

Breakdown of Mucosal Immunity in the Gut and Resultant Systemic Sensitization by Oral Antigens in a Murine Model for Systemic Lupus Erythematosus¹

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Secreted IgA plays a pivotal role in the mucosal immunity to maintain the front line of body defense. We found that the level of fecal IgA was dramatically decreased in aged (NZB × NZW)F₁ (BWF₁) mice developing lupus nephritis, whereas levels in similarly aged New Zealand Black (NZB) and New Zealand White (NZW) mice remained unchanged compared with young mice. The number of cells obtained from Peyer's patches was markedly decreased in aged BWF₁ mice. Aged BWF₁ mice showed increased susceptibility to pathogenic bacterial infection. Furthermore, oral administration of OVA failed to inhibit secondary IgG response induced by systemic immunization, suggesting defective oral tolerance in aged BWF₁ mice. A significant amount of orally administered OVA was incorporated directly into the intestinal lamina propria in aged BWF₁ mice whereas it was mainly localized in subepithelial domes and interfollicular region in Peyer's patches in young mice. T cells obtained from renal and pulmonary lymph nodes of aged BWF₁ mice that had been orally administered with OVA showed an Ag-specific T cell proliferation, whereas those from young BWF₁, aged NZB, and aged NZW mice did not. Interestingly, aerosol exposure to OVA of aged BWF₁ mice, which had been orally administered with the same Ag, provoked an eosinophil infiltration in the lung. These results demonstrate that mucosal immunity in the gut is impaired and oral Ags induce systemic sensitization instead of oral tolerance in the development of murine lupus. *The Journal of Immunology*, 2005, 174: 5499–5506.

Isotype IgA is the most abundant Ig in mucosal secretions and plays an important role for the body defense to protect against microbial infection at mucosal surfaces (1, 2). It is generally believed that Peyer's patches (PP)³ are the major site for IgA⁺ B cell development in the gut, although this dogma has been recently challenged by Fagarasan and Honjo (3). IgA⁺ B cells migrate to the draining mesenteric lymph nodes (LNs) where they differentiate into plasma blasts and target the gut lamina propria through the thoracic duct and blood. Another source of IgA in the gut is B1 cells in the peritoneal cavity. Kroese et al. (4, 5) reported that approximately half of IgA⁺ cells in the intestinal lamina propria were derived from B1 cells. IgA production of by B1 cells is T cell-independent and requires the presence of commensal microflora (6). Furthermore, commensal bacteria bind mostly B1 cell-derived intestinal IgA, and less so B2 cell-derived intestinal IgA (7). B1 cells, therefore, might play a crucial role for preventing systemic invasion by intestinal bacteria.

(NZB × NZW)F₁ (BWF₁) mice spontaneously develop systemic autoimmune disorders resembling systemic lupus erythematosus (SLE) in humans (8). It is characterized by production of a variety of IgG autoantibodies and massive immune complex deposition in glomeruli in the kidney. Marked mononuclear cell infiltration in the target organs including the thymus, kidney, and lung is another characteristic in aged BWF₁ mice developing lupus nephritis. The mechanisms for systemic autoimmune or allergic responses in SLE remain to be elucidated. We previously demonstrated an ectopic and high expression of B lymphocyte chemoattractant (BLC)/CXCL13 in the target organs and defective B1 cell homing to the peritoneal cavity in aged BWF₁ mice, a murine model for SLE (9, 10). These findings prompted us to investigate the status of mucosal immunity in the gut in aged BWF₁ mice. We found an impaired intestinal mucosal immunity including an increased susceptibility to bacterial infection, defective oral tolerance, and systemic sensitization by orally administered Ags in aged BWF₁ mice developing lupus nephritis. Pathological significance of impaired mucosal immunity in the gut in the development of SLE is discussed.

Materials and Methods

Mice

BWF₁, New Zealand Black (NZB), and New Zealand White (NZW) mice, originally obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained under specific pathogen-free condition in our animal facility at the University of Tokyo. Female mice age 7–10 wk were used as young mice, whereas 8- to 10-mo-old mice with moderate to severe proteinuria (300–1000 mg/dl, Albustix; Bayer) were used as aged mice. All animal experiments complied with the standards defined in the Guideline for Care and Use of Laboratory Animals of the University of Tokyo.

Quantitative ELISA immunoassay for fecal IgA

Fecal pellets (100 mg) were placed into 1.5 ml of microcentrifuge tubes, 1 ml (10 w/v) of PBS added, and incubated at room temperature for 15 min.

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³ Abbreviations used in this paper: PP, Peyer's patch; BLC, B lymphocyte chemoattractant; LN, lymph node; SLE, systemic lupus erythematosus; NZB, New Zealand Black; NZW, New Zealand White; KLH, keyhole limpet hemocyanin; BAL, bronchoalveolar lavage.

Samples were vortexed, left to settle for 15 min, revortexed until all material was suspended, then centrifuged at 12,000 rpm for 10 min. The supernatant was removed and stored at -80°C or immediately tested on ELISA for IgA using mouse IgA ELISA Quantitation kit (Bethyl Laboratories). Microtiter plates were coated with goat anti-mouse IgA affinity-purified Ab and incubated for 60 min. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and each well was blocked with 200 μl of 50 mM Tris, 0.15 M NaCl, and 1% BSA (pH 8.0) for 30 min. Plates were washed with PBST, 100 μl of test samples and standards was added per well, and incubated for 60 min. Plates were washed with PBST and 100 μl of HRP-labeled goat anti-mouse IgA Fc-specific Abs was added to each well and incubated for 60 min. Plates were washed, developed for 30 min with HRP substrate (3,3',5,5'-tetramethylbenzidine), stopped with H_2SO_4 , and read at 450 nm with a Emax precision microplate reader (Molecular Devices).

Flow cytometry

The following mAbs from BD Pharmingen were used: FITC-conjugated anti-CD4 (GK1.5), anti-CD5 (53-7.3RRH), and anti-CD11b (M1/70); PE-conjugated anti-CD8 α (53-6.7), anti-CD11c (HL-3), and anti-B220 (RA3-6B2); and allophycocyanin-conjugated anti-B220 (RA3-6B2). A single cell suspension was isolated from PP and nucleated cells were counted using a hemocytometer. After Fc blocking with anti-CD16/CD32 Ab (BD Pharmingen) for 10 min, lymphoid cells were stained with: 1) FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and allophycocyanin-conjugated anti-B220 Abs; 2) FITC-conjugated anti-CD5 and PE-conjugated anti-B220 Abs; or 3) FITC-conjugated anti-CD11b and PE-conjugated anti-CD11c Abs. Flow cytometric analyses of lymphoid cells were performed using an Epics Elite cell sorter (Coulter Electronics).

Immunohistochemical and immunofluorescent study

Intestinal tissue fragments contain PP were embedded in Tissue-Tek OCT compound (Miles) and frozen in liquid nitrogen. Cryostat sections (7- μm thick) were preincubated with Block Ace (Dainippon Pharmaceutical) and then incubated with rat anti-mouse B220 Ab (RA3-6B2, 2 $\mu\text{g}/\text{ml}$; BD Pharmingen) for 30 min followed by incubation with biotin-conjugated goat anti-rat IgG (5 $\mu\text{g}/\text{ml}$; Cedarlane Laboratories). The sections were washed three times with PBS and then incubated with avidin-biotin peroxidase complexes (Vector Laboratories). The color development was achieved by Vectastain AEC substrate kit (Vector Laboratories) according to the manufacturer's instructions and the sections were counterstained with hematoxylin. Endogenous peroxidase activity was blocked with 0.2% H_2O_2 and 0.05% NaN_3 in distilled water for 10 min at room temperature. For staining tight junction proteins, cryostat sections of the intestine obtained from young and aged BWF₁ mice were incubated with rabbit anti-zonula occluden protein 1 (zo-1) Ab (Zymed Laboratories), followed by incubation with PE-conjugated goat anti-rabbit IgG and observed under fluorescent microscopy (AX-80; Olympus).

Infection experiments

Escherichia coli B41 (O101:K-, K99, F41) was obtained from the American Type Culture Collection (no. 31619). *E. coli* B41 was inoculated on a Trypticase Soy Agar (BD Biosciences) plate, which was then incubated for 24 h at 37°C . The bacteria were washed off this plate with 5 ml of sterile PBS. A suspension of *E. coli* B41 was prepared in this way and sterile DMSO was added to a final concentration of 10% v/v. The culture was dispensed aseptically in 500 μl of portions and rapidly frozen in liquid nitrogen. The viable count of all *E. coli* suspensions was measured at the time of inoculation. Bacterial suspensions were recovered from frozen storage and resuspended to comprise 10^9 CFU in 100 μl of PBS. Each mouse was given 100 μl of bacterial suspensions directly into the stomach using a ball-tipped gastroesophageal needle for 3 consecutive days. Infected mice were observed daily and mortality was recorded.

Induction of oral tolerance

Induction of systemic unresponsiveness to OVA (Sigma-Aldrich) was performed as previously described (11). Briefly, mice were given 25 mg of OVA in 250 μl of PBS by gastric intubation on day 0. Control mice received PBS. On days 7 and 21, mice were immunized and challenged s.c. with 100 μg of OVA in 100 μl of CFA (Difco). OVA-specific Ab in the serum was measured 7 days after the second s.c. immunization.

OVA-specific serum Abs by ELISA

Anti-OVA Ab titers in serum samples were determined by ELISA as previously described (11). Briefly, ELISA plates (Corning Life Sciences) were coated overnight at 4°C with 1 mg/ml OVA in PBS. Blocking was done

with 200 μl of 1% BSA in PBS for 1 h at 37°C . Serial dilutions of serum in 1% BSA/PBS were prepared and 100 μl was added per well in duplicate. Following incubation at 37°C for 4 h, HRP-labeled goat anti-mouse IgG Fc-specific Abs (Bethyl Laboratories) were added and incubated overnight at 4°C . Color was developed with 1.1 mM ABTS (Sigma-Aldrich) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H_2O_2 .

Oral administration of Alexa 488-labeled OVA

Two hundred microliters of Alexa 488-labeled OVA solution (3.3 mg/ml, fluorescence to protein ratio), a kind gift from Dr. T. Uchida (National Institute of Infectious Diseases, Tokyo, Japan), was orally administered to young and aged BWF₁ mice, and cryosections were prepared 3 h after administration. Localization of incorporated labeled OVA was analyzed under fluorescent microscope (AX-80; Olympus).

OVA-induced T cell proliferation assay

OVA (25 mg) in 250 μl of PBS was administered intragastrically three times weekly. One week after the last administration, spleen and LNs (axillary, pulmonary, mesenteric, renal, and inguinal) were removed aseptically. Single cell suspensions were obtained using fine-mesh screens (Cell Strainer; BD Biosciences). A total of 4×10^5 cells were cultured in the presence of OVA or keyhole limpet hemocyanin (KLH, 200 $\mu\text{g}/\text{ml}$) for 5 days at 37°C in 5% CO_2 in atmosphere. On day 5, each well was pulsed with WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) [2H]tetrazolium salt; Polysciences) for 5 h and optical absorbance was measured.

Induction of allergic response to OVA

OVA-induced pulmonary eosinophilia was generated as previously described by Kawasaki et al. (11) with a slight modification. Briefly, mice were first immunized i.p. with 50 μg of OVA plus alum (Wako Pure Chemical) on days 1 and 8, then exposed to aerosol inhalation of 1% OVA for 15 min on days 22, 23, 24, 28, and 29 to induce an allergic response in the lung. To detect the systemic sensitization by oral Ags in aged BWF₁ mice, mice were orally administered with 50 μg of OVA in PBS on days 1 and 8, and then exposed to aerosol inhalation of 1% OVA as described. Mice were killed 48 h after the last aerosol challenge and subjected to histologic examination.

Bronchoalveolar lavage (BAL) and histologic examination

Forty-eight hours after the last aerosol exposure, the lungs were lavaged via a tracheal cannula with 0.7 ml of PBS three times. The recovered BAL fluid was immediately centrifuged (1000 rpm, 2 min, 4°C), and cells in BAL fluid were washed and resuspended in 1 ml of PBS. The number of cells was determined by hemocytometer. Samples were applied to glass slides by cytocentrifugation, air-dried for 10 min, and then subjected to Diff-Quick staining kit (International Reagents). The percentage of eosinophils, lymphocytes, neutrophils, and macrophages were determined by counting at least 200 cells/slide using standard morphologic criteria.

Lung specimens were fixed in 10% neutrally buffered Formalin and paraffin embedded. Deparaffinized sections (5- μm thick) were stained with H&E and analyzed under a light microscope.

Statistical analysis

Statistical analysis was performed using Student's *t* test or log-rank test. The 95% confidence limit was taken as significant.

Results

Defective IgA secretion into the gut lumen and increased susceptibility to bacterial infection in aged BWF₁ mice

The level of fecal IgA was dramatically decreased in most of aged BWF₁ mice developing lupus nephritis compared with young BWF₁ mice (Fig. 1C). In 14 of 17 aged BWF₁ mice, IgA levels were almost zero whereas similarly aged NZB and NZW mice showed a comparable level of fecal IgA to young BWF₁ mice (Fig. 1, A and B). The decreased level of fecal IgA level was detected as early as 5- to 6-mo-old before the establishment of severe renal failure (data not shown). Serum IgA level is also decreased in aged BWF₁ mice compared with young BWF₁, aged NZB, and aged NZW mice (data not shown).

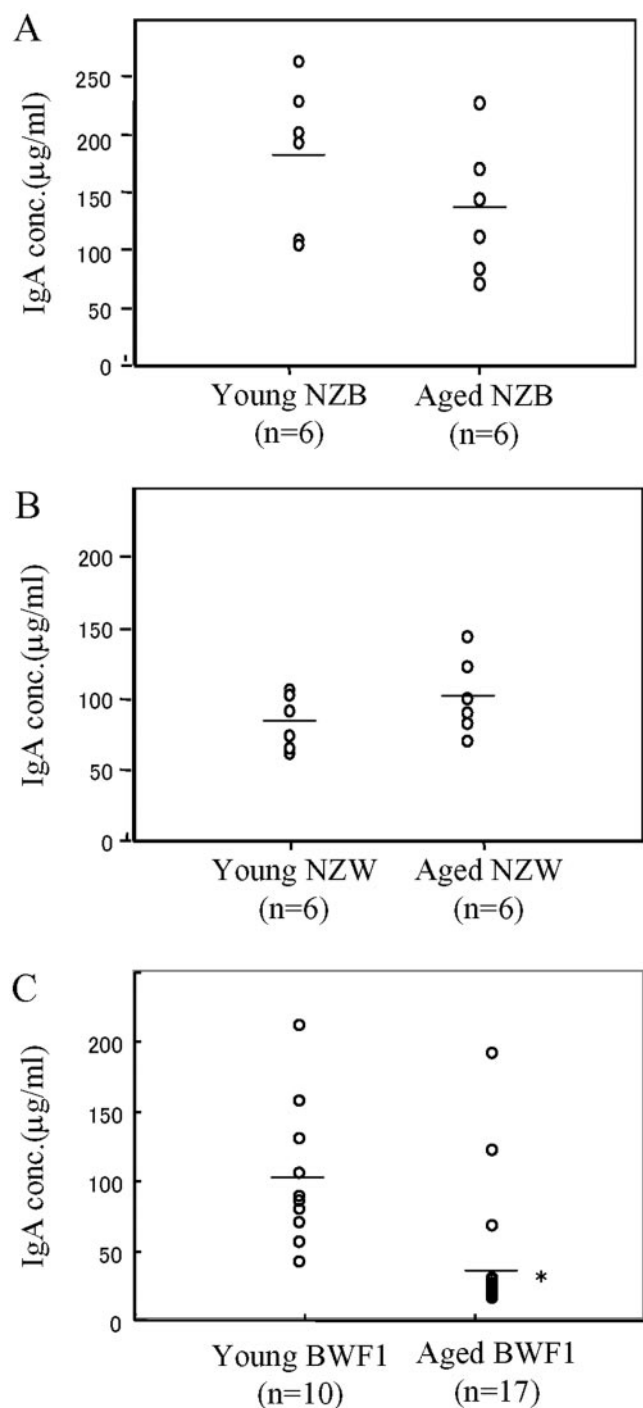


FIGURE 1. Defective IgA secretion in aged BWF₁ mice developing lupus nephritis. IgA concentrations in the feces of young and aged NZB ($n = 6$) (A), NZW ($n = 6$) (B), and BWF₁ ($n = 10$ and 17) (C) mice were determined by ELISA. Each IgA level (○) of an individual mouse is shown and horizontal bars represent median values. Statistical analysis was performed by Student's *t* test. *, $p < 0.03$.

Decreased cell number in PP in aged BWF₁ mice

Total cell count obtained from PP in aged BWF₁ mice was markedly decreased compared with those in young BWF₁, aged NZB, and aged NZW mice (Fig. 2B), whereas the number of PP in aged BWF₁ mice is similar to that in young BWF₁ mice (Fig. 2A). The absolute cell number of CD4⁺ cells and B220⁺ cells recovered from PP was decreased in aged BWF₁ mice (Fig. 2, C and D).

Increased susceptibility to bacterial infection in aged BWF₁ mice

Aged BWF₁ mice showed a higher mortality than young BWF₁ mice when intragastrically infected with pathogenic *E. coli* strain (B41), whereas young BWF₁ mice were resistant to the bacterial infection (Fig. 3). Control aged BWF₁ mice without bacterial inoculation were alive during the experimental period. Similarly aged NZB and NZW mice also showed resistance to the bacterial infection (data not shown).

Breakdown of oral tolerance in aged BWF₁ mice

We next investigated oral tolerance in aged BWF₁ mice developing lupus nephritis. OVA-specific IgG production was suppressed in young BWF₁ mice that had been orally administered with OVA before systemic immunization (Fig. 4A), demonstrating that oral tolerance was induced in young BWF₁ mice. Oral administration of OVA did not inhibit KLH-specific IgG production in young BWF₁ mice (data not shown). In contrast, the suppression of IgG production was not observed in aged BWF₁ mice, suggesting a breakdown of oral tolerance in aged BWF₁ mice developing lupus nephritis (Fig. 4B). This was not due to impaired general immune responses in aged BWF₁ mice because they produced comparable amount of anti-OVA Ab to that in young BWF₁ mice when immunized with OVA together with alum. Furthermore, oral tolerance was intact in similarly aged NZB and NZW mice (Fig. 4, C and D).

Direct incorporation of Alexa 488-labeled OVA into the lamina propria in aged BWF₁ mice

To investigate the route of orally administered protein Ag in aged BWF₁ mice developing lupus nephritis, Alexa 488-labeled OVA was orally administered. The labeled OVA was localized mainly in the subepithelial dome and interfollicular region in PP in young BWF₁ mice (Fig. 5Aa), whereas a significant amount of labeled OVA was directly incorporated into the lamina propria as well as in the subepithelial dome in aged BWF₁ mice (Fig. 5, Ab and Ac). It was unlikely that direct incorporation of Alexa 488-OVA into the lamina propria in aged BWF₁ mice was attributed to intestinal epithelial dysfunction because the tight junction protein (zo-1) was intact in the intestinal mucosa (Fig. 5B).

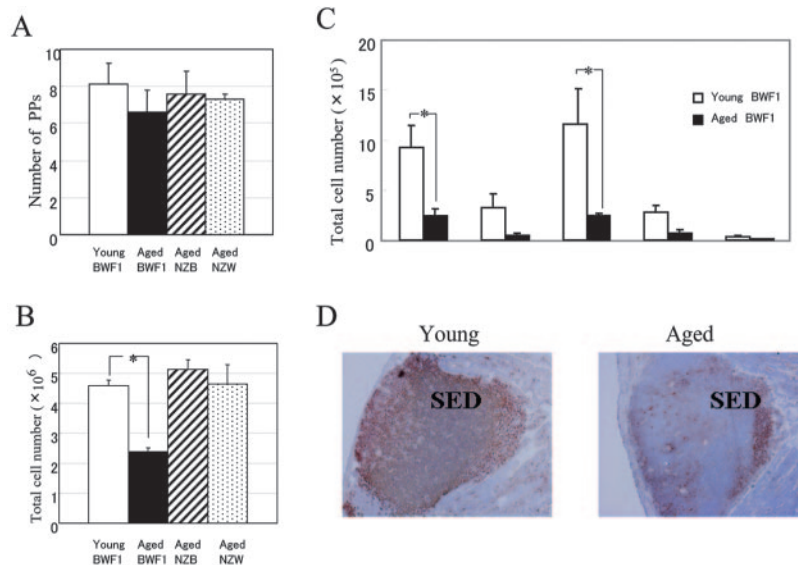
Oral administration of OVA resulted in systemic sensitization in aged BWF₁ mice

T cells obtained from renal and pulmonary LNs of aged BWF₁ mice, which had been orally administered with OVA, showed an Ag-specific T cell proliferation whereas T cells from young BWF₁, aged NZB, and aged NZW mice did not (Fig. 6). These LNs are usually enlarged in aged BWF₁ mice due to mononuclear cell infiltration in the lung and kidney. It should be noted that T cells from superficial LNs such as axillary and inguinal LNs were not sensitized by oral administration of OVA in aged BWF₁ mice.

Respiratory inhalation of OVA induced eosinophil infiltration in aged BWF₁ mice that had been orally sensitized with the same Ag

Allergic sensitization by orally administered OVA in aged BWF₁ mice was examined in an OVA-induced asthma model (Fig. 7). In the positive control in which young BWF₁ mice had been injected i.p. with OVA plus alum and then subjected to aerosol inhalation of OVA, there was a marked increase in total cell number, mostly eosinophils in BAL fluids (Fig. 7A). In contrast, young BWF₁ mice that had been orally administered with OVA and then exposed

FIGURE 2. Decreased cell number in PP of aged BWF₁ mice developing lupus nephritis. **A**, Number of PP in young and aged BWF₁ and similarly aged NZB and NZW mice. Mean ($\pm$ SE) in three mice per group is shown. **B**, Total cell numbers obtained from PP. Mean ($\pm$ SE) for $n = 3$ is shown. Statistical analysis was performed by Student's *t* test. *, $p < 0.02$. **C**, FACS analysis on cells obtained from PP. Total cell number of each cellular subset (mean $\pm$ SE) for $n = 3$ is presented. *, $p < 0.03$. **D**, Immunohistochemical staining of PP. Cryosections were stained with anti-B220 mAb as described in *Materials and Methods*. Original magnification $\times 100$. SED, subepithelial dome.



aerosol inhalation of OVA did not show any inflammatory response. Surprisingly, aged BWF₁ mice that had been orally administered with OVA and then exposed to aerosol inhalation of OVA showed a significant increase in the number of eosinophils in BAL cells (Fig. 7A). In contrast, aged BWF₁ mice that had been orally administered with OVA and then exposed to KLH inhalation showed no eosinophil infiltration, suggesting Ag specific allergic sensitization in the lung. Aged BWF₁ mice just exposed to OVA aerosol inhalation showed no response either. Furthermore, similarly aged NZB and NZW mice that had been orally administered with OVA and then exposed to aerosol inhalation of OVA did not show eosinophil infiltration in BAL cells (Fig. 7A).

Eosinophils were also observed in the lung parenchyma in aged BWF₁ mice that had been orally administered with OVA and then exposed aerosol inhalation of OVA, whereas mononuclear, but no eosinophils, were present in untreated aged BWF₁ mice developing lupus nephritis (Fig. 7, Ba and Bb). No cellular infiltration was observed in aged NZB and NZW mice even when orally admin-

istered with OVA and exposed to OVA aerosol inhalation (Fig. 7, Bc and Bd).

Discussion

In the present study, we found that secreted IgA in the gut lumen was severely decreased in aged BWF₁ mice developing lupus nephritis. Secreted IgA derives from both B1 and B2 cells. It is generally believed that B2 cells class-switch from IgM to IgA in the germinal centers in PP in the presence of cytokines, in particular TGF- β (12), although this view has been recently challenged by studies by Fagarasan et al. (13). In aged BWF₁ mice, the total cell number of CD4 T cells and B2 cells obtained from PP was markedly decreased compared with young BWF₁ mice although the number of PP itself remained unchanged. RT-PCR analysis showed that the level of TGF- β in PP was not decreased in aged BWF₁ mice except in one of six mice compared with young BWF₁ mice (data not shown). It is, therefore, likely that low level of fecal IgA in the gut lumen is partly attributed to decreased T cell-dependent IgA production by B2 cells in PP.

In contrast, the precise mechanism for class-switch in B1 cells remains to be elucidated. It is reported that approximately half of IgA⁺ cells in the intestinal lamina propria were derived from B1 cells (4). It has been recently suggested that IgA secreted into the gut lumen by B1 cells may play a crucial role for preventing systemic invasion by intestinal bacteria (6, 7). We previously reported that B1 cell homing to the peritoneal cavity was impaired in aged BWF₁ mice due to the aberrant high expression of BLC/CXCL13 in the target organs and also to the decreased number of peritoneal macrophages, which are the major cell source for BLC/CXCL13 in the peritoneal cavity (10). We also demonstrated in transfer experiments that a smaller number of B1 cells migrated to PP and mesenteric LNs in aged BWF₁ mice than in young BWF₁ mice (10). Our results are consistent with the previous studies demonstrating that BLC/CXCL13 is critical for B1 cell homing to the peritoneum and for B1 and B2 cell homing to PP as well as a decrease in T15-containing Ab production in CXCL13-deficient mice (14, 15). Recruitment of follicular Th cells, which express CXCR5 and can promote IgA production by B cells, may also be hampered in aged BWF₁ mice (16). It is, therefore, also likely that aberrant B1 cell recruitment and localization due to ectopic and high expression of BLC/CXCL13 in the target organs in aged BWF₁ mice may result in defective IgA secretion by B1 cells into

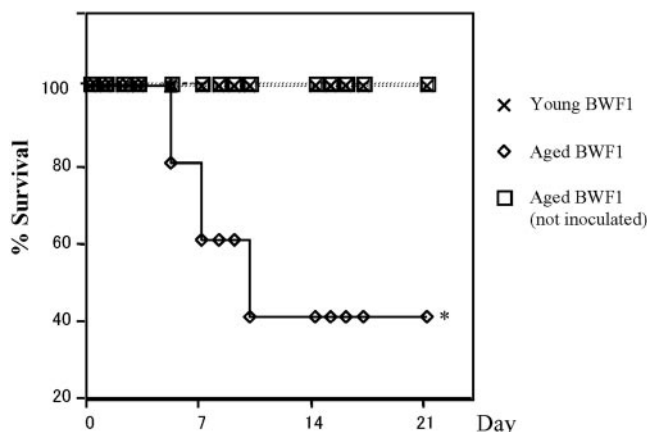
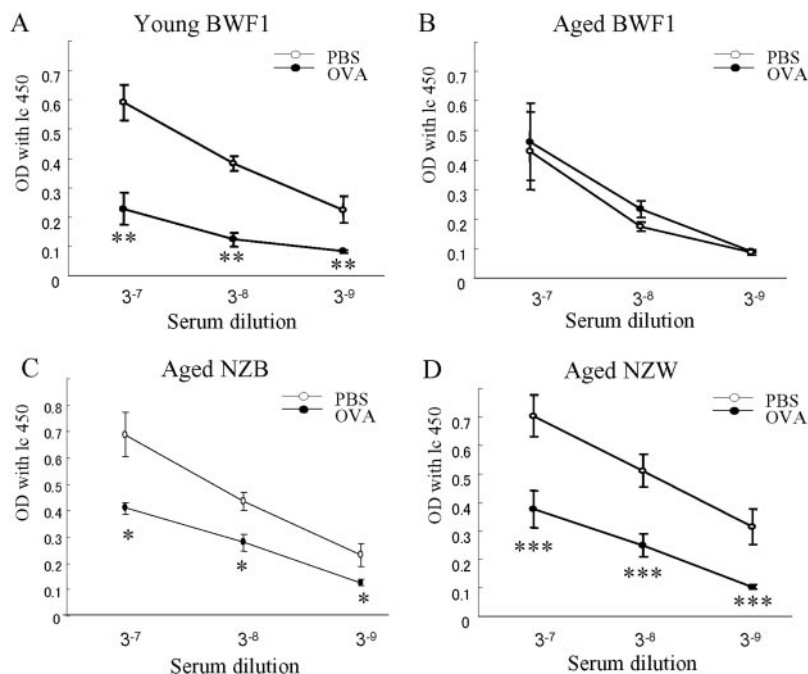


FIGURE 3. Increased susceptibility to bacterial infection in aged BWF₁ mice. Mice were infected with *E. coli* (10^9 CFU B41 strain) on days 1, 2, and 3 and mortality was monitored daily. Each group consisted of five mice: $\times$, young BWF₁; $\diamond$, aged BWF₁ infected with *E. coli*; $\square$, aged BWF₁ without infection. Significantly higher mortality was observed in aged BWF₁ mice than in young BWF₁. Representative data from three experiments are presented. Statistical analysis of mortality data was performed by log-rank test. *, $p < 0.05$.

FIGURE 4. Defective oral tolerance in aged BWF₁ mice. Young BWF₁ (A), aged BWF₁ (B), aged NZB (C), and aged NZW (D) mice were orally administered with 25 mg of OVA (●) or PBS (○) on day 0 and immunized s.c. with 100 μ g of OVA in CFA on days 7 and 21 ($n = 4$). The serum concentration of OVA-specific IgG Ab was determined on day 28 by ELISA. The results are expressed as the mean ($\pm$ SD). Representative data from three experiments are presented. Statistical analysis was performed by Student's *t* test. *, $p < 0.01$; **, $p < 0.005$.



the gut lumen. Impaired mucosal immunity in aged BWF₁ mice is not simply attributed to generalized immune dysfunctions by renal failure or moribund status because systemic Ab production and CD4 T cell proliferation in the particular lymphoid organs were intact in aged BWF₁ mice and also because the decreased level of fecal IgA was readily detectable as early as age 5–6 mo before the establishment of severe renal failure.

Aberrant B1 cell trafficking would also affect IgM natural Ab production by B1 cells. T15⁺ anti-phosphorylcholine Ab that is produced exclusively by B1 cells (17) is decreased in aged BWF₁ mice (data not shown). Defective IgA in the gut lumen and decreased IgM natural Ab would cause more chance for bacterial and viral infection, consistent with our data showing more susceptibil-

ity to weak pathogenic bacteria. Penetration of commensal bacterial Ag would then provoke vigorous immune responses by B1 cells, which have specificity for polysaccharides such as phosphorylcholine, lipids, and proteins of bacterial components (18–20). B1 cells have also been considered to be involved in autoantibody production including anti-DNA Abs that is a hallmark in SLE (21–23). Unlike mammalian DNA, bacterial DNA has potent immunologic effects that lead to polyclonal B cell activation as well as the production of specific Abs in mice (24). It is also reported that bacterial DNA induces anti-dsDNA Ab cross-reactive to mammalian dsDNA in autoimmune prone mice such as BWF₁ mice (25). It is, therefore, tempting to speculate that impaired IgA secretion in the gut lumen and low level of IgM natural Abs in aged BWF₁

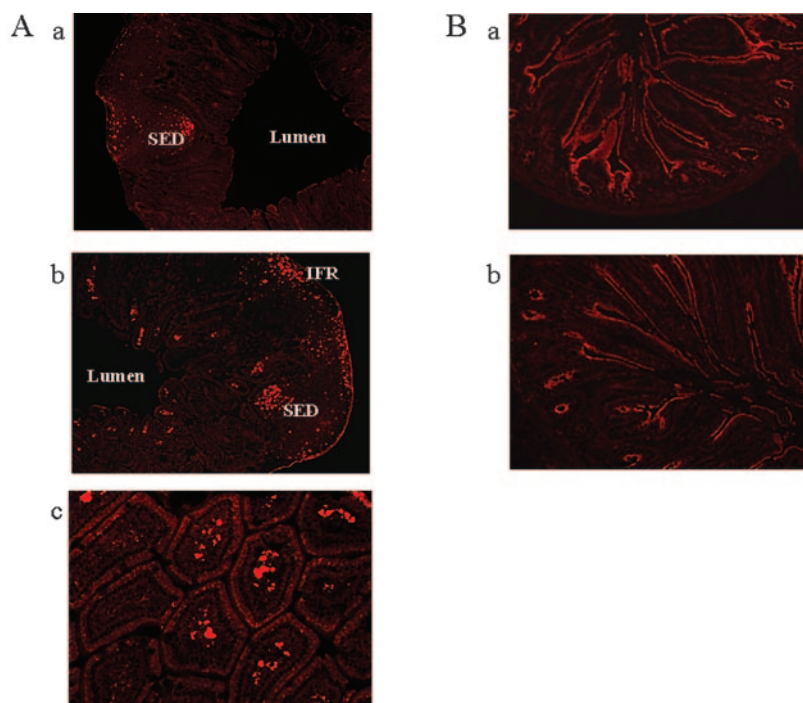


FIGURE 5. Direct incorporation of Alexa 488-labeled OVA into the lamina propria in aged BWF₁ mice. A, Alexa 488-labeled OVA was administered intragastrically to young (a) and aged (b and c) BWF₁ mice and cryostat sections prepared 3 h after administration were analyzed under a fluorescent microscope. The labeled OVA was localized mainly in subepithelial dome (SED) of intestinal mucosa in young BWF₁ mice, whereas a significant amount of the labeled OVA was directly incorporated into the lamina propria as well as subepithelial dome in aged BWF₁ mice ($\times 100$) in greater magnification in c ($\times 200$). B, Cryostat sections of the intestine obtained from young (a) and aged (b) BWF₁ mice were incubated with rabbit anti-zo-1 Ab, followed by incubation with PE-conjugated goat anti-rabbit IgG and observed under a fluorescent microscope.

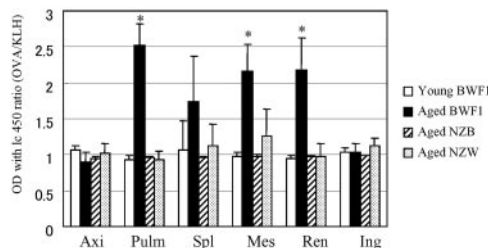


FIGURE 6. Systemic sensitization by orally administered Ag. Young and aged BWF₁, aged NZB, and NZW mice were intragastrically given 25 mg of OVA twice on days 0 and 7, then single cell suspensions were obtained on day 14 from spleen (Spl), axillar (Axi), pulmonary (Pulm), renal (Ren), mesenteric (Mes), and inguinal (Ing) LNs. A total of 4×10^5 cells were stimulated with OVA or KLH (200 μ g/ml) for 5 days and pulsed with WST-1 for the last 5 h to measure the optical absorbance. Results are presented as the mean ($\pm$ SE) of $n = 4$. Representative data from three experiments are presented. Statistical analysis was performed by Student's *t* test. *, $p < 0.05$.

mice due to aberrant B1 cell trafficking may result in penetration of commensal bacteria into systemic immune system and induction of vigorous antibacterial DNA Ab production, which cross-reacts to mammalian DNA.

Breakdown of oral tolerance and systemic sensitization by orally administered OVA in aged BWF₁ mice was another interesting finding. Oral tolerance is historically and originally described as inhibition of Ab production by oral preadministration of protein Ag (26, 27). In this sense, impaired oral tolerance does exist as a phenomenon in aged BWF₁ mice developing lupus nephritis. However, the precise mechanism for oral tolerance has not been fully elucidated at present.

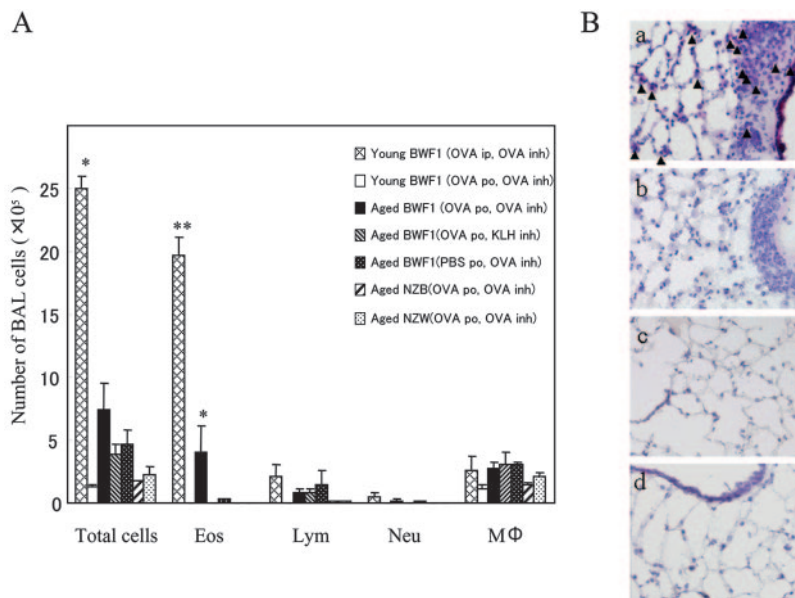
It was previously reported that regulatory T cells were induced in PP upon oral administration of protein Ags (28–31). It was also demonstrated that Ag-specific T cells were deleted in PP in high-dose oral tolerance (32, 33). However, this idea for a pivotal role of PP in oral tolerance was challenged by several studies demonstrating that oral tolerance could be induced independently of PP (34, 35). Spahn et al. (36) further demonstrated that mesenteric LNs were critical for the induction of high-dose tolerance in the absence of PP. It was also demonstrated that the spleen played an important role for oral tolerance (37, 38). These results favor the

idea that mesenteric LNs and/or spleen are critical lymphoid organs for the induction of oral tolerance although these results do not exclude a physiologic role of PP in the induction of oral tolerance.

In contrast, recent studies have revealed that dendritic cells can induce Ag-specific unresponsiveness or tolerance in lymphoid tissues (39, 40). Possible roles of dendritic cells in oral tolerance are also suggested (41, 42). There are distinct subsets of dendritic cells in PP, which have different cell surface phenotypes and chemotactic activities (43). Furthermore, accumulating data suggest that dendritic cells in the lamina propria might be crucial for induction of tolerance to fed Ags (44, 45). It should be noted that mesenteric, renal and pulmonary, but not axillar and inguinal LNs, were sensitized by orally administered OVA in aged BWF₁ mice. Lymphatic flow in the intestine is directed to the mesenteric LNs and then to the thoracic duct to enter the systemic circulation. T cells in the mesenteric LNs were sensitized in aged BWF₁ mice, whereas those in young BWF₁, aged NZB, and NZW mice were not, suggesting that sensitized T cells were first generated in the mesenteric LNs in aged BWF₁ mice. Direct incorporation of Alexa-OVA into the lamina propria in aged BWF₁ mice may suggest that dendritic cells localized in the lamina propria capture the Ag and migrate to the mesenteric LNs to activate OVA-specific T cells in aged BWF₁ mice. Villous M cells may be involved in this process (46). Because it is unlikely that OVA-loaded dendritic cells migrate to the pulmonary and renal LNs, OVA-specific T cells are presumably activated in the mesenteric LNs and enter the systemic circulation through thoracic duct to distribute in inflamed renal and pulmonary LNs in which various chemokines and adhesion molecules are expressed on high endothelial venules (47).

It is also suggested that oral tolerance is associated with IFN- γ production at priming step (48, 49), although it is also demonstrated that oral tolerance can be induced in IFN- γ -deficient mice (50, 51). In this context, characterization of dendritic cells present in the lamina propria, PP, mesenteric LNs, and spleen is essential to elucidate the mechanism for impaired oral tolerance in aged BWF₁ mice developing lupus nephritis. Chemokine/chemokine receptor expression on dendritic cells in the sites as described and kinetic study on dendritic cell trafficking as well as T cell-dendritic cell interaction are of particular interest.

FIGURE 7. Eosinophil infiltration by OVA inhalation in aged BWF₁ mice presensitized orally with the same Ag. **A**, Analysis on BAL cells. Mice were administered intragastrically with 25 mg of OVA twice on days 0 and 7 and subjected to aerosol exposure to the same Ag on days 22, 23, 24, 28, and 29 (15 min for each exposure with 1% OVA in PBS). BAL cells were collected 2 days after the last aerosol exposure and analyzed for each cellular subset using Diff-Quick. The data are expressed as the mean $\pm$ SE ($n = 5$). Eos, eosinophils; Lym, lymphocytes; Neu, neutrophils; M ϕ , macrophages. *, $p < 0.03$; **, $p < 0.01$. Representative data from three experiments are presented. **B**, H&E staining of the lungs obtained from aerosolized BWF₁ mice after oral administration of OVA (**a**), untreated aged BWF₁ mice (**b**), aerosolized aged NZB mice with oral administration (**c**), and aerosolized NZW mice with oral administration (**d**). It should be noted that many eosinophils (arrowheads) were observed in aged BWF₁ mice that had been orally administered with OVA and aerosolized to the same Ag, but not in untreated aged BWF₁ mice. Magnification, $\times 100$.



Interestingly, aerosol exposure to OVA in aged BWF₁ mice that had been orally administered with OVA provoked a significant eosinophil infiltration in the lung as well as in BAL fluid, although mice did not show any clinical manifestation for asthma. Dendritic cells have been shown to be the principal APCs in the lung and they are rapidly recruited to the airway epithelia following various stimuli and then migrate to the draining LNs within 48 h (52, 53). Thus, OVA primed T cells in pulmonary LNs most likely differentiated to Th2 cells in the presence of OVA-loaded dendritic cells migrated from respiratory mucosa. This finding provides an important implication to understand systemic mononuclear cell infiltration or allergic responses observed in SLE. Breakdown of mucosal immunity in the gut may generate preallergic status in remote immunologic sites such as the respiratory tract and the skin. It is, in fact, reported that serum IgE level is elevated in female SLE patients (54).

Collectively, we have first demonstrated the defective mucosal immunity in the gut during the development of murine lupus and the resultant systemic sensitization by orally administered Ag. These findings would highlight the crucial role of the defective mucosal immunity in the pathogenesis of lupus and provide a unique therapeutic potential by improving mucosal immunity in the gut.

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Disclosures

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