lesions [2] through Th2-type cell infiltration has been reported. IL-4 [3] and/or the costimulatory molecule CD86 (B7-2) [4,5] have been shown related to AD lesions. IL-12 [6] and/or the costimulatory molecule CD80 (B7-1) [7] may possibly have important involvement in the Th1 dominant pathway. The Th1 pathway subsequent to bacterial and viral infection may function to alleviate AD [8,9]. Inverse association between response to tuberculin [10] and atopic disorders has been demonstrated, indicating tuberculin positive response [11,12] with Th1 predominance to possibly be a rare atopic disorder.

The streptococcal preparation, OK-432, a penicillin-inactivated and lyophilized preparation of the low-virulence strain, Su *Streptococcus pyogenes*, has been shown to have immunomodulatory and possibly therapeutic properties [13,14]. This preparation has also been found a potent inducer of IL-12 [15]. The immunomodulatory action of OK-432 [15] was stronger than immunopotentiator bacillus Calmette-Guerin (BCG). Of nosological interest is the Th1 pathway by which bacterial or viral agents may possibly down-regulate Th2 components [12] in atopic disorders. Clinical trial results for allergic rhinitis indicate the possibility of symptom inhibition by the bacterial agent, OK-432 [16], which accordingly may have potential clinical application in immunotherapy of atopic disorders. Actually, OK-432 topical application [17] to human AD has been shown very effective for treating this disorder, though considerably more trials should be conducted for definite confirmation of efficacy.

NC/Nga (NC) mice were produced in 1957 by Kondo [18] under ordinary conditions. The NC mice contracted dermatitis similar to human AD, but in the absence of any specific pathogens (SPF), no dermatitis was detected. Mites, *Myobia musculi*, inhabit mice as parasites (SLC Inc., Hamamatsu,

Japan) to possibly lead to AD-like lesions. To date, in most human AD patients, *Dermatophagoides* mites [19] have been shown to cause AD. In conventional NC mice, over-production of Th2 cytokines such as IL-4 and elevated serum IgE antibodies [18] beyond the age of 8 weeks were evident. Such mice should thus be useful as an atopic dermatitis model.

The mechanism for contracting AD lesions and Th2 polarization in mice remains to be determined. Study was made here to determine whether the streptococcal preparation, OK-432, is responsible for the shifting of Th2 predominance to Th1 predominance in NC/Nga mice.

2. Materials and methods

2.1. Mice

Male NC/Nga mice 5 weeks of age were purchased from SLC Japan (Shizuoka, Japan) and maintained under clean conditions. The animals, housed individually, were provided standard chow and mineral water in a conventional room without air control.

2.2. Reagents

The following chemicals were used: 0.5 KE OK-432, Picibanil[®], Chugai Pharmaceutical Ltd., Japan, a lyophilized preparation of group A *Streptococcus*, type 3. Su strain, inactivated with penicillin G. OK-432 concentration was expressed in Klinische Einheit units (KE, clinical unit) with 1 KE of OK-432 equal to 0.1 mg dried streptococci, rat anti-mouse CD80 (B7-1) monoclonal antibodies (Serotec Ltd., Oxford, UK), rat anti-mouse CD86 (B7-2) monoclonal antibodies (Serotec Ltd., Oxford, UK), rat anti-mouse IL-4 monoclonal antibodies (Serotec Ltd., Oxford, UK), rat anti-mouse IL-12p40 monoclonal antibodies (R & D System Inc., USA), and rabbit anti-phosphorylated STAT4 (Zymed Laboratories Inc., USA) and p-STAT6 (Cell Signaling Technology Inc., USA), EnVision peroxidase immunostaining kit (DAKO, Denmark), and mouse IgE ELISA kit (Yamasa Shouyu Co., Chooshi, Japan).

2.3. Evaluation of the severity of dermatitis

This evaluation was made essentially according to the scoring method modified by Hiroi et al. [20] as follows: no symptoms, 0; mild scratching, 1; moderate scratching and/or mild hair loss, 2; severe scratching and/or severe hair loss, 3. Only mice with scores of more than 2 were used in this study.

2.4. Examination procedure

The examination began at 10 weeks of age with the complete set of mice, when mild hair loss corresponding to a score of more than 2 was noted. Mice were used in the following numbers: six mice at 5 weeks of age as the negative control (controls) and six at 10 weeks, four at 13 weeks and six at 15 weeks of age as non-treated mice and four at 3 weeks of OK-432 treatment and six at 5 weeks of treatment for immunohistochemical and RT-PCR study, respectively. A volume of 0.2 ml (0.01 KE/mouse) to 0.25 ml (0.0125 KE/mouse) 0.5 KE OK-432, diluted with 10 ml sterilized saline (optimal dose of OK-432, 0.01 KE as reported by Fujimoto et al. [15] this being the most effective for activating the Th1 pathway), were injected intracutaneously and/or subcutaneously under diethyl ether anesthesia, at six sites from the head to back (Fig. 1) every other day for 3 or 5 weeks. At 5, 10, 13 and 15 weeks, the animals were sacrificed. Skin specimens were obtained from the heads and backs of the animals, and for cases of OK-432 treatment, obtained at just 48 h following final injections, frozen immediately at -20°C in tissue compound Tissue-Tek[®] (Miles Inc., USA) and stored at -80°C .

2.5. Immunohistochemical staining

This process was conducted according to the polymer-complex method (peroxidase labelled polymer conjugated to anti-rabbit and mouse immunoglobulins) of the EnVision system (DAKO, Denmark) for IL-4/IL-12p40, CD80/86 and p-STAT4/p-STAT6. For the

negative control, no polymer second antibodies were used.

Briefly, 7 μm -thick cryostat sections from skin specimens were placed on resin coated glass and fixed in cold acetone for 5 min. The primary antibodies, IL-4, IL-12p40, CD80, CD86, p-STAT4 and p-STAT6, diluted with Tris buffered saline (TBS, pH 7.6) to a final concentration of $25\times$, were incubated with EnVision polymer secondary antibody reagent for 1 h at room temperature. The sections were pre-blocked intracellular peroxidase containing blocking agent that was 0.03% H_2O_2 for 5 min. The sections were incubated with the primary and secondary antibody complex for 1 h at room temperature. After being washed with TBS, all tissue samples were incubated with a chromogenic substance, diaminobenzidine (DAB). Chromogenic reaction was terminated with water followed by hematoxylin counter staining.

2.6. Positive cell number

Positive cells for IL-4/CD86 and IL-12p40/CD80 antibodies were counted with a grid in the eyepiece of a microscope, at a magnification of $100\times$ in the upper dermis just beneath the dermo-epidermal junction. Counting was done in two fields for each specimen, using an eyepiece grid (one field = 1.1926 mm^2) and the result as shown in Fig. 7 expressed as average number of positive cells per specimen.

2.7. Statistical analysis

The Student's *t*-test was carried out for this purpose with $P < 0.05$ taken as significant.

2.8. RT-PCR for TARC, IL-4/IL-12p40 and STAT4/STAT6

2.8.1. mRNA extraction

Total RNA from 50 to 100 mg weight of whole skin samples from head and back of the animals was extracted with manufactureral procedure protocol using Fast RNA kit Green (BIO 101 Inc., USA). RNA

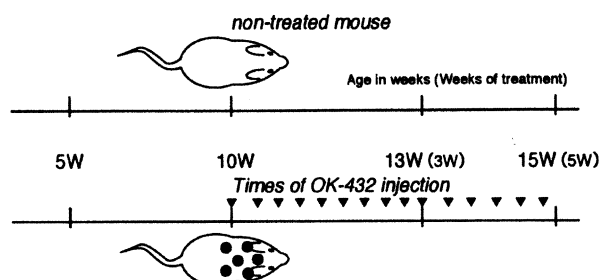


Fig. 1 Time course and sites of OK-432 injection indicated as (●) on mouse back.

concentration was measured and expressed as $\mu\text{g}/\mu\text{l}$ using spectrometer ND-1000 (NanoDrop Technologies Inc., USA).

2.8.2. RT-PCR and primers

Reverse transcription (RT) was carried out with 2 μg of total RNA using oligo primer and a ready to go RT-PCR Beads kit (Amersham Inc., UK). To amplify specific cDNA, the following primers were used for the PCR reactions: mouse IL-4 (371 bp, ref. NM021283, Maxim Technology Inc., USA), 5'-CACTGACGGCACAGAGCTATTGATG, 3'-TCATGGTGCAGCTTTTCGATGAATC, mouse IL-12p40 (412 bp, ref. NML008352), 5'-TTCAATCAGGGCTTCGTAGG, 3'-TAGACAAGCACTGCCGTC, mouse STAT4 (354 bp, ref. NB43780), 5'-CCTGGGTGGACCAATCTGAA, 3'-CTCGCAGGATCGTCAGCGAA, mouse STAT6 (1300 bp, ref. XM012540), 5'-TCACTGGGGGACCGGATCCGGGATCTT, 3'-GCGCTCAGCTCGGGCCTACACGCATTA, mouse TARC (600 bp, [21]), 5'-AACTTCACCACTAGTAGACA, 3'-GGTTCGCTGTAGTGCATAA, mouse GAPDH (320 bp, ref. XM284785), 5'-GCCAAGGCATCCATGACAACCTTTGG, 3'-GCCTGCTTCACCACTTGATGTC. Amplification was conducted for 35 cycles at 94 °C for 1 min (for STAT6, pre-85 °C denaturation) at 55 °C for 1 min and 72 °C for 1 min in all PCR reactions, except for the 40-cycle PCR reaction for TARC and IL-4. PCR amplified fragments were fractionated on 1.5% agarose gel.

3. Results

3.1. Evaluation

OK-432 treatment was concluded to remarkably suppress the development of skin lesions (Fig. 2) in 10 of 12 OK-432-treated mice, none of which developed lesions of grade more than 2. In contrast, all non-treated mice had score of 3 at 15 weeks (Fig. 2). Serum IgE in non-treated NC/Nga mice at 15 weeks exceeded 10×10^4 ng/ml and in mice treated for 5 weeks, slightly decreased (8×10^4 ng/ml) though not significantly. Five-week-old mice samples and negative controls showed no cellular staining by any antibody used. In compounded specimens of skin from some non-treated mice, a few mites could be detected.

3.2. Histology of the skin

The skin of 5 weeks NC control mice was thin with only one to a few layer epidermal cells present (Fig. 3a). In non-treated NC mice 15 weeks of age (Fig. 3b), extensive acanthosis was evident with strong cellular infiltrates in the dermis. Histological

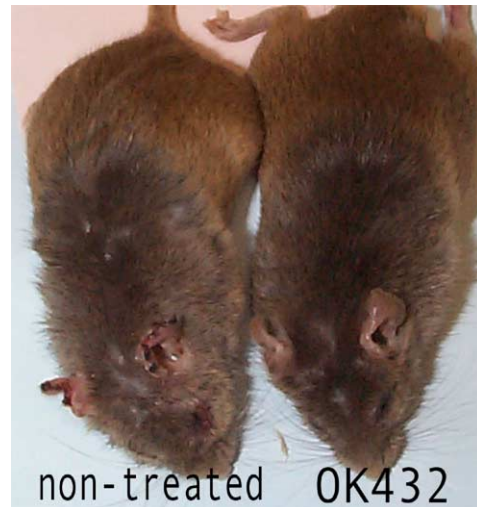


Fig. 2 Clinical features. Remarkable improvement in nearly intact head and back hair with OK-432 treatment at 5 weeks (at 15 weeks age) compared with non-treated NC mice (at 15 weeks age), with severe hair loss and impaired body growth.

examination of OK-432 5-week-treated mice (15 weeks of age) (Fig. 3c) showed reduced mild acanthosis with three to five layers of epidermal cells with mild dermal infiltrates.

3.3. TARC mRNA expression for clinical evaluation (Fig. 4)

All the non-treated NC mice samples (six cases represented) expressed TARC mRNA from their whole skin. But, except only one mouse, that clinically showing no improvement, five in six OK-432 treated mice expressed no TARC mRNA (data shown those of five mice including one non-improved mouse) (Fig. 4).

3.4. Results for non-treated NC/Nga mice

3.4.1. CD86/IL-4

Extensive CD86 positive cellular infiltrates were particularly evident in non-treated mice at 15 weeks, at which time, CD86 positive cells were 92 ± 12 (mean $\pm$ S.D.)/field (Fig. 7).

In non-treated mice at 10 weeks, IL-4 positive cellular infiltrates were abundant, as also noted at 13 and 15 weeks. At 15 weeks (Fig. 5a), the number of IL-4 positive cells of non-treated mice was 58 ± 13 (mean $\pm$ S.D.)/field (Fig. 7).

3.4.2. CD80/IL-12p40

CD80 positive cell number in non-treated mice at 10, 13 and 15 weeks (34 ± 12 (mean $\pm$ S.D.)/field)

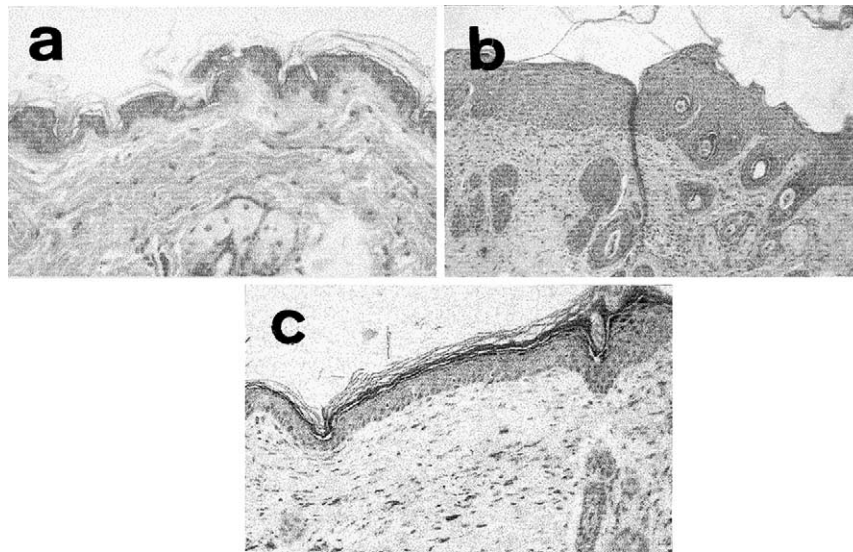


Fig. 3 Histology of skin. (a) Non-affected NC mice at 5 weeks of age showing thin epidermis and no cellular infiltration. (b) Non-treated NC mouse skin at 15 weeks of age showing extensive acanthosis with cellular infiltrates but (c) OK-432 treated skin with reduced acanthosis even with the presence of dermal infiltrates.

was basically the same (Fig. 7). No CD80-positivity in the epidermis could be found in any sample.

A small number of IL-12p40 positive cells were noted to infiltrate the upper dermis of 10-week-old mice. At 13 weeks, the number of IL-12p40 positive cells was the same as at 10 weeks. IL-12p40 positive cell number (Fig. 6a) in 15-week-controls showed no change (14 ± 4 (mean $\pm$ S.D.)/field) (Fig. 7).

3.5. Results for OK-432-treated NC/Nga mice

3.5.1. CD86/IL-4

The value 28 ± 7 (mean $\pm$ S.D.)/field of CD86 positive cells at 5-week-treatment was considerably less than that for non-treated mice ($P < 0.05$) (Fig. 7).

In OK-432-treated mice for 3 and 5 weeks (24 ± 9 (mean $\pm$ S.D.)/field), IL-4 positive cellular infiltrates (Fig. 5b) decreased significantly ($P < 0.05$), compared to non-treated mice (Fig. 7).

3.5.2. CD80/IL-12p40

From 3-week (38 ± 7 (mean $\pm$ S.D.)/field) to 5-week OK-432 treatment (46 ± 8 (mean $\pm$ S.D.)/field), CD80 positive cellular infiltrates increased, but the extent was essentially the same as in the non-treated group ($P > 0.05$) (Fig. 7).

In OK-432 5-week-treated mice, many IL-12p40 positive cells (Fig. 6b) (43 ± 9 (mean $\pm$ S.D.)/field) were present (Fig. 7), and their number increased ($P < 0.05$), beyond that in non-treated mice.

3.5.3. p-STAT6/p-STAT4 immunohistochemical staining

In 15-week non-treated mice, extensive p-STAT6 positive cell infiltration into lesional upper dermis was apparent (Fig. 8a). In non-treated mice at 15 weeks, p-STAT4 positive cells were seen infrequently or were absent (Fig. 8c).

At the sites of skin lesions in OK-432 treated mice, p-STAT6 positive cell infiltration (Fig. 8b) was slight. OK-432-treated mice showed many p-STAT4 positive cells (Fig. 8d).

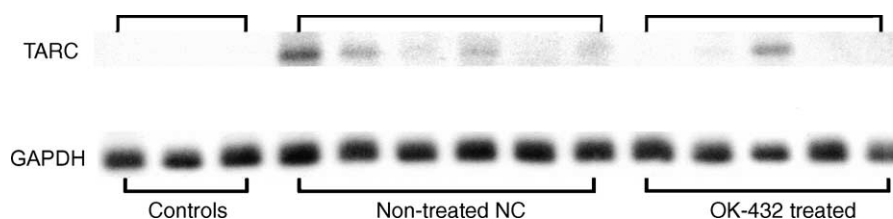


Fig. 4 Th2 chemokine TARC mRNA evaluation. All non-treated NC mice (represented six mice data) express in positively TARC mRNA in whole skin but all OK-432-treated mice, except one without improvement, and non-affected (5 weeks of age) mice, no TARC mRNA expression present by RT-PCR methods.

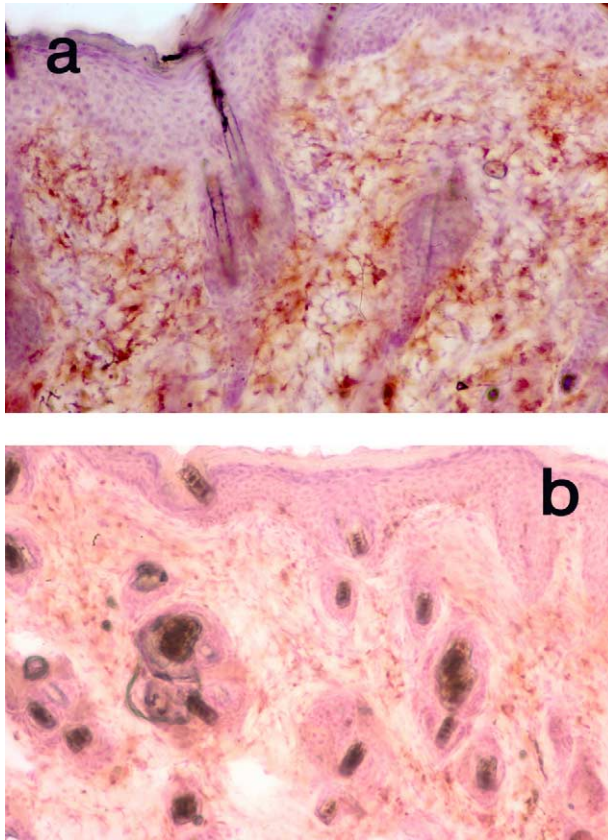


Fig. 5 Immunohistochemical staining of IL-4. (a) Extensive IL-4 positive cells in 15-week non-treated mice (original magnification, 100 $\times$). (b) Reduced number of IL-4 positive cells in OK-432 5-week-treated mice (original magnification, 100 $\times$).

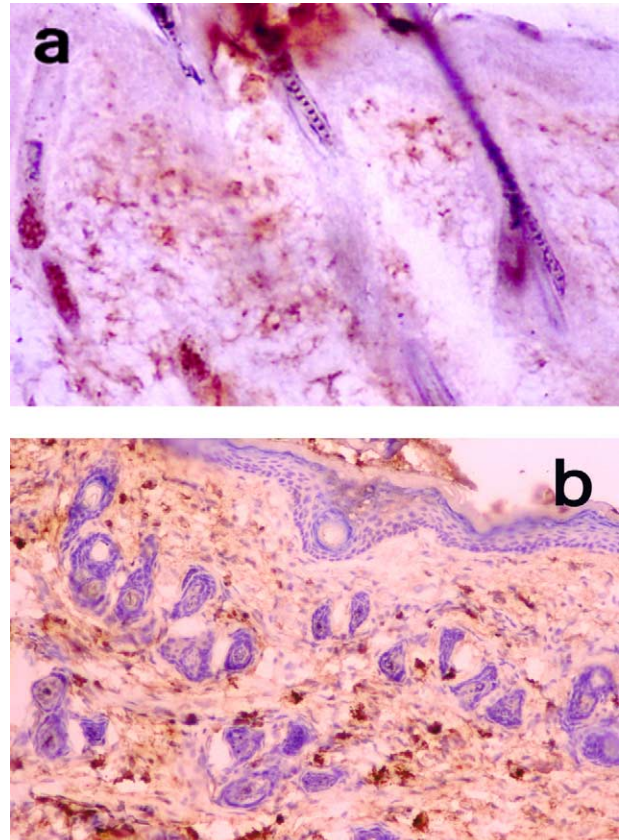


Fig. 6 Immunohistochemical staining of IL-12p40. (a) Moderate infiltration of IL-12p40 positive cells in 15-week non-treated mice (original magnification, 100 $\times$). (b) Many IL-12p40 positive cells in OK-432 5-week-treated mice (original magnification, 100 $\times$).

3.6. RT-PCR analysis

3.6.1. IL-4/IL-12p40 mRNA (Fig. 9)

IL-4 mRNA expression in whole skin may be very slight, and thus, would not differ significantly in any of the three groups. In all OK-432-treated mice, IL-4 mRNA expression was virtually absent. IL-12p40 mRNA expression was essentially the same for the control, non-treated and OK-432-treated mice groups, though expression in two OK-432 treated mice could weakly detected (Fig. 9).

3.6.2. STAT6/STAT4 mRNA expression (Fig. 10)

STAT4 mRNA expression in OK-432-treated mice was somewhat greater compared to non-treated mice, but that of STAT6 mRNA in OK-432-treated mice had been reduced considerably compared to those of non-treated. STAT6 mRNA is shown by the present results to be expressed in control mice, though STAT6 expresses are present in normal tissue and cells [22]. The reason for this may be clarified through additional study (Fig. 10).

4. Discussion

It is clearly evident from the present study that at 5 weeks following injection of the bacterial agent, OK-432, head and back skin lesions clinically improved quite remarkably in 10 of 12 OK-432 treated NC/Nga mice. Serum Th2 chemokine TARC has been taken as indicator of the clinical severity of AD patients [23] and high expression of TARC in basal epithelium of NC mice [24] with skin lesions is consistent with the findings of the present study in which TARC mRNA expression from whole skin from the head and back decreased in the improved AD-like lesions of OK-432 treated mice. Immunohistochemical staining results indicated cellular infiltrates at OK-432 treated sites to undergo a shift from Th2 predominance with abundant IL-4/CD86 to Th1 predominance with abundant IL-12p40/CD80, compared to non-treated NC/Nga mice. In NC/Nga mice, systemic deficient Th1 response to bacterial stimulation has recently been shown to lead to Th2-dominance with excessive production of IgE and AD-like dermatitis [25]. Th2 response is required

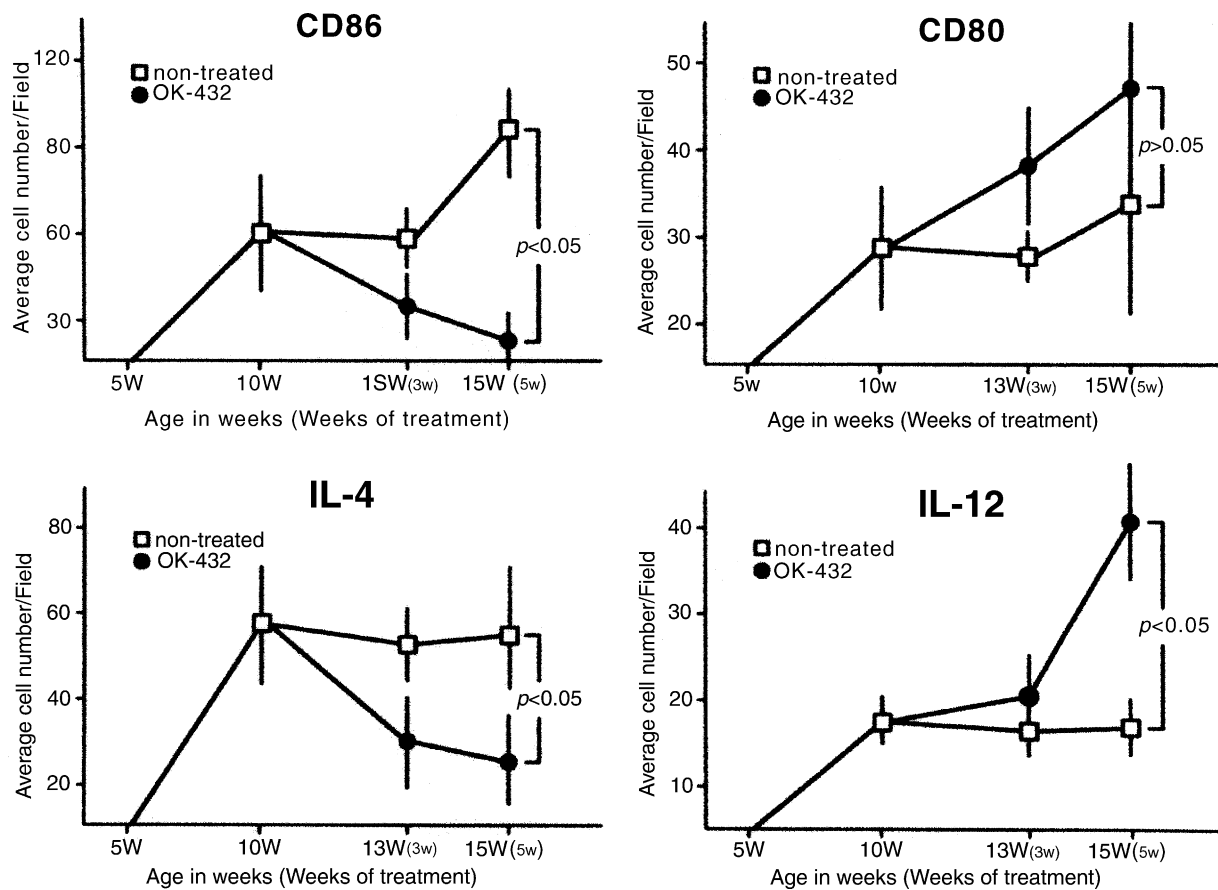


Fig. 7 Change in numbers of cells positive to CD80/CD86 and IL-4/IL-12p40 antibodies during course of OK-432 treatment and non-treated NC/Nga mice.

for the production of antibodies that react various antigens such as mites and pollen. Th1 response is essential for host defense against bacterial [12] and/or viral infection [26]. Although Th1 and Th2 immune response are considered to crossregulate each other, the results of this study indicate the down-regulation of Th2 components through induced Th1 response to Th1-inducer OK-432 application to possibly be one means for the control of atopic disorders.

The costimulatory molecule, CD86 (B7-2), present in activated T/B cells, resting monocytes and Langerhans cells [27], is expressed in AD lesional skin [4]. This molecule may thus bear some significant relation to AD lesions [4,5] CD86 over-expression may augment antigen content in the skin with consequently excessive cutaneous DTH (delayed type hypersensitivity) [28] through CD4 T-lymphocyte activation. During OK-432 treatment, considerable reduction in CD86 positive cellular infiltration compared to non-treated mice was noted, suggesting that initial activation of the Th2 inflammatory pathway through the CD86 molecule may be possibly inhibited by OK-432 application. CD80-positive cel-

lular infiltrates in non-treated and OK-432-treated mice, in contrast to CD86, showed no distinct differences during the course of application. The present results and recent observations would suggest CD86 and CD80 to have overlapping functions [7] and the amplified expression of IL-12 in dermal cellular infiltrates to possibly be mediated through decrease in CD86-positive cellular infiltration.

IL-12 production, which has a central role in promoting Th1 response, was the result of cell activation stimulated by agents such as bacteria or bacterial products [15]. BCG has been shown to induce Th1 polarized response regulated by IL-12 and IFN- γ production and OK-432, a potent multi-cytokine inducer, has been found a specifically strong inducer of IL-12 [15]. This, in turn, may augment the innate immune response of Th1 by stimulating IL-12. Atopic patients may be incapable of sufficient IL-12 production, this possibly resulting in enhanced chronic allergic inflammation. The importance of IL-12 in regulating allergic immune response is substantiated by considerable data obtained with animal models for studying allergic airway inflammation [29] and results from studies on

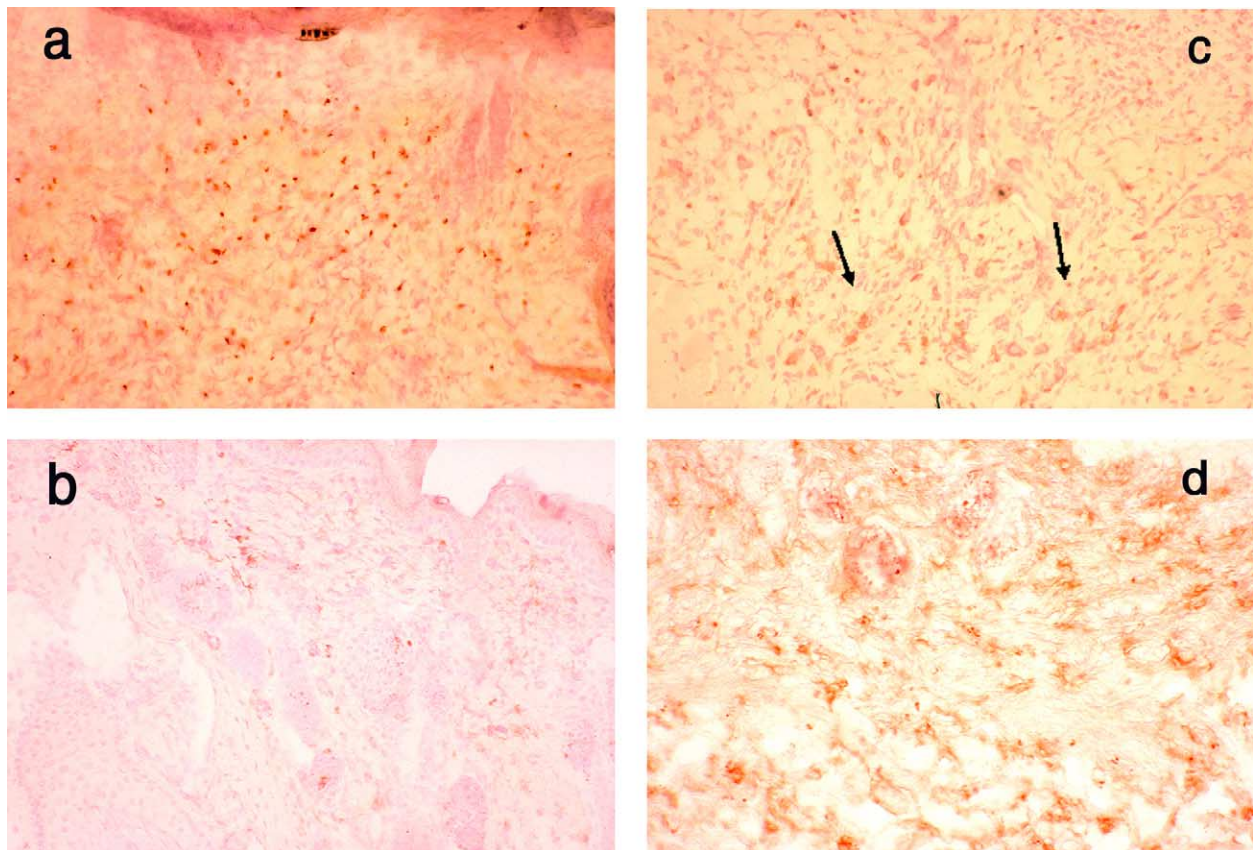


Fig. 8 p-STAT6/p-STAT4 immunohistochemical staining. (a) Extensive p-STAT6 positive cellular infiltrates particularly evident in non-treated mice at 15 weeks. (b) In OK-432-treated NC mice, p-STAT6 positive cellular infiltrates have decreased significantly, compared to non-treated mice (original magnification, 100 $\times$). (c) p-STAT4 positive cells (arrows) were few in non-treated mice but (d) extensive p-STAT4 positive cellular infiltrates particularly evident in Ok-432-treated mice at 5 weeks treatment (original magnification, 200 $\times$).

varicella zoster virus infections [26] in human AD indicate Th1 cytokine IL-12 to be capable of preventing antigen-induced hyper-response and Th2 cytokine expression. The Th2 cytokines, IL-4 and IL-5, are responsible for IgE antibody production and IL-4 may be required for IgE synthesis [30]. IL-12

suppresses the synthesis of IgE from lymphocytes stimulated by IL-4 in humans [31]. The inducement of IL-12 positive cellular infiltration subsequent to OK-432 administration as well as reduction in IL-4 positive cell infiltration may serve to very effectively reduce AD lesions by lessening the polariza-

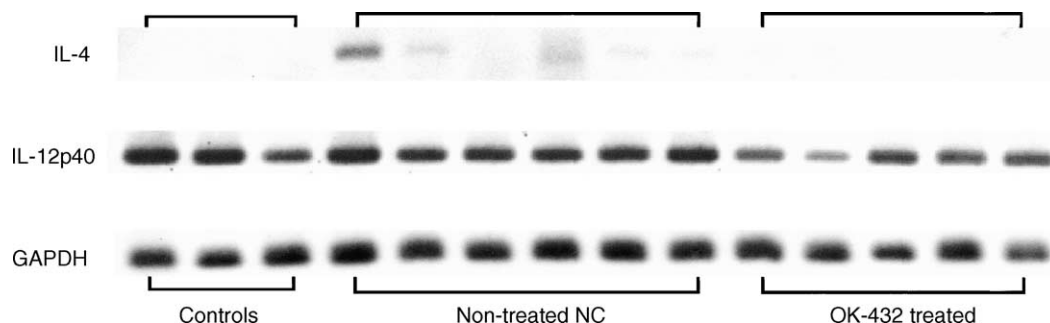


Fig. 9 mRNA expression for IL-4/IL-12p40. Expression of IL-4 mRNA in whole skin detected in all non-treated NC mice, so very slight that expression in the three groups, and that in OK-432-treated mice could not be detected. Expression of IL-12p40 basically the same for control, non-treated and OK-432-treated groups. Even that in OK-432-treated mice was weak in some cases.

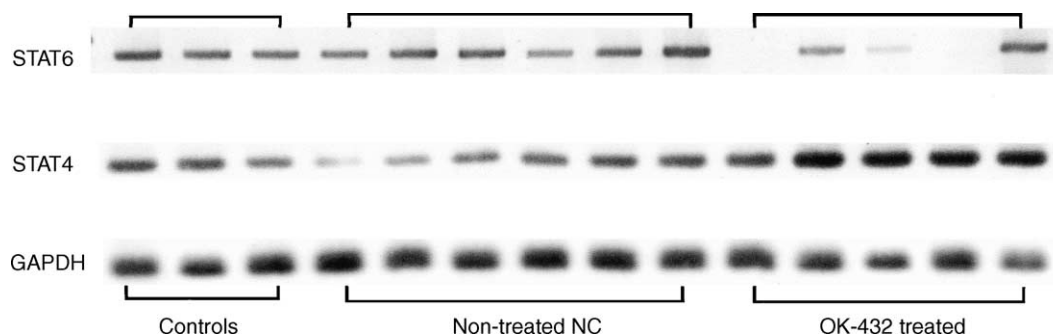


Fig. 10 mRNA expression for STAT6/STAT4. Strong STAT6 mRNA expression in all non-treated NC mice but in OK-432-treated NC mice, expression was reduced. STAT4 mRNA in OK-432-treated mice can be seen greater than in non-treated mice.

tion of Th response toward Th2-dominance. IL-4 is promoted mostly by the costimulatory molecule [32], CD86, while CD80 serves only slightly to do so. IL-4 mRNA expression in all OK-432 treated mice, even with very little IL-4 mRNA expression, could not be detected in whole skin by RT-PCR, suggesting OK-432 application to possibly reduce Th2 regulatory response through IL-4 via STAT6 pathways.

Signal transducer and activator of transcription (STAT) 4 is a key molecule in intracellular signal transduction activated by IL-12 stimulation [33,34]. The low response of NC/Nga mouse [35] to IL-12 as a result of reduced phosphorylation of STAT4 may lead to defective IFN- γ production and may be one cause for high IgE production. Results with STAT4-deficient mice [36] demonstrate STAT4 essential for mediating response to IL-12 in lymphocytes and regulating the differentiation of Th1 and Th2 cells. STAT6 [33] regulates response to allergic stimuli in the development of Th2 cells. STAT6 activation must be tyrosine-phosphorylated by JAK kinase, followed by nuclear translocation. Down-stream signaling pathways from the IL-4R complex with IL-13R include activation of STAT6 signaling pathways. IL-4R and STAT6 positive cells are considerably fewer in non-atopic than atopic subjects [37]. Immunohistochemical staining in this study indicated cellular infiltration with IL-4 and p-STAT6 expression to be significantly less in OK-432-treated mice and IL-12p40 and p-STAT4 positive cellular infiltrates in OK-432-treated mice, to be remarkably more than in non-treated mice. Results obtained by p-STAT6/p-STAT4 immunohistochemistry and mRNA expression, indicate OK-432 application to possibly induce inhibition of Th2 response and cause a shift to Th1 predominance. The inducement of IL-12p40 positive cellular infiltrates and enhanced expression of STAT4 mRNA were evident, but IL-12p40 mRNA expression from whole skin of OK-432 treated mice was essentially

the same or less than for the controls and non-treated groups. IL-12p40 mRNA expression is considerable in keratinocytes [38], where IL-12p40 proteins may not be detectable in the epidermis by immunohistochemistry and this may possibly explain the above.

Streptococcal preparation OK-432 is shown by the present study to bring about abundant IL-12p40/CD80 as well as Th1 predominance in NC/Nga mice skin lesions. OK-432 would appear from the present results effective for treating AD. More effective therapy for up-regulating Th1 response through IL-12 production in atopic disorders should be established.

Antimicrobe peptides play key roles in the innate immune system and provide the first line of defense against microbe invasion of the skin. Cytokine production occurs early through innate immune response towards infected microbes such as *Streptococcus*. Okamoto et al. [39] showed lipoteichoic acid (LTA) to be a major component of OK-432. LTA and/or DNA from *Streptococcus* microbes may induce innate immunity and recently, lipoteichoic acid (LTA), products of *Streptococcus* have been shown to possibly induce activation of the toll-like receptor 4/MD-2 innate pathway [40], which in turn, activates Th1 type immunoregulatory response. Reduction in Th2 response in AD lesions through innate immunoregulatory pathways induced by bacterial agents such as OK-432 should be studied in detail and the results used to establish safer and more effective methods for the treatment of AD.

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References

- [1] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature* 1996;383:787–93.
- [2] Grewe M, Bruijnzeel-Koomen CA, Schopf E, Thepen T, Langeveld-Wildscheld AG, Ruzicka T, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 1998;19:359–61.
- [3] Renz H, Jujo K, Bradley J, dementico J, Gelfand EW, Leung DV. Enhanced IL-4 production and IL-4 receptor expression in atopic dermatitis and their modulation by interferon- γ . *J Invest Dermatol* 1992;99:403–8.
- [4] Ohki O, Yokozeki H, Katayama I, Umeda T, Azuma M, Okumura K, et al. Functional CD86 (B7-2/B70) is predominantly expressed on Langerhans cells in atopic dermatitis. *Br J Dermatol* 1997;136:838–45.
- [5] Jirapongsananuruk O, Hofer MF, Trumble AE, Norris DA, Leung DY. Enhanced expression of B7.2 (CD86) in patients with atopic dermatitis: a potential role in the modulation of IgE synthesis. *J Immunol* 1998;160:4622–7.
- [6] Trinchieri G. Interleukin-12: cytokine produced by antigen-presenting cells with immunoregulatory function in the generation of T-helper type 1 and cytotoxic lymphocytes. *Blood* 1994;84:4008–27.
- [7] Natesan M, Razi-Wolf Z, Reiser H. Costimulation of IL-4 production by murine B7-1 and B7-2 molecules. *J Immunol* 1996;156:2783–91.
- [8] Farooqi IS, Hopkin JM. Early childhood infection and atopic disorder. *Thorax* 1998;53:927–32.
- [9] Boner AL, Valletta EA, Bellanti JA. Improvement of atopic dermatitis following natural measles virus infection. Four case reports. *Ann Allergy* 1985;55:605–8.
- [10] Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorders. *Science* 1997;275:77–9.
- [11] Gruber C, Kulig M, Bergmann R, Guggenmoos-Holzmann I, Wahn U. Delayed hypersensitivity to tuberculin, total immunoglobulin E, specific sensitization and atopic manifestation in longitudinally followed early Bacille Calmette-Guerin-vaccinated and non-vaccinated children. *Pediatrics* 2001;107:e36.
- [12] Kinjo Y, Kawakami K, Uezu K, Yara S, Miyagi K, Koguchi Y, et al. Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40. *J Immunol* 2002;169:323–9.
- [13] Kimura I, Ohnoshi T, Yasuhara S, Watanabe T, Sugiyama M, Hirai K. Immunotherapy in human lung cancer using *Streptococcus* agent OK-432. *Cancer* 1976;37:2201–3.
- [14] Inui S, Asada H, Wataya M, Kaneda M, Miyamoto T, Itami S, et al. OK-432 therapy for recalcitrant warts. *J Dermatol* 1997;24:301–5.
- [15] Fujimoto T, Duda RB, Szilvasi A, Chen X, Mai M, O'Donnem MA. Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state. *J Immunol* 1997;158:5619–26.
- [16] Enomoto M, Shirakawa T, Dake Y, Hopkin JM. Preliminary trial for atopy intervention with microbe extracts. *Clin Immunol* 1999;32:454–9 (in Japanese).
- [17] Horiuchi Y. Effect of streptococcal preparation OK-432 for atopic dermatitis. In: Proceedings of the 67th Shizuoka regional meeting of Japanese dermatological association. 2000.
- [18] Matsumoto M, Ra C, Kawamoto K, Watanabe P, Tsukamoto T. IgE hyper production through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga mice, a model for human atopic dermatitis. *J Immunol* 1999;162:1056–63.
- [19] Okada M, Terui T, Honda M, Tanaka M, Chikama R, Tabata N, et al. Cutaneous late phase reaction in adult atopic dermatitis patients with high serum IgE antibody to *Dermatophagoides farinae*: correlation with IL-5 production by allergen-stimulated peripheral blood mononuclear cells. *J Dermatol Sci* 2002;29:73–84.
- [20] Hiroi J, Sengoku T, Morita K, Kishi S, Sato S, Ogawa T, et al. Effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga mice. *Jpn J Pharmacol* 1998;76:175–83.
- [21] Hiroyama T, Iwama A, Nakamura Y, Nakauchi H. Fractalkine shares signal sequence with TARC: gene structures and expression profiles of two chemokine genes. *Genomics* 2001;75:3–5.
- [22] Nishio H, Matsui K, Tsuji H, Tamura A, Suzuki K. Immunolocalisation of the janus kinases (JAK)-signal transducers and activators of transcription (STAT) pathway in human epidermis. *J Anat* 2001;198:581–9.
- [23] Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001;107:535–41.
- [24] Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999;104:1097–102.
- [25] Habu Y, Seki S, Takayama E, Ohkano T, Koike Y, Ami K, et al. The mechanism of a defective IFN-gamma response to bacterial toxins in an atopic dermatitis model, NC/Nga mice and the therapeutic effect of IFN-gamma, IL-12, or IL-18 on dermatitis. *J Immunol* 2001;166:5439–47.
- [26] Fujimura T, Yamanashi R, Masuzawa M, Fujita Y, Katsuoka K, Nishiyama S, et al. Conversion of the CD4⁺ T cell profile from TH2-dominant type to TH1-dominant type after varicella-zoster virus infection in atopic dermatitis. *J Allergy Clin Immunol* 1997;100:274–82.
- [27] Rattis FM, Peguet-Navarro J, Staquet MJ, Dezutter-Dembuyant C, Curtellemont P, Rodzinski G. Expression and function of B7-1 (CD80) and B7-2 (CD86) on human epidermal Langerhans cells. *Eur J Immunol* 1996;26:449–53.
- [28] Gaspari AA, Burns R, Nasir A, Ramirez D, Barth RK, Haidaris CG. CD86 (B7-2), but not CD80 (B7-1), expression in the epidermis of transgenic mice enhances the immunogenicity of primary cutaneous *Candida albicans* infections. *Infect Immun* 1998;66:4440–9.
- [29] Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Will-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995;182:1527–36.
- [30] Romagnani S, Maggi E, Del Prete GF, Parronchi P, Macchia D, Tiri A, et al. Role of interleukin 4 and gamma interferon in the regulation of human IgE synthesis: possible alterations in atopic patients. *Int Arch Allergy Appl Immunol* 1989;88:111–3.
- [31] Kishiwa M, Gately M, Gaubler U, Chizzonite R, Fargeas C, Despesse G. Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J Clin Invest* 1992;90:262–6.
- [32] Freeman G, Boussiotis VA, Anumanthan A, Bernstein GM, Ke XY, Rennert PD, et al. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* 1996;2:532–42.
- [33] Wurster AL, Tanaka T, Grusby MJ. The biology of Stat4 P-41 and Stat6. *Oncogene* 2000;19:2577–84.

- [34] Schindler H, Lutz MB, Rollinghoff M, Bogdan C. The production of IFN- γ by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J Immunol* 2001;166:3075–82.
- [35] Matsumoto M, Itakura A, Tanaka A, Fujisawa C, Matsuda H. Inability of IL-12 to down-regulate IgE synthesis due to defective production of IFN- γ in atopic NC/Nga mice. *J Immunol* 2001;167:5955–62.
- [36] Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996;382:174–7.
- [37] Christodoulopoulos P, Cameron L, Nakamura Y, Lemiere C, Muro S, Dugas M, et al. TH2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activation of transcription 6 expression. *J Allergy Clin Immunol* 2001;107:586–91.
- [38] Yawalker N, Limat A, Brand CU, Braathen LR. Constitutive expression of both subunits of interleukin 12 in human keratinocytes. *J Invest Dermatol* 1996;106:80–3.
- [39] Okamoto M, Ohe G, Oshikawara T, Furuichi S, Nishikawa H, Tano T, et al. Enhancement of anti-cancer immunity by a lipoteichoic acid related molecule isolated from a penicillin-killed group A *Streptococcus*. *Cancer Immunol Immunother* 2001;50:408–16.
- [40] Okamoto M, Oshikawara T, Tano T, Ohe G, Furuichi S, Nishikawa H, et al. Involvement of Toll-like receptor 4 signaling in interferon- γ production and anti-tumor effect by streptococcal agent OK-432. *J Nat Cancer Inst* 2003;95:316–26.

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