Oral Tolerance Increased the Proportion of CD8+ T Cells in Mouse Intestinal Lamina Propria

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Background: Oral tolerance is defined by the inhibition of immune responsiveness to a protein previously exposed via the oral route. Protein antigens exposed via the oral route can be absorbed through the mucosal surfaces of the gastrointestinal tract and can make physical contact with immune cells residing in the intestinal lamina propria (LP). However, the mechanisms of oral tolerance and immune regulation in the intestines currently remain to be clearly elucidated. **Methods:** In order to determine the effect of oral protein antigen intake (ovalbumin, OVA) on the intestinal LP, we assessed the expression profile of the T cell receptor and the co-receptors on the cells from the intestines of the tolerant and immune mouse groups. **Results:** We determined that the proportion of OVA-specific B cells and $\gamma\delta$ T cells had decreased, but the CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ T cells were increased in the LP from the tolerant group. The proportion of CD8+ T cells in the spleen did not evidence any significant differences between treatment groups. **Conclusion:** These results indicate that CD8+ T cells in the intestinal LP may perform a regulatory role following antigen challenge via the oral route.
[Immune Network 2008;8(2):46-52]

INTRODUCTION

Oral tolerance is the immunological phenomenon by which the mucosal immune system maintains a hypo-responsive condition against a number of proteins previously exposed via the oral route (1). Commensal bacteria and dietary antigens induce immune tolerance in the intestine under physiological conditions. Protein antigens ingested via the oral route can be absorbed through the mucosal surfaces of the gastrointestinal tract, making physical contact with immune cells residing in the intestinal lamina propria (LP). Many cell types and cytokines have been reported to be involved in the mechanism of oral tolerance (e.g. regulatory T cells, TGF-β, IL-10, $\gamma\delta$ T cells and CD8+ T cells) (2), and several mechanisms have been proposed for the oral tolerance induction via the intestinal tract ranging from the deletion of antigen-specific T cells (3,4), to induction of anergy (5), immune deviation (6) and supression by Tregs (7,8) or by other cells.

The mucosal surface is the major portal of entry for external antigens. Such a mucosal defense system is comprised of gut-lining epithelial cells, M cells, associated lymphoid tissues, and the lamina propria (9). Although many immune cells reside within the lamina propria in the intestines, the functional role of cells residing in the lamina propria remains unclear.

In order to evaluate the effects of antigen challenge on the lamina propria via oral route after tolerance induction or systemic immunization, we induced oral tolerance followed by systemic immunization with OVA in BALB/c mice. After 3 weeks of OVA-feeding, we isolated the spleen and intestinal lamina propria from the mice and determined the proportion of B cells and T cells, especially $\gamma\delta$ T cells and CD8+ T cells, via flow cytometric analysis.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Daehan Biolink (Eumsung, Korea). All mice were bred and housed under specific pathogen-free conditions. All procedures were approved by the Animal Care and Use Committee of the Ewha

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This work was supported by Ewha Medical Alumni Association.

Keywords: oral tolerance, CD8 T cell, lamina propria, ovalbumin

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Oral antigen administration, immunization, and oral challenge

Oral tolerance to OVA was induced by allowing mice *ad libitum* access to 1% OVA (Grade II, Sigma) solution dissolved in drinking water for 5 consecutive days (Fig. 1). Immunization with OVA was conducted via the intraperitoneal injection of 100 ug of OVA adsorbed to 1 mg of aluminum hydroxide gel (Sigma) (10). For OVA oral challenge, the mice were treated with 20 mg of OVA in 100 ul of water via the oral route.

LP cell isolation

LP cells were isolated from the small intestines of 6～12 week-old BALB/c mice. In brief, as previously reported (11), small intestine segments were incubated with FACS buffer (PBS containing 10% fetal calf serum (FCS), 20 mM HEPES, 100 U/ml penicillin, 100 g/ml streptomycin, 1 mM sodium pyruvate, 10 mM EDTA, and 10 g/ml polymyxin B) for 30 min at 37°C in order to remove the epithelial cells, and were extensively washed with PBS. The segments were then digested with 400 U/ml of collagenase D (Roche, Germany) and 10 g/ml of DNase I (Roche) in RPMI 1,640/10% FCS with 45～90 minutes of continuous stirring at 37°C. EDTA was added (10 mM), and the cell suspension was incubated for an additional 5 min at 37°C. After washing, the cells were subjected to density-gradient centrifugation in 40%/75% Percoll (approximate density is 1.058 g/ml and 1.093 g/ml, respectively). The cells harvested from the interface were washed and utilized as LP leukocytes for the assays.

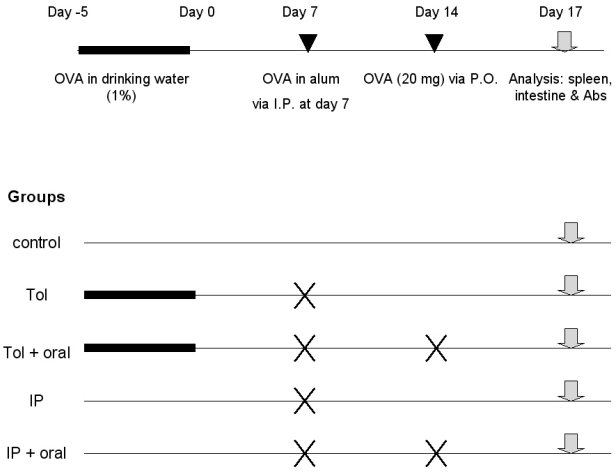

Figure 1. Schedule for the induction of tolerization and experimental groups. A group of six BALB/c mice were administered a 1% OVA solution in drinking water for 5 consecutive days (day -5 to 0) in the case of the "Tol" and "Tol+oral" groups. On Day 7, the mice were immunized with 100 ug of OVA in alum via the intraperitoneal route in the "Tol," "Tol+oral," "IP" and "IP+oral" groups, represented "X" in this figure. Seven days after immunization, the mice were challenged with 20 mg of OVA via the oral route in the "Tol+oral" and "IP+oral" groups (also represented "X"). Three days later (on day 17), the sera, intestinal LP, and spleen were harvested for analysis.

ELISA

OVA-specific IgG and IgA antibodies were quantified via ELISA. OVA (20 ug/ml) and BSA (20 ug/ml) were coated on ELISA plates with 0.1 M bicarbonate buffer (pH 9.6) followed by blocking with 2% skim milk solution. The sera from the mice were diluted and added into each well. Secondary antibodies were HRP-conjugated anti-mouse Ig (Biorad) and biotinylated anti-mouse IgA (BD Pharmingen, San Diego, CA) antibodies. Streptavidin-HRP and TMB substrate were obtained from BD Pharmingen. The serum titers were defined as the lower dilution necessary to render an OD value higher than 0.1 for IgA and 3.0 for IgG after background subtraction.

FACS analysis

The cells were stained with antibodies for 20 min on ice in FACS buffer. Anti-mouse CD3-Cy5 (145-2C11), anti-mouse CD4-FITC (GK1.5), and anti-mouse CD8α-PE (53-6.7) antibodies were from DiNonA Inc. (Korea). Anti-mouse CD8β-PE (H35-17.2), anti-mouse TCR β chain (H57-597), anti-mouse B220-Cy5.5 (RA3-6B2), anti-mouse $\gamma\delta$ TCR (GL3) antibodies were from BD Pharmingen. FITC-conjugated OVA was prepared using a PD-10 desalting column (Amersham). The cells were analyzed using a flow cytometer (BD, Mountain View, CA).

RESULTS

Oral administration of OVA suppresses OVA-specific IgA and IgG production

The mice were divided into five groups. "Tol" group of mice were forced to drink OVA containing water before systemic immunization with OVA via intraperitoneal (IP) route. "IP" group of mice were induced systemic immunization without oral tolerance induction. After 1 week of IP injection 20 mg of OVA was challenged via oral route ("oral") to observe the local change of intestinal lamina propria in oral tolerance induced mice and systemic immunization group. The mice

were decapitated and their sera were collected. Antibody titers from the tolerant group of mice were lower than those of the immunized groups (Fig. 2). IgA or IgG titers were not affected by OVA oral challenge. Therefore, we confirmed the OVA-drinking mice induced the oral tolerance by the result which anti-OVA antibody titer did not increase in OVA challenging group of mice in OVA-drinking followed by systemic challenge ("Tol+oral" group in Fig. 2A and B).

Oral challenge of OVA did not increase the OVA-specific B cells from LP in the tolerance-induced group

The cells were isolated from the spleen or the LP and incubated with the FITC-conjugated OVA solution as well as anti-B220 or anti-mouse IgA antibodies. The tolerant group and IP-only group of mice did not evidence any difference in the percentage of OVA+ B cells. However, after OVA challenge

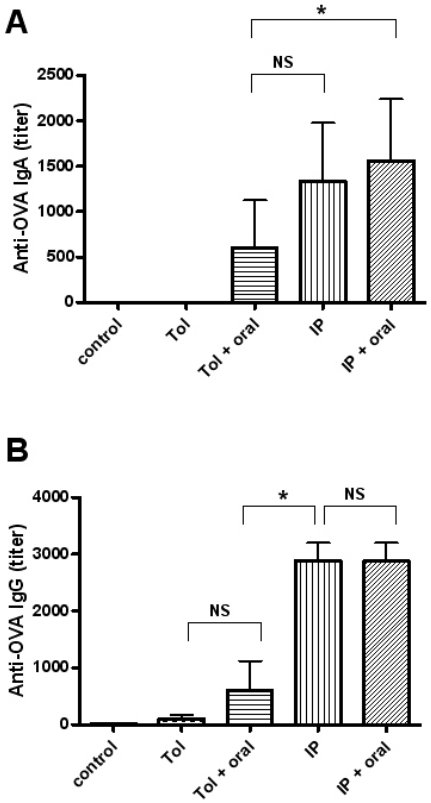

Figure 2. Supression of OVA-specific IgA and IgG production by oral tolerance. Control group, Tol group, Tol+oral group, IP group, and IP+oral groups (n=6) were prepared as described in Figure 1. (A) OVA-specific IgA antibody titers in the sera were determined via ELISA. (B) OVA-specific IgG antibody titers in the sera were determined via ELISA. The data are expressed as the means±SEM. The cells were isolated from more than 6 mice in each experiment. When necessary, a two-group comparison was conducted using Student's t-test. A p value<0.05 was considered to be statistically significant (*).

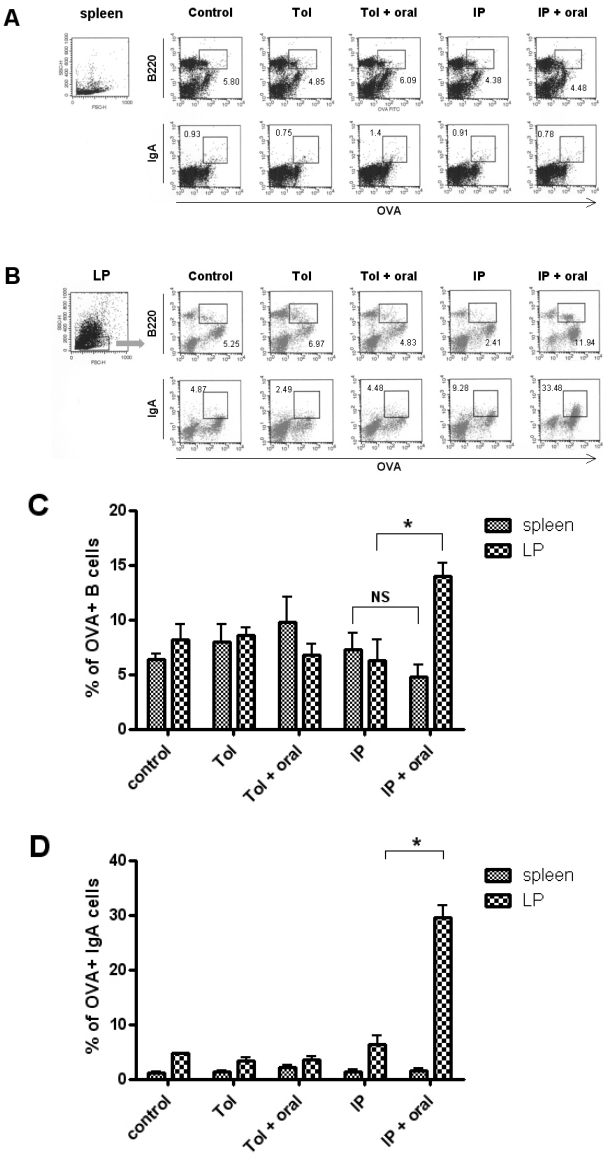

Figure 3. Percentage of OVA+ B cells and IgA+ cells in LP decrease in OVA intake after tolerance induction. Spleen cells (A) and LP cells (B) were harvested from the spleen or the intestinal lamina propria and stained with OVA-FITC, anti-mouse B220-Cy5.5 or anti-mouse IgA-PE antibodies and analyzed by flow cytometer. Each is one representative flow cytometric analysis of one mouse. (C) Cells from 6 mice were stained with OVA-FITC and anti-mouse B220 antibody and analyzed. (D) Cells from 6 mice were stained with OVA-FITC and anti-mouse IgA antibody and analyzed. Data are expressed as the means±SEM (*p<0.05 using Student's t-test).

via the oral route, the proportion of OVA+ B cells were increased in the IP-only group in the LP but did not increase the OVA+B cells nor OVA+IgA+ cells in the tolerant group (Fig. 3B, C, and D). This result showed that after systemic immunization with OVA followed by local challenge increased the OVA+ B cells ("IP+oral" in Fig. 3C and D) but not in the oral tolerance induction group ("Tol+oral" in Fig 3C and D). Although the titers of anti-OVA antibody did show difference among the group, OVA+ cells from spleen cells did not show significant difference between the treatment groups (Fig. 3A, C, and D).

The effect of oral tolerance and immunization on the proportions of LP granulocytes

In order to locate the possible candidate cells for the induction of tolerance in the LP, we collected the LP and spleen cells and analyzed with flow cytometer. The fraction of granulocytes was gated by size (forward scatter) and granularity (side scatter) in the flow cytometer (Fig. 4A). For the granulocytes, oral challenge with OVA did not showed statistically significant difference among the groups in the spleen or LP (Fig. 4B).

The effect of oral tolerance and immunization on the proportions of $\gamma\delta$ T cells in the LP

In order to locate the possible candidate cells for the induction of tolerance in the LP, we stained the LP and spleen cells with anti-mouse $\gamma\delta$ TCR antibody and anti-CD3 antibody (Fig. 5A). The immunization group showed increased percentage of $\gamma\delta$ T cells as compared with the tolerant group but did not have statistical significance (Fig. 5B).

CD8+ T cells increase in the tolerance induction group

Cells were stained with anti-mouse CD8β antibody for the detection of CD8$\alpha\beta$+ T cells or stained with anti-mouse CD8α antibody for the detection of CD8$\alpha\alpha$+ T cells (Fig. 6A and B) (12). We determined that the proportion of CD8$\alpha\beta$+ T cells in the LP increased after oral challenge in the tolerant group but were reduced in the immunized mouse group (Fig. 6D). In addition, CD8$\alpha\alpha$+ T cells in LP increased after oral challenge in the tolerant group (Fig. 6C).

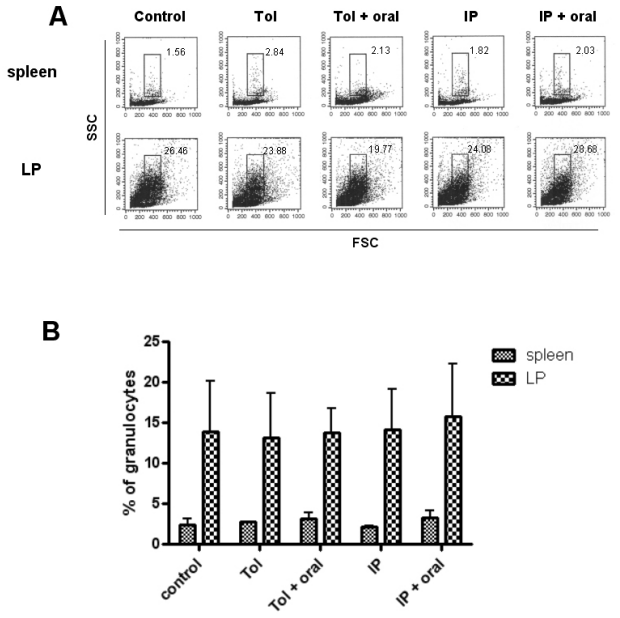

Figure 4. Percentage of granulocytes in the spleen as well as LP did not show significant difference among the treatment groups. (A) Granulocytes were gated according to distinct forward scatter (FSC) and side scatter (SSC) properties in the flow cytometer analysis. (B) Proportions of granulocytes from 6 mice were analyzed using Student's t-test. Data are expressed as the means±SEM.

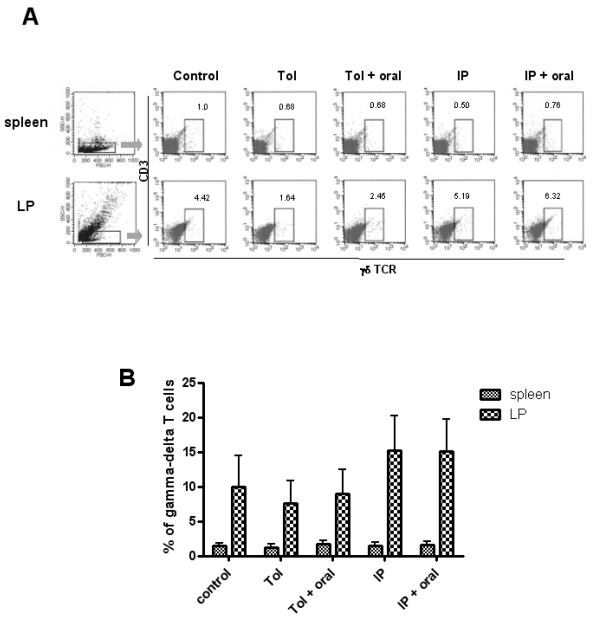

Figure 5. Percentage of $\gamma\delta$ T cells in the spleen as well as LP did not show significant difference among the treatment groups. (A) Cells were stained with anti-$\gamma\delta$ TCR antibody and anti-CD3 antibody, and analyzed by flow cytometer. Each is one representative flow cytometric analysis of one mouse. (B) Proportion of $\gamma\delta$ TCR+ cells from 6 mice were analyzed using Student's t-test. Data are expressed as the means±SEM.

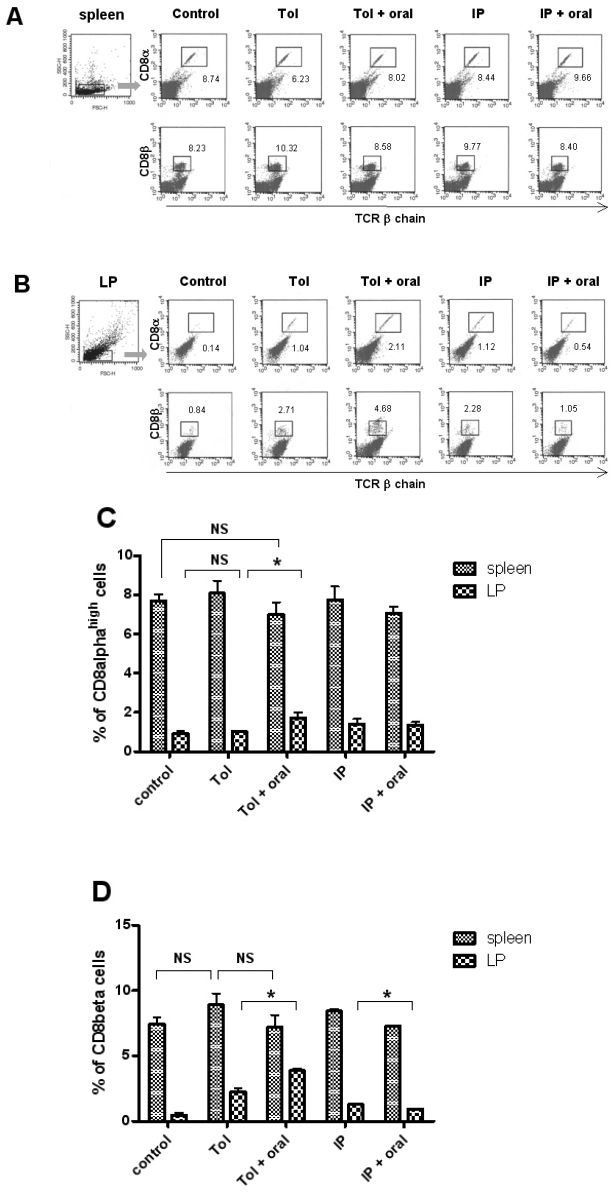

Figure 6. Percentage of CD8$\alpha\beta$ and CD8$\alpha\alpha$ cells in the mouse LP increases after oral OVA challenge in the tolerant group but not in the immunized group. (A) Spleen cells were stained with anti-TCR β chain and anti-CD8α or anti-CD8β antibodies, and analyzed by flow cytometer. Each is one representative flow cytometric analysis of one mouse. (B) LP cells were stained with anti-TCR β chain and anti-CD8 α or anti-CD8β antibodies, and analyzed with flow cytometer. Each is one representative flow cytometric analysis of one mouse. (C) For the detection of CD8$\alpha\beta$ cells, cells were stained with anti-mouse CD3, anti-mouse CD4 and anti-mouse CD8β antibodies. (D) For the detection of CD8$\alpha\alpha$ cells, the cells were stained with anti-mouse CD8 α antibody rather than anti-mouse CD8β antibody. On the flow cytometer, CD8α^{high} expressed cells were considered to be CD8$\alpha\alpha$ cells. Data are expressed as the means±SEM (*$p<0.05$ using Student's t-test).

However, the proportion of CD8$\alpha\alpha$+ T cells or CD8$\alpha\beta$+ T cells from the spleen did not evidence any significant difference among the treatment groups.

DISCUSSION

To assess the effects of oral antigen challenge on the LP after oral tolerance induction, we administered OVA challenge via the oral route into the oral tolerance-induced followed by immunization ("Tol") or immunization-only ("IP") BALB/c mice and compared the cell proportion in the intestinal LP. Oral challenge with OVA antigen induced an increased proportion of CD8$\alpha\beta$ T cells as well as CD8$\alpha\alpha$ T cells, but did not increase the $\gamma\delta$ T cells in the LP from the tolerant BALB/c mouse group. We first induced oral tolerance by challenging OVA in drinking water for 5 days, and then we immunized mice with OVA via IP route for systemic immunization. As we expected, oral tolerance group of mice did not much increase of anti-OVA antibody (Fig. 2). Then we proceed to observe the differences of cell population in the LP, where the oral antigens first encounter, among the treatment groups, to find possible candidate cells for the regulatory mechanism.

LP, a layer of connective tissue underlying the mucosal epithelium, is the inductive and effective site for mucosal immunity, and contains T cells, activated IgA+ B cells, DC, macrophages, NK cells, eosinophils, and mast cells (9). The T cells are principally CD4+ T cells (60 to 70%) and CD8+ T cells (~30%) primarily expressing TCR$\alpha\beta$. The majority of CD8+ T cells are detected as intraepithelial lymphocytes in the intestines.

The mechanism inherent to oral tolerance involves the induction of regulatory T cells and the induction of clonal anergy or deletion. However, it has been previously reported that the antigen dosage determines the form of induction in the case of oral antigen administration: low doses of antigen drives regulatory cell-mediated tolerance, and high doses of antigen often result in the anergy or deletion of specific T cells in the gut (13). In the case of regulatory cell-mediated tolerance, T cells secrete immune modulatory cytokines including transforming growth factor (TGF)-β, IL-10, and IL-4. Such regulatory CD4+CD25+ T cells and TGF-β perform a complementary role in oral tolerance, partially via the regulation of the expansion of antigen-specific CD4+ T cells (14).

Although there is debate over the role of CD4+ versus CD8+ T cells as mediators of oral tolerance, many studies proposed that oral tolerance mediated by CD8+ T cells. For

example, the mechanism of CD8+ T cells participating in oral tolerance was to producing IL-4 or IL-10 (15) and TGF-β (16). These CD8+ suppressor T cells were separated from cytotoxic CD8+ T cells and may be distinct functional subsets of CD8+ T cells (17). Because here we did not define the increased population of CD8+ T cells in the LP, it needs to determine the characteristic of CD8+ T cells in oral tolerance for further study.

In mice, a significant proportion (10 to 50%) of IEL are TCR $\gamma\delta$+, whereas in humans the proportion is approximately 10% (18). A significant proportion of TCR $\gamma\delta$+ and TCR $\alpha\beta$ + are CD8 $\alpha\alpha$+rather than TCR $\alpha\beta$+CD8 $\alpha\beta$+, which are predominant T cells present in the peripheral blood and spleen (19). Although the $\gamma\delta$ TCR-expressing T cells may also perform a role in the induction of oral tolerance (20), the proportion of $\gamma\delta$ T cells did not increase in the tolerant group in this study (Fig. 5). Such a result could be explained in that the cells collected in this study were principally from the LP, not from the epithelial layer of the intestine in which the intraepithelial lymphocytes (IELs) reside. Many $\gamma\delta$ expressing T cells reside as IELs, and thus lower amounts of $\gamma\delta$ T cells would be separated from the LP in such a cell separation process.

Because another systemic effect of oral tolerance on peritoneal inflammation was characterized as the mobilization of lymphocytes and bone marrow eosinopoiesis (21), we compared the proportional changes of granulocytes in LP after oral OVA challenge. However, we did not observed significant changes of the proportion of granulocytes in the LP among the treatment groups after oral OVA (Fig. 4A) in this experimental design.

In summary, our result indicated that the proportion of CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ T cells were increased in the LP from the tolerant group but not in the immunized group. Because oral OVA challenge did not induce systemic immune response, as reflected by serum IgA or IgG induction in tolerant group (Fig. 2) and did not induce local immune response to OVA reflected by OVA+B cells in the LP of tolerant mice (Fig. 3), such increases in the levels of CD8+ T cells in the intestinal LP after oral challenge with the antigen in the tolerant group may perform a regulatory role.

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