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GATA-3 regulates contact hyperresponsiveness in a murine model of allergic dermatitis

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ABSTRACT

Contact hypersensitivity (CHS) is thought to be associated mainly with the activation of T helper (Th) type 1 cells. However, evidence also suggests that Th type 2 cells (Th2) and cytokines play roles in the development of CHS in humans. The present study examines the Th2 response during the development of CHS in response to 2,4,6-trinitrochlorobenzene (TNCB) in GATA-3-transgenic (GATA-3 Tg) mice.

GATA-3 Tg mice were immunized with 7% TNCB applied to abdominal shaved skin. Seven days later, the mice were challenged with 1% TNCB applied to the left ear. Ear swelling, cytokine production in the skin of the ear, and the levels of IgE, IgG1 and IgG2a were measured. Furthermore, we examined the effects of medical treatment on TNCB-induced contact dermatitis using this model. The ear-swelling responses of TNCB-sensitized/challenged GATA-3 Tg mice were significantly greater than those of similarly treated wild-type (WT) mice. The expression of both IL-5 and IL-13 in TNCB sensitized/challenged skin tissues and the IgE response after challenge were obviously increased in the GATA-3 Tg mice, whereas the expression of IFN- γ was identical in the challenged skin tissues of GATA-3 Tg and WT mice. When TNCB-sensitized GATA-3 Tg mice were treated with a high dose of tacrolimus, ear swelling was not significantly decreased, compared with the results in WT mice.

These results suggest that GATA-3-induced Th2-dominant responses play a critical role in the pathogenesis of allergic types of dermatitis, such as atopic dermatitis, and may lead to useful new drug development in the future.

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Introduction

The GATA transcription factors are major regulators of the hematopoietic and immune systems. Among them, GATA-1, -2, and -3 play crucial roles in the development of erythroid cells, hematopoietic stem and progenitor cells, and Th2 cells, respectively. GATA-3 is a novel transcription factor that mediates Th2 differentiation (Zhang et al. 1997; Zheng and Flavell 1997). GATA-3 plays an essential role in the earliest steps of T cell development

(Ting et al. 1996; Hattori et al. 1996). In addition to its essential role in T-cell development, GATA-3 is also a Th2 differentiation factor (Farrar et al. 2001). Naive CD4⁺T cells express low levels of GATA-3 mRNA. The expression of GATA-3 is significantly up-regulated in cells that differentiate along the Th2 lineage (Zhang et al. 1997; Zheng and Flavell 1997), and it is indispensable for Th2 development. GATA-3 transactivates the IL-5 and IL-13 promoter, but appears to have limited effects on IL-4 gene transcription (Zheng and Flavell 1997; Lavenu-Bombléd et al. 2002; Zhang et al. 1998).

We generated GATA-3 Tg mice and demonstrated that the expressions of IL-5 and IL-13, the number of eosinophils in the bronchoalveolar lavage fluid, and the levels of serum IgE increased in these mice after active sensitization to ovalbumin aerosol (Tamauchi et al. 2004). Also, we recently determined that a Th2 type immunoresponse was involved in a Th1 type-dependent metal allergy (Niiyama et al. 2010). Because Th2 type immunity was thought to be involved in a contact dermatitis model and

Abbreviations: CHS, contact hypersensitivity; TNCB, 2,4,6-trinitrochlorobenzene; FK506, tacrolimus.

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drug responses, we examined these reactions using a GATA-3 Tg mouse model. These results suggested that GATA-3-induced Th2-dominated responses play key pathogenetic roles in atopic dermatitis (AD), atopic asthma, and allergic rhinitis. To clarify the role of GATA-3 in allergic dermatitis, we investigated the Th2-dominant response during contact hypersensitivity (CHS) reactions in GATA-3 Tg mice exposed to 2,4,6-trinitrochlorobenzene (TNCB).

Materials and methods

Mice

GATA-3 transgenic mice were established as described (Tamauchi et al. 2004). C57BL/6 mice with a RAG2^{-/-} background were housed under specific pathogen-free conditions and had free access to a commercial diet and water. The mice were studied at 8–15 weeks of age. Animals were cared for and handled according to our institutional guidelines and our institutional review board approved all procedures associated with the study.

Immunization and challenge

The mice were immunized by an epicutaneous application of 100 μ L of 7% 2,4,6-trinitro-1-chlorobenzene (TNCB; Tokyo Kasei Co., Tokyo, Japan) in acetone to abdominal shaved skin. Seven days later, the mice were challenged by applying 20 μ L of 1% TNCB to both sides of the left ear. Ear swelling was measured using a dial thickness gauge (Ozaki Engineering) under light ether anesthesia at various time points thereafter. For the time-course analysis of the ear-swelling reactions, ear thickness was usually measured 3, 6, 9, 12, 18, 24 and 48 h after the challenge.

RT-PCR analysis of extracts from skin tissue

Sensitized or unsensitized WT and GATA-3-Tg mice were killed at 18 h after challenge with TNCB by cervical dislocation. The excised ears were homogenized with ISOGEN (Nippon Gene Co.), and the total RNA was extracted according to the manufacturer's instructions. The following amplification program was used: 1 cycle of 95 °C for 9 min, followed by 40 cycles of 94 °C for 1 min, 60 °C for 1.5 min, 72 °C for 2 min (for Ick-GATA-3) or 40 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min (for β -actin) and a final cycle of 72 °C for 7 min. The enforced GATA-3 primer was applied as described, and the other primers were those for β -actin (5'-5'-TGGATCCTGTGGCATCCATGAAAC-3'; 3'-5'-TAAACGAGCTCAGTAACAGTCCG-3').

Histological examination

Ear skin specimens were excised at 24 h after applying TNCB, fixed in 10% formalin, then stained as follows. The numbers of mononuclear cells and granulocytes, such as neutrophils and eosinophils, infiltrating the dermis were evaluated by staining with hematoxylin and eosin. The number of mast cells infiltrating the dermis was evaluated by staining with toluidine blue. Sections were examined at a magnification of 400 $\times$. At least 10 fields were examined for each lobe. The results are expressed as the numbers of cells per mm².

Quantification of cytokine levels in extracts from skin

Extracts from the ear tissues were prepared for ELISA as described by Ferguson et al. (1994). Briefly, the ears were excised at 18 h after the TNCB application and immediately homogenized with 500 μ L of 0.1% Tween 20 in PBS. The samples were quickly

frozen in liquid nitrogen, thawed in a 37 °C water bath, sonicated for 15 s, and sedimented by centrifugation for 5 min at 13,000 $\times$ g. The supernatants were stored at -80 °C before cytokine assay. Interleukin-4, IL-5, and IFN- γ were assayed using ELISA kits (Endogen) according to the manufacturer's instructions. Interleukin-13 was assayed using a mouse IL-13 Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

Measurement of serum concentrations of total IgE, IgG1, and IgG2a

The total IgE, IgG1 and IgG2a antibody titers in the serum of sensitized/challenged mice were measured using a sandwich ELISA, as described previously (Tamauchi et al. 2004). Serum samples and control serial dilutions were incubated in 96-well microplates coated with anti-mouse antibody against each isotype. Biotin-labeled rat mAb against mouse IgE, IgG1 or IgG2a mAb was added for the second incubation. After a thorough wash, the plates were incubated with streptavidin-horseradish peroxidase. Color developed using a peroxidase reaction in TMB substrate was measured at 450 nm using an ELISA plate reader.

Passive transfer of CHS sensitivity

To determine the effect of GATA-3-transgenic-purified CD4⁺ T cells on the elicitation of CHS, GATA-3 Tg or WT mice were sensitized with TNCB. Lymph node cells were drained on day 7. Single cell suspensions were prepared by gentle teasing and were washed three times with PBS. These cells were passed through a column packed with nylon fiber (Wako Pure Chemicals). CD4⁺ T cells were positively selected by magnetic sorting using Dynabeads and DTACHaBEAD mouse CD4. The CD4⁺ T cells were usually >95% pure according to flow cytometry. CD4⁺ T cells (2 $\times$ 10⁶/mouse) were intravenously transferred into RAG2^{-/-} mice. The ears of recipient mice were challenged immediately thereafter with TNCB, and the swelling response was measured 6, 12, 18, 24 and 48 h later.

Drugs and treatment

Tacrolimus ointment (20 mg of 0.1% FK506) and tacrolimus in powder was a kind gift by Fujisawa Pharmaceutical Co. (Osaka, Japan), and tacrolimus in powder was reconstituted in absolute ethanol to a concentration of 10 mg/mL and stored until use at -80 °C. Dilutions were made in sterile PBS for intraperitoneal administration. 9-Fluoro-11 β ,17,21-trihydroxy-16 β -methylpregna-1,4-diene-3,20-dione 17-valerate (betamethasone valerate, Rinderon-V) and (+)-7 α -Chloro-11 β ,17,21-trihydroxy-16 α -methyl 1,4-pregnadine-3,20-dione 17,21-dipropionate (alclometasone dipropionate, Almeta) were purchased from Shionogi Pharmaceuticals Co. Ltd. (Osaka, Japan). ($\pm$)-[2-[4-(3-Ethoxy-2-hydroxypropoxy) phenylcarbamoyl]ethyl] dimethylsulfonium *p*-toluene sulfonate (suplatast tosilate, IPD-1151T) and dexamethasone propionate (Methaderm ointment) was purchased from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). 4-(1-Methylpiperidine-4-ylidene)-4H-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10(9H)-one monofumarate (ketotifen fumarate, Zaditen) was purchased from Chiba-Giegy Ltd. (Tokyo, Japan).

N-(2-Benzhydroxyethyl)-*N,N*-dimethylamine (diphenhydramine, Restamine) was purchased from Kowa Ltd. (Tokyo, Japan). Tacrolimus, Rinderon-V, Almeta and Restamine ointments were applied to the inner and outer sides of the ears 20 min before the challenge. The controls received Vaseline ointment. Suplatast (1 mg/60 kg) and ketotifen (60 mg/60 kg) were dissolved in PBS and injected (i.p.) into the mice 20 min after TNCB re-stimulation. A second injection was performed 24 h later.

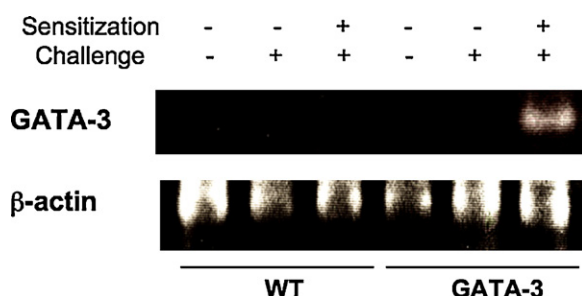


Fig. 1. Expression of GATA-3 in TNCB-sensitized/challenged transgenic mice. RT-PCR analysis shows the expression of GATA-3 mRNA from skin tissue extracts in non-immunized wild-type (WT) and GATA-3-Tg mice and at 18 h after challenge with TNCB in sensitized or unsensitized WT and GATA-3-Tg mice. GATA-3 mRNA was expressed in sensitized GATA-3-Tg mice. In contrast, GATA-3 mRNA expression was undetectable in unsensitized WT and GATA-3-Tg mice.

Statistical analysis

Data were expressed as the mean $\pm$ SD and were statistically analyzed using the Student *t*-test.

Results

Expression of GATA-3 mRNA in extracts of skin tissue from GATA-3 Tg mice challenged with TNCB

We evaluated GATA-3 expression using RT-PCR in skin tissue from GATA-3 Tg and WT mice treated or not with TNBS. GATA-3 mRNA was detected in skin tissue extracts of TNCB-sensitized/challenged GATA-3 Tg mice but not in those of WT mice. In contrast, the expression of GATA-3 mRNA was undetectable in non-sensitized/challenged WT and GATA-3 Tg mice (Fig. 1). Moreover, GATA-3 mRNA expression was detectable in skin tissue extracts from sensitized/challenged GATA-3 Tg mice but was undetectable in skin tissue from non-sensitized/non-challenged GATA-3 Tg and WT-mice (Fig. 1).

Up-regulation of ear swelling response to TNCB in GATA-3 Tg mice

We evaluated the capacity of GATA-3 Tg mice to develop a CHS reaction in response to TNBS. Fig. 2a shows that the CHS response to TNBS increased remarkably and that the early phase of CHS to TNBS was increased in GATA-3 Tg mice, compared with WT mice. Ear swelling peaked at 18 h after challenge in GATA-3 Tg mice, and the ear swelling response continued to be visible at more than 48 h after the challenge in GATA-3-Tg mice (Fig. 2a). However, TNCB elicited a typical DTH response in the non-treated left ears of these mice that was indistinguishable from that in normal mice (data not shown).

Histopathology of TNCB-induced CHS in GATA-3 Tg mice

Since the CHS to TNCB in GATA-3 Tg mice increased at 18 h after the challenge, we histologically analyzed the responses in GATA-3 Tg and WT mice sensitized with TNCB. The inflammatory response was very severe, with infiltration by eosinophils, monocytes/macrophages, and mast cells. Fig. 2b shows severe edema in the challenged skin of sensitized-GATA-3 Tg, but not in that of WT mice. In contrast, the inflammatory response in the challenged skin of WT mice was mild, with the diminished infiltration of mononuclear cells and neutrophils. The numbers of infiltrating eosinophils and neutrophils in the skin were significantly higher in sensitized/challenged GATA-3 Tg mice than in similarly treated WT mice (Fig. 2c).

Cytokine production in ear tissues of TNCB-challenged GATA-3 transgenic mice

The levels of both IL-5 and IL-13 were significantly higher in the supernatants of skin tissues obtained from TNCB-sensitized/challenged GATA-3-Tg mice than in similarly treated WT mice. The levels of IL-5 and IL-13 in the TNCB-sensitized/challenged GATA-3 Tg mice were 2- to 3-fold higher than those in the control WT mice. On the other hand, IL-4 and IFN- γ production did not differ significantly between the TNCB-sensitized/challenged GATA-3 Tg mice and the WT mice (Fig. 2d).

Elevation of serum IgE in TNCB-sensitized GATA-3 Tg mice challenged with TNCB

Because IgE is thought to be important in the pathogenesis of CHS, GATA-3 and WT mice were sensitized with TNCB. Eighteen hours later, the mice were challenged with TNCB; the serum concentrations of IgE, IgG1 and IgG2a were then measured using ELISA kits. Neither the non-sensitized/challenged nor the non-sensitized/non-challenged mice produced detectable levels of serum IgE (data not shown). The TNCB-sensitized/challenged GATA-3 Tg mice produced significantly more serum IgE than did the TNCB-sensitized/challenged WT mice (Fig. 2e). The serum IgG1 and IgG2a levels elicited in this experiment were comparable between the TNCB-sensitized/challenged GATA-3 Tg and the TNCB-sensitized/challenged WT mice (Fig. 2e).

Effect of CD4⁺ T cells purified from GATA-3-Tg mice on the transfer of CHS

We examined whether GATA-3-dependent CD4⁺ cells enhance the response of ear swelling in TNCB-sensitized GATA-3 Tg mice. CD4⁺ T cells from the lymph nodes of WT and GATA-3-Tg mice that had been immunized with TNCB were transferred into RAG2^{-/-} mice, and the RAG2^{-/-} mice were then challenged with TNCB. The ears of RAG2^{-/-} mice with CD4⁺ cells transferred from immunized GATA-3 Tg mice were more swollen than those of the immunized WT mice at 6, 12, 18, 24, and 48 h after elicitation. Ear swelling peaked at 18 and 24 h after elicitation with TNCB in the GATA-3 Tg and WT mice, respectively (Fig. 3a). These results suggested that GATA-3-dependent CD4⁺ cells from TNCB-sensitized GATA-3 Tg mice regulated the ear swelling response more significantly than that of WT mice. A histological examination showed that the edema was severe in skin lesions from RAG2^{-/-} mice given CD4⁺ splenic T cells from TNCB-sensitized GATA-3 Tg mice, but not in those from RAG2^{-/-} mice that received such cells from sensitized WT mice. The obvious inflammatory response of RAG2^{-/-} mice given CD4⁺ T cells from TNCB-sensitized GATA-3 Tg mice included the considerable infiltration of eosinophils, neutrophils and mononuclear cells. In contrast, the inflammatory response was mild in the skin of both the RAG2^{-/-} mice that received CD4⁺ T cells from TNCB-sensitized GATA-3 Tg and WT mice, and the infiltration of mononuclear cells, eosinophils and neutrophils was diminished. RAG2^{-/-} mice that received CD4⁺ T cells from TNCB-sensitized GATA-3 Tg mice had significantly more eosinophils and neutrophils than those given such cells from similarly treated WT mice (Fig. 3b). Immunoglobulin E is thought to be important in the pathogenesis of CHD. However, IgE was undetectable in the serum of RAG2^{-/-} mice regardless of whether they received CD4⁺ T cells from TNCB-sensitized GATA-3 or WT mice (data not shown). The levels of IL-5 and IL-13 were significantly higher in supernatants from skin tissues from TNCB-challenged RAG2^{-/-} mice given CD4⁺ T cells from TNCB-sensitized GATA-3 Tg mice, than in those from RAG2^{-/-} mice given cells from TNCB-sensitized WT mice. However, the levels of IL-4 and IFN- γ did not differ in the supernatants of inflammatory

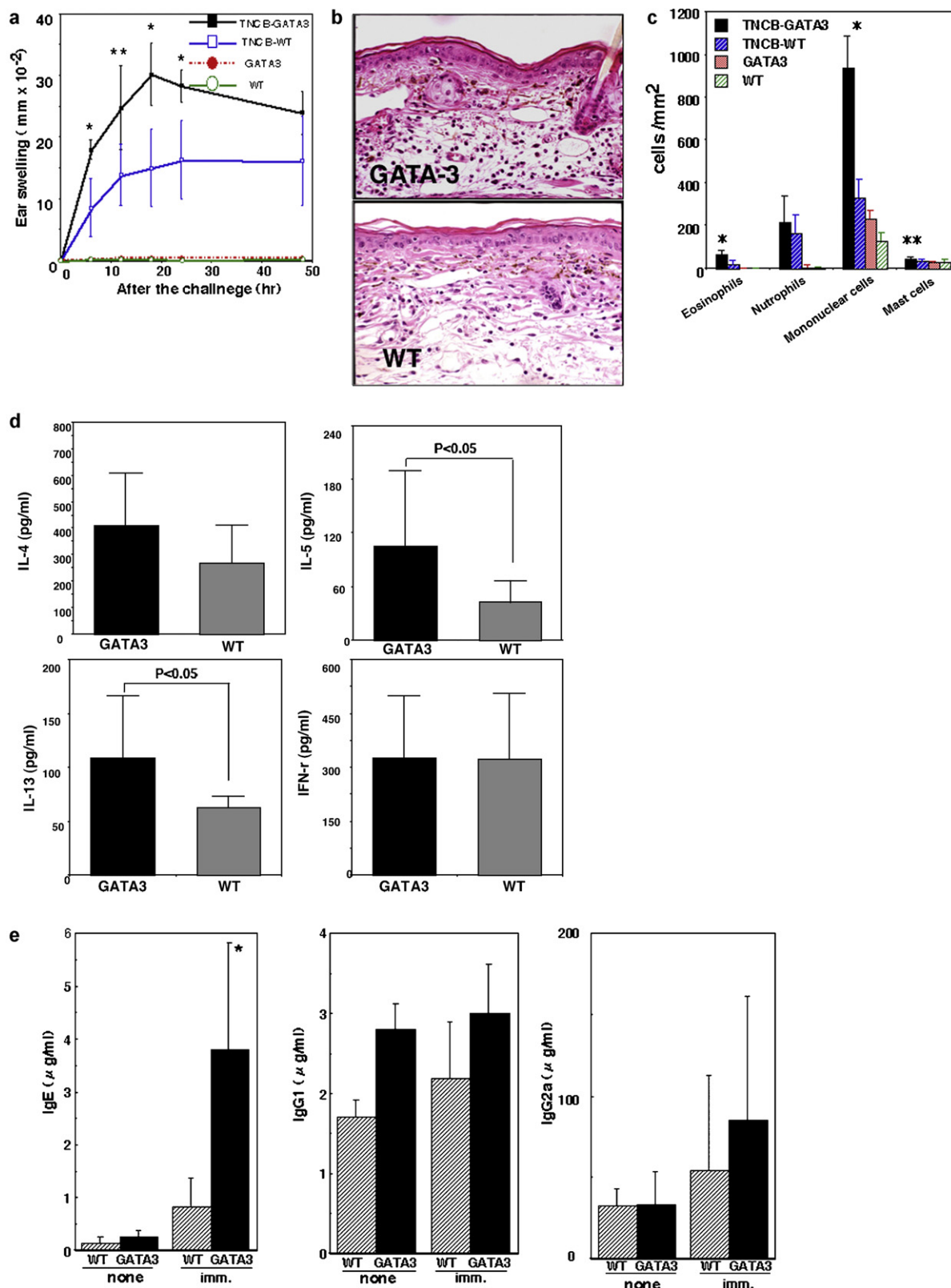


Fig. 2. Immunological response of TNBC-sensitized GATA-3 Tg after elicitation with TNBC. (a) TNBC-challenged GATA-3 Tg mice had significantly greater ear-swelling responses than TNBC-challenged wild-type (WT) mice at 6, 12, 18, and 24 h after elicitation. GATA-3-Tg and WT mice were contact-sensitized by a single topical painting with 7% TNBC or were not sensitized. Seven days after immunization, both mice were challenged. Ear swelling reached a maximum at 18 and 24 h for the GATA-3 Tg and WT mice, respectively. Data are the mean ± SD of 5 and 10 mice per group, respectively, and are representative of three independent experiments. **P* < 0.01, significantly different compared with WT mice. ***P* < 0.05 vs. TNBC-challenged WT mice. (b) Sections were stained with hematoxylin and eosin. And then the sections were observed at a magnification of 400×. (c) Distribution of cell types in skin from GATA-3 Tg and WT mice challenged with 1% TNBC. The bars represent the numbers of eosinophils, neutrophils, monocytes/macrophages, and mast cells. Data are the mean ± SD for groups of 10 mice. **P* < 0.01 and ***P* < 0.05 vs. TNBC-challenged WT mice. (d) Cytokine levels in skin tissue supernatants in TNBC-challenged GATA-3 Tg and WT mice. Data are the mean ± SD for groups of three mice and are representative of three independent experiments. (e) Serum IgE, IgG1 and IgG2a levels in pre-immunized WT and GATA-3 Tg mice and at 18 h after TNBC challenge in sensitized WT and GATA-3 Tg mice. Data represent the mean ± SD for groups of 5 mice and are representative of three independent experiments. **P* < 0.01 vs. TNBC-challenged WT mice.

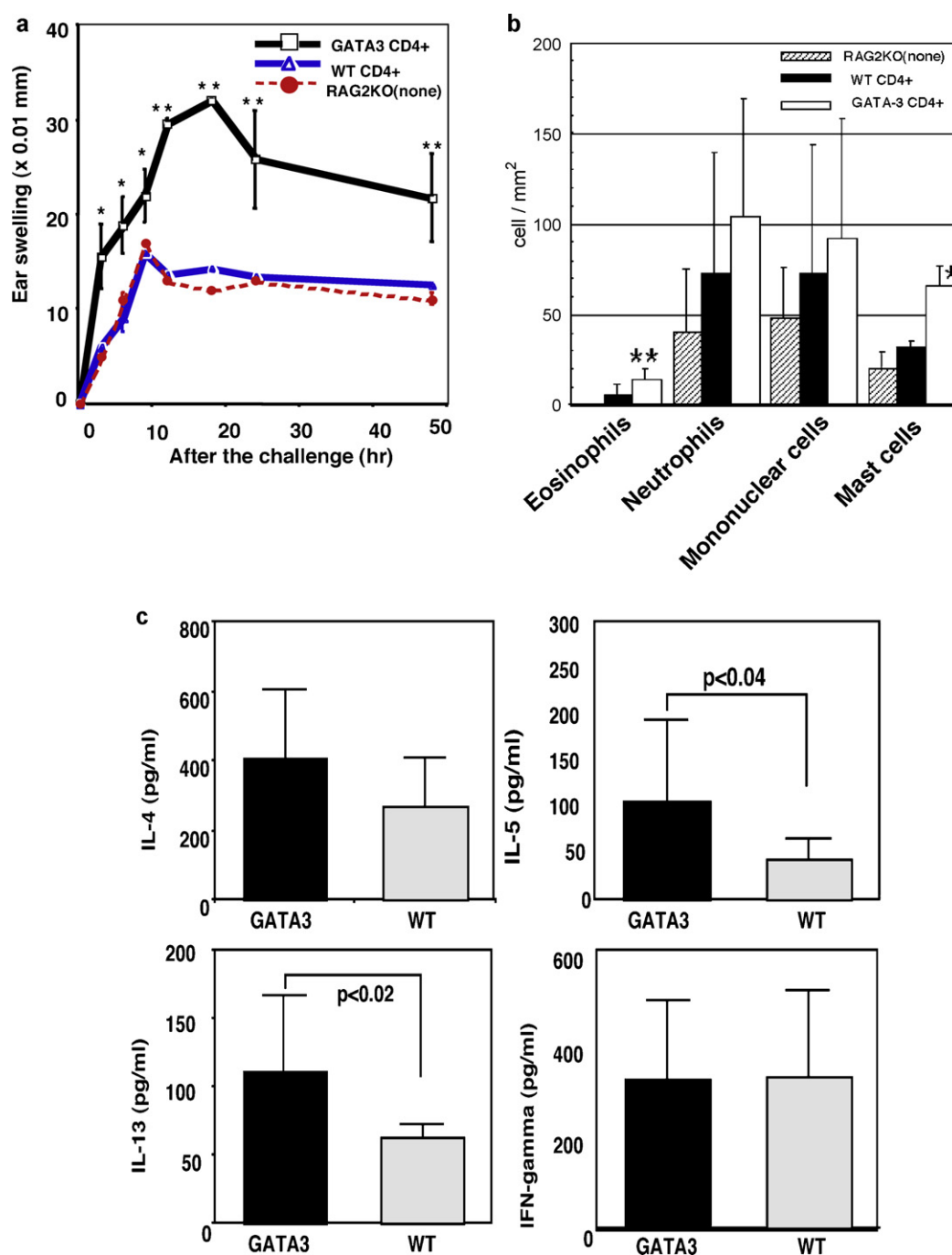


Fig. 3. Immune response of Rag-2^{-/-} mice with CD4⁺ cells transferred from the lymph nodes of TNBC-sensitized GATA-3 Tg mice. CD4⁺ T cells (2×10^6) from TNBC-sensitized GATA-3 or WT mice were intravenously transferred into Rag-2^{-/-} mice. One day later, the Rag-2^{-/-} mice were challenged with TNBC. (a) Increases in the ear thickness of Rag-2^{-/-} mice carrying CD4⁺ lymph node cells from TNBC-sensitized GATA-3 Tg or WT mice were measured at 6, 12, 18, 24, and 48 h after challenge. CD4⁺ T cells from TNBC-sensitized GATA-3 Tg (□) and TNBC-sensitized WT (Δ) mice; non-transferred Rag-2^{-/-} mice (●). **P* < 0.05 and ***P* < 0.01 vs. Rag-2^{-/-} mice carrying with CD4⁺ T cells from TNBC-sensitized WT mice. (b) At 18 h after the TNBC-challenge, the ear lobes were fixed in 10% formalin, stained with hematoxylin and eosin, and examined under a microscope. **P* < 0.01 and ***P* < 0.01 compared with non-transferred Rag-2^{-/-} mice group. (c) Cytokine levels in skin tissue supernatants from Rag-2^{-/-} mice carrying CD4⁺ T cells from TNBC-sensitized GATA-3 Tg or WT mice.

ear lesions of Rag-2^{-/-} mice given TNBC-sensitized CD4⁺ cells from either GATA-3 Tg or WT mice (Fig. 3c).

Topical tacrolimus reduced ear swelling in Th1-dominant WT mice

We investigated the effects of tacrolimus, suplastat, ketotifen, Almet and Restamine on TNBS-induced ear swelling by the administration of these agents at 20 min prior to the TNBS re-challenge. As shown in Table 1, a single abdominal administration of tacrolimus

significantly reduced the ear swelling of WT mice (92%), compared with that in GATA-3 Tg mice (60%), after the TNBC challenge at 18 h (Fig. 4 and Table 1). Furthermore, a single topical application of 0.1% tacrolimus ointment more obviously reduced ear swelling in the WT mice than in the GATA-3 Tg mice (Fig. 4). We also confirmed that the intraperitoneal administration of tacrolimus conferred the same effects in both types of mice (not shown data). Treatment with suplastat tosylate inhibited the ear swelling responses by approximately 50% in the GATA-3 mouse group and the WT mouse group.

Table 1

Effect of tacrolimus ointment on ear thickness in mice with TNCB-induced contact dermatitis.

Drug	% Reduction	
	GATA-3	WT
Tacrolimus (FK506)	60.0	92.0
Suplatast	53.6	45.4
Ketotifen	13.3	11.5
Restamine	9.3	0.0
Rinderon	75.0	18.3
Almeta	92.0	51.0
Methaderm	90.7	57.1

GATA-3 Tg mice were sensitized by applying 7% TNCB to their abdomens; 1% TNCB was then applied to their ears 1 week later, and ear swelling was measured after 18 h. Ointment (20 mg; tacrolimus, Restamine®, Rinderon®-V, Almeta®, Methaderm®) was applied to the ears followed by challenge with 1% TNCB 20 min later. Suplatast tosilate (60 mg/Kg) and Ketotifen (0.03 mg/Kg) were administered 20 min before TNCB re-stimulation. A second injection was performed 4 h later. As a control, white petrolatum (Vaseline) was used.

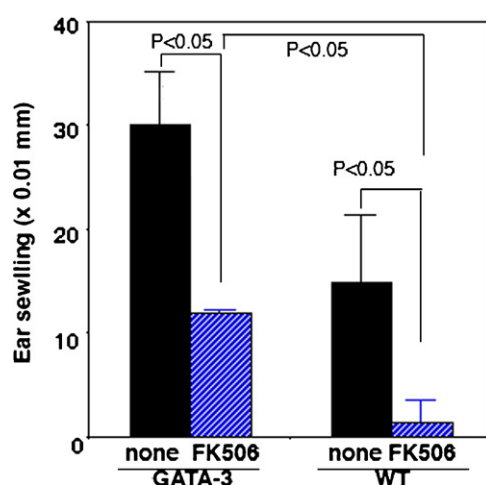


Fig. 4. Tacrolimus significantly reduced ear swelling. Sensitization was performed by applying 7% TNCB solution to the abdomen of each GATA-3 Tg mouse. One week later, the 1% TNCB solution was applied to the ear and the ear swelling reaction was measured at 18 h after the challenge. Alternatively, the ear was treated with 20 mg of ointment (tacrolimus [0.1%]; FK506), and a challenge with the TNCB solution was performed 20 min later. As a control, white petrolatum (Vaseline) was used.

Suplatast tosilate is an antiallergic agent that is capable of suppressing Th2 type immunoresponses related to IgE synthesis and chemical release. In the present study, however, suplatast tosilate had a more drastic suppressive effect on ear swelling in the GATA3-Tg mice than in the WT mice. However, a slight controlling effect was observed in the GATA3-Tg mice. Ketotifen and Restamine did not have a suppressive effect on antigen-induced ear swelling in GATA-3 and WT mice. In contrast, Almeta, Rinderon and Methaderm inhibited CHS more strongly in GATA-3 Tg mice than in WT mice. Tacrolimus and Almeta are immunosuppressive and steroid agents that have powerful, differential inhibitory effects on the late phases of cutaneous allergy as a result of a T cell-dependent mechanism involved in hypersensitivity. However, tacrolimus ointment had a greater controlling effect for Th1 type immune reactions than for Th2 type immune reaction. Tacrolimus reduced erythema, edema, scaling and excoriation and was effective against the clinical signs and histological changes in the skin in this model.

Discussion

Th1 cells and their cytokines are believed to play important roles in the CHS reaction, although other evidence indicates that Th2 cells in mouse skin can induce a cellular inflammatory response with

faster kinetics (Yokozeki et al. 2000; Muller et al. 1993). Here, we examined whether the Th2-type immune response is also involved in the pathogenesis of the CHS response. We recently established that GATA-3 transcription factor regulates the Th2-type immune response but does not effectively suppress the Th1-type response *in vivo* (Tamauchi et al. 2004). Therefore, we used a GATA-3 Tg mouse model to study the roles of Th2 cytokines, IL-4, IL-5 and IL-13, in CHS. The ear swelling response in the GATA-3 Tg mice was significantly enhanced at 18 h after the challenge, compared with that in the WT mice. The ear swelling response continued until 48 h after the challenge. The kinetics of ear swelling in WT and GATA-3 Tg mice were similar, but the magnitude of the response was 3-fold higher in the GATA-3 Tg mice. Terui et al. (2001) reported that Th2-mediated ear swelling peaked at 24 h after antigen the challenge, whereas Th1-mediated ear swelling peaked at 48 h in the TCR-Tg mouse model using OVA. Our results indicated that the kinetics of ear swelling in GATA-3 Tg mice after the challenge was reflected in the Th2-type immune response elicited by TNCB-sensitization and in the Th1-type response, which was not suppressed by the Th2-type response. Furthermore, RT-PCR identified Th1 and Th2 cytokine mRNA during the late stage of the ear swelling response, whereas the Th1 profile was undetectable at the early stage (data not shown). The results did not show it, but the 1–6 h and a late auricular swelling response were observed to the 24 h lesson an early auricular swelling response. We induced OVA-specific contact sensitivity to the GATA-3/TCR-Tg mouse which was immunized with OVA and Alum. An early ear swelling response was observed 1–6 h after OVA re-stimulation, and a late ear swelling response was observed 24 h after antigen re-stimulation. The GATA-3 transcription factor may be involved not only during the early stage response (in an OVA-specific IgE antibody-dependent manner), but also during the late response. This evidence indicated that both the Th1 and the Th2-mediated immune responses are key factors in establishing CHS in GATA-3 Tg mice; in this model, the Th2 cytokines are important factors involved in the induction of CHS.

Both IL-5 and IL-13 were up-regulated, and eosinophils, monocytes/macrophages, and mast cells infiltrated the skin lesions. IL-5 is considered a key factor in the growth, differentiation, and activation of eosinophils (Sanderson et al. 1988). IL-5 Tg mice develop obvious eosinophilia that is abrogated by anti-IL-5 antibody (Tominaga et al. 1991; Dent et al. 1990; Coffman et al. 1989). The upregulated IL-5 production in GATA-3 Tg mice can be explained by the direct binding of GATA-3 to the IL-5 promoter region (Zhang et al. 1997, 1998). We speculated that IL-5 induced eosinophil infiltration into skin lesions. Satoh et al. (2002) recently reported that the infiltration of eosinophils in the skin lesions of AD patients is involved in the P-selectin expression on endothelial cells. Our results showed that the Th2 cell and Th2 cytokine reactions arising within the lesional skin of patients with AD are mimicked in GATA-3 Tg mice. Mast cells are involved in the pathogenesis of atopy and allergic diseases (Hattori et al. 1996; Lavenu-Bombled et al. 2002). Mast cells secrete IL-4, IL-5, and IL-13 in response to the antigen cross-linking of IgE or by non-IgE-mediated mechanisms. In this manner, the early production of cytokines by mast cells might initiate a shift in the immune response towards Th2.

The hyperproduction of IgE in GATA-3 Tg mice challenged with TNCB is probably dependent on IL-13, rather than IL-4, in our system. Interleukin-4 determines B cell differentiation to produce IgE and IgG1 and was believed to have the sole responsibility for IgE production in the mouse (Finkelman et al. 1988). However, recent studies have demonstrated that IgE can be generated in IL-4-deficient mice, indicating that other factors are involved in murine IgE production (Punnonen et al. 1993; McKenzie et al. 1993; Zurazski and de Vries 1994). The discovery of IL-13 and its ability to induce IgE in human B cells highlights the complexity of the

regulation of IgE. The levels of IgE in the serum of IL-13 Tg mice were significantly higher than those in non-Tg mice (Emson et al. 1998). Moreover, IgE production is elevated in IL-4-deficient mice carrying the IL-13 transgene, suggesting that IL-4 is not required for IL-13-dependent IgE production in mice (Terui et al. 2001). The production of IL-13 was enhanced in GATA-3 Tg mice after TNCB challenge, whereas IL-4 was not elevated, compared with controls. GATA-3 might regulate IL-13 gene expression in Th2-skewed naive T cells since all IL-5-producing cells express IL-13, but not vice versa (Yamashita et al. 2002; Kishikawa et al. 2001). This means that IL-13 production is correlated with that of IL-5 under Th2-skewed conditions *in vitro*. In fact, enhanced IL-5 production was associated with the up-regulation of IL-13 in sensitized GATA-3 Tg mice challenged with TNCB. The level of IgG1 in the serum of TNCB-sensitized/challenged GATA-3 Tg mice was similar to that of WT mice, although the immunoglobulin subtype was Th2-dependent. We speculated that the induction of CHS in this study was mild, compared with that reported by others (Yokozeki et al. 2000), so the level of IgG1 in the serum was not obviously enhanced. Asherson et al. (1996) reported that IL-4 plays an important role in the pathogenesis of CHS. They speculated that the IL-4 secreted from mast cells or B220⁺ cells binds to α/β T cells, and thus induces them to produce IL-4 or other cytokines that increase the expression of adhesion molecules on endothelial cells, which play a critical role in the induction of CHS. In contrast, Berg et al. (1995) induced CHS in IL-4-deficient mice using 4-ethoxyl methylene-2-phenyl-2-oxazolin-5-one (Oxa). Traidl et al. (1999) reported that the complete loss of endogenous IL-4 expression in BALB/C mice is associated with an impaired manifestation of CHS to 2,4-dinitrochlorobenzene, but not to Oxa. Yokozeki et al. (2000) recently established STAT6-deficient mice and demonstrated that STAT6 plays a central role in IL-4- and IL-13-mediated biological responses. Moreover, they reported that the CHS response to TNCB, 2,4-dinitrofluorobenzene or Oxa was significantly reduced with a delayed peak in STAT6-deficient mice. To clarify the profile of cytokines in ear tissues after a challenge with TNCB, we examined the cytokine levels in skin tissue supernatants in TNCB-challenged/sensitized GATA-3 Tg and WT mice. Significant amounts of IL-5 and IL-13 were produced from the skin tissue supernatants of TNCB-challenged sensitized GATA-3 Tg mice, compared with the WT mice, but the levels of IL-4 and IFN- γ did not differ significantly. We previously described the profile of Th2 cytokine production in GATA-3 Tg mice after antigen-stimulation (Tamauchi et al. 2004), and the results were similar in the model used in the present study.

GATA-3 is required for the transcription of Th2 cytokines, but recent *in vitro* studies have suggested that GATA-3 does not equally control the production of all Th2 cytokines. For instance, several investigators have found that ectopic GATA-3 expression is sufficient to drive IL-5 expression, but not IL-4 expression, in non-Th2 cells (Zhang et al. 1997; Zheng and Flavell 1997). The present study identified a limited effect of GATA-3 on IL-4 expression *in vivo*, since GATA-3 overexpression did not contribute to increased IL-4 production in sensitized T cells (Zhang et al. 1999; Lee et al. 2000), whereas IL-5 and IL-13 production was completely GATA-3 dependent. However, in dominant-negative GATA-3 transgenic mice, IL-4 production is reduced by as much as that of IL-5 under polarizing culture conditions (Yamashita et al. 2004). These results indicated that GATA-3 is indispensable for both IL-4 and IL-5, or that the GATA-3 requirement for cytokine production is similar between committed Th2 cells *in vitro* and *in vivo*. This issue could be resolved by investigating the chromatin remodeling status at these regulatory regions in Th2 cell lines and in fresh T cells isolated from sensitized GATA-3 Tg mice (Simon et al. 1993; Andersson et al. 1992).

We examined whether the ear swelling response is dependent on CD4⁺ cells using Rag2^{-/-} mice transferred with CD4⁺ T cells from

TNCB sensitized-GATA-3 or WT mice. Ear swelling peaked at 18 h after the TNCB challenge, and IL-5 and IL-13 cytokine production in the tissue supernatants and the serum IgE levels were enhanced in TNCB-sensitized/challenged GATA-3 Tg mice. In this model, ear swelling was dependent on Th2-type CD4⁺ cells and cytokines, but the possibility that the Th1-type response was not associated with ear swelling after the TNCB challenge cannot be eliminated.

We also assessed the effect of tacrolimus on ear swelling in GATA-3 Tg mice. Tacrolimus ointment (20 mg) alleviated ear swelling in WT mice more than in GATA-3 Tg mice, but higher doses were equally suppressive in both types of mice. Studies of tacrolimus *in vivo* have so far failed to clearly explain its immunosuppressive activity. Tacrolimus works by inhibiting cytokine or chemokine production by Th cells. The potentiating effect of tacrolimus might be a consequence of selective inhibitory activity towards the Th1 subset, while at higher doses, the selectivity towards the Th1 subset is lost and the Th subsets are non-specifically suppressed (Yamashita et al. 1996; Koyama et al. 1999; data not shown). In contrast, the primary Th2 response to *Ascaris suum* extracts *in vivo* is inhibited by a high dose of tacrolimus, while the secondary Th2 response is potentiated (Fujino et al. 1998). How tacrolimus influences granulocyte function and accumulation remains unknown.

Although one report has indicated that tacrolimus reduces IL-8 expression in human eosinophils (Hendey et al. 1992), it did not appear to affect eosinophil recruitment for delayed worm expulsion in a murine parasite model. Tacrolimus inhibits the motility of neutrophils and the production of destructive superoxide radicals (Burnett et al. 1994), according to a recent report indicating that tacrolimus does not directly suppress neutrophil chemotaxis induced by several chemotactic factors. Taken together, these results suggest that the suppression of Th1-induced inflammatory skin responses induced by tacrolimus is mediated via its effects on granulocytes as well as on Th cells, although the results of the present study are inconclusive regarding this point. Our data indicated that ear swelling was more Th2-dependent in GATA-3 Tg mice and Th1-dependent in WT mice. The drug is also effective for treating patients with chronic severe allergic disease, such as bronchial asthma and AD (Wollenberg et al. 2008; Morishita et al. 2005). IgE antibody may play an important role in the development of these conditions.

In this study, supatast did not inhibit antigen-induced ear swelling via a T-cell mediated response. However, Matsuura et al. showed that chronic treatment with supatast significantly inhibited airway hyperresponsiveness, preventing immunized animals from producing specific IgE antibody, since supatast has been shown to selectively suppress the production of IgE antibody in mice without affecting that of IgM and IgG antibodies (Niiyama et al. 2010). Also, the suppression of the ear swelling response with steroids was remarkably stronger in GATA-3Tg mice than in WT mice (Table 1).

The mechanism of suppression is unclear, but Umland et al. (1997) suggested that glucocorticoids inhibit the production of the IL-4 and IL-5 involved in the Th2 immune response. Glucocorticoids inhibit IFN- γ production, which is a Th1 type immune response, but the Th2 type suppression of the immune response is stronger. These facts suggest that the Th2 type immune response is more strongly involved in the ear swelling response in the GATA-3Tg mice.

In our system, TNCB-induced ear swelling in GATA3-Tg mice was not an IgE-independent response because TNCB-immunized CD4⁺ cells from GATA3-Tg mice transferred into Rag2^{-/-} mice induced an IgE-independent ear swelling response.

The expression of GATA-3 is rapidly up-regulated during CD4⁺ CD45RA⁺ naive human T cell differentiation towards Th2 commitment and during the allergen-specific reactivation of peripheral CD4⁺ CD45RO⁺ Th2 memory cells established from AD patients,

whereas T cells from non-AD patients display equally rapid GATA-3 down-regulation under identical conditions of allergen stimulation (Ting et al. 1996). These findings suggest that GATA-3 is a key determinant of AD. Thus, the GATA-3-transgenic murine model is relevant to understanding the physiological significance of the roles of Th2 cells and Th2 cytokines in allergic skin diseases such as AD.

In summary, GATA-3 is essential for the induction of CHS. GATA-3 mediates CHS by playing a pivotal role in Th2 differentiation, resulting in the subsequent stimulation of IgE synthesis and the infiltration of eosinophils and mast cells in the skin. Using these models, we confirmed the usefulness of understanding the mechanism of development and that of a potential therapeutic drug for the treatment of AD. The knowledge gleaned from our murine model will be highly relevant to understanding the physiological significance of the Th2 response observed in chronic allergic diseases such as AD.

GATA-3 Tg mice harboring various promoters, such as CD2 and CD4, have a shifted Th2 response and produce IL-4, IL-5 and IL-13; furthermore, GATA-3 is expressed in T cells without specific stimulation. These GATA-3 Tg mice develop a high incidence of thymic lymphoma and other disorders (Tamauchi et al. 2004), but the GATA-3 Tg mice in the present study did not exhibit such diseases. Our model animals may also be useful for developing a new generation of therapeutics for the treatment of CHS and/or AD. Thus, we believe that GATA3-Tg mice are a useful model for investigating the pathogenesis of CHS and/or AD and for the development of treatments for diseases that depend on the Th2 type immune reaction. This mouse model can also be used to examine allergic inducibility or the effects of medicines for various kinds of allergens as well as specific allergens.

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