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Evidence of GATA-3-dependent T_h2 commitment during the *in vivo* immune response

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Abstract

The transcription factor GATA-3 has been shown to play an important role for the *in vitro* induction of T_h2 cells. To clarify how the *in vivo* immune response is governed under GATA-3 function, we generated double-transgenic mice by crossing GATA-3 transgenic mice with ovalbumin (OVA)-specific TCR transgenic mice. After immunization with OVA, the double-transgenic mice showed increased expression of GATA-3 in antigen-reactive fresh $CD4^+$ T cells, and higher production of IL-5 and IL-13 in cultured spleen cells in the presence of cognate antigen without any polarizing conditions for T_h2 cells. Moreover, the immunized double-transgenic mice showed a higher increase of *in vivo* secretion of IL-5 and IL-13 in bronchoalveolar lavage fluid after OVA aerosol challenging. The serum levels of OVA-specific IgG1, IgE and IgA antibodies were much higher in the immunized double-transgenic mice than TCR transgenic mice. These findings provide direct evidence that antigen-stimulated $CD4^+$ T cells in the immunized mice have already been committed into T_h2 cells producing IL-5 and IL-13 selectively through enhanced GATA-3 expression *in vivo*, thereby inducing higher production of antigen-specific antibody for three isotypes other than IgM.

Introduction

Past *in vitro* studies have shown that mature $CD4^+$ T cells are functionally divided into two distinct subsets—T_h1 and T_h2, based on their cytokine production pattern (1–5). T_h1 cells produce IFN-γ and transforming growth factor-β, whereas T_h2 cells are characterized by the production of IL-4, IL-5, IL-10 and IL-13. Both T_h1 and T_h2 are derived from common naive $CD4^+$ T cells by a multi-step process after antigen stimulation (1,5,6). Although the molecular mechanism governing this process still remains to be resolved, it has been gradually clarified that various potent factors such as cytokines and transcription factors influence T_h1 and T_h2 development (6–11). For instance, IL-12 induces the differentiation of naive T cells to the T_h1 effector phenotype by activation of transcription factor Stat4 and, in contrast, T_h2 cell development is mediated by Stat6 activation through IL-4 signaling.

Among transcription factors associated with T_h2 cell development, GATA-3 is selectively expressed in the T cell lineage from the early developing stage in the thymus (12,13). In

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mature peripheral T cells, GATA-3 expression is detectable in $CD4^+$ naive T cells at a low level and is exclusively increased in T_h2 cells during the *in vitro* developmental process, while it is extinguished in T_h1 cells (14,15). This exclusive expression of GATA-3 in T_h2 cells is considered to play a role in T_h2-specific function and/or cytokine gene expression (16). *In vitro* studies demonstrated that the ectopic overexpression of GATA-3 in cell lines results in an increase of products of T_h2 cytokine genes or enhancement of their promoter activities (17,18). Conversely, a decrease in T_h2 cytokine production has been shown in cell lines expressing antisense GATA-3 or dominant-negative GATA-3 (14,15,17). However, these studies have not clearly shown whether GATA-3 protein can underlie the transcription of all the T_h2 cytokine genes in a similar manner, although T_h2 cells coordinately produce gene products such as IL-4, IL-5 and IL-13. For instance, it has been clearly demonstrated that GATA-3 acts directly on the IL-5 promoter (17,19,20), while involvement of GATA-3 for promoting IL-4 expression has been controversial.

In contrast to the extensive *in vitro* studies, little is known about whether and how GATA-3 substantially functions for the immune response *in vivo* through T_h2 cytokine production. Limited reports are available on the *in vivo* role of GATA-3 during immune responses in animals. A recent study using transgenic mice with dominant-negative mutant GATA-3 demonstrated a decreased potential of T_h2 cytokine production (such as IL-4, IL-5 and IL-13) after *in vitro* CD3 cross-linking by mAb on T cells of the mutant mice (21). In the mutant mice, antigen-specific IgE antibody was also reduced, while other Ig isotopes were not analyzed. However, this report did not clarify whether T_h2 cytokine-producing cells are virtually induced *in vivo* from antigen-reacting naive T cells in the immunized body. Very recently, Nawijn *et al.* generated GATA-3 transgenic mice and showed that despite of the increased potential of T_h2 cytokine production in culture, the antigen-specific IgG1 in post-immunized transgenic mice is comparable to that of wild-type mice, while the total serum level of IgE is increased (22).

To demonstrate direct evidence that antigen-reacted T cells are committed to be T_h2 cells in a GATA-3-mediated manner during *in vivo* immune response to antigen-specific antibody production, we crossed GATA-3 transgenic mice with antigen-specific TCR transgenic mice. In these double-transgenic mice, T_h2 cell functions governed by GATA-3 are conspicuously detectable because most T cells in the mutant mice express CD4 and react with ovalbumin $(OVA)_{323–339}$ peptide in the context of $I\text{-}A^d$ restriction (23). Using the mutant mice, we demonstrated that OVA immunization induces the increased expression of IL-5 and IL-13 in a GATA-3-dependent fashion in spleen T cells *in vivo*, and enhances the serum level of antigen-specific antibodies of three isotypes, IgG1, IgE and IgA, in comparison with TCR single-transgenic mice.

Methods

Transgenic mice

Murine GATA-3 cDNA was kindly provided by Dr M. Yamamoto (Tsukuba University School of Medicine, Tsukuba, Japan). The 1.65-kb *Bgl*II fragment of mouse GATA-3 cDNA was cloned into *Bam*HI sites of vector pw120 containing the potent *lck* distal promoter and human growth factor poly(A) sequence. The 7.2-kb *Not*I fragment was injected into pronuclei of C57BL/6 fertilized oocytes. For genotyping the pups, tail DNA was extracted by a DNA Isolation Kit (Gentra, Minneapolis, MN) and examined by PCR using the following primers: GATA-3 sense, 5′-TCTCAC-TCTCGAGGCAGCATGA-3′ and GATA-3 anti-sense, 5′-GGT-ACCATCTCGCCGCCACAG-3′. As a control, Igβ chain (B29) was detected using primers: B29 sense, 5′-ATGAAGACA-YAGYGACTCTTCGGA-3′; B29 anti-sense, 5′-ATTTGCAGAT-TCCAACAAGTCTCT-3′. These primers were set up from the 3′ site of exon 3 and 5′ site of exon 4 respectively. The hemizygous GATA-3 transgenic (Tg) mice were crossed with the homozygous type of another transgenic mouse for TCR recognizing $OVA_{323–339}$ peptide in the context of $I\text{-}A^d$ restriction (23). These double-transgenic and non-GATA-3 TCR transgenic mice were designated as GATA-3/TCR-Tg and WT/TCR-Tg mice respectively in this study.

RT-PCR

To examine expression of the GATA-3 gene in T cells, $CD4^+$ T cells or $B220^+$ B cells were isolated from the spleen of mutant mice after immunization with OVA plus alum 3 times every other week. For isolation of the subsets, spleen cells were treated with magnetic beads coated with anti-CD4 or anti-B220 (Dynabeads mouse CD4 and B220) and subsequently treated with DTACHaBEAD mouse CD4 or B220 (Dynal, Oslo, Norway) respectively. After checking the purity by FACScan, the cells recovered from the beads were subjected to isolate of total RNA using TRIzol reagent (Life Technologies, Rockville, MD). Reverse transcription was carried out with Superscript II reverse transcriptase (Life Technologies). The primers for detection of the GATA-3 transcription were described above. The cDNA of β-actin was amplified by PCR using the primers: β-actin sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; β-actin anti-sense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Antibodies and flow cytometric analysis

Phycoerythrin-conjugated anti-CD4 mAb (BD PharMingen, San Diego, CA) was used for cell-surface staining of spleen cells. For intracellular staining of GATA-3, isolated CD4 spleen cells were treated by Cytofix/Cytoperm (BD PharMingen), and were then incubated with anti-GATA-3 mAb (Hg-3-31; Santa Cruz Biotechnology, Santa Cruz, CA) and FITC-labeled anti-mouse IgG1 mAb (BD PharMingen) as a second step.

Immunoblotting

To detect the expression of GATA-3 protein, total nuclear protein extracts were prepared from spleen cells of the immunized transgenic mice with OVA plus alum. Then, the protein extracts were separated by SDS–PAGE under reducing conditions and the proteins transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) (24). After blocking in Tris–HCl (pH 7.6), the membrane was incubated with GATA-3 mAb (1:200) and horseradish peroxidase-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) at room temperature for 2 h. After further washing, blots were developed using the enhanced chemiluminescence system (Pierce, Rockford, IL) and X-ray film.

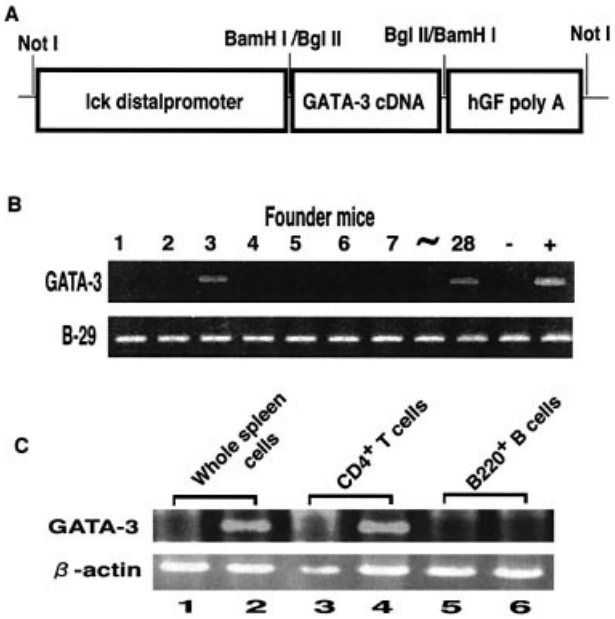

Fig. 1. Expression of the GATA-3 transgene in GATA-3 transgenic and (GATA-3 × TCR) double-transgenic mice. (A) Schematic diagram of the *lck*–GATA-3 transgene. The 7.2-kb *Not*I fragment containing the *lck* distal promoter, mouse GATA-3 cDNA and the human growth factor poly(A) sequence was used as the transgene. (B) The transgene, mouse cDNA of GATA-3, was identified in the founder tail by PCR using sense and anti-sense primers as described in Methods. (C) RT-PCR analysis was performed for the cells obtained from WT/TCR-Tg (lanes 1, 3 and 5) and GATA-3/TCR-Tg mice (lanes 2, 4 and 6). Lanes 1and 2 were from whole spleen cells, lanes 3 and 4 from purified $CD4^+$ T cells, and lanes 5 and 6 from $B220^+$ B cells.

Cytokine production assay of culture supernatants

Cytokines secreted in the culture supernatants were determined by sandwich ELISA as described above. Spleen cells obtained from the immunized mice were cultured with OVA (250–1000 μg/ml) for 48 h, and supernatants recovered from the culture were assayed using ELISA kits for IL-4, IL-5, IFN-γ (Endogen, Cambridge, MA) and IL-13 (R & D Systems, Abingdon, UK).

Immunization and challenging protocol

GATA-3/TCR-Tg and WT/TCR-Tg mice were immunized i.p. with 50 μg OVA plus 1 mg alum on days 0 and 7. For analyzing *in vivo* cytokine production in bronchoalveolar lavage fluid (BALF), the immunized mice were challenged by exposure to an aerosol of 1% OVA in saline for 20 min. At 24 h after the last inhalation challenge, BALF (1 ml of PBS) was collected.

Estimation of cytokines and eosinophils in BALF

To determine cytokine concentrations in BALF, the contaminating cells in BALF were removed by centrifugation at 1200 r.p.m. for 5 min. The supernatant was subjected to sandwich ELISA for cytokines using the commercial kit systems described above. The cell numbers in 100-μl aliquots of BALF were counted under a microscope. BALF cells were prepared by cytospin and were stained with Diff-Quik (International Reagents, Tokyo, Japan) to distinguish eosinophils based on morphology and staining characteristics. Eosinophil peroxidase (EPO) activity was measured by modified methods of Strath *et al.* (25). Briefly, the substrate was added to samples in microplate wells and the reaction was stopped by the addition of 50 μl of 4 M sulfuric acid after incubation at room temperature for 30 min. The absorbance was then determined at 450 nm.

Measurement of OVA-specific Ig in sera

OVA-specific antibodies in the sera of immunized mice were measured by sandwich ELISA as described previously (21); each serum sample as well as serial dilutions of the control sample was cultured in 96-well microtiter plates coated with OVA (100 μg/ml; Sigma, St Louis, MO). After incubation, biotin-labeled rat mAb against mouse IgM, IgG1, IgG2a, IgE and IgA (Zymed, South San Francisco, CA) were added for the second incubation. After washing, the plates were incubated with streptavidin–horseradish peroxidase (PharMingen). Finally, the plates were subjected to peroxidase reaction in TMB substrate solution for color development, which was determined at 450 nm with an ELISA plate reader. Standard curves for anti-OVA antibody were generated using hyperimmune serum from BALB/c mouse with a titer of 10^5.

Statistical analysis

The statistical significance of the data was determined by Student's *t*-test. $P < 0.05$ was considered as significant.

Results

Generation of GATA-3 transgenic mice and their double-transgenic mice with OVA-specific TCR transgenic mice

In order to clarify the *in vivo* role of the transcription factor GATA-3 in the immune response, we generated GATA-3 transgenic mice by injecting murine GATA-3 cDNA ligated with the *lck* promoter into C57BL/6 fertilized eggs (Fig. 1A). Integration of the GATA-3 transgene was detected in two of the pups born from the DNA-injected eggs independently (Fig. 1B). The GATA-3 transgene was transmitted into the offspring of two transgenic lines, in which their cell number and proportion of T and B cells in the spleen and thymus did not differ between the transgenic mice and wild-type littermates (data not shown).

To determine GATA-3 function in the *in vivo* immune response conspicuously, GATA-3 transgenic mice were crossed with OVA-specific TCR transgenic mice in which most T cells express CD4 in the context of $I\text{-}A^d$-restricted TCR αβ (23). These ($GATA\text{-}3^+$ × TCR) double-transgenic mice were referred to as GATA-3/TCR-Tg mice while GATA-3 transgene negative of TCR transgenic mice were referred to as WT/TCR-Tg mice. Since homozygous TCR transgenic mice (BALB/c) were crossed with heterozygous GATA-3 transgenic mice (C57BL/6, B6), the F_1 mice of both GATA-3/TCR-Tg and WT/TCR-Tg possess the (B6 × BALB/c) background. As shown in RT-PCR analysis (Fig. 1C), GATA-3 expression was clearly found in whole spleen cells and in $CD4^+$ T cells of GATA-3/TCR-Tg mice (Fig. 1C, lanes 2 and 4), while such bands were almost undetectable in $CD4^+$ T cells of WT/TCR-Tg littermates (Fig. 1C, lanes 1 and 3). Since GATA-3

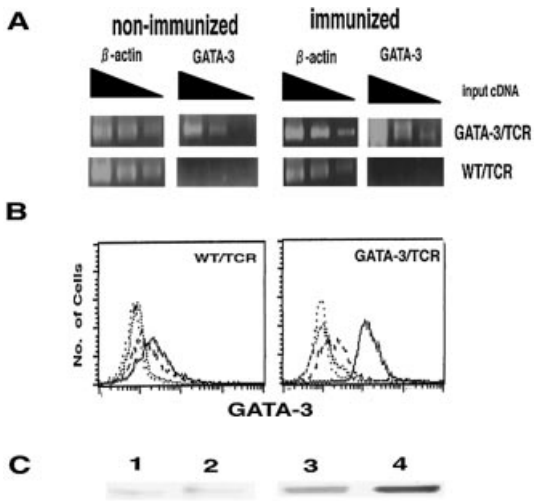

Fig. 2. Increased expression of GATA-3 in immunized GATA-3/TCR mice. CD4+ spleen T cells were obtained from GATA-3/TCR-Tg and WT/TCR-Tg mice with and without OVA immunization 72 h after the last booster. These cells were submitted to RT-PCR for the expression for GATA-3 and cytokine. (A) Semi-quantitative RT-PCR analysis of GATA-3 expression was performed by serial 10-fold dilutions of the representative input cDNA of CD4+ spleen T cells from GATA-3/TCR-Tg (upper column) and WT/TCR-Tg (bottom column) mice with (right) and without (left) immunization. (B) Histograms showed the intracellular expression of GATA-3 in purified CD4+ cells from immunized (—) and non-immunized (…) GATA-3/TCR-Tg (right) and WT/TCR-Tg mice (left). Secondary antibody alone is indicated as dashed lines (non-immunized: ……, immunized: ——). (C) Western blotting for GATA-3 expression was performed using total nuclear protein extracts of CD4+ spleen T cells from two transgenic lines with or without OVA immunization. Lanes 1 and 2: WT/TCR-Tg mice without or with immunization; lanes 3 and 4: GATA-3/TCR-Tg mice without and with immunization respectively.

expression is very low in wild-type mice, the GATA-3 bands shown in lanes 2 and 4 are considered to be almost exogenous.

Enhancement of GATA-3 expression in fresh T cells of post-immunized GATA-3/TCR double-transgenic mice

It is known that GATA-3 expression is enhanced in T cells after stimulation *in vitro* (14). To determine GATA-3 expression of the immunized cells in GATA-3/TCR-Tg mice, the mice were immunized i.p. with OVA and alum. Forty-eight hours after the last immunization, GATA-3 expression was compared between isolated CD4+ spleen T cells from immunized and non-immunized GATA-3/TCR-Tg mice. Transcription of the GATA-3 including endogenous and exogenous genes was higher in the immunized GATA-3/TCR-Tg mouse than non-immunized one (Fig. 2A), but was not almost undetectable on WT/TCR-Tg mice. At the protein level, GATA-3 expression in the immunized GATA-3/TCR-Tg mouse was also clearly shown to increase in CD4+ T cells by cytoplasmic staining and by immunoblotting in the cell lysates in comparison to non-immunized cells (Fig. 2B and C).

In the present study, we estimated total GATA-3 expression without distinguishing endogenous and exogenous expression because constitutive expression of the GATA-3 transgene may in part affect endogenous GATA-3 expression. In fact, there is a report showing that exogenous GATA-3 by retrovirus infection can activate endogenous GATA-3 expression (11). At the same time, constitutive expression of the GATA-3 transgene under the control of the *lck* promoter may also promote IL-4 expression, which is predicted to induce the early expression of endogenous GATA-3 (14). Thus, the increase of total GATA-3 expression was the main focus in this study for investigating the effect of GATA-3 on the immune response.

Increased production of IL-5 and IL-13 in non-polarized cultured cells of immunized double-mutant mice

The spleen cells obtained from the immunized transgenic mice were cultured at various doses of OVA for 48 h without polarizing conditions for T_h1/T_h2 subsets. Under such culture conditions, IL-5 and IL-13 in the culture supernatants of spleen cells were increased in GATA-3/TCR-Tg mice; the production of IL-5 and IL-13 was 2- to 10- and 10- to 12-fold higher in GATA-3/TCR-Tg mice respectively than in WT/TCR-Tg mice (Fig. 3A). In contrast, another important player among T_h2 cytokines, IL-4, showed almost equivalent levels in the culture supernatant between GATA-3/TCR-Tg and WT/TCR-Tg mice. A similar tendency of cytokine production was observed when isolated CD4+ T cells were cultured in the presence of spleen antigen-presenting cells and OVA (Fig. 3B), but this increased pattern was reduced when CD4+ cells were eliminated from spleen cells (Fig. 3C, right). These results indicate that in the immunized GATA-3/TCR-Tg mice, GATA-3 may be important in transactivation of IL-5 and IL-13 genes, but appears to have only limited effects on IL-4 gene transcription as was also indicated in *in vitro* studies of polarizing culture conditions (15,19,20). In GATA-3 Tg mice produced without backcrossing with TCR-Tg mice, the amounts of individual cytokines in the culture were significantly lower than in the GATA-3/TCR-Tg mice (data not shown).

Further in vivo *evidence of increased production of IL-5 and IL-13 in immunized GATA-3/TCR-Tg mice*

For further direct evidence that the *in vivo* secretion of IL-5 and IL-13 is virtually increased in immunized GATA-3/TCR-Tg mice, immunized TCR-Tg mice with and without the GATA-3 transgene were challenged with OVA by inhalation. Twenty-four hours after OVA aerosol challenging, BALF was recovered from anesthetized mice. BALF obtained from the challenged GATA-3/TCR-Tg mice showed a higher increase of IL-5 and IL-13. However, no statistical difference in IL-4 amounts was found in BALF between GATA-3/TCR-Tg and WT/TCR-Tg mice, as was expected from the culture supernatants of immunized double-transgenic spleen cells (Fig. 4A).

At the same time, we examined whether infiltration of eosinophils is increased in bronchoalveolar tracts in these mice because IL-5 is known to possess unique functions for eosinophils such as cell proliferation, development and their mobilization (26–28). The total eosinophil cell number in BALF was 5- to 10-fold higher in the challenged GATA-3/TCR-Tg mice than in WT/TCR-Tg mice (Fig. 4). The increase in the cell population in BALF was mainly due to eosinophils (data not shown). EPO activity, which is considered to correlate with the number of eosinophils, was 2- to 3-fold higher in BALF of GATA-3/TCR-Tg than WT/TCR-Tg mice (Fig. 4B).

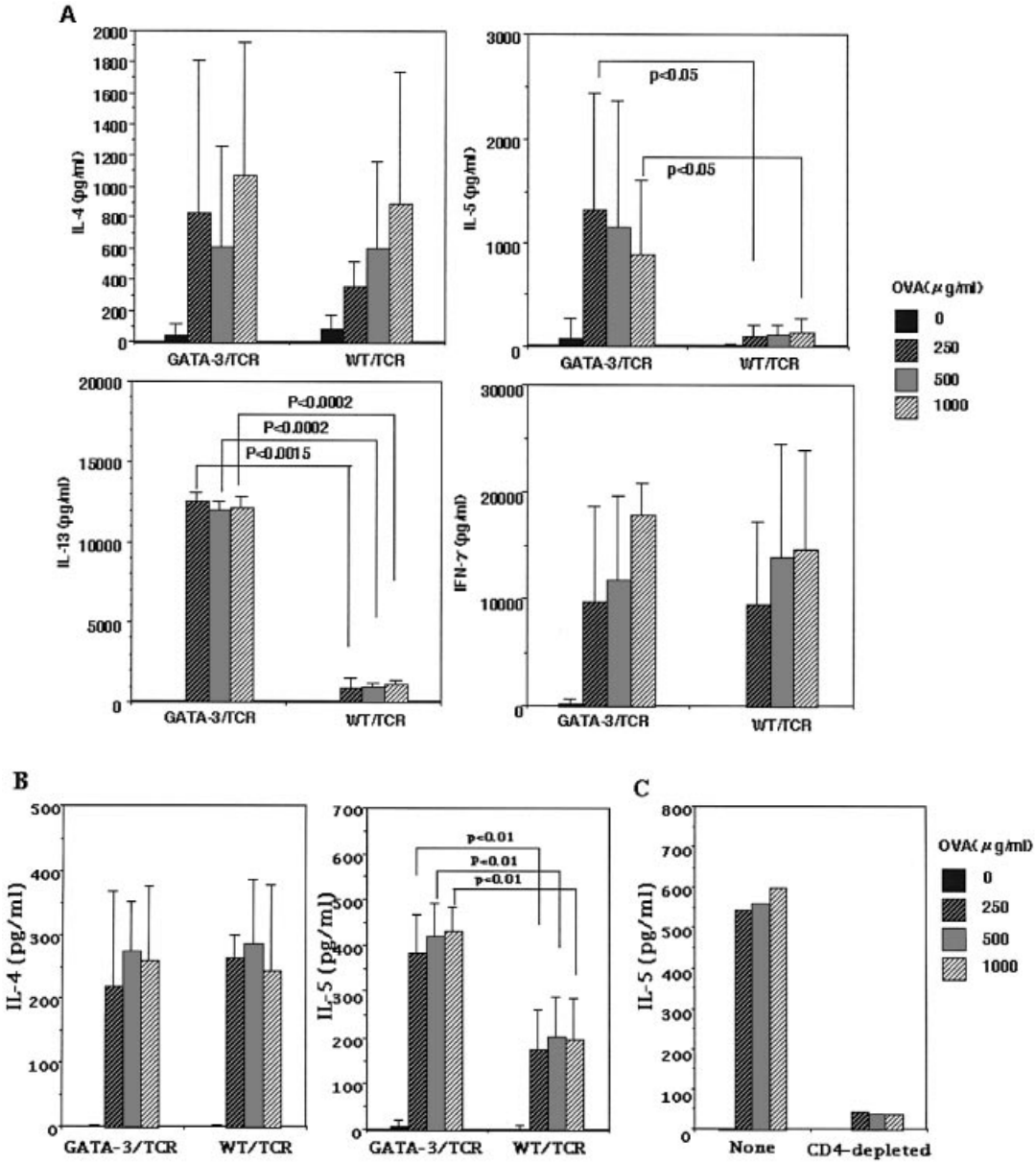

Fig. 3. Increased production of IL-5 and IL-13 in spleen cells of immunized GATA-3/TCR-Tg mice without polarizing culture. Cytokine production of the whole spleen (A) and purified $CD4^+$ cells (B) was obtained from immunized GATA-3/TCR-Tg and WT/TCR-Tg mice, and these were re-stimulated with OVA *in vitro* without any polarizing reagents for 48 h of culture. The cytokine production for IL-4, IL-5, IL-13 and IFN-γ in culture supernatants was measured by ELISA. (C) IL-5 production was markedly reduced in the culture supernatant of CD4-depleted spleen cells that were treated with anti-CD4 antibody-coated beads.

Enhancement of OVA-specific IgA, IgG1 and IgE antibody in immunized double-transgenic mice

T_h2 cytokines induce antigen-recognizing B cells to develop into antibody-producing cells for isotype Ig such as IgG1 and IgE, as well as IgM. In GATA-3/TCR-Tg mice receiving OVA immunization, IL-4 production in antigen-reacting T cells was not highly increased in comparison to WT/TCR-Tg mice (Fig. 3). Then, we examined whether antibody production of Ig isotypes other than IgM is much more enhanced in immunized GATA-3/TCR-Tg mice under the limited elevation of IL-4 production (Fig. 5). To address this issue, we measured the serum level of antigen-specific antibody in GATA-3/TCR-Tg mice i.p. immunized with OVA and alum.

As depicted in Fig. 5, the serum levels of OVA-specific antibody of IgG1 and IgE isotypes were largely elevated in immunized GATA-3/TCR-Tg mice in comparison with WT/TCR-Tg mice. This result was predicted in the immunized GATA-3/TCR-Tg mice because *in vitro* studies have shown that IL-5

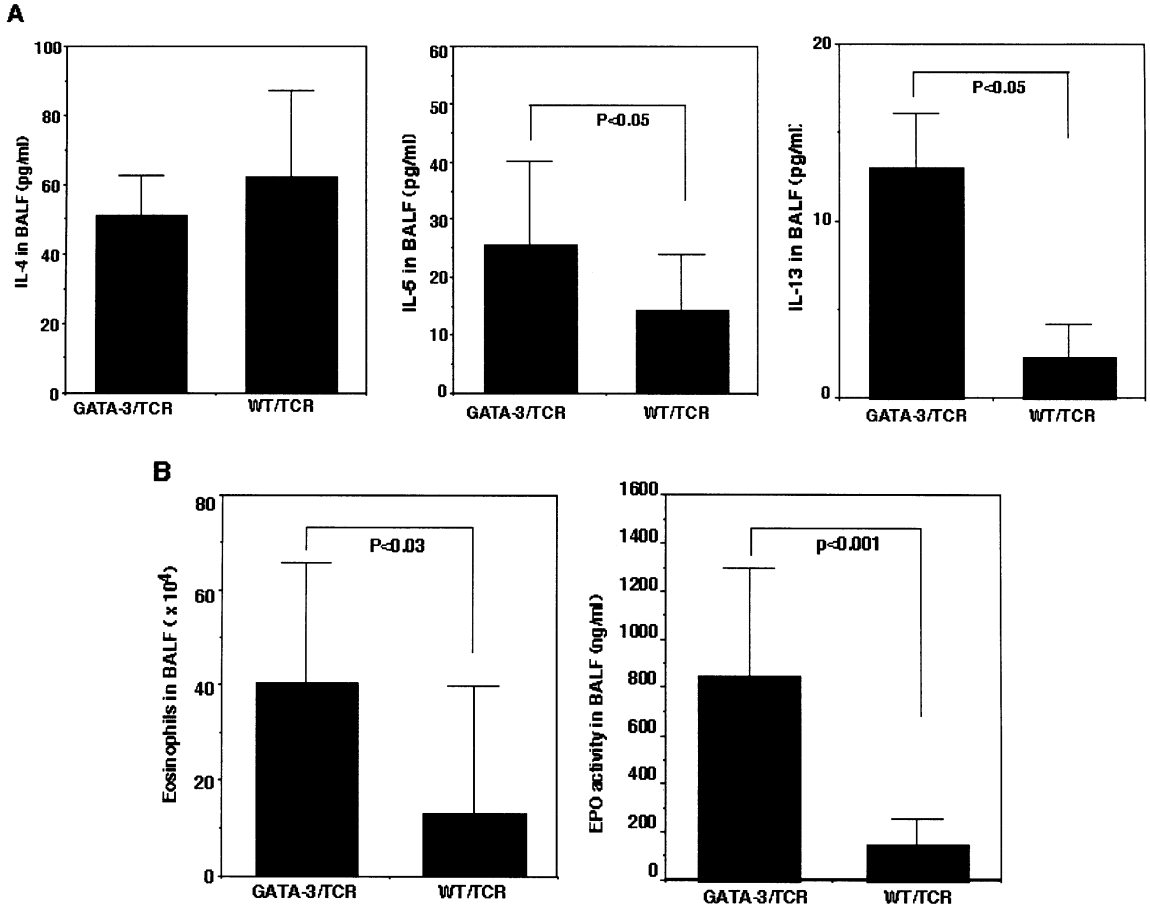

Fig. 4. Increase of T_h2 cytokine and eosinophils in BALF of immunized GATA-3/TCR-Tg mice. Immunized GATA-3/TCR-Tg and WT/TCR-Tg mice were challenged with OVA in the airway. BALF was obtained 24 h after aerosol challenge from the anesthetized mice. (A) Estimation of cytokines, IL-4, IL-5 and IL-13, in BALF by ELISA. $^*P < 0.05$. (B) The number of eosinophils and EPO activity in BALF.

and/or IL-13 can replace IL-4 at least in part for both IgG1 and IgE synthesis (29–32). The serum IgA level was also enhanced in the i.p. immunized GATA-3/TCR-Tg mice. This finding is not inconsistent with the past reports showing the involvement of IL-5 for IgA production in both *in vitro* and *in vivo* systems (33–35), although these reports were mainly obtained by using B cells receiving local mucosal stimulation. Collectively, the present results indicate that the enhanced serum levels of antigen-specific IgG1, IgE and IgA are dependent on the increased production of certain T_h2 cytokines, including IL-5 and IL-13, which are induced by overexpression of GATA-3 in GATA-3/TCR-Tg mice.

Discussion

Past *in vitro* studies revealed that the transcription factor GATA-3 contributes to enhanced T_h2 cytokine production in T_h2 cell lines presumably through interacting with the promoter of IL-4, IL-5 and IL-13 genes (14,17,19,36,37). In a reciprocal experiment, antisense GATA-3 in the T_h2 clone inhibited expression of the T_h2 cytokine gene (14). However, there is little *in vivo* evidence about whether GATA-3 is virtually involved in the selective commitment of the activated T cells into T_h2 cytokine-producing cells in the immunized individuals as seen in the polarized culture conditions.

In this study, we generated GATA-3 transgenic mice and crossed them with OVA-specific TCR transgenic mice, in which most T cells are activated with OVA antigen in the immunized mice. In the GATA-3/TCR double-transgenic mice immunized with OVA, the spleen cells were strongly induced to produce IL-5 and IL-13 in an antigen-specific manner, when they were cultured with OVA in the absence of any polarizing reagents such as rIL-4 and anti-IL-12 (Fig. 3). Since T_h2 cytokine production is not clearly inducible without polarizing culture conditions in T cells of wild-type mice, our results suggest that T_h2 cell development is virtually induced *in vivo* in a GATA-3-dependent manner. In fact, fresh T cells of the immunized double-transgenic mice showed detectable mRNA expression of T_h2 cytokines, although the expression level was low (data not shown). Moreover, increased amounts of IL-5 and IL-13 were found in BALF in the GATA-3/TCR double-transgenic mice immunized by exposure to OVA aerosol in comparison to WT/TCR-Tg littermates. These results demonstrate direct evidence that the *in vivo* immunized T cells have already been committed to be producers of particular T_h2 cytokines prior to the *in vitro* re-stimulation due to the highly expressed GATA-3.

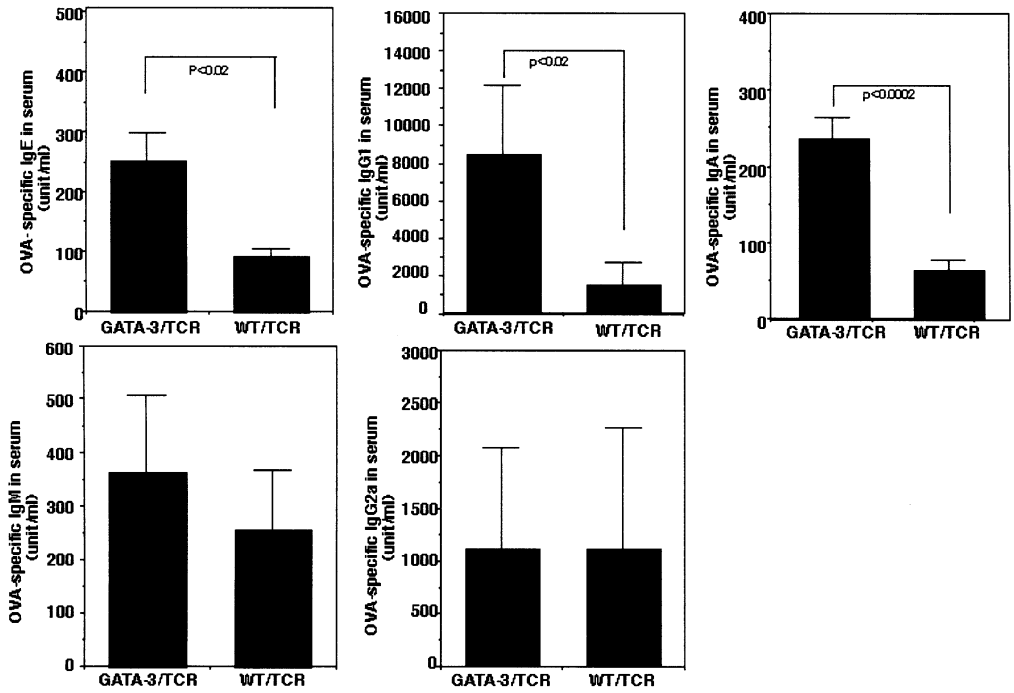

Fig. 5. Increase of serum antigen-specific antibody in immunized double-transgenic mice. The sera were obtained from GATA-3/TCR-Tg and WT/TCR-Tg mice 10 days after the second immunization with OVA plus alum, and were submitted to ELISA for isotypes of antigen-specific antibodies, IgM, IgG1, IgGa, IgA and IgE, as described in Methods.

Recently, Nawijn *et al.* generated a GATA-3 transgenic mouse under regulation of the CD2 promoter, and showed that naive T cells obtained from the non-immunized transgenic mice can produce higher IL-4, IL-5 and IL-13 in culture supernatants upon stimulation with anti-CD3 antibody than those of non-transgenic littermate (22). Under T_h2-polarizing culture conditions, these T cells showed transgene-dependent enhancement of cytokine production for IL-5 and IL-10, but not for IL-4 or IFN-γ. However, they did not examine whether or not T cells in their GATA-3 transgenic mice possessed cytokine production potential *in vivo* after immunization. As a reciprocal study, Zhang *et al.* generated a dominant-negative mutant of GATA-3, and revealed reduced IL-5 and IL-13 in the BALF after aerosol challenge in immunized mice than in non-transgenic littermates (21). Although they demonstrated a minimum requirement for GATA-3 on *in vivo* T_h2 cytokine production, it remains to be clarified whether GATA-3 expression is virtually involved in the increase of T_h2 cytokine production following immune responses. In our immunized double-transgenic mice, we clearly showed the good correlation of the immunization-dependent increase of GATA-3 expression with the enhanced T_h2 cytokine production *in vivo*, which is correlated to antigen-specific antibody production.

GATA-3 is known to be required for the transcription of T_h2 cytokines, but recent *in vitro* studies have suggested that GATA-3 does not equivalently control all T_h2 cytokine production. For instance, several reports documented that ectopic expression of GATA-3 is sufficient to drive IL-5, but not IL-4, expression in non-T_h2 cells (17,19,20,36). In the present study, a limited *in vivo* effect of GATA-3 on IL-4 expression has been evident by showing that GATA-3 overexpression does not contribute increased IL-4 production in the sensitized T cells (Figures 3 and 4), while IL-5 and IL-13 production was completely GATA-3 dependent. However, in dominant-negative GATA-3 transgenic mice, IL-4 production is reduced as much as IL-5 under polarizing culture conditions (21). These results suggest that GATA-3 is indispensable for both IL-4 and IL-5 cytokine production, but a quantitative and/or qualitative requirement of GATA may be different between IL-4 and IL-5, or the requirement of GATA-3 for cytokine production may be different between *in vitro* and *in vivo* committed T_h2 cells. This issue may be resolved by investigating the chromatin remodeling status on these regulatory regions in T_h2 cell lines and freshly isolated T cells from the immunized GATA-3/TCR-Tg mice.

In addition to IL-4, the T_h1 cytokine IFN-γ in the spleen culture supernatants appeared not to be statistically different between the immunized GATA-3/TCR-Tg and WT/TCR-Tg mice (Fig. 3A). Based on the past *in vitro* experimental results, IFN-γ production might be expected to decrease in GATA-3-overexpressing mice because ectopic GATA-3 expression reduced IFN-γ production and IL-12Rβ in T_h1 cells (14,18,20). In our immunized transgenic mice, however, IL-12Rβ expression was not significantly different between WT/TCR-Tg and GATA-3/TCR-Tg mice (data not shown). Enhanced GATA-3 expression may contribute in part to the reduction of IFN-γ only in already committed T_h1 cells, but not direct to the naive T cells in the immunized mice, although the mechanism is unclear. At the same time, we do not deny a possibility that IFN-γ is secreted from non-T cells stimulated by OVA immunization, recovering the reduced IFN-γ production.

Each T_h2 cytokine is known to promote the development of activated B cells into antibody-forming cells with differential specificity for Ig isotype production. Among T_h2 cytokines, IL-

4 has been considered to play a key role for both IgE and IgG1 production in immune response (38). However, recent studies showed that IL-4 is not always essential for promoting Ig production; IgE synthesis occurs in the absence of IL-4, which is rescued by the constitutive expression of IL-13 (30,31). IL-13 is also reported to contribute to the production of the IgG1 isotype through GATA-3-mediated up-regulation, although the promoting activity was lower than that of IL-4 (39). In addition to IL-13, IL-5 was shown to be involved in isotype class switching to IgG1 and IgE (29–32). Moreover, IgA production has been reported to be increased by IL-5 alone or its combination with IL-4, IL-6 or transforming growth factor-β *in vitro* (33,34,40), or in IL-5 transgenic mice (41). In our immunized double-transgenic mice, antigen-specific antibody production of IgG1, IgE and IgA was largely enhanced in accordance with the increased expression of IL-5 and IL-13. This is the first evidence that three antigen-specific isotype Ig, IgG1, IgA and IgE, were enhanced in the serum in a GATA-3-mediated manner, although IL-4 production is not particularly increased. It will be interesting to know whether IL-5 and IL-13, via the GATA-3 effect, can be replace IL-4 function in the immune response. For this, generation of triple-mutant mice (IL-4 deficient mice × GARA-3/TCR-Tg mice) may be useful.

IgA is dominant in the mucosal Ig isotypes, and its production is effectively induced by antigen stimulation at the mucosal sites with numerous IgA^+ cells and producing cells (42). In the same area, IL-5-expressing T cells are observed at a high frequency (43,44). In our immunized GATA-3/TCR-Tg mice, the serum level of IgA was enhanced when they were immunized peritoneally, but not at a mucosal site (data not shown). Thus, it is assumed that IgA production may be highly induced in the presence of sufficient amounts of IL-5 without regard to the immunizing process and/or sites.

In addition to antibody production, IL-5 is also known to be a key cytokine in the regulation of the biological functions of eosinophils such as cell proliferation, differentiation and migration (26–28). Consequently, IL-5 plays a protective role against parasitic infection and may promote eosinophilic inflammation in allergic diseases including asthma (45,46). In our double-transgenic mice immunized with OVA, EPO as well as IL-5 was increased in BALF after challenging with OVA (Fig. 4) or diarrhea after OVA feeding (data not shown). Thus, the double-transgenic mouse recognizing OVA may be a good model for the study of the molecular mechanism of dietary-driven allergy and their therapies.

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Abbreviations

BALF	bronchoalveolar lavage fluid
EPO	eosinophil peroxidase
OVA	ovalbumin
Tg	transgenic

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