

Propionibacterium acnes vaccination induces regulatory T cells and Th1 immune responses and improves mouse atopic dermatitis

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Abstract: Atopic dermatitis (AD) is a chronic disease characterized by a polarized Th2 immune response. *Propionibacterium acnes* (*P. acnes*) has been shown to elicit strong Th1 immune responses. We hypothesized that the host immune response to *P. acnes* will prevent the development of AD. To demonstrate this hypothesis, we investigated the effect of *P. acnes* vaccination on AD that occurs in keratin 14/driven caspase-1 transgenic mouse. Vaccination with low dose of *P. acnes* successfully prevented clinical manifestations in the skin of AD mice associated with systemic and cutaneous increased

expression of Th1-type cytokines but without suppression of Th2 cytokines. Interestingly, the numbers of IFN- γ ⁺T cells, FoxP3⁺CD4⁺CD25⁺T cells (nTreg) and IL-10⁺T cells (Tr1) were significantly increased in the spleen. *P. acnes* vaccination has effects to alter the cytokine milieu and may be useful for the improvement of atopic symptom.

Key words: atopic dermatitis – IFN- γ -regulatory T cell – K14/caspase-1 transgenic mouse – *Propionibacterium acnes*

Accepted for publication 30 July 2010

Background

Atopic dermatitis (AD) is a chronic disease characterized by a pre-dominant expression of Th2-type cytokines. Th2-type-dominant cytokine expression is associated with increased cellular infiltration and overproduction of IgE (1). On the other hand, Th1 cells mainly secrete IL-12 and IFN- γ that counteract the effect of Th2 cells, suppress IgE production and stimulate IgG expression. This observation suggests that control of Th1/Th2 imbalance may improve AD. Th1 cells may be induced by some infectious agents including *Propionibacterium acnes* (*P. acnes*). *P. acnes* is an aerotolerant anaerobic gram-positive bacteria, present in skin flora of healthy individuals, that plays an important role in the pathogenesis of acne (2). *P. acnes* is known as an inducer of Th1-type immune response (3).

Questions addressed

K14/caspase-1 transgenic mouse (KCASP1Tg) develops AD characterized by recalcitrant itching dermatitis with increased expression of Th2-type cytokines (4–6). We investigated the therapeutic effects of vaccination with low-dose *P. acnes* upon AD manifestations, cytokine profile and the involvement of regulatory T cells in KCASP1Tg.

Experimental design

Vaccination of AD model mice with *P. acnes*

KCASP1Tg was treated with heat-killed *P. acnes* (66 μ g) injection twice a week between 4 and 14 weeks of age (*P. acnes*-Tg). PBS-treated KCASP1Tg (Mock) and wild-type C57/BL6 (WT) were used as control mice (Appendix S1). Skin manifestation usually appears in KCASP1Tg mice after the age of 8 weeks. Skin manifestations were examined every 2 weeks between 8 and 14 weeks, and the affected skin areas were calculated as percentage of the whole body area. Skin specimens were also sampled at 14th week and stained with haematoxylin & eosin and toluidine blue.

Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM). The statistical difference between variables was calculated by the Kruskal–Wallis one-way analysis of variance. $P < 0.05$ was considered as significant.

Results

Cutaneous manifestations and histopathological findings

KCASP1Tg (Mock) developed erosive dermatitis on the face at 8th week and showed severe scratching. Skin lesions spread rapidly to the ears, neck and trunk (Fig. 1a). The erosive lesions worsened and became hairless lichenoid dermatitis mixed with acute lesions. In contrast, *P. acnes*-Tg developed significantly milder dermatitis, and the total involved skin area was significantly less than in Mock after 10 weeks (Fig. 1b). Histopathologically, the 14-week-old Mock showed marked inflammatory reactions with acanthosis, hyperkeratosis and marked cellular infiltration. The inflammatory changes and the number of mast cells were significantly decreased in the *P. acnes*-Tg compared to Mock (Fig. 1c).

Conclusions

This study demonstrated a novel candidate for the improvement of AD by vaccination with *P. acnes*. Vaccination with the extract of this microorganism improved skin lesions by acting as an immune response modifier. Antigen from *P. acnes* activates IL-12p40 promoter after binding to TLR2 (2,7). IL-12 binds to its receptor (IL-12R) and activates the transcription factors STAT-4 and T-bet, and then T-bet binding to the promoter of IFN- γ gene stimulates the production of this Th1-polarizing cytokine (8,9). In this study, the cytokine production was evaluated in spleen cells from each group of mice, and it was found that IFN- γ -positive T cells are significantly elevated in *P. acnes*-Tg (Appendix S1 and Fig. S2). In addition, the mRNA expressions of IFN- γ , IL-12 and T-bet were also significantly

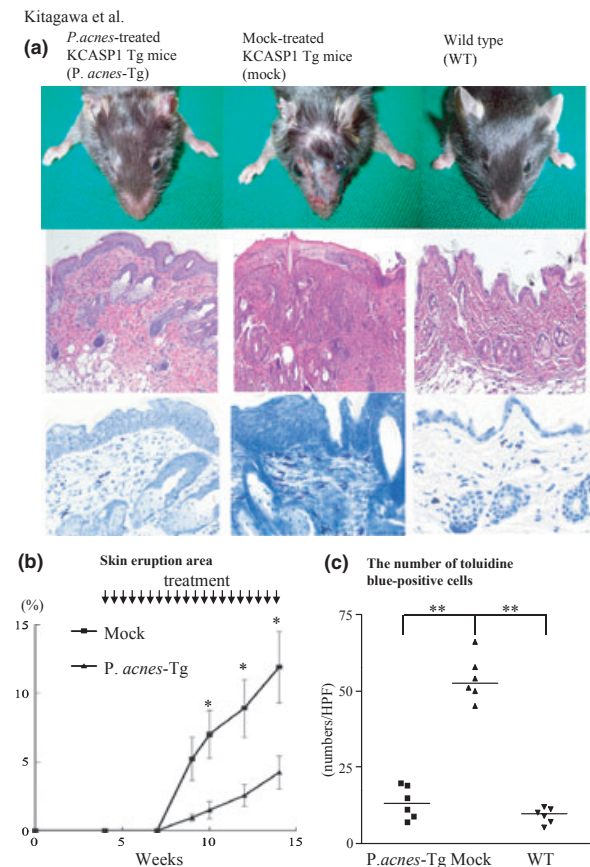


Figure 1. (a) Skin facial manifestations at 14th week of age. Severe dermatitis was observed on the face in Mock. In contrast, *P. acnes*-treated mice (*P. acnes*-Tg) showed mild dermatitis limited to the perioral areas. Histologically, Mock showed severe skin lesions including acanthosis and hyperkeratosis in the epidermis and marked fibrosis with dense mononuclear cell infiltration. *P. acnes*-Tg showed mild inflammatory reactions and limited epidermal acanthosis (H&E section). The infiltration of mast cells was also increased in the skin from Mock compared to *P. acnes*-Tg (toluidine blue stain). (b) Time course and skin areas with disease. The diseased skin area increased from 8 weeks after birth in Mock, but it was less in *P. acnes*-Tg. Data are expressed as the mean $\pm$ SE. * $P < 0.05$. (c) The toluidine-blue-positive mast cell numbers per HPF were significantly increased in Mock compared to WT; the degree decreased to the level of WT in animals treated with *P. acnes*. Data are expressed as the mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$.

elevated in the spleen cells from *P. acnes*-Tg compared to control mice (Appendix S1, Figs S1 and S3). In our previous report, injection of 1mg of *P. acnes* into KCASP1Tg caused death of mouse owing to severe liver injury (4). However, in this experiment, vaccination with low dose of *P. acnes* increased the differentiation of T

cells expressing Th1-type cytokines without liver injury (data not shown). *P. acnes* treatment did not affect the elevated IL-4 mRNA expression in KCASP1Tg, suggesting that *P. acnes* therapy does not simply switch Th2 into Th1-type cells.

Th17 and Treg cell are also important immunoregulators. IL-17A mRNA and IL-17A-positive cells are significantly elevated in Mock, suggesting the involvement of Th17 immune response in the pathogenesis of dermatitis (Figs S1 and S2). However, we found that Th17 expression is not affected by *P. acnes* treatment. The expression of IL-17A receptor is also a critical factor for IL-17 signal transduction. The expression of IL-17A receptor was decreased in the skin of Mock compared to that of WT (Fig. S3), suggesting that there is a negative feedback following increased IL-17A production in KCASP1Tg. *P. acnes* vaccination showed no effects on IL-17A receptor expression. Therefore, it appears that IL-17 is involved in the development of dermatitis, but it is not affected by *P. acnes* therapy.

Regarding regulatory T cells, nTreg cells were slightly decreased in the spleen of Mock compared to WT. However, *P. acnes* therapy increased nTreg in KCASP1Tg (Fig. S2). A bacterial TLR2 agonist has been shown to induce nTreg *in vivo* (10); therefore, *P. acnes* vaccination may increase nTreg via TLR2 (7).

TGF- β is a pleiotropic cytokine that may increase the number of Treg. This study showed that vaccination with *P. acnes* significantly augmented TGF- β mRNA expression compared to untreated mice (Figs S1 and S3), suggesting that the suppression of TGF- β expression plays a role in the development of dermatitis in KCASP1Tg and that TGF- β is a key factor to control dermatitis.

P. acnes treatment significantly increased the number of Tr1 cells compared to WT (Fig. S2). We also analysed mRNA expression in spleen cells and skins (Figs S1 and S3) and found that the mRNA expression of IL-10 is significantly enhanced in *P. acnes*-Tg compared to that in Mock and WT. In humans, IL-10 released by Tr1 cells is critical for the immune control of atopic diseases (11,12). A recent study has shown that *P. acnes*-soluble polysaccharide suppresses type1 hypersensitivity late-phase reaction in mice (13). However, further investigation is warranted to clarify the mechanism of Treg induction by *P. acnes*.

In brief, we showed in this study that *P. acnes* vaccination ameliorates AD by generating natural and induced Tregs and by inducing Th1 cytokines in a mouse model that spontaneously develops intrinsic AD. This study indicates that *P. acnes* immunotherapy may be a potent therapeutic approach for AD.

Disclosures

The authors have no financial conflict of interest.

References

- Del Prete G. Allergy 1992; **47**: 450–455.
- Kurokawa I, Danby F W, Ju Q *et al.* Exp Dermatol 2009; **18**: 821–832.
- Sugisaki H, Yamanaka K, Kakeda M *et al.* J Dermatol Sci 2009; **55**: 47–52.
- Yamanaka K, Tanaka M, Tsutsui H *et al.* J Immunol 2000; **165**: 997–1003.
- Yoshimoto T, Mizutani H, Tsutsui H *et al.* Nat Immunol 2000; **1**: 132–137.
- Konishi H, Tsutsui H, Murakami T *et al.* Proc Natl Acad Sci U S A 2002; **99**: 11340–11345.
- Kim J, Ochoa M T, Krutzik S R *et al.* J Immunol 2002; **169**: 1535–1541.
- Romagnani S Immunology 2004; **112**: 352–363.
- Yoshimoto T, Takeda K, Tanaka T *et al.* J Immunol 1998; **161**: 3400–3407.
- Liu H, Komai-Koma M, Xu D *et al.* Proc Natl Acad Sci U S A 2006; **103**: 7048–7053.
- Akdis M, Akdis C A. J Allergy Clin Immunol 2007; **119**: 780–791.
- Yamanaka K, Yuta A, Kakeda M *et al.* J Allergy Clin Immunol 2009; **124**: 842–845.
- Squaiella C C, Longhini A L, Braga E G *et al.* Immunol Lett 2008; **121**: 157–166.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

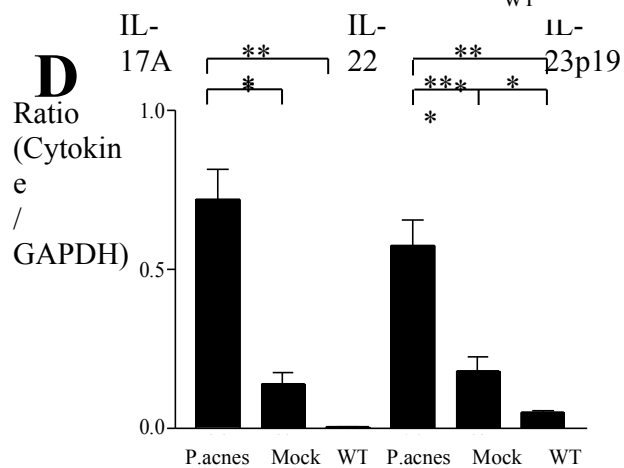
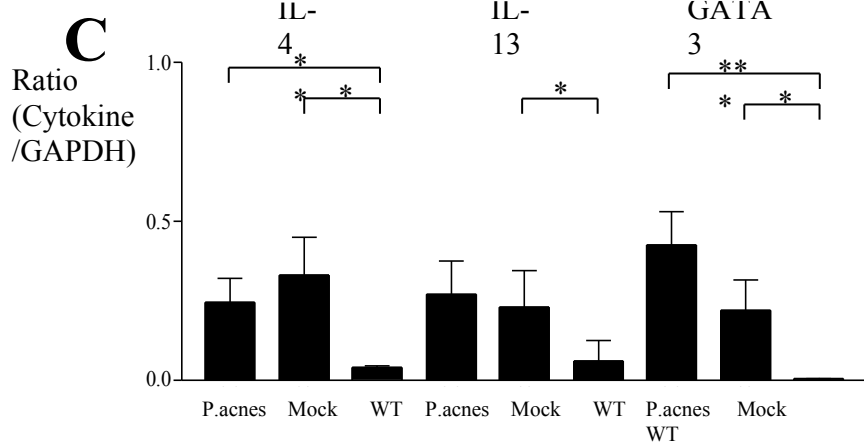
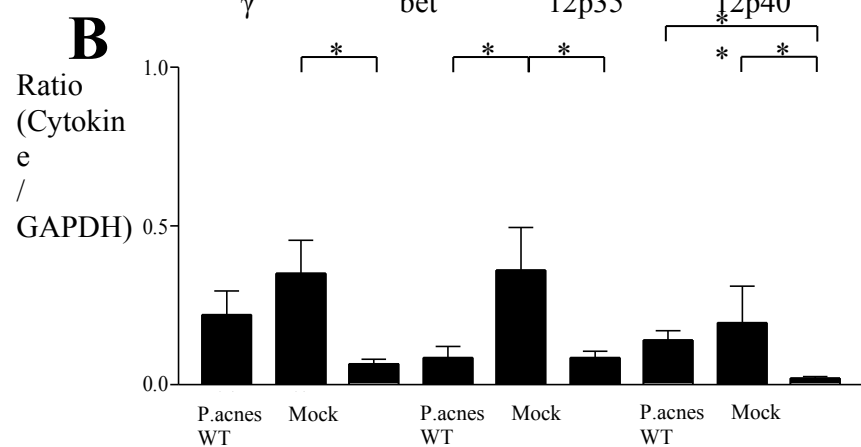
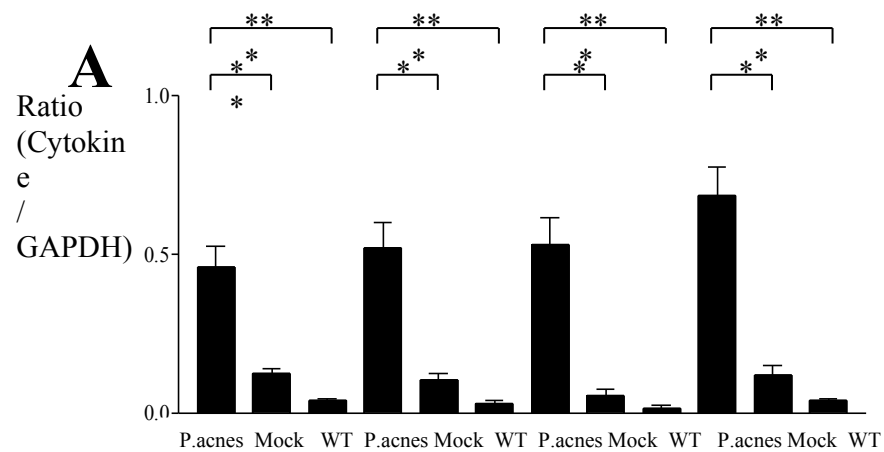
Figure S1. mRNA expression in spleen cells.

Figure S2. Intracellular cytokine and Foxp3 staining in spleen cells.

Figure S3. mRNA expression in the skin lesions.

Appendix S1. Materials and methods.

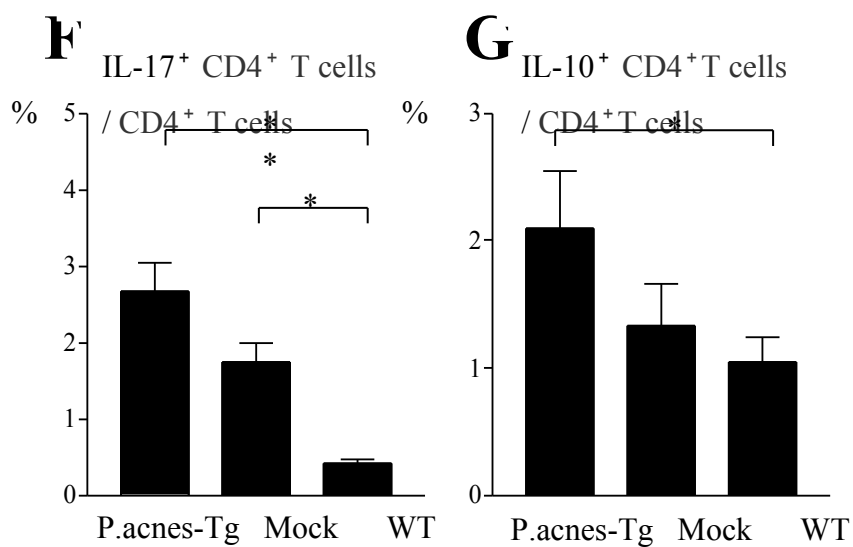
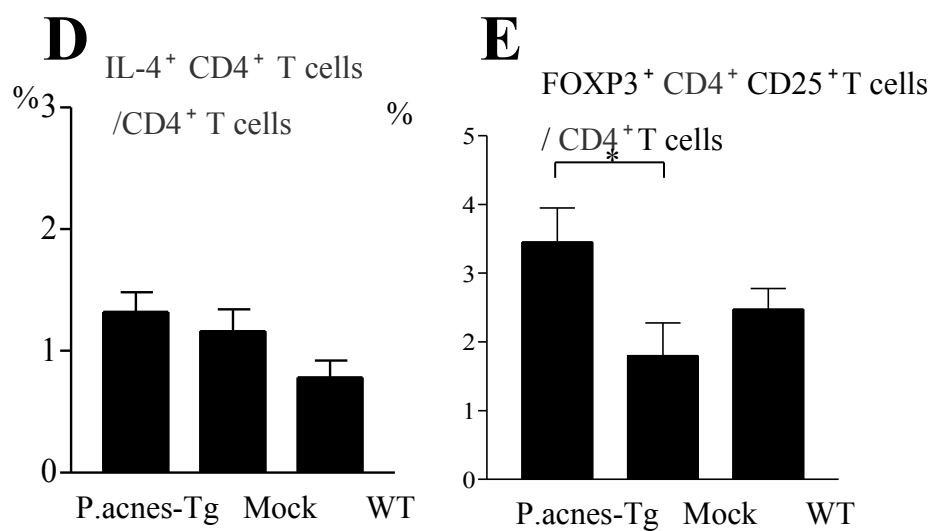
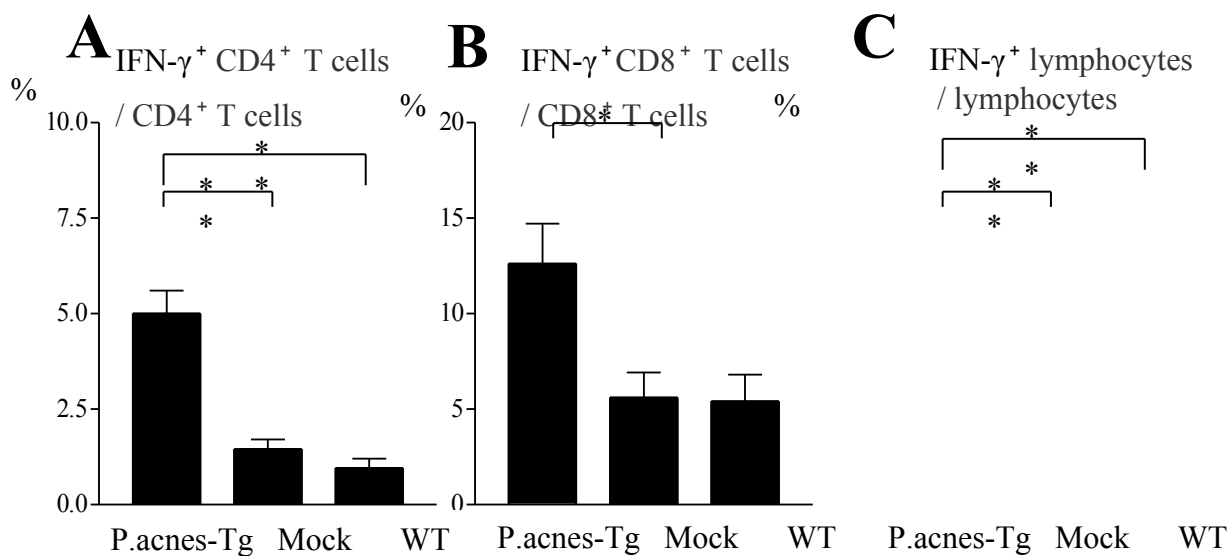
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SI 2. mRNA expression in spleen cells.

The spleens were sampled at 14th week of age. mRNA was extracted from non-stimulated spleen cells. Quantitative RT-PCR was performed to investigate cytokine mRNA expression in spleen cells.

A. Expression of Th1 cytokines mRNAs including IFN- γ , T-bet, IL-12p35, IL-12p40 was significantly increased in *P. acnes*-Tg compared to that in Mock or WT. **B.** mRNAs of Th2 cytokines (IL-4, GATA3) remained unchanged even after the treatment with *P. acnes*. Expression of IL-13 mRNA was significantly decreased in *P. acnes*-Tg compared with Mock. **C.** mRNA expression of IL-17A, IL-22, and IL-23p19 mRNA was increased in *P. acnes*-Tg and Mock compared with WT. **D.** mRNA of IL-10 and TGF- β was significantly enhanced in *P. acnes*-Tg compared to Mock and WT. *P<0.05, **P<0.01, ***P<0.001



SI 3. Intracellular cytokine and Foxp3 staining in spleen cells.

The spleens were removed at 14th week, and stained with antibodies to

CD4, CD25, FoxP3, and the fluorescence profile were analyzed by flow cytometry.

Cells were also cultured with anti-mouse CD3e and CD28 antibodies in the presence of brefeldin A for 8h. IFN-g, IL-4, IL-10, and IL-17A production in T cells were analyzed.

A, B, C, The percentages of IFN-g+CD4+, IFN-g+CD8+, and IFN-g+ lymphocytes

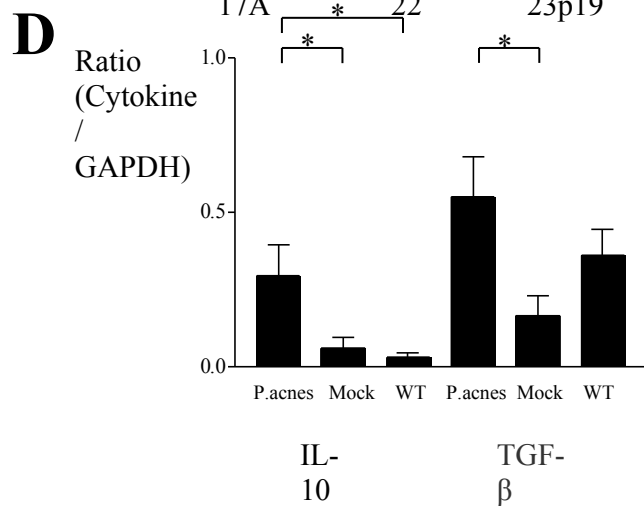
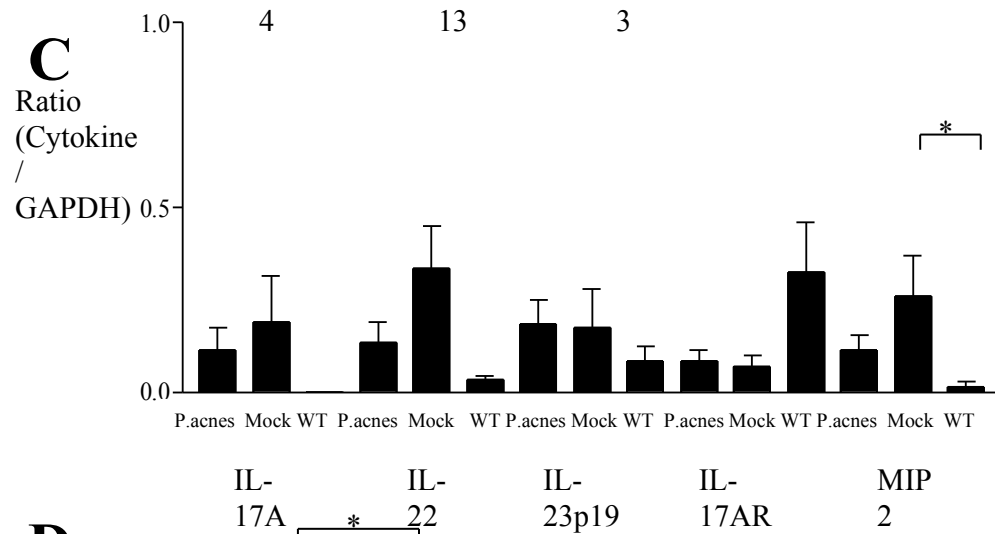
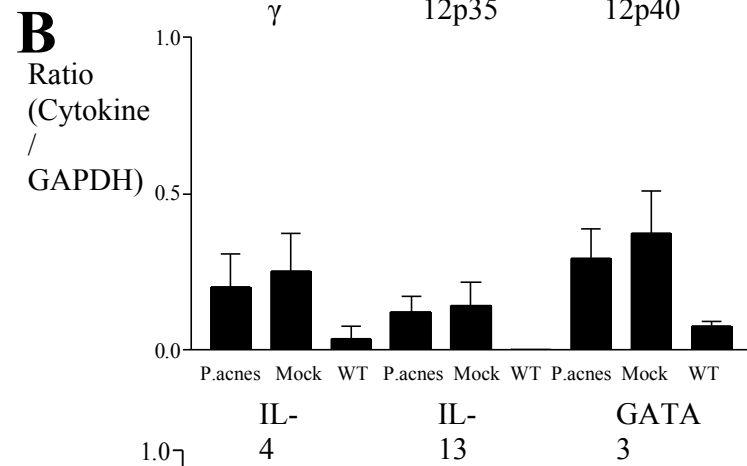
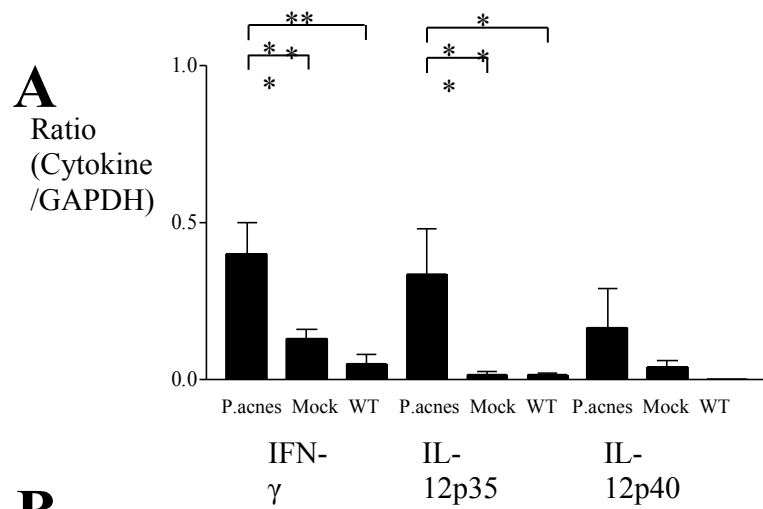
were higher in P. acnes-Tg than in Mock. **D**, The percentage of IL-4+CD4+ cells remained

unchanged in the three groups. **E**, The percentage of FoxP3+CD4+CD25+ T cell population was

significantly higher in P. acnes-Tg than that in Mock. **F**, The percentage of IL-17+CD4+ T cells

was increased in P. acnes-Tg and Mock compared with WT. **G**, The percentage of IL-10+CD4+ T

cells was higher in P. acnes-Tg than in WT. *P<0.05, **P<0.01



SI 4. mRNA expression in the skin lesions.

The cytokine profile of skin lesions was similar to that of spleen cells.

A, mRNA expression of IFN- γ and IL-12p35 was significantly increased in P. acnes-Tg than in Mock or WT. **B**, No significant changes were found in IL-4, IL-13, or GATA3 mRNA among the three groups. **C**, The mRNA expression of IL-17A, IL-22, and IL-23 did not differ significantly in the skin lesions from P. acnes-Tg and Mock. The mRNA of IL-17A receptor was less in P. acnes-Tg and Mock than in WT, and that of MIP2 was slightly decreased in P. acnes-Tg than in Mock. **D**, the expression of IL-10 and TGF- β mRNA in P. acnes-Tg was significantly elevated compared with those in Mock. *P<0.05, **P<0.01, ***P<0.001

Atopic dermatitis mouse model

KCASP1Tg is a keratin 14 promoter driven caspase-1 transgenic mouse that develops itching dermatitis with acute erosive lesions followed by chronic lichenoid lesions from face to body and extremities with plasma IgE and histamine elevation (1-3). The skin lesions show mononuclear cells and mast cells infiltration and dermal fibrosis. These findings fulfilled the diagnostic criteria of Hanifin & Rajka (4) and U.K. working party (5) for AD except for a history of asthma or hay fever. In all experiments, age-matched female C57/BL6 mice (SLC Shizuoka, Japan) were used as controls.

P. acnes

P. acnes was cultured in brain-heart infusion medium supplemented with L-cysteine and Tween-80 as we reported previously (6). The harvested bacteria were washed with sterile distilled water, killed by heating at 60°C for 60 min, and then lyophilized.

P. acnes treatment

Heat-killed *P. acnes* (66µg) was injected intra-peritoneally into 4 week-old KCASP1Tg mice, and then continued twice a week for 10 weeks. We set up three groups of mice: Mock-treated (20µl PBS was intra-peritoneally injected) KCASP1Tg (Mock, n=10), *P. acnes*-treated KCASP1Tg (*P. acnes*-Tg, n=9), and wild-type C57/BL6 (WT, n=8). All mice were kept under SPF conditions. Animal care was performed according to ethical guidelines, and the experimental protocol was approved by the Institutional Board Committee for Animal Care and Use of Mie University.

Histopathological study

The skin lesions were sampled at 14th weeks of age. Samples were embedded in paraffin and stained with haematoxylin & eosin and toluidine blue. The numbers per mm² of mast cells were counted in randomly selected high power fields. The numbers of mast cells were counted in randomly selected high power fields(×400).

Cell isolation and preparation

The spleens were obtained at 14th week, and single cell suspensions were prepared. After passing through a 70-µm-pore mesh, cells were washed with PBS and resuspended with 5 ml of PBS. After Ficoll (SIGMA, St. Louis, MO, USA) separation, spleen cells were resuspended in RPMI containing 10% FBS.

In vitro stimulation for cytokine production

Spleen cells were cultured in RPMI 1640 medium with 10% (v/v) FBS (5% of murine serum was used for IL-10 analysis), 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Spleen cells were seeded at 2×10^6 cells/ml (1 ml/well) in 24-well culture plates (Costar, corning, NY, USA) supplemented with 1 µg/ml of anti-mouse CD3e (BD Pharmingen, San Jose, CA, USA), 2 µg/ml anti-mouse CD28 (BD Pharmingen) and 1 µg/ml brefeldin A (Biolegend, San Diego, CA, USA). The cells were incubated for 8 h at 37°C in a 5% CO₂ atmosphere.

Cell surface antigen and intracellular cytokine staining

The cell surface antigen and intracellular cytokines were stained according to the formal Cell Surface Immunofluorescence Staining Protocol and Intracellular Cytokine Staining Protocol (BioLegend). Briefly, for the detection of IFN-γ, IL-4, and IL-17, the cells were stained with PE/Cy5 anti-mouse CD4 (BioLegend). After treatment with Fixation Buffer (BioLegend) and Permeabilization Wash Buffer (BioLegend), cells were treated with PE-conjugated anti-mouse IL-17A, IL-4, or FITC-conjugated anti-mouse IFN-γ (BioLegend). For the detection of IL-10, stimulated cells were stained with FITC anti-mouse CD4 (BioLegend), and PE-conjugated anti-mouse IL-10 (Miltenyi Biotec, Bergisch Gladbach, Germany). Fluorescence profiles were analyzed by flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA, USA).

FoxP3 Intracellular Staining

The cells were initially stained with FITC anti-mouse CD4 and PE/Cy5 anti-mouse CD25, and fixed in FoxP3 Fix/Perm solution (BioLegend). The cells were then stained with PE conjugated anti-mouse FoxP3 antibody (BioLegend). Fluorescence profiles were analyzed by flow cytometry using FACSCalibur.

Analysis of cytokine mRNA expression

The mRNA was extracted from spleen cells without stimulation and skin lesions using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions: 1 ml of homogenate was mixed with 200 µl of chloroform, and then centrifuged. The aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifuging, the precipitate was washed with 70% ethanol (Nacalai Tesque) and the RNA was suspended in 40 µl RNase-free water. The concentration was measured at 260 nm absorbent, and the quality was confirmed by electrophoresis. cDNA was synthesized from 2 µg of mRNA using an archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure transcriptional activity in spleen cells and skin lesions. A 25-µl reaction mixture containing 1 µg of cDNA, 900 nM of each primer, and 250 nM of TaqMan probe was mixed with 12.5 µl of TaqMan Master Mix (AB). Quantitative RT-PCR for cytokine transcripts was performed using prequalified primers and probes for IL-4, IL-10, IL-12p35, IL-12p40, IL-13, IL-17A, IL-22, IL-23p19, IL-17AR, MIP2, T-bet, GATA3, IFN-γ, and GAPDH (AB). The $\Delta\delta C_t$ method was used to standardize the transcripts to GAPDH, and the ratio to that of control mice was calculated.

References

1. Yamanaka K, Tanaka M, Tsutsui H *et al.* Skin-specific caspase-1-transgenic mice show cutaneous apoptosis and pre-endotoxin shock condition with a high serum level of IL-18. *J Immunol* 2000; **165**: 997-1003.
2. Yoshimoto T, Mizutani H, Tsutsui H *et al.* IL-18 induction of IgE: dependence on CD4⁺ T cells, IL-4 and STAT6. *Nat Immunol* 2000; **1**: 132-7.
3. Konishi H, Tsutsui H, Murakami T *et al.* IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion

independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci U S A* 2002; **99**: 11340-5.

4. Hanifin J M, Rajka G. diagnostic features of atopic dermatitis. *Acta Dermatovenereol Suppl(Stockh)* 1980; **92**: 44-7.

5. Williams H C, Burney P G, Hay R J *et al*. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br J Dermatol* 1994; **131**: 383-96.

6. Tsutsui H, Matsui K, Kawada N *et al*. IL-18 accounts for both TNF- α and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. *J Immunol* 1997; **159**: 3961-7.