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Heat-killed BCG and *Mycobacterium kansasii* Ag85B combined vaccination ameliorates dermatitis in a mouse model of atopic dermatitis by inducing regulatory T cells

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Running head: hkBCG and Ag85B vaccination ameliorates atopic dermatitis

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Bulleted statements:**What's already known about this topic?**

Secretory protein Ag85B and *Bacillus Calmette-Guerin* (BCG) are Th1 cytokine inducers.

KCASP1Tg were recognized as AD model mice.

What does this study add?

Mycobacterium kansasii Ag85B protein and heat-killed BCG combined vaccination ameliorates dermatitis in KCASP1Tg by inducing regulatory T cells.

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Abstract

Background: Atopic dermatitis (AD) is a recurrent inflammatory skin disease characterized by dominant Th2-cytokine response. *Bacillus Calmette-Guerin* (BCG) has been used for preventing tuberculosis, and is regarded as a strong Th1 cytokine inducer. Ag85B is a secretory protein present in mycobacterium species that induces Th1 cytokine production.

Objectives: We investigated the effects of combined vaccination of heat-killed BCG (hkBCG) and *Mycobacterium kansasii* Ag85B in an AD mouse model.

Methods: As AD model, K14 promoter-derived caspase-1 overexpressing mice (KCASP1Tg) were used. The mice received a combination therapy: hkBCG at the 3rd week of age and Ag85B twice a week for 11 weeks from the 4th week, Ag85B mono-therapy from the 4th week, hkBCG mono-therapy at the 3rd week, or control saline. Areas of skin lesions, cytokine mRNA expression and serum IL-18 and IgE levels were analyzed. Foxp3⁺ regulatory T cells (iTreg), IL-10 producing T cells (Tr1), and IFN- γ / IL-4/ IL-17 producing T cells were evaluated in the spleen.

Results: Saline treated mice and hkBCG mono-therapy mice spontaneously developed severe dermatitis. However, combined therapy with hkBCG and Ag85B significantly suppressed the development of skin lesions and mast cell infiltrations. Elevations of the serum IgE and IL-18 levels were significantly suppressed with combined therapy. Mice treated with hkBCG and Ag85B had normal number of iTreg in the spleen, and decreased number of both IL-4 and IL-17 producing CD4⁺T cells. The effect of Ag85B mono-therapy was limited.

Conclusions: Combined vaccination with hkBCG and Ag85B decreases AD skin lesions by inducing regulatory T cells; suggesting that this vaccination as a potent and novel therapeutic strategy for AD.

Key words: atopic dermatitis, KCASP1Tg, Ag85B, heat-killed BCG, regulatory T cells

Introduction

Atopic dermatitis (AD) is a common allergic skin disorder characterized by recurrent pruritic inflammatory skin lesions. Several factors including immunological imbalance are involved in the pathogenesis of AD. Of these, the Th2 cytokines IL-4, IL-5, IL-13, and Ag-specific IgE play important roles. A dominant Th2 cytokine response has been described in AD. However, IFN- γ produced by Th1 cells is also involved in the mechanism of chronic AD¹. In addition to Th1/Th2 responses, increased number of T cells producing IL-17² and IL-10³ have been shown to play critical roles, and infiltration of IL-17 producing Th17 cells has been described in AD skin lesions².

The prevalence of AD is steadily increasing, especially in industrialized countries⁴. The “hygiene hypothesis” is an attractive theory that was proposed to explain the increased incidence of allergic diseases⁵. Epidemiological studies have demonstrated an inverse correlation between positive tuberculin responses and subsequent development of atopic disorder⁶. There are many studies suggesting that not only bacterial infections, but also bacterial antigen exposure are able to inhibit the development of allergic diseases⁷.

Bacillus Calmette-Guerin (BCG), a live attenuated *Mycobacterium bovis* (*M. bovis*), is commonly used for the vaccination against tuberculosis. BCG is also recognized as a strong Th1 immune response inducer⁸. Live BCG settlement in the lung prevented allergen-induced airway eosinophilia and the development of airway hyperresponsiveness in mice^{9,10}. The microbial stimulation induces regulatory T cells (Treg); suppressing both Th1 and Th2 types of inflammation¹¹. *M. vaccae* significantly reduces allergic pulmonary inflammation by inducing Treg that secretes IL-10 and TGF- β ¹². Inducible Foxp3⁺CD25^{high}CD4⁺ regulatory T cells (iTreg) and inducible allergen specific IL-10 producing CD4⁺ T cells (Tr1) have been implicated in the suppression of allergic disorders¹³⁻¹⁵.

Ag85B is a 30-kDa major secretory protein present in mycobacterium species. Immunization with Ag85B DNA¹⁶ or purified Ag85B protein¹⁷ induces a strong antigen-specific CD4⁺ T cell and IFN- γ response¹⁸. Recently, we reported that plasmid DNA encoding Ag85B derived from *M. kansasii* inhibits immediate type hypersensitivity responses in association with increased iTreg in the skin¹⁹.

We have previously established a keratin14 promoter-driven caspase-1 transgenic mouse (KCASP1Tg) that overexpresses caspase-1 in keratinocytes²⁰. KCASP1Tg constitutively secretes endogenous IL-1 β and IL-18. The dermatitis is characterized by acute erosive lesions followed by chronic lichenoid lesions. The findings fulfill the diagnostic criteria of Haniffin & Rajka²¹ and that of the U.K. working party²² except for the history of

asthma or hay fever. Therefore, we used the KCASP1Tg mice as AD models.

In the present study, we investigated whether the combination of hkBCG and *M. kansasii* Ag85B vaccination suppresses AD-like skin lesions in KCASP1Tg. We found that this vaccination ameliorates skin lesions and suppressed cytokine production. These observations suggest that combined administration of hkBCG and Ag85B is a promising therapeutic approach for AD.

Materials and Methods

Mice

3-week-old female KCASP1Tg and age-matched wild-type C57/BL6 mice (Japan SLC, Shizuoka, Japan) were used in the experiments. KCASP1Tg mice were used as models of AD^{20, 23, 24}. All experimental procedures were conducted in accordance with Guidelines for Animal Experiment of Mie University and the protocol was approved by the Ethical Committee of Mie University.

Vaccination with heat-killed BCG and *Mycobacterium kansasii* Ag85B

M. bovis BCG (Japan BCG Laboratory, Tokyo, Japan) was used. Heat-killed BCG (hkBCG) was prepared by heating at 121°C for 20 min and lyophilized in normal saline. 100µl of normal saline containing 10µg of hkBCG was administered by intraperitoneal injection (i.p.) at 3rd week of age. Control mice and Ag85B-alone treated mice received physiological saline.

The recombinant Ag85B protein from *M. kansasii* was a generous gift from Maruho Pharmaceuticals (Kyoto, Japan), and 100µl of Ag85B (100µg/ml) was administered by i.p. twice a week from 4th to 14th week of age. Control mice and hkBCG mono-therapy mice received physiological saline. All products were kept frozen until the use for injection.

Study groups

Mice were divided into four groups (n = 10 in each group): 1) hkBCG/Ag85B combined therapy group: KCASP1Tg mice were initially treated with hkBCG by i.p. on 3rd week, followed by Ag85B i.p. twice a week for 11 weeks from the 4th week; 2) Ag85B monotherapy group: KCASP1Tg mice were treated with Ag85B i.p. twice a week for 11 weeks from the 4th week; 3) hkBCG monotherapy group: KCASP1Tg mice were treated with hkBCG by i.p. on 3rd week; 4) Control group: KCASP1Tg mice treated with physiological saline i.p. The therapeutic regimen is depicted in Fig. 1a.

Clinical Manifestations

Skin manifestations were expressed as the percentage of areas with skin lesions of the total-body surface. Skin lesions and the total body surface area were evaluated by marking on lucent plastic film, and they were examined on weeks 4, 5, 6, 8, 10, 12, and 14.

End-point evaluation

Sampling of blood, skin, and organs was performed at 14th week of age. About 10×10mm forehead skin was excised and divided into two specimens; one was for histopathological evaluation and another for mRNA extraction. Serum samples were stored at -20°C until analysis. The cervical lymph nodes were excised for mRNA extraction, and spleen was processed for flow cytometric analysis.

Histological evaluation

The skin lesion samples were fixed in 10 % formaldehyde, embedded with paraffin, and stained with standard haematoxylin & eosin (H&E), or toluidine blue to detect mast cells. Dermal mast cells were counted as the number of toluidine-blue positive cells in ten randomly selected high power fields on each specimen using a light microscope.

Measurement of serum IgE and IL-18

The serum levels of IgE and IL-18 were measured using specific ELISA kits (IgE: Yamasa-shoyu, Chiba, Japan, IL-18: MBL, Nagoya, Japan) according to the manufacture's instructions.

Isolation and Preparation of mononuclear cells from spleen

Single cell suspensions from spleen were prepared by squeezing with two sterile glass slides, washing with PBS, and passed through a 40µm nylon mesh cell strainer (BD Falcon, Sparks, MD, USA). Mononuclear cells were obtained by density gradient centrifugation over Histopaque (Sigma, St. Louis, MO, USA) according to manufacture's instruction.

Analysis of Foxp3+ Treg

Cell surface antigen staining and intracellular staining for analysis of iTreg were performed by using Foxp3 Fix/Perm Buffer Set (BioLegend, San Diego, CA, USA). Briefly, cells were re-suspended in 100µl PBS containing 1% FCS, and were stained with FITC anti-mouse CD4 and PE/Cy5 anti-mouse CD25 (BD pharmingen, San Diego, CA). Cells were then fixed in BioLegend's Foxp3 Fix/Perm solution and stained with PE anti-mouse Foxp3 (BioLegend) antibody. Fluorescence profiles were analyzed by FACSCalibur (BD biosciences, San Jose, CA, USA).

Intracellular cytokine staining

Cells were suspended in RPMI 1640 (2×10^6 cells/well) with 10 % FCS, 2mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, and cultured in a 24-well culture plate (Coster, NY, USA). For the analysis of IL-10 expression, 10% FCS was replaced with 5% mouse serum. The cells were stimulated with 2µg/ml anti-mouse CD3e and 4µg/ml CD28 (BD Pharmingen) and cultured for 12 hours. For the last 8 hours of culture, 1 µg/ml brefeldin A (BioLegend) was added. The cells were washed twice in cold PBS and incubated for 30 min at 4°C with 0.5µg of anti-CD16/32 mAb, and followed by staining with PE/Cy5 anti-mouse CD4 antibody (BD Pharmingen). The cells were fixed and permeabilized using intracellular staining kit (BioLegend). Subsequently, cells were stained with PE anti-mouse IL-4, IL-10, IL-17A, and/or FITC anti-IFN-γ antibodies. Analysis was performed using FACSCalibur.

mRNA preparation and cDNA synthesis from lesional skin and cervical lymph node

Skin and cervical lymph node samples were snap frozen in liquid nitrogen and homogenized. Total RNA was extracted using Isogen (Nippon-Gene, Tokyo, Japan). mRNA concentrations were calculated based on the absorbance at 260nm by spectrophotometry. cDNA was synthesized by reverse transcribed from 2µg of mRNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA).

Quantitative Real-Time PCR analysis for cytokine production

Sequence-specific primers and probes for TaqMan® quantitative PCR analysis were purchased from Applied Biosystems (Carlsbad, CA, USA). PCR was performed on ABI 7700. One-µL of cDNA was used for each PCR analysis. cDNA concentrations were normalized by

those of GAPDH. The $\Delta\delta\text{Ct}$ method was used to normalize transcripts to calculate fold induction relative to transcripts of GAPDH levels.

Statistical analysis

Results are expressed as mean $\pm$ SEM values. Statistical analysis was carried out using Mann-Whitney U test to compare the areas of skin lesions. The mast cell count, the levels of IgE and IL-18, and cytokine expression were compared between groups by Kruskal-Wallis nonparametric test using Dunn's multiple comparison test as post hoc analysis. $P < 0.05$ was considered as statistically significant.

Results

hkBCG and Ag85B combined therapy suppresses the development of AD-like dermatitis in KCASP1Tg

KCASP1Tg treated with physiological saline or hkBCG mono-therapy spontaneously developed erosive dermatitis on the face from the 8th week of age (Fig.1b, Fig.2a). The skin lesions spread to ears, neck, extremities and trunk, and mice intensively scratched the skin lesions; that was characterized by acute erosive lesion (Fig. 2a). At the chronic phase, hairless lichenoid dermatitis, multiple skin ulcers, and re-epithelization were observed. In contrast, the group that received hkBCG/Ag85B combination therapy showed limited periocular dermatitis and showed significantly decreased dermatitis compared with the saline-treated group. Ag85B monotherapy showed limited inhibitory effects (Fig. 1b and 2a).

Histopathological findings

Saline-treated and hkBCG alone treated KCASP1Tg showed marked inflammatory mononuclear cells and mast cell infiltration accompanied with epidermal hyperplasia and dermal fibrosis (Fig. 2b). However, hkBCG/Ag85B combination therapy group showed very mild inflammatory changes. The suppressive effect of Ag85B monotherapy was limited on histopathological changes. The toluidine blue stained mast cell count significantly increased in the skin lesions of KCASP1Tg (Fig. 2c, 2d). hkBCG/Ag85B combination therapy significantly decreased the number of mast cells in the skin lesions. Ag85B monotherapy only showed moderate suppressive effects on mast cell infiltration but not at statistically significant level.

Serum IgE and IL-18 levels

IgE level was significantly elevated in saline treated KCASP1Tg compared with that of WT. hkBCG/Ag85B combination therapy significantly reduced IgE levels. However, there was no significant difference in serum IgE levels between KCASP1Tg and Ag85B, or hkBCG monotherapy groups (Fig. 3a). The serum IL-18 level in KCASP1Tg was also significantly elevated compared with WT. Mice receiving hkBCG/Ag85B combination therapy had significantly decreased IL-18 levels, but not those treated with Ag85B or hkBCG monotherapy (Fig. 3b).

Foxp3+Treg in the spleen

The saline treated KCASP1Tg had significantly decreased percentage of FoxP3+ Treg (iTreg) among CD4⁺ T cells compared with WT. hkBCG/Ag85B combination therapy induced significant increase in iTreg ratio close to that observed in WT (Fig.4a, 4f). However, Ag85B or hkBCG monotherapy failed to increase iTreg ratio and remained close to that observed in KCASP1Tg.

IFN- γ , IL-4, IL-17, and IL-10 production from splenocytes

The ratio of IFN- γ , IL-4, IL-17, and IL-10 producing T cells in CD4⁺ T cells was significantly increased in KCASP1Tg compared with that in WT (Fig. 4b-e). hkBCG/Ag85B combination, Ag85B monotherapy, and hkBCG monotherapy group showed no significant change in IFN- γ producing cell ratio (Fig. 4b). In contrast, the number of IL-4 producing CD4⁺ T cells was significantly suppressed in both combined therapy and Ag85B monotherapy groups (Fig. 4c). The IL-10 producing cell ratio was elevated in all of the four KCASP1Tg mice compared with WT, and the combination therapy further augmented the ratio of IL-10 producing cells compared with the saline-treated KCASP1Tg. Ag85B and hkBCG monotherapy showed no effect in IL-10 production (Fig. 4d). The IL-17 producing cell ratio in CD4⁺T cell was elevated in KCASP1Tg, and it was significantly suppressed by the combination therapy, but not by Ag85B or hkBCG monotherapy (Fig. 4e). The representative flow cytometry data of each group was shown in Figure. 4f-i.

Cytokine mRNA expressions in skin lesions

IFN- γ , IL-4, IL-13, TSLP, IL-10, and IL-17 mRNA expressions in the skin lesions were elevated in KCASP1Tg compared with WT. The skin expression of GATA-3 mRNA was moderately elevated in KCASP1Tg compared with WT. hkBCG /Ag85B combination therapy downregulated Th2 cytokines (IL-4, IL-13, TSLP) mRNA expression in the skin but

not at significant level. However, the combination therapy but not Ag85B monotherapy significantly suppressed the GATA-3 mRNA expression. Additionally, IL-10 mRNA in the skin of KCASP1Tg was elevated compared with WT, and the combination therapy further enhanced IL-10 mRNA expression but not at significant level. The IL-17 mRNA expression was also significantly elevated in KCASP1Tg compared with that in WT. hkBCG/Ag85B combination therapy decreased IL-17 mRNA expression but the difference did not reach the statistically significance. IFN- γ mRNA level remained unchanged during these therapies. mRNA level for TGF- β showed marginal increase by the combination therapy (Fig. 5).

Cytokine mRNA expressions in lymph nodes

IFN- γ mRNA expression level in AD mice was slightly decreased compared with WT, but unchanged by the treatment. IL-4 mRNA level was increased in control non-treated AD compared with WT, and showed decreased tendency by the combination therapy. IL-17 mRNA expression was increased in AD model, but unchanged by the treatment (Fig. 6).

Discussion

Th1/Th2 imbalance has been ascribed to be a critical factor in the pathogenesis of allergic diseases. AD is associated with high levels of serum IgE, and Th2 cytokines. The recent identification of Th17 and Tregs introduced a new multi-factorial balance concept in the mechanism of allergic diseases²⁵. AD model mouse, KCASP1Tg showed decreased number of iTreg, and increased production of IFN- γ , IL-10, and IL-17 in addition to IL-4. AD has been previously thought to be a Th2 dominant disease, but subsequent studies have shown that Th1^{1, 26} and Th17² cytokines are interconnected. These results showing high concentration of cytokines including Th2, Th1 and Th17 in KCASP1Tg are compatible to the phenomena observed in human AD.

In the present study, we demonstrated remarkable effects of hkBCG and *M. kansasii* Ag85B combination therapy for the prevention of AD. Both hkBCG and Ag85B are known as strong Th1 cytokines inducers. The combination therapy was associated with significant decrease in IL-4, IL-13 and GATA-3 expressions. The roles of IL-17 in AD have not been so far well defined. This therapy surprisingly downregulated the expression of IL-17. The mechanism of IL-4 and IL-17 suppression remains still unclear. However, hkBCG/Ag85B combination therapy increased iTregs and IL-10+CD4+T cell, and moderately skin IL-10 mRNA expression, implicating IL-10 producing Tr1 in this suppressive mechanism. IL-10 is a strong anti-inflammatory cytokine, and shows antigen specific and non-specific tolerance

induction via locally production of IL-10²⁷⁻²⁹. It is conceivable that increase of iTreg and Tr1 contributed to the suppression of dermatitis in our present immunotherapy, resulting in subsequent suppression of IL-4 and IL-17.

KCASP1Tg showed increased expression of IFN- γ . In the present study, skin lesions of KCASP1Tg were in chronic phase mixed with exacerbation. Therefore, it was expected to observe elevated IFN- γ and Th2 cytokines in KCASP1Tg. Interestingly, the strong Th1 inducer showed no obvious additional augmentation in IFN- γ expression. This result can be explained by the fact that IFN- γ expression might already reach a plateau in KCASP1Tg, and /or by the fact that further IFN- γ induction is suppressed by negative feedback due to induction of IL-10 and Tregs.

Exacerbation of AD is associated with increased cellular infiltration including dermal mast cells³⁰ and lymphocytes. Recently, we reported novel functions for mast cell chymase³¹ and granzyme B released from CD8⁺ T cells³² in the maturation of IL-18. Increased IL-18 level directly reflects systemic Th2 dominance²³, therefore, the suppression of skin cellular infiltration by combined vaccinations is a key factor for AD treatment.

Control of the number and function of mast cells is indispensable for successful AD immunotherapy. A significant amount of stem cell factor as well as IL-18 is released from lesional epidermis in AD. Stem cell factor activates and stimulates proliferation of dermal mast cells. IL-18 stimulates mast cells and promotes the formation of a Th2 milieu in the skin. Thus, suppression of dermatitis contributes to the decrease in the number of dermal mast cells. Interestingly, Treg and mast cell interaction was reported *in vivo* experiments³³. CD4⁺CD25⁺Foxp3⁺ Tregs are known to directly suppress mast cell degranulation³⁴. The increased Treg number and decreased mast cell counts observed in the combination therapy group may be related.

In Japan, a strong inverse association between delayed hypersensitivity to *Mycobacterium tuberculosis* and atopy has been reported among school children⁶. On the other hand, BCG has been proposed as a potential therapeutic tool for AD³³. Repeated antigen exposure is required for tolerance induction during immunotherapy. However, repeated live BCG vaccination, due to the waxy cell wall of BCG, may induce chronic inflammatory reactions such as granuloma and ulcer formation in the skin area of vaccination, and it may even produce some scar³⁵. The *in vivo* activity of released cell wall lipids of *Mycobacterium bovis* BCG is mainly due to trehalose mycolates. Ag85B is the secretory protein present in *Mycobacterium* species including BCG, that can induce a strong Th1-dominant response¹⁸. We have previously shown that the intraperitoneal administrations of plasmid DNA encoding *M. kansasii* Ag85B cDNA inhibits immediate type

hypersensitivity by inducing iTreg, IL-10, and TGF- β ¹⁹. In addition, Ag85B DNA vaccine was found to suppress airway inflammation by inducing Th1 immune deviation in mice asthma model³⁶. In our present study, Ag85B protein monotherapy showed limited beneficial effect. However, Ag85B therapy with preceding hkBCG resulted in successful clinical and immunological improvement of AD in mouse model. Almost all Japanese schoolchildren are vaccinated with BCG. Repeated BCG vaccination is difficult for the clinical use due to the risk for granuloma and ulcer formation, but it is unlikely for Ag85B. Less granuloma formation by repeated vaccination is necessary; therefore, the BCG and repeated Ag85B combination will be preferable.

Interestingly, in our present study, Ag85B protein, but not DNA vaccine, reduces the development of AD proportionally with the increase in the number of iTreg. The mechanism of this effect is uncertain at present, but we speculate that TLR-2 signaling may be involved. The recognition of mycobacterial components by TLR-2 on dendritic cells has been implicated in mycobacterium-induced Treg cell responses³⁷. In addition, it has been reported that TLR-2 stimulation inhibits airway eosinophilia³⁸, that TLR-2 ligand induces IL-10 and regulatory T cells³⁹, and that TLR-2 induces proliferation of iTreg⁴⁰. Further, Ag85B may stimulate TLR-2⁴¹. We have recently reported that *propionibacterium acnes*, a strong Th1 inducer, induces IL-10 and iTreg through TLR-2 pathway⁴². Further investigation is required to identify the mechanisms of iTreg induction.

In summary, we found that hkBCG and Ag85B vaccination suppresses the development of AD-like dermatitis and modulates the expression of cytokines (IL-4, IL-17), at least in part due to the induction of iTreg in KCASP1Tg. One limitation of this study is that it was performed in mice and that observations in mice cannot be easily extrapolated to human disorders. However, in some countries, BCG vaccination has been performed during childhood or certain generation, thus booster effect of the additional Ag85B administration has the potential to ameliorate Th2 immunological profile. Ag85B vaccine in combination with the currently used BCG vaccine is a promising therapeutic approach for AD.

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Figure legends

Figure 1. hkBCG and Ag85B combined vaccination decreases AD-like skin lesions in KCASP1Tg.

(a) Experimental design used to investigate the effects of hkBCG and Ag85B combined vaccination on KCASP1Tg. KCASP1Tg were treated with saline alone (control), hkBCG at the 3rd week of age and followed by Ag85B twice a week from the 4th to 14th week of age by i.p. injection (hkBCG / Ag85B), Ag85B twice a week from the 4th to 14th week of age by i.p. injection (- / Ag85B), or hkBCG at the 3rd week of age (hkBCG/ -). (b) The areas of skin lesions were evaluated on weeks 4th, 5th, 6th, 8th, 10th, 12th and 14th. KCASP1Tg treated with physiological saline and hkBCG monotherapy spontaneously developed erosive dermatitis on the face from the 8th week of age, and the skin lesions then gradually spread. hkBCG and Ag85B combined treatment group showed only modest dermatitis and resulted in a significant inhibition of erosive dermatitis compared with saline-treated KCASP1Tg. In the Ag85B-alone treatment group, there was inhibitory tendency but not at significant level. Data are expressed as mean $\pm$ SEM of 10 mice per group. * $p < 0.05$ compared with saline-treated control group.

Figure 2. hkBCG and Ag85B combined treatment improved clinical manifestation, skin inflammation, and mast cell infiltration in KCASP1Tg.

Representative clinical and histopathological features of KCASP1Tg facial skin lesion and that of WT are shown. a) Clinical photos, b) haematoxylin and eosin (H&E) staining; original magnification x100, c) toluidine blue staining; original magnification x200, d) The number of infiltrated mast cells in the dermis from random 10-fields in each specimen was counted in

high power fields. Values are mean $\pm$ SEM (n = 10). * P< 0.05, *** P< 0.001.

Figure 3. Serum IgE and IL-18 levels.

The serum levels of (a) IgE and (b) IL-18 in saline-treated control KCASP1Tg were significantly higher compared with WT. hkBCG and Ag85B combined vaccination significantly suppressed both of them. Results are shown as mean $\pm$ SEM. * P<0.05 **P<0.01 ***P<0.001

Figure 4. Inducible Treg and cytokine production in CD4 T cells from spleen.

The percentage of (a) Foxp3⁺CD25^{high} CD4⁺ cells (b) IFN- γ ⁺ CD4⁺ cells (c) IL-4⁺ CD4⁺ cells (d) IL-10⁺ CD4⁺ cells (e) IL-17⁺ CD4⁺ cells in total CD4⁺ T cells from spleen in saline treated KCASP1Tg, KCASP1Tg receiving combination therapy, Ag85B alone-treated KCASP1Tg, hkBCG alone-treated KCASP1Tg, and WT are shown. Saline-treated KCASP1Tg significantly decreased the percentage of iTreg in CD4⁺ T cells as compared with that of WT, and combination vaccinated group showed recovery in the number of iTreg. Such increase in the percentage of iTreg was not observed in Ag85B or hkBCG alone-treated mice (a). The percentages of IFN- γ , IL-4, IL-10, and IL-17 producing CD4⁺ T cells were significantly increased in KCASP1Tg compared with WT. Regarding IFN- γ , there was no significant change between the four groups (b). IL-4 producing CD4⁺ T cells were significantly suppressed in the mice treated with combination therapy, as well as in Ag85B alone-treated group compared to control KCASP1Tg (c). The percentage of IL-10 producing CD4⁺ T cells was significantly increased in the group receiving combination therapy. (d). The percentage of IL-17 producing cells was decreased in the group receiving combination therapy compared to control KCASP1Tg (e). Data are expressed as mean $\pm$ SEM. * P<0.05 **P<0.01 ***P<0.001

The representative flow-cytometry data of each group was shown (f-i). CD4⁺ T cells were gated and stained with FoxP3/ CD25 (f), IFN- γ / IL-4 (g), IFN- γ / IL-17 (h), and IL-10 (i). The percentage of cytokine producing CD4⁺ cells in total CD4⁺ T cells is indicated (f-i).

Figure 5. Cytokine mRNA expressions in the facial skin lesion.

mRNA expressions of IFN- γ , IL-4, IL-13, TSLP, IL-10, and IL-17 in the facial skin were up-regulated in the saline-treated KCASP1Tg compared with WT. Treatment with hkBCG and Ag85B significantly decreased the GATA-3 mRNA expression, and the mRNA expression of IL-4, IL-13 and TSLP tended to decrease. Additionally, IL-10 mRNA level was increased, and that of IL-17 was decreased in hkBCG- and Ag85B-treated mice compared to saline-treated KCASP1Tg. IFN- γ mRNA level remained unchanged. mRNA for TGF- β showed marginal increase by the combination therapy. Data are expressed as mean $\pm$ SEM. * P<0.05 **P<0.01 ***P<0.001

Figure 6. Cytokine mRNA expressions in cervical lymph node.

mRNA expressions of IFN- γ , IL-4, IL-13, GATA-3, IL-10, IL-17, and TGF- β in cervical lymph node were measured. IFN- γ mRNA was unchanged by the treatment compared to non-treated control AD mice (a). IL-4 mRNA expression was increased in control AD, and was slightly decreased by the combination treatment (b). IL-13 mRNA level was unchanged (c). Combined therapy significantly decreased the GATA-3 mRNA level compared with control group (d). IL-10 mRNA expression was slightly increased in BCG/ Ag85B combination therapy (e). IL-17 mRNA expressions were increased in non-treated AD, and was unchanged by the treatment (f). TGF- β level was unchanged among five groups (g). Data are expressed as mean $\pm$ SEM. * P<0.05 **P<0.01 ***P<0.001

Figure 1.

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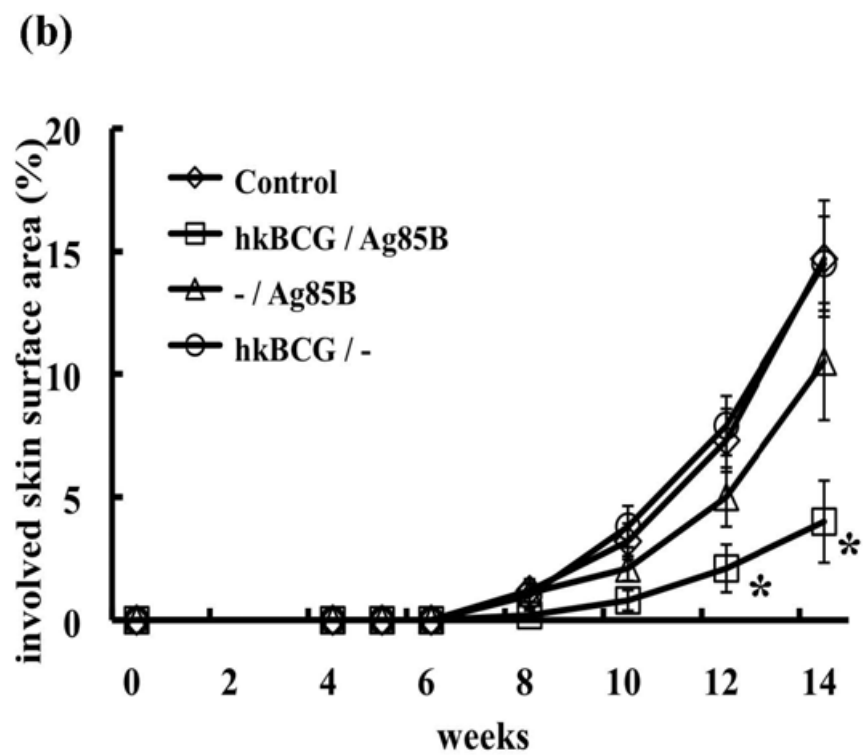
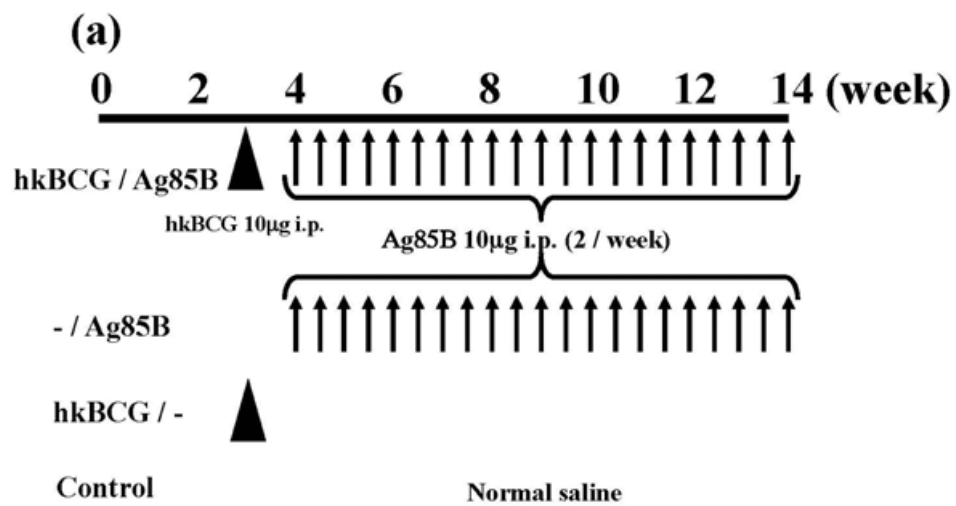
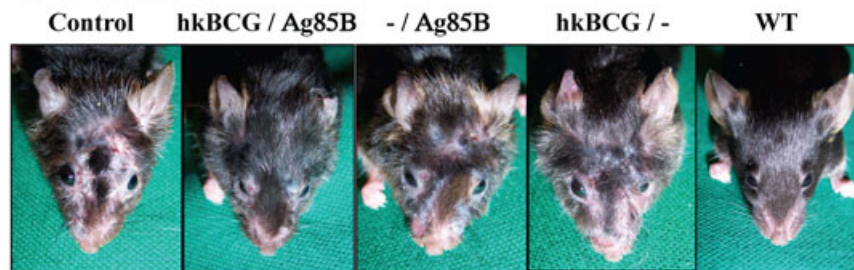


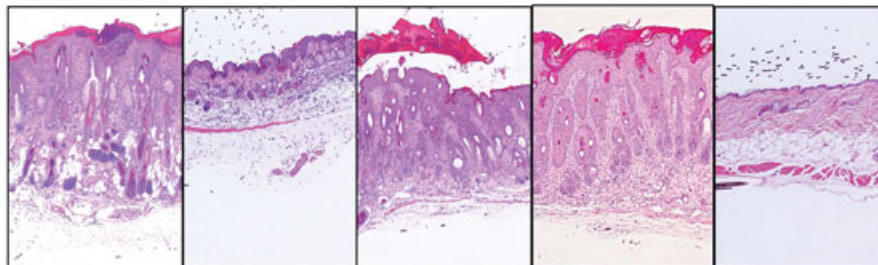
Figure 2.

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(a) Clinical



(b) H & E



(c) Toluidine blue

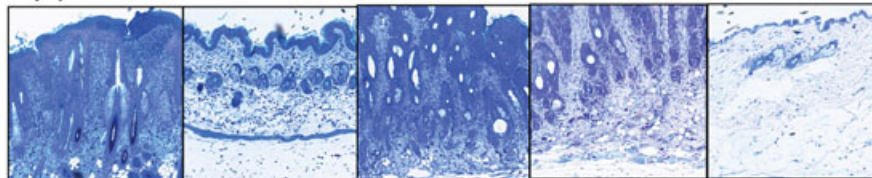


Figure 2.

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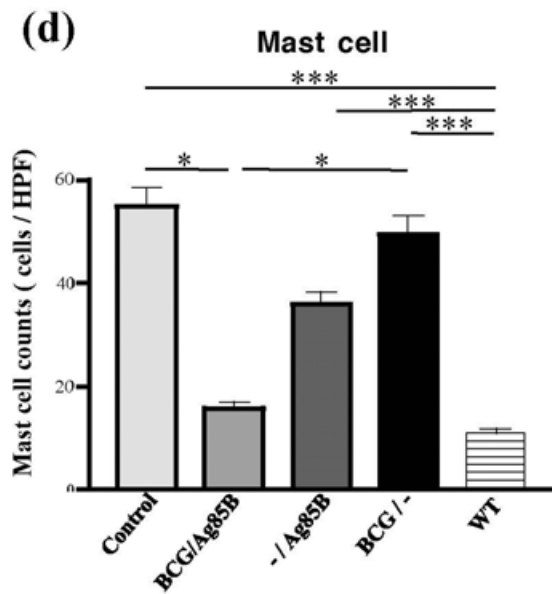


Figure 3.

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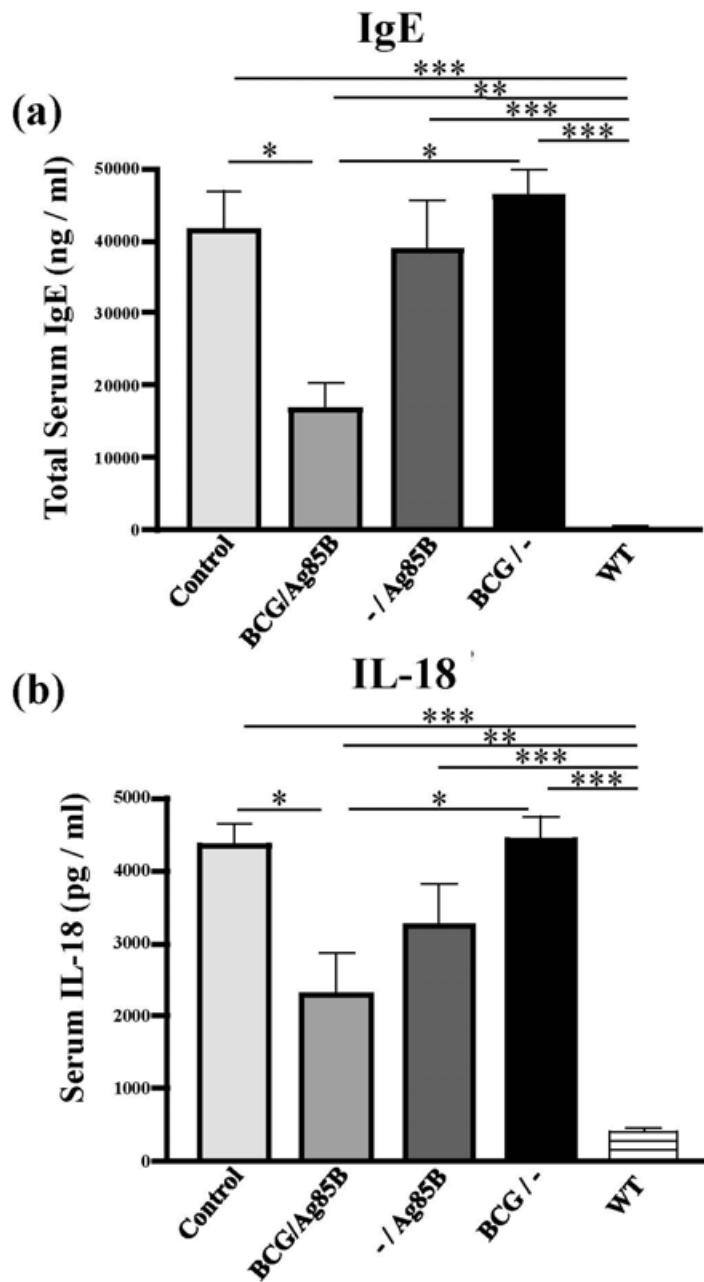
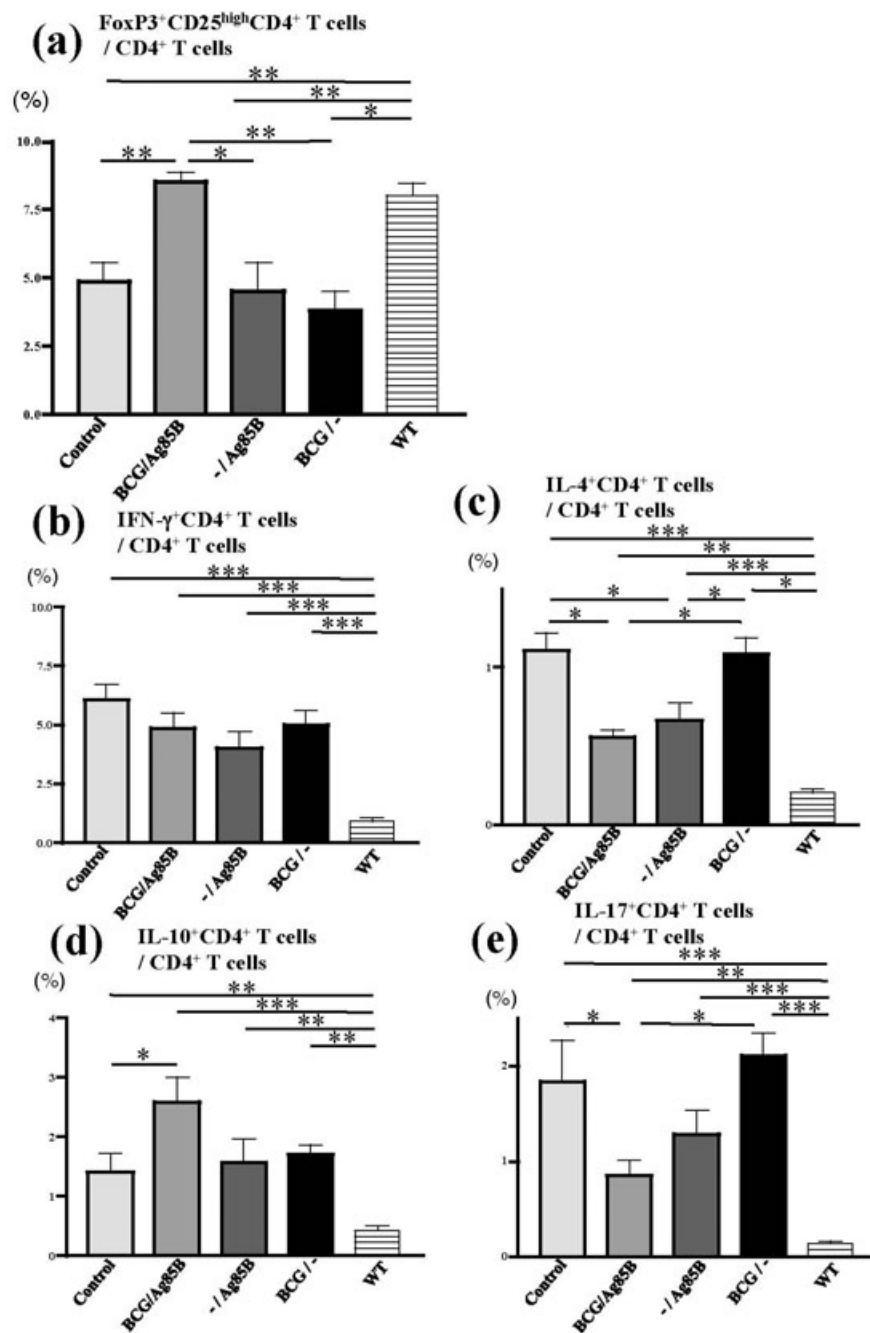


Figure 4.

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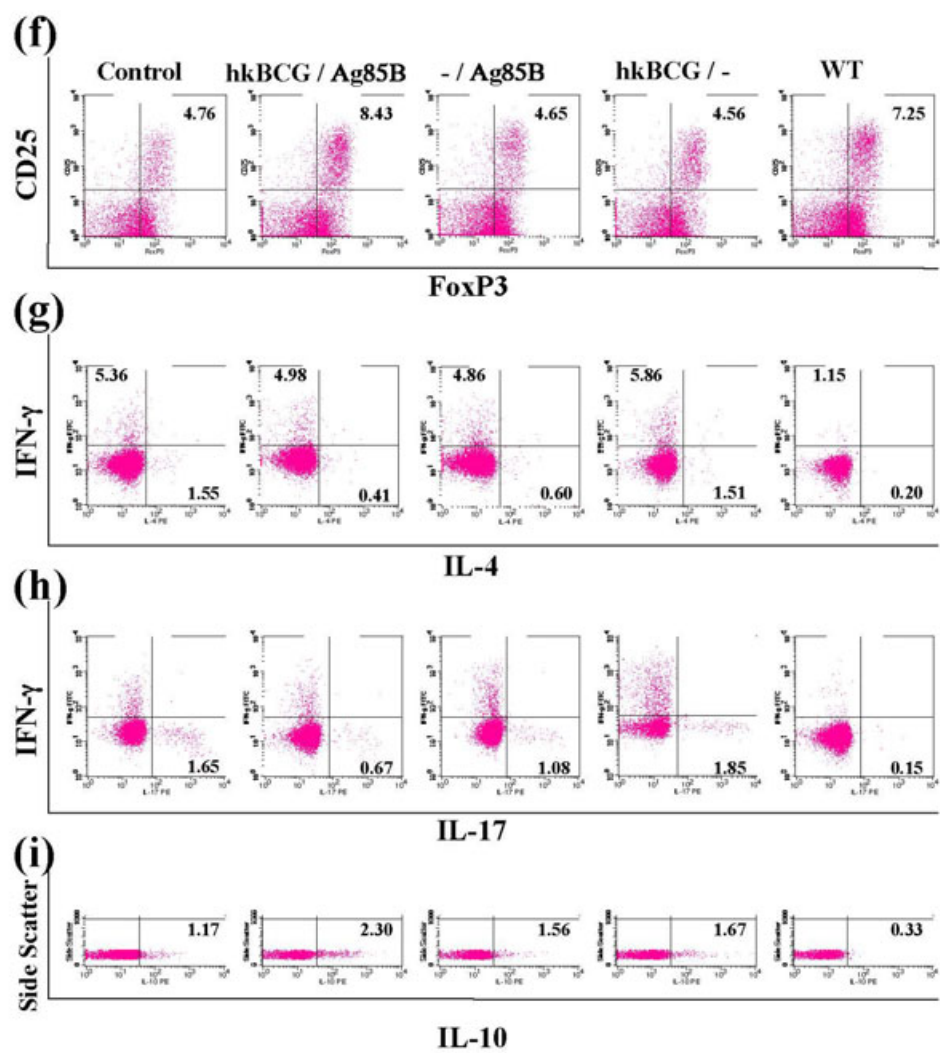


Figure 5.

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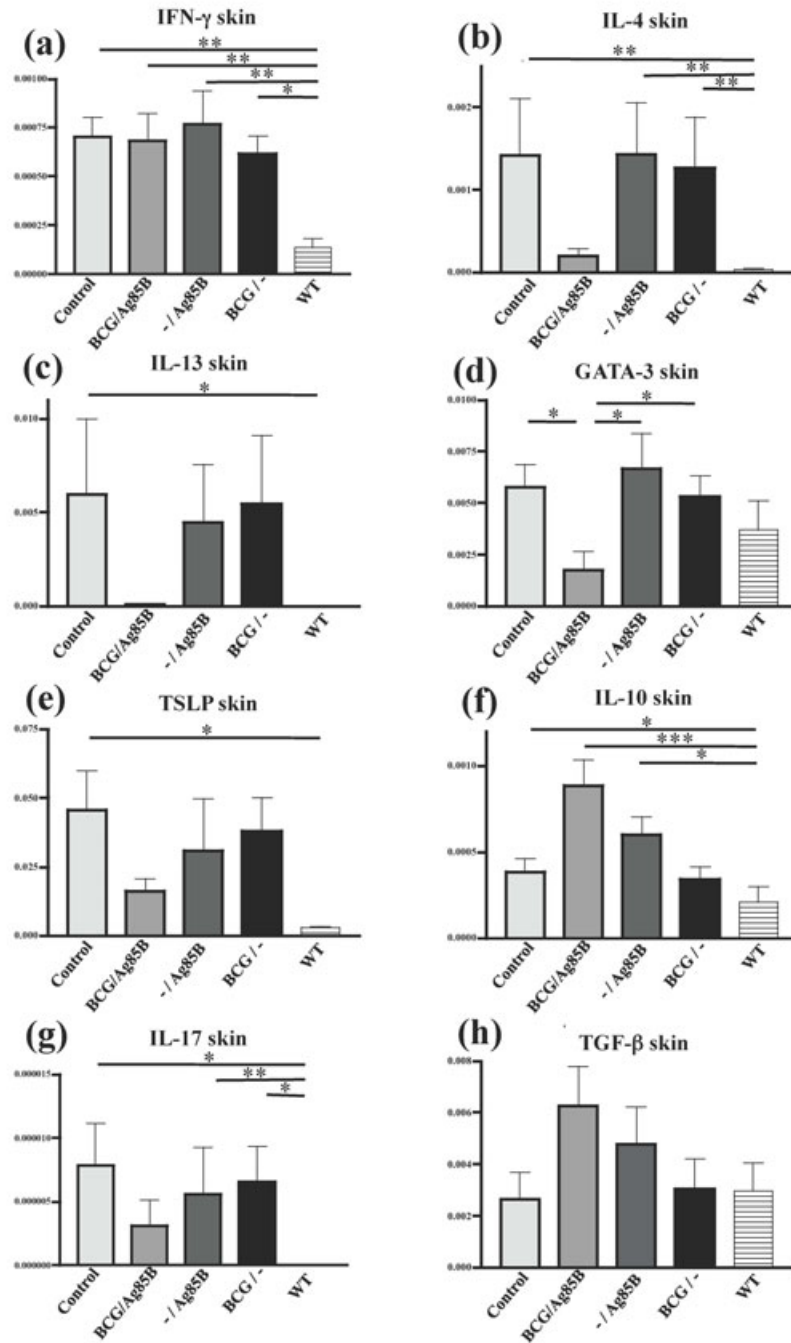


Figure 6.

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