



Intestinal commensal bacteria promote T cell hyporesponsiveness and down-regulate the serum antibody responses induced by dietary antigen

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ABSTRACT

Colonization of the gut by commensal bacteria modulates the induction of oral tolerance and allergy. However, how these intestinal bacteria modulate antigen-specific T cell responses induced by oral antigens remains unclear. In order to investigate this, we used germ-free (GF) ovalbumin (OVA)-specific T cell receptor transgenic (OVA23-3) mice.

Conventional (CV) or GF mice were administered an OVA-containing diet. Cytokine production by CD4⁺ cells from spleen (SP), mesenteric lymph nodes (MLN) and Peyer's patches (PP) was evaluated by ELISA, as was the peripheral antibody titer. T cell phenotype was assessed by flow cytometry.

CD4⁺ cells from the SP and MLN of CV and GF mice fed an OVA diet for 3 weeks produced significantly less IL-2 than the corresponding cells from mice receiving a control diet, suggesting that oral tolerance could be induced at the T cell level in the systemic and intestinal immune systems of both bacterial condition of mice. However, we also observed that the T cell hyporesponsiveness induced by dietary antigen was delayed in the systemic immune tissues and was weaker in the intestinal immune tissues of the GF mice. Intestinal MLN and PP CD4⁺ T cells from these animals also produced lower levels of IL-10, had less activated/memory type CD45RB^{low} cells, and expressed lower levels of CTLA-4 but not Foxp3 compared to their CV counterparts. Furthermore, GF mice produced higher serum levels of OVA-specific antibodies than CV animals. CD40L expression by SP CD4⁺ cells from GF mice fed OVA was higher than that of CV mice. These results suggest that intestinal commensal bacteria promote T cell hyporesponsiveness and down-regulate serum antibody responses induced by dietary antigens through modulation of the intestinal and systemic T cell phenotype.

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1. Introduction

A large number of commensal bacteria colonize the gastrointestinal tract of mammals. These organisms, which are unique in being able to reside in the gut without being eliminated, despite being identified as non-host antigens, play a key role in inducing the development and maintaining the homeostasis of the intestinal immune system [1–6].

The intestinal mucosa is also constitutively exposed to food-derived antigens and therefore maintains an appropriate immune response to these antigens. This means that most oral antigens are tolerated through down-regulation of systemic and peripheral

antigen-specific cellular and humoral responses, a process known as oral tolerance. If tolerance to an orally administered antigen is either not established or breaks down, the result is food hypersensitivity or food allergy [7,8]. Previous studies have suggested that intestinal commensal bacteria are an important factor in the induction of oral tolerance and in the development of allergy. As a consequence, germ-free (GF) animals, which lack indigenous bacteria in their gastrointestinal tract, provide valuable tools for analyzing the direct modulation of the immune system by such bacteria. Indeed, when such mice are intraperitoneally immunized with an antigen plus adjuvant, more antigen-specific antibodies are produced in the serum of GF mice than in that of specific-pathogen free (SPF) animals [9,10]. In addition, it has been reported that oral administration of various antigens prior to immunization with those same antigens induces oral tolerance in conventional (CV) mice but not in GF mice [1,9,11]. Other studies suggest that oral tolerance can be induced in GF mice but occurs to a lesser extent or for a shorter duration than in CV mice [12,13]. One hypothesis for the dysregulation of the induction of oral tolerance or allergic sensitization in GF

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mice is that there is a Th2-skewed immune response in these animals [9,10]. It has also been suggested that the reduction in T cells observed in Peyer's patches (PP) [1] and the impaired function of regulatory T cells in mesenteric lymph nodes (MLN) [11] contribute to the impaired induction of oral tolerance in GF mice. However, the above studies were mostly limited either to evaluating serum antibody production and systemic cellular responses of GF mice following oral and/or intraperitoneal sensitization by the antigen, or to assessing immune parameters in naïve GF mice which had never been sensitized with antigens. Therefore, it remains unclear how intestinal commensal bacteria directly modulate the responses and functions of intestinal and systemic antigen-specific T cells, which act as the effector cells in several steps of the immune response to dietary antigen, to cause either oral tolerance or food allergy.

OVA23-3 mice are an ovalbumin (OVA)-specific T cell receptor transgenic strain that has a larger population of OVA-responsive CD4⁺ T cells than wild-type BALB/c mice, allowing easier evaluation of OVA-specific T cell responses. We have previously established a GF line of this mouse strain to investigate the modulation of antigen-specific T cell responses to dietary antigen by intestinal commensal bacteria [14]. We have also shown that intestinal *Bifidobacterium* association in GF OVA23-3 mice leads to the down-regulation of dietary antigen-specific immune responses in the small intestine [15]. In contrast, under conventional breeding conditions, OVA23-3 mice have been reported to serve as an experimental model for oral tolerance and food allergy. Indeed, oral tolerance can be induced in spleen (SP) T cells from these mice when fed an OVA-containing diet over a long period [16,17]. It has also been reported that OVA23-3 mice produce OVA-specific antibodies in the serum and develop intestinal inflammation in a Th2-dependent manner after intake of an OVA-containing diet [18,19]. However, it remains unknown how intestinal commensal bacteria regulate oral tolerance and dietary antigen-induced allergic reactions in this mouse model.

In this study, we therefore investigated the direct effects of intestinal commensal bacteria on the intestinal and systemic antigen-specific T cell responses and the serum antibody responses induced by oral administration of the antigen, comparing the immune response of CV mice with that of GF OVA23-3 animals. Our results demonstrate that the commensal bacteria promote intestinal and systemic T cell hyporesponsiveness and suppress the serum antibody response induced by exposure to dietary antigen.

2. Materials and methods

2.1. Animals

OVA23-3 (+/–) mice were used in this study. F1 animals were produced by crossbreeding GF OVA23-3 (+/+) and BALB/c mice (Clea Japan, Tokyo, Japan). The animals were maintained in sterile vinyl isolators, with their GF condition being confirmed monthly based on aerobic and anaerobic cultures and Gram stains of fresh feces from the mice in each isolator. CV OVA23-3 (+/–) mice were also produced by crossbreeding CV OVA23-3 (+/+) and BALB/c mice (Clea Japan). CV and GF OVA23-3 (+/–) mice at 8–14 weeks of age were administered a 50 kGy γ -ray-irradiated OVA diet which consisted of a purified diet supplemented with 20% dry egg-white (AIN-76 compound feed with casein substituted by egg-white) for 1–4 weeks. The control mice were administered a 20% casein-containing purified diet. All mice were allowed to drink sterile deionized water *ad libitum*. All animal experiments were carried out at the animal facility of Nihon University College of Bioresource Sciences, in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' established by the College of Bioresource Sciences, Nihon University.

2.2. Cell preparation

Single-cell preparations were prepared from the SP, MLN and PP of experimental mice. Briefly, SPs were physically dissociated and filtered in RPMI1640 medium, after which the erythrocytes were lysed with BD Pharm Lyse™ Lysing Buffer (BD Biosciences, San Diego, CA, USA).

MLN and PP were cut into small fragments, and incubated for 50 min at 37 °C in RPMI1640 medium containing 10% FCS, 1 mg/ml of collagenase D (Roche Diagnostics, Indianapolis, IN, USA) and 0.1 mg/ml of DNase I (Roche Diagnostics). Cell pellets were additionally incubated with HBSS (Ca and Mg free; Sigma Aldrich, St. Louis, MO, USA) containing 5 mM EDTA and 5% FCS for 10 min at 37 °C. Single-cell suspensions were filtered through a cell strainer (70 μ m Nylon, BD Biosciences) and re-suspended in 10% FCS-RPMI medium after centrifugation.

CD4⁺ cells were isolated using an anti-mouse CD4 monoclonal antibody conjugated to magnetic microbeads and autoMACS™ (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's protocol. The purity of the CD4⁺ cells was confirmed as being >90% by flow cytometry.

2.3. Cytokine production assay

CD4⁺ cells (5×10^5 cells) from the SP, MLN, and PP were added to 48-well flat-bottom plates. These were then cultured for 48 h with 50 μ g/ml mitomycin C (Sigma Aldrich)-treated SP cells (2×10^6 cells) isolated from non-experimental BALB/c mice as antigen-presenting cells (APCs) in the presence of OVA (5 mg/ml) in 1 ml of RPMI1640 medium containing a final concentration of 5% FCS.

The amount of cytokine in the cell-culture supernatants was assayed by sandwich ELISA. For measurement of interleukin (IL)-2 and IL-4, purified rat anti-mouse IL-2 (JES6-1A12) and IL-4 (BJD4-1D11) monoclonal antibodies, and biotinylated rat anti-mouse IL-2 (JES6-5H4) and IL-4 (BJD6-34G2) monoclonal antibodies were used, all of which were purchased from BD Biosciences. The amount of IL-10 was measured using a BD OptEIA mouse ELISA set (BD Biosciences) according to the manufacturer's instructions.

2.4. Measurement of serum antibody titer

Serum samples were collected from each mouse and stored at –20 °C. The level of total IgE and OVA-specific IgE/IgG1/IgG2a was measured by sandwich ELISA. For measurement of total IgE, purified rat anti-mouse IgE (R35-92; BD Biosciences) was used as a capture antibody, and biotinylated anti-mouse IgE (LO-ME-2; AbD Serotec, Oxford, UK) was used as a detection antibody. For measurement of OVA-specific IgE, IgG1 and IgG2a, OVA was coated on a 96-well plate to capture OVA-specific antibodies, and biotinylated anti-mouse IgE or goat anti-mouse IgG1 and IgG2a (Southern Biotechnology, Birmingham, AL, USA) was used as the detection antibody. The OVA-specific antibody was obtained from the serum of mice immunized with 4 intraperitoneal injections of OVA absorbed with alum (for evaluation of OVA-specific IgE and IgG1) at intervals of 1.5 weeks. The titers of the OVA-specific IgE and IgG1 in standard serum were arbitrarily assigned a value of 10,000 unit/ml.

2.5. Flow cytometry analysis

Cells (1×10^6) from the SP, MLN, and PP were washed with FACS buffer (Dulbecco's modified Eagle's medium (Sigma Aldrich) containing 1% bovine serum albumin, 10 mM HEPES, 2 mM sodium pyruvate, 10^{-3} mM EDTA, 0.01% Na₃N₃), and then incubated with anti-CD16/CD32 (93; eBioscience, San Diego, CA, USA). Cell surface markers were stained with fluorescein isothio-

cyanate (FITC)-conjugated anti-CD45RB (C363.16A; eBioscience), and PerCPy5.5-conjugated anti-CD4 (RM4-5; BD Biosciences) for 15 min on ice and then washed. For staining intracellular molecules, cells were subsequently fixed and permeabilized using the appropriate solution supplied with the BD Cytofix/Cytoperm

Fixation/Permeabilization kit (BD Biosciences) according to the manufacturer's instructions, and then stained with phycoerythrin (PE)-conjugated anti-fork head box P3 (Foxp3) (FJK-16s; eBioscience) or anti-CD152 (cytotoxic T lymphocyte-associated antigen 4, CTLA-4; UC10-4F10-11, BD Biosciences) for 30 min on ice.

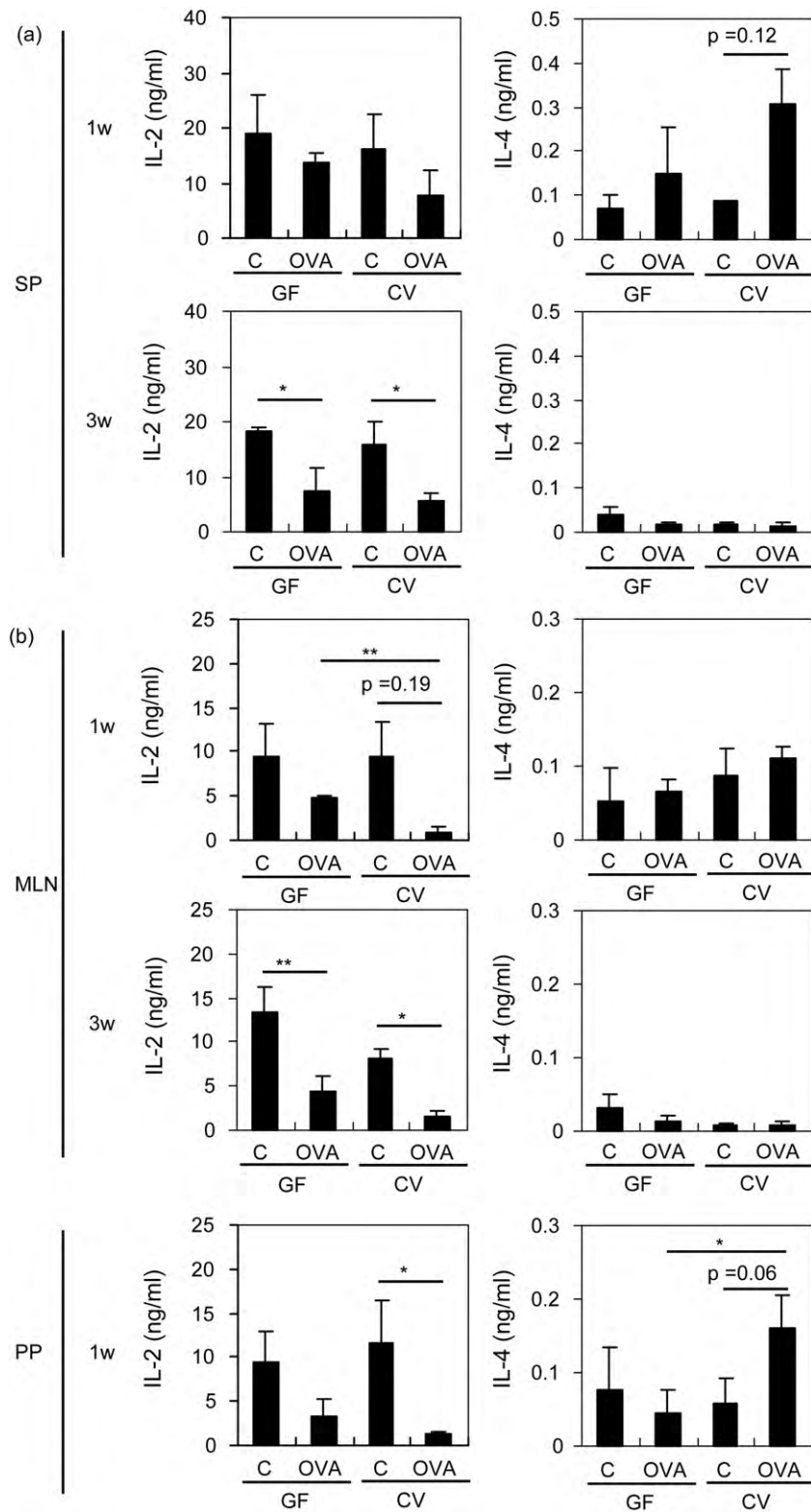


Fig. 1. Cytokine production by systemic and intestinal CD4⁺ cells from CV and GF mice fed an OVA diet for differing time periods. CD4⁺ cells were isolated from the SP (a), MLN and PP (b) of CV and GF mice fed a casein (C) or OVA diet for 1 or 3 weeks. 5×10^5 CD4⁺ cells were co-cultured with 5 mg/ml of OVA and 2×10^6 cells of mitomycin C-treated splenocytes from non-experimental BALB/c mice as APCs. Culture supernatants were collected at 48 h, and the concentration of IL-2 and IL-4 was measured by ELISA. The values represent mean $\pm$ S.D. of 3 independent experiments. Significant differences between the experimental groups are denoted by * $p < 0.05$ and ** $p < 0.01$.

To determine the expression of CD40L on CD4⁺ T cells, SP CD4⁺ cells (1×10^5 cells) were cultured with mitomycin C-treated Thy1.2 (CD90.2)⁺ SP cells (4×10^5 cells) from non-experimental BALB/c mice in the presence of OVA (5 mg/ml) and biotin-conjugated anti-CD40L (MR1) or Armenian Hamster IgG (isotype control) for 20 h. Cultured cells were then stained with FITC-conjugated anti-CD4, streptavidin-PECy7 and propidium iodide.

Cell acquisition was performed on a FACS Canto with FACS DiVa software (BD Biosciences). Data were analyzed with FlowJo software (Tree star, Inc., Ashland, OR, USA)

2.6. Statistics analysis

Data are represented as mean $\pm$ S.D. Statistical significance was calculated using SPSS software (SPSS Ver.13.0, SPSS, Chicago, IL,

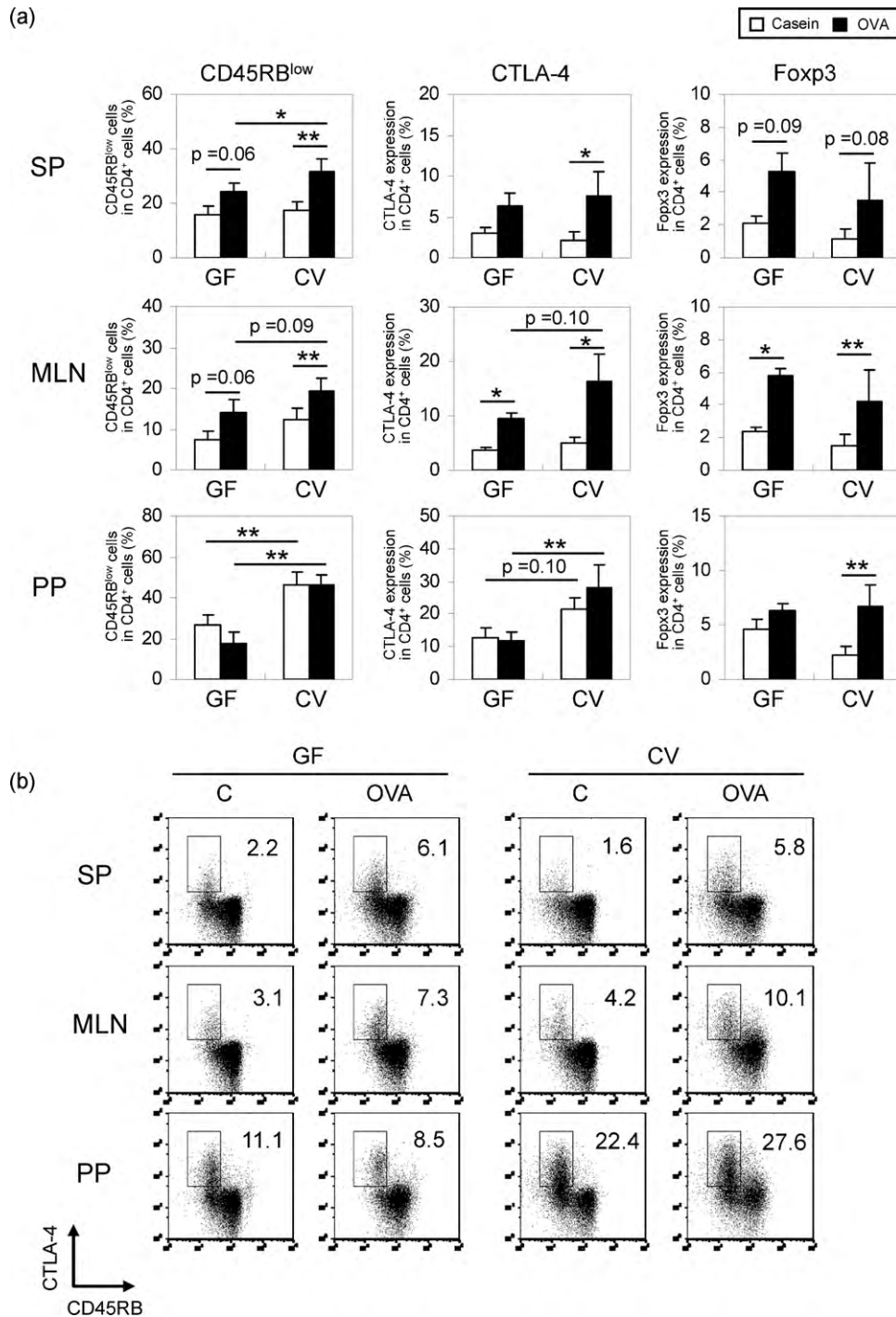


Fig. 2. Expression of regulatory makers by CD4⁺ cells of CV and GF mice fed an OVA diet. SP, MLN and PP cells were isolated for FACS analysis from CV and GF mice fed a casein or OVA diet for 1 week. The cells were stained with PerCPy5.5-conjugated anti-CD4 and FITC-conjugated anti-CD45RB, and PE-conjugated anti-Foxp3 or anti-CTLA-4. (a) Results show the frequency of CD45RB^{low} cells and intracellular Foxp3⁺ and CTLA-4⁺ cells gated on CD4⁺ cells. (b) Typical patterns of the dot plots representing CD45RB and CTLA-4 gated on CD4⁺ cells. Data are shown as mean $\pm$ S.D. (a) and are representative (b) of 3 independent experiments. Significant differences between the experimental groups are denoted by * $p < 0.05$ and ** $p < 0.01$.

USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test or Dunnett's T3 test. Differences were considered statistically significant for values of $p < 0.05$.

3. Results

3.1. Impaired hyporesponsiveness of T cells from GF mice receiving dietary antigen compared to those in CV mice

To examine whether intestinal commensal bacteria produce a change in the cytokine profiles of systemic and intestinal T cells over the course of antigen sensitization and tolerance induction, we purified CD4⁺ cells from both CV and GF mice fed either an OVA or a control diet for 1–3 weeks (Fig. 1).

SP CD4⁺ cells from both CV and GF mice fed the OVA diet for 3 weeks produced significantly lower levels of IL-2 but similar levels of IL-4 compared with the corresponding cells from animals that received the control diet (Fig. 1a), thereby demonstrating the hyporesponsiveness of the systemic T cells in both the CV and GF mice. At the earlier (1 week) time-point, the SP CD4⁺ cells from the CV mice, but not the GF animals, showed a tendency to decreased IL-2 production in the OVA-fed group (Fig. 1a). In addition, there was a tendency for SP CD4⁺ cells from CV mice to produce higher levels of IL-4 than those from GF mice. These data suggest that the induction of Th1 tolerance and Th2 effector responses in splenic T cells in GF mice might be delayed compared with those in CV animals.

Intestinal MLN CD4⁺ cells from both GF and CV mice on the OVA diet showed decreased production of IL-2 (Fig. 1b). Notably, however, IL-2 production by the MLN CD4⁺ cells from the GF mice fed the OVA diet for 1 week was higher than that from the corresponding CV mouse cells (Fig. 1b). In addition, cytokine production by PP CD4⁺ cells from the CV mice alone was significantly decreased after exposure to dietary OVA (Fig. 1c). Taken together, these data are indicative of an impaired Th1 tolerance in intestinal CD4⁺ T cells from GF mice receiving dietary antigen compared to those from CV mice.

3.2. The production of IL-10, and the proportion of CD45RB^{low} and CTLA-4⁺ fractions in intestinal CD4⁺ cells from GF mice were lower than those from CV mouse cells

To examine differences in the phenotype and function of T cells from both CV and GF mice, we determined the CD4⁺ cell levels of the regulatory cytokine IL-10 and cellular markers such as CD45RB (a marker which distinguishes naïve from activated/memory phenotypes), Foxp3 (a master transcriptional factor for regulatory T cells) and CTLA-4 (a co-stimulatory molecule which induces negative feedback signals in T cells).

The proportion of CD45RB^{low} (activated/memory phenotype) cells in the PP CD4⁺ population from GF mice was constitutively lower than that seen in CV mice, even after exposure to the OVA diet (Fig. 2a). The proportion of CD45RB^{low} cells in the SP and MLN CD4⁺ populations was increased in both CV and GF mice on the OVA diet. Interestingly, however, the frequency of CD45RB^{low} cells in these SP and MLN CD4⁺ populations from GF mice administered OVA was lower than that seen in CV mice. We also observed that the expression pattern of CTLA-4 on the intestinal CD4⁺ cells of the CV and GF mice was similar to that seen in the CD45RB^{low} population. However, we could not detect any significant differences in the expression of this molecule by SP CD4⁺ cells of CV and GF mice fed OVA (Fig. 2a), although the frequency of CTLA-4⁺ cells in the CD4⁺ population was lower than the frequency of CD45RB^{low} cells. In addition, the proportion of CD45RB^{low}CTLA-4⁺ cells in the PP and MLN CD4⁺ populations from GF mice was lower than that of CV mice (Fig. 2b). However, the expression of Foxp3 in CD4⁺ cells from the systemic and intestinal tissues of GF mice fed the OVA diet was comparable to that of CV mice (Fig. 2a).

A smaller amount of IL-10 was produced by MLN and PP CD4⁺ cells from GF mice fed the OVA diet for 1 week than by the corresponding cells from CV animals, although this difference was not significant (Fig. 3). The level of IL-10 production by SP CD4⁺ cells from GF mice fed the OVA diet was comparable to that of CV mice (Fig. 3). No IL-10 production could be detected from any CD4⁺ cells from mice fed the OVA diet for 3 weeks (data not shown). These results suggest that the production of regulatory cytokines and the proportion of activated/memory cells in the intestinal CD4⁺ population are lower in GF mice than in CV animals.

3.3. Serum antibody responses to dietary OVA are stronger in GF mice than in CV mice

To examine the effect of intestinal colonization by commensal bacteria on dietary antigen-induced serum antibody responses, both CV and GF OVA23-3 mice were administered an OVA or a control diet for 4 weeks, after which serum samples were collected. Consistent with a previous report [19], the total level of IgE in the serum of CV mice fed the OVA diet for 4 weeks was significantly higher than that of those mice fed the control diet (Fig. 4). In addition, OVA-specific IgE and IgG1 were detected in the CV mice administered the OVA diet but not in mice fed the control diet (Fig. 4).

We could not detect any significant differences in total IgE levels between the CV and GF mice fed the OVA diet (Fig. 4), although there was a tendency for OVA-specific IgE and IgG1 levels to be higher in the serum of the GF mice (Fig. 4). Furthermore, we observed that some of the GF mice fed the OVA diet had detectable levels of OVA-specific IgG2a antibody, whereas little of this antibody was detected in CV mice, even after feeding with OVA (Fig. 4). These data suggest

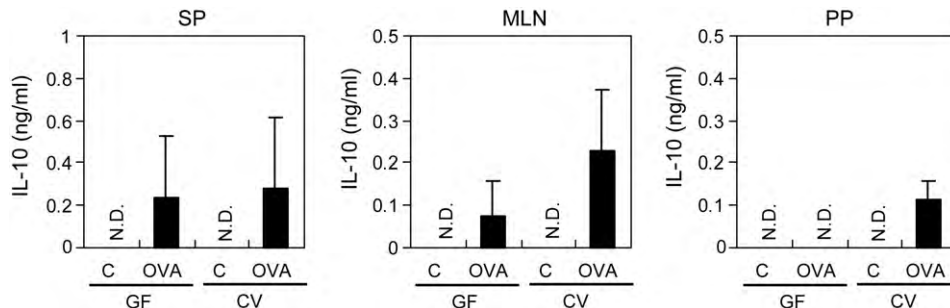


Fig. 3. IL-10 production by CD4⁺ cells from CV and GF mice fed an OVA diet. CD4⁺ cells were isolated from the SP, MLN and PP of CV and GF mice fed a casein (C) or OVA diet for 1 week. 5×10^5 CD4⁺ cells were co-cultured with 5 mg/ml of OVA and 2×10^6 cells of mitomycin C-treated splenocytes from non-experimental BALB/c mice as APCs. Culture supernatants were collected at 48 h, and the concentration of IL-10 was measured by ELISA. The values represent mean $\pm$ S.D. of 3 independent experiments.

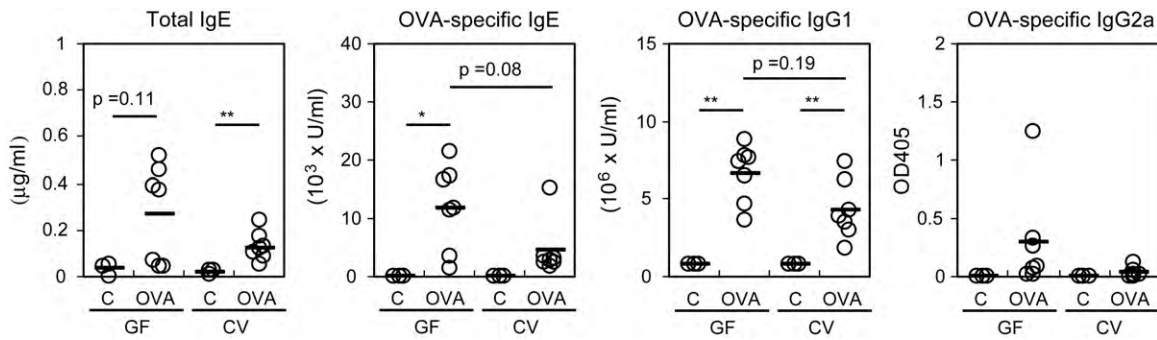


Fig. 4. Antibody titer in the serum of CV and GF mice fed an OVA diet. Serum samples were individually collected from CV and GF mice fed an OVA diet for 4 weeks. The concentration of total IgE, OVA-specific IgE, IgG1 and IgG2a was measured by ELISA. The values represent the mean ± S.D. Data are representative of two independent experiments, $n = 3$ –8 mice per group. Significant differences between the experimental groups are denoted by * $p < 0.05$ and ** $p < 0.01$.

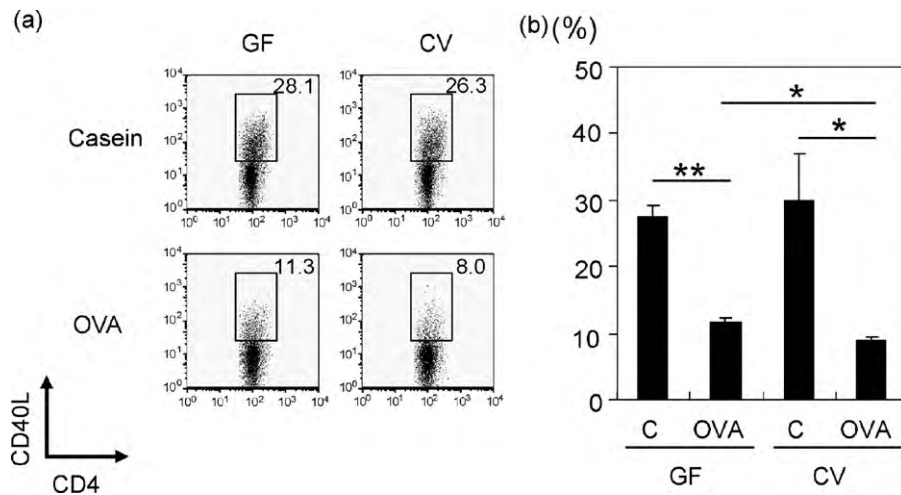


Fig. 5. CD40L expression on splenic CD4⁺ cells from GF mice fed an OVA diet decreased to a lesser extent than that of the corresponding cells from CV mice. CD4⁺ cells were isolated from the SP of CV and GF mice fed a casein (C) or OVA diet for 3 weeks. These CD4⁺ cells (1×10^5 cells) were then cultured with mitomycin C-treated Thy1.2 (CD90.2)⁺ SP cells (4×10^5 cells) from non-experimental BALB/c mice in the presence of OVA (5 mg/ml) and biotin-conjugated anti-CD40L (MR1) or matched-isotype control antibody (Armenian Hamster IgG) for 20 h. Cultured cells were then stained with FITC-conjugated CD4, streptavidin-PECy7 and propidium iodide (PI). Cell acquisition was performed on a FACS Canto with FACS DiVa software. Data are shown as CD40L expression gated on CD4⁺ cells in the live population based on PI staining. Data are representative dot plots (a) and mean ± S.D. (b) of 3 independent experiments. Significant differences between the experimental groups are denoted by * $p < 0.05$ and ** $p < 0.01$.

that more pronounced Th1- and Th2-dependent serum antibody responses are induced in GF mice following oral antigen administration than in CV mice.

3.4. Expression of CD40L on CD4⁺ T cells from GF mice was higher than that in CV mice

We obtained conflicting results with regards to the lower levels of the Th2 cytokine IL-4 produced by SP cells from GF mice, although there was a higher antibody production in their serum compared to that of CV mice. We therefore considered the possibility that intestinal commensal bacteria modulate other factors besides cytokine balance in CD4⁺ T cells to down-regulate the systemic antibody response. In order to investigate this further, we focused on the CD40L molecule which binds to CD40 on B cells and is known to be induced by antigen stimulation. Previous studies have demonstrated that splenic CD4⁺ cells in a state of oral tolerance show reduced expression of this molecule.

SP CD4⁺ cells from both CV and GF mice fed a control diet displayed up-regulated expression of CD40L following in vitro OVA stimulation (Fig. 5), indicating that naïve T cells respond to primary stimulation by dietary antigen by producing increased levels of this molecule. CD4⁺ cells from CV mice fed an OVA diet for 3 weeks expressed lower levels of CD40L than the corresponding cells from GF mice (Fig. 5), although both CV and GF CD4⁺ cells

displayed decreased expression of this molecule following dietary administration of OVA. These data suggest that commensal bacteria promote down-regulation of the expression of CD40L induced by dietary antigen.

4. Discussion

In this study, using germ-free OVA-specific T cell receptor transgenic OVA23-3 mice, we have provided evidence that intestinal commensal bacteria play a role in the modulation of antigen-specific immune responses responsible for oral tolerance and food allergy. More specifically, the commensal organisms promote T cell hyporesponsiveness and suppress the serum antibody response following exposure to dietary OVA.

It is still unclear how commensal bacteria regulate the peripheral events of oral tolerance, including antigen-specific T cell responses in the intestinal immune system. Previous studies have mainly evaluated serum antibodies and systemic immune responses after oral administration of antigen in GF mice, or have determined the number and function of regulatory T cells from naïve GF BALB/c mice. Here we provide evidence for the direct modulation of OVA-specific T cell responses in the intestinal MLN and PP by intestinal commensal bacteria using GF OVA23-3 mice fed an OVA-containing diet. Our results reveal that T cell hyporesponsiveness induced by dietary antigen is delayed systemically and is

weaker in the intestinal immune tissues of GF mice compared to CV mice, suggesting decreased induction of oral tolerance at the T cell level in the former case (Fig. 1). In contrast to these findings, Walton et al. have shown that T cell tolerance is intact in GF mice, based on the use of a model in which OVA-transgenic T cells are transferred from SPF mice into GF or SPF BALB/c mice [20]. However, as their experimental paradigm used T cells derived from SPF donor mice, they could not rule out the possibility of OVA-specific T cells being directly or indirectly stimulated by intestinal commensal bacteria. In contrast, we analyzed OVA-specific T cells from GF mice which had never been exposed to any viable bacteria. Thus, the present study provides the first definitive evidence for hyporesponsiveness of OVA-transgenic T cells from GF mice being induced by dietary antigen.

Our results also show that intestinal and systemic CD4⁺ T cells from GF mice have a lower proportion of activated/memory cells (CD45RB^{low}) (Fig. 2), although the key suppressive factor for T cell tolerance in the OVA23–3 mouse model is still not clear. It is well known that CD4⁺ T cells can be divided into two populations representing naïve (CD45RB^{high}) and activated/memory (CD45RB^{low}) phenotypes based on their CD45RB expression. In general, these cells have distinct roles in the transfer model of colitis [21,22] and show distinct patterns of cytokine production [23]. Using SP cells from naïve OVA23–3 mice, we also confirmed that CD4⁺CD45RB^{low} cells produced virtually no IL-2 (below the detection limit) following stimulation by OVA in vitro. In contrast CD4⁺CD45RB^{high} cells produced high levels of this cytokine (data not shown). Consequently, we consider that the difference in the proportion of these cell populations between CV and GF mice might be responsible for the impaired T cell hyporesponsiveness observed following oral administration of OVA in GF mice. There are two possible explanations for this outcome: the first is a difference in the suppressive ability of the CD4⁺CD45RB^{low} fraction between CV and GF mice, and the second is a difference in the ability of the CD4⁺CD45RB^{high} cells of CV and GF mice to produce IL-2.

CD4⁺CD25⁺Foxp3⁺ T cells, which exist within the CD45RB^{low} fraction, comprise the most important regulatory T cell population, acting to induce or maintain oral tolerance [24]. Interestingly, some studies have reported a decreased number or frequency of these regulatory T cells in GF mice, and have also suggested that their function is impaired [11,25,26]. However, in the present study, we could not find any significant differences in the frequency of Foxp3⁺ cells in the PP and MLN CD4⁺ populations between CV and GF mice, although we have still not evaluated the suppressive function of regulatory T cells in GF mice. In addition, Asai et al. suggested that the hyporesponsiveness of CD4⁺ T cells induced by long term feeding of OVA in OVA23–3 mice was not mediated by the CD4⁺CD25⁺ regulatory cells in vitro [16]. Therefore, commensal bacteria might have little involvement with CD4⁺CD25⁺ cells in their promotion of T cell hyporesponsiveness induced by oral antigen in the OVA23–3 model. Interestingly, we observed that the proportion of CTLA-4⁺CD45RB^{low} but not Foxp3⁺ cells in the intestinal CD4⁺ population was lower in GF mice. We consider that the population of intestinal Foxp3⁺CTLA-4⁺ cells but not the Foxp3⁺CTLA-4⁺ CD4⁺ population might be fewer in number in GF mice, given that we confirmed that the CTLA-4⁺ subpopulation of CD4⁺ cells was larger than the Foxp3⁺ or CD25⁺ subpopulations (Fig. 2a and data not shown), and most Foxp3⁺CD4⁺ cells expressed CTLA-4 (data not shown). Thus, our study suggests that intestinal commensal bacteria may induce the generation or expansion of Foxp3⁺ regulatory T cells such as the IL-10-producing T regulatory type 1 (Tr1) cells [27] or CD25⁺CD45RB^{low}CD4⁺ cells [28]. In fact, we confirmed that intestinal CD4⁺ cells in GF mice produced lower levels of IL-10 than those in CV mice (Fig. 3), indicating that intestinal bacteria may contribute to the induction of Tr1 cells. In addition, it has been reported that CTLA-4 is required for the induction of T cell anergy [29,30].

CTLA-4 on T cells acts to suppress T cell responses through down-regulation of the expression of co-stimulatory molecules on APCs [31,32]. Therefore, commensal bacteria might also be involved in T cell hyporesponsiveness induced by dietary antigen through modulation of the expression of CTLA-4.

In terms of the second possibility, at least, we consider that the higher proportion of CD4⁺CD45RB^{high} cells in the intestinal tissues of GF mice compared to CV mice is responsible for the impaired hyporesponsiveness of T cells after oral administration of OVA, although we have not yet examined the relative IL-2 producing abilities of CD4⁺CD45RB^{high} cells from CV and GF mice. Further investigation is required to examine the function and mechanisms of generation or expansion of activated/memory type (CD45RB^{low}) and CTLA-4⁺ T cells modulated by intestinal commensal bacteria.

Previous studies have also suggested that GF mice are more susceptible to allergic sensitization than CV mice [9,10]. Our present study, using a model in which the antigen was exclusively fed, without any systemic sensitization by intraperitoneal or intravenous administration, has revealed that higher serum antibody responses (OVA-specific IgE, IgG1 and IgG2a) are induced in GF mice than in CV animals (Fig. 4). Thus, our results suggest that GF mice are more susceptible to a dietary antigen-induced serum antibody response. We have shown that the level of Th2-dependent antibodies is higher in the serum of GF mice fed an OVA diet. However, IL-4 production by SP cells from GF mice was lower than that of CV mice, in spite of the fact that splenic T cell responses play important roles in the regulation and induction of serum antibody responses. To explain such contradicting phenomena, we have demonstrated here that there is a higher expression of CD40L on SP CD4⁺ cells in GF mice fed OVA compared to that in CV mice. This molecule may be responsible for the higher serum Th2 type antibody production in this model. Indeed, it has been reported that the interaction of CD40-CD40L on T and B cells can induce activation and class switching in B cells, resulting in antibody production [33]. Therefore, our data suggest that colonization by commensal bacteria regulates antibody responses to dietary antigen through the modulation of surface molecules, as well as a consequence of altered cytokine balance on CD4⁺ T cells.

In conclusion, we have demonstrated that intestinal colonization by commensal bacteria promotes T cell tolerance and down-regulates serum antibody responses by modulating antigen-specific T cell responses. These data support the hypothesis that these bacteria act in the development and maintenance of the normal intestinal immune system to induce oral tolerance and prevent food allergy. It will be interesting for future studies to examine the interactions between intestinal commensal bacteria and APCs which recognize the bacteria or dietary antigens and present them to direct T cell responses.

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