

Oral Administration of Lipopolysaccharides Activates B-1 Cells in the Peritoneal Cavity and Lamina Propria of the Gut and Induces Autoimmune Symptoms in an Autoantibody Transgenic Mouse

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Summary

About a half of the antierythrocyte autoantibody transgenic (autoAb Tg) mice, in which almost all B cells are detected in the spleen, lymph nodes, and Peyer's patches, but not in the peritoneal cavity, suffer from autoimmune hemolytic anemia. The occurrence of this disease is strongly linked to production of autoAb by activated peritoneal B-1 cells in the Tg mice. In this study, we have shown that oral administration of lipopolysaccharides (LPS) activated B-1 cells in the lamina propria of the gut as well as the peritoneal cavity in the healthy Tg mice and induced the autoimmune symptoms in all the Tg mice. The activation of peritoneal and lamina propria B-1 cells by enteric LPS is found not only in the anti-RBC autoAb Tg mice and normal mice but also in the *aly* mice which congenitally lack lymph nodes and Peyer's patches. These results suggest that B-1 cells in the two locations may form a common pool independent of Peyer's patches and lymph nodes, and can be activated by enteric thymus-independent antigens or polyclonal activators such as LPS. The induction of autoimmune hemolytic anemia in the Tg mice by enteric LPS through the activation of B-1 cells in the lamina propria of gut and in the peritoneal cavity suggests that B-1 cells and bacterial infection may play a pathogenic role in the onset of autoimmune diseases.

Functional Ig genes are created by somatic DNA rearrangement of sub-exon DNA segments: V-D-J rearrangement for the heavy chain and V-J rearrangement for the light chain gene (1). Such a random genetic event on one hand contributes to the diversity of Ig repertoire, but on the other hand produces autoreactive B cells that recognize and potentially react with self-antigens. Studies on transgenic (Tg)¹ mice expressing Igs against self-antigens have clearly shown that autoreactive B cells are eliminated (clonal deletion) (2–4), and/or functionally inactivated (clonal anergy) (4–6). Without such proper selection of autoreactive B cells (self-tolerance), these B cells would appear *in vivo* and produce autoantibodies (autoAbs), which are involved with the pathogenesis of autoimmune diseases. Several lines of studies suggest that genetic and/or environmental factors such as nutrition and infection may be involved in breakage of the self-tolerance and induction

of autoimmune diseases (7–9). However, the precise mechanism of the occurrence of autoimmune diseases remains elusive due to their enormous complexity.

A key to clarify the mechanism of autoimmune diseases lies in creating suitable animal models for defining the nature of the break-down of immunological tolerance. Previously, we established and characterized a Tg mouse line carrying the Ig genes derived from the anti-red blood cell (RBC) autoAb 4C8 (4). In these mice, almost all B cells are deleted in the periphery but a normal number of B-1 cells survived in the peritoneal cavity, the sequestered compartment from erythrocytes. About 50% of the animals of this transgenic line suffer from autoimmune hemolytic anemia. Production of the autoantibody by peritoneal B-1 cells was shown to be responsible for autoimmune hemolytic anemia in the Tg mice because apoptotic death of the B-1 cells by exposure to RBCs completely cured autoimmune hemolytic anemia (10). It was puzzling why only a half of the Tg mice develop the autoimmune symptoms whereas the remaining half look completely healthy even though they have the same genetic

¹ Abbreviations used in this paper: autoAb, autoantibodies; GALT, gut-associated lymphoid tissues; HBS, Hank's balanced solution; Tg, transgenic.

background and the same number of B-1 cells. Although some environmental factors such as infection are suspected to be involved in the activation of B-1 cells and the subsequent onset of the autoimmune disease in the Tg mice, this possibility remains to be tested.

B-1 cells consist of B-1a cells with the Ly-1 (CD5) antigen and B-1b cells without the Ly-1 antigen. B-1a and B-1b cells have the same characters except for expression of the Ly-1 antigen (11). B-1 cells are distinguished from conventional B cells by the anatomical localization, surface phenotypes, and functional characteristics (12, 13). B-1 cells are virtually absent from lymph nodes and present at a low frequency in spleen; however, they constitute the major fraction of B cells in the peritoneal cavity. Several important questions have to be settled as to the origin, function, and development of B-1 cells. Although adoptive transfer studies suggest that B-1 cells and conventional B cells have different developmental pathways through distinct progenitor cells (14–17), *in vitro* studies showed that B-1 cells may derive from conventional B cells (18). In addition, it remains to be tested whether B-1 cells are directly involved in the pathogenesis of autoimmune diseases (12, 13, 19). It is also unknown why the peritoneal cavity contains a large number of B-1 cells.

Peritoneal B cells have been suggested to interact with gut-associated lymphoid tissues (GALT), composed of Peyer's patches, mesenteric lymph nodes, lamina propria lymphocytes and intraepithelial lymphocytes (20). B cells in GALT preferentially produce and transport IgA into the gut lumen (21). Moreover, lymphocytes in GALT are known to have a unique homing pattern as follows: committed cells leave the Peyer's patches after antigenic stimulation, and migrate to mesenteric lymph nodes and via the thoracic duct into the blood circulation to the spleen; and finally many of these cells return to GALT (22). However, the lineage and origin of B-1 and conventional B cells in GALT as well as the peritoneal cavity remain to be clarified.

In this study, we report that the oral administration of LPS induced the autoimmune disease in all the non-symptomatic autoAb Tg mice by activation of peritoneal B-1 cells. We have also shown that B-1 cells in the peritoneal cavity form a common pool with B-1 cells in lamina propria of the gut, independently from Peyer's patches and lymph nodes, serving as a part of the mucosal immune system for defending the invasion of microorganisms via gut lumen. Taken together, it is likely that enteric bacterial antigens activate GALT and peritoneal B-1 cells, which eventually causes the autoimmune disease in the Tg mice.

Materials and Methods

Mice. Homozygous Tg mice for the heavy and light chain genes of the anti-RBC autoAb were established and maintained as described (4). By mating homozygous heavy and light chain Tg mice, we could get double Tg mice which carry both heavy and light chain transgenes of the autoAb. Normal littermates were also obtained in this procedure. *Aly* mice (23) were kindly given by Nippon Sinyaku Co., Ltd. (Kyoto, Japan). In this study mice were used at 8–12 wk after birth.

LPS Administration. LPS (*Salmonella minnesota*, [T4270] and

Escherichia coli Serotype 026B6 [L8274a] Sigma Chemical Co., St. Louis, MO) was purchased and dissolved in PBS. In case of oral administration of LPS, 500 μ l of LPS solution was injected into the gut of mice through a polyethylene tube of 1 mm diameter. In case of intramuscular and intraperitoneal administration of LPS, 500 μ l of LPS solution was injected into the back and abdominal regions of the mice, respectively, with a needle.

Detection for Ab-producing Cells. The number of IgM-producing cells was measured by enzyme-linked immunospot (Elispot) assay as described previously (10) for detection of anti-RBC autoAb producing cells except that plastic plates were coated with 100 μ l of goat anti-mouse IgM Ab (Cappel Laboratories, Cochranville, PA) at the concentration of 10 μ g/ml in PBS.

Preparation of Cells from Each Compartment of GALT. Under diethyl ether anesthesia, mice were sacrificed and lymphoid cells from GALT were isolated according to the procedures reported previously (24, 25). At first, after removing feces from the gut, all Peyer's patches and mesenteric lymph nodes were separated and lymphoid cells were isolated by gentle rubbing with a steel mesh in Hank's balanced solution (HBS) supplemented with 5% FCS. Next, the gut epithelium was stripped by treating the inverted gut segments with HBS, followed by shaking the gut for 15 min at 37°C in HBS containing 5 mM EDTA Na₂ without Ca⁺² and Mg⁺². After two cycles of this procedure, the cells in the medium were collected for intraepithelial lymphocytes suspension. The gut segments were digested by 0.015% collagenase (Wako, Tokyo, Japan) in PBS containing 2% FCS for 75 min at 37°C and cells suspensions were obtained for lamina propria lymphocytes. These two suspensions were passed through a glass wool column and the lymphoid cells were separated from epithelial cells with a Ficoll-isopaque gradient centrifugation (450 g, 30 min, 4°C) to obtain the final, intraepithelial, and lamina propria lymphocytes, respectively.

Flow Cytometry. Single cell suspensions of 10⁶ mononuclear cells were pelleted and resuspended in 10 μ l of normal mouse serum for blocking the nonspecific binding of antibodies. After 10 min incubation, one of first-reagent antibodies, i.e., rat anti-mouse B220 (RA3-6B2) mAb, mouse anti-Thy-1 (IgM class) mAb (ICN Biomedicals Inc., Costa Mesa, CA), anti-Mac-1 mAb (M1/70.15.115.5HL) or biotinylated-anti CD23Ab (gift of Dr. Yagita and Dr. Hirano, Juntendo University, Tokyo, Japan) at the appropriate dilution in PBS was added directly. After incubation, cells were washed twice with 1 ml of PBS containing 5% FCS and 0.05% sodium azide. Then phycoerythrin-conjugated anti-mouse IgM antibody (Southern Biotechnology Associates, Birmingham, AL) or fluorescein-conjugated mouse anti-rat κ mAb (Immunotech, Marseilles, France), was added as second-reagent antibody, in 10 μ l of PBS at appropriate concentrations, under the same conditions. After excluding dead cells by propion-iodine staining and gating the lymphoid cells in forward and scatter analysis, 10⁴ viable lymphoid cells were analyzed on FACScan® (Becton Dickinson & Co., Mountain View, CA) and were plotted on quadruple logarithmic scales.

Immunohistochemistry. Identification of lymphocyte surface markers was carried out by the avidin-biotin-peroxidase complex technique (26). Under diethyl ether anesthesia, mice were killed by exsanguination, and Peyer's patches and the small intestine were sliced and embedded with Tissue Tek. O.C.T. compound (Miles Inc., West Haven, CT) and frozen in liquid nitrogen. The frozen sections were cut by cryostat at 5 μ m in thickness, and fixed in acetate for 10 min at 4°C. Then these sections were immersed in 0.228% periodic acid solution for 15 s at 30°C to block endogenous peroxidase activity (27). To block nonspecific binding between avidin and lectin-like substances on the specimen, slides were in-

cubated with the avidin/biotin blocking kit (SP2001; Vector Laboratories, Inc., Burlingame, CA) for 60 min at room temperature. Finally, all sections were rinsed three times in PBS and soaked in 0.05M Tris-HCl buffer, pH 7.6, peroxidase (0.015%), and 0.03% NiCl₂ as a substrate enhancer for 2–10 min. In control preparations, primary antibodies were replaced by a biotin solution and avidin/biotin/peroxidase complex only without primary antibodies; control staining was all negative.

Results

Administration of LPS Induces Autoimmune Disease in Anti-RBC AutoAb Tg Mice In the previous study, we have demonstrated that ~50% of anti-RBC autoAb-Tg mice develop hemolytic anemia due to the autoAb production by peritoneal B-1 cells, whereas the remaining half are almost completely healthy (4). This result suggests that environmental factors especially infection may be involved in the activation of peritoneal B-1 cells and the occurrence of the autoimmune disease. To test this possibility, we used LPS of *S. minnesota*, which may mimic bacterial infection by serving as polyclonal activator of B cells. We administered LPS orally, intramuscularly, or intraperitoneally into nonanemic Tg mice, whose hematocrit values were >40%. Regardless of the routes of LPS administration, anemia was induced in all the Tg mice that received LPS (Fig. 1). In case of intramuscular or intraperitoneal injections of LPS, lower doses of LPS (<100 μg/mouse) could induce hemolytic anemia in the Tg mice but higher doses of LPS (>100 μg/mouse) were lethal, probably because of the hemorrhagic necrosis (28). On the other hand, oral administration of LPS induced hemolytic anemia in a dose-dependent manner up to 200 μg/mouse in the nonanemic Tg mice. The different dose effects of LPS between oral administration and systemic injection may depend, at least in part, on different absorption rates of LPS.

Activation of B-1 Cells Is Responsible for Autoimmune Symptoms in LPS-treated Tg Mice To see whether the activation of peritoneal B-1 cells is involved in the LPS-induced autoimmune disease shown in the naturally occurring autoimmune hemolytic anemia, we measured the number of anti-RBC Ab producing cells in the spleen, bone marrow, and peritoneal cavity of the LPS-treated Tg mice. Unexpectedly, only oral administration of LPS increased dose dependently the number of Ab-producing cells in the peritoneal cavity whereas intraperitoneal or intramuscular injection of LPS did not activate peritoneal B cells (Fig. 2). On the other hand, B cells in the spleen and bone marrow were activated by systemic administration of LPS but did not respond to oral administration of LPS. The same results were obtained when we used LPS of *E. coli* (data not shown).

To clarify the character of B cells activated by LPS, we examined B cells in the spleen and peritoneal cavity of the Tg mice by flow cytometry. After intramuscular or oral injection of LPS, the majority of B cells (B220⁺, IgM⁺) in the spleen were CD23⁻ (Fig. 3). Peritoneal B cells after intramuscular or oral administration of LPS were also almost all Mac-1⁺ and CD23⁻. These results suggest but do not demonstrate that LPS-activated B cells in the Tg mice may be B-1 cells but not conventional B cells. As spleen B-1 cells

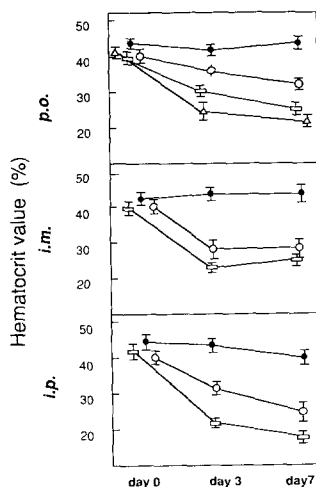


Figure 1. Injection of LPS aggravates hemolytic anemia dose-dependently. *Salmonella* LPS, suspended in PBS, was administered orally (p.o.), intramuscularly (i.m.), or intraperitoneally (i.p.) to nonanemic Tg mice. Hematocrit value of the peripheral blood from each mouse was measured at days 0, 3, and 7. The bars indicate the standard deviation of the values of the three to five mice. Amounts of LPS used per mouse are: ●, 0 μg; ○, 10 μg; □, 100 μg; △, 200 μg.

are indistinguishable from activated conventional B cells by the surface phenotype, an undetectable number of residual conventional B cells might have proliferated in response to LPS stimulation in spleen. It is safe to conclude, however, that activated B cells in both the spleen and the peritoneal cavity after LPS injection have the same surface phenotypes as B cells in the nontreated Tg mice.

To confirm the pathogenic role of B-1 cells in induction of the autoimmune disease in LPS-stimulated anti-RBC autoAb-Tg mice, we established male F-1 mice by crossing anti-RBC autoAb-Tg mice with CBA/N mice which bear the X-linked immunodeficiency (*xid*) mutation. Mice with the *xid* mutation lack B-1 cells (29) and show profound defects in producing the antibody in response to immunization with thymus-independent type-2 antigens such as protein-free LPS and hapten-polysaccharide conjugates. However, the response to protein antigens and thymus-independent type-1 antigens

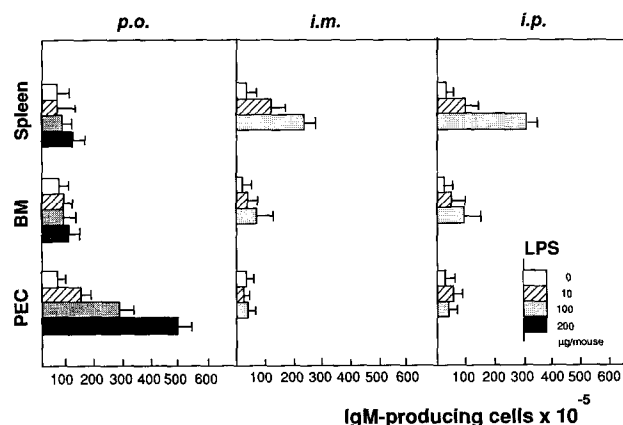


Figure 2. Peritoneal B cells are activated by oral administration of LPS, but not by intramuscular or intraperitoneal injection of LPS. 7 d after oral, intramuscular, or intraperitoneal injection of *Salmonella* LPS to the nonanemic transgenic mice, the number of IgM producing cells in 10⁵ cells of spleen, bone marrow (BM) or peritoneal cavity (PEC) was measured by Elispot as described in experimental procedures. Each experiment was carried out in three Tg mice and the bars show the standard deviation.

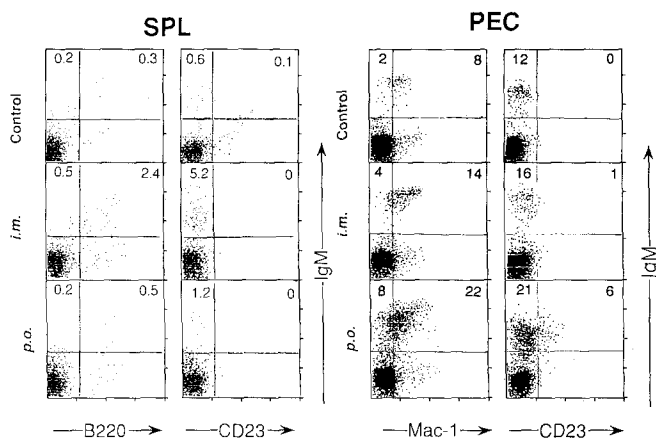


Figure 3. LPS-activated B cells in Tg mice are mostly B-1 cells. *Salmonella* LPS (100 μ g/mouse), was administered to Tg mice intramuscularly or orally. After 7 d, spleen and peritoneal cells were stained with antibodies specific to B220, IgM, CD23 and analyzed with FACScan[®] (Becton Dickinson & Co.). The number in each figure shows the percentage of B cells (B220⁺, IgM⁺) or B-1 cells (Mac-1⁺, IgM⁺, CD23⁻).

such as lipoprotein-containing LPS is normal (30). Indeed, in anti-RBC autoAb-Tg/*xid* mice, neither B-1 nor conventional B cells existed in the peritoneal cavity and the spleen (Fig. 4). As the controls the Tg mice contained only B-1 cells (B220^{low}, IgM^{high}, CD23⁻) in the peritoneal cavity, while CBA/N mice contained only conventional B cells (B220⁺, IgM⁺, CD23⁺) in the spleen and peritoneal cavity. In ad-

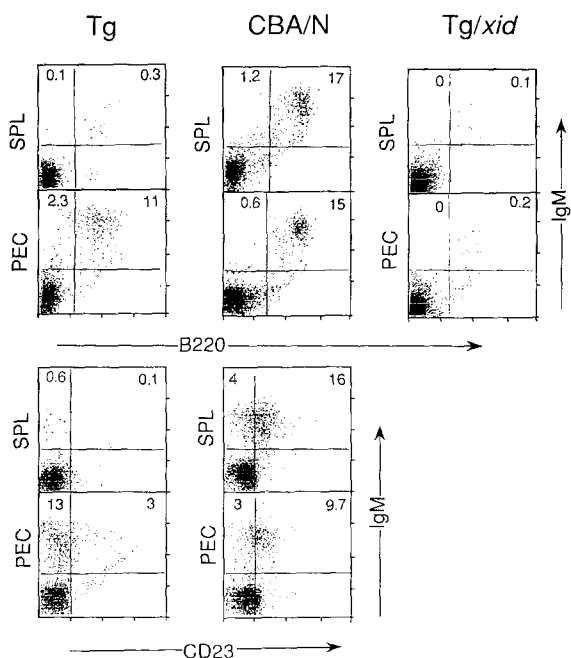


Figure 4. Few B cells exist in the peritoneum as well as in the spleen in Tg/*xid* mice. Spleen or peritoneal lymphocytes were collected from Tg/*xid*, Tg, and CBA/N mice, stained with antibodies against B220, IgM, and CD23, and analyzed with FACScan[®]. Numbers indicate the percentage of B cells or B-1 cells.

Table 1. Occurrence of Hemolytic Anemia by LPS Administration

	Tg/ <i>xid</i>	Tg	CBA/N
<i>i.m.</i>	0/3	3/3	0/3
<i>p.o.</i>	0/3	3/3	0/3

100 μ g LPS in PBS were injected orally (*p.o.*) or intramuscularly (*i.m.*) into three each of Tg/*xid*, Tg, and CBA/N male mice. At day 7, the occurrence of hemolytic anemia was examined by measuring the hematocrit value of the peripheral blood from each mouse. Mice with a hematocrit value <40% were diagnosed as hemolytic anemia. LPS (*E. coli* Serotype 026B6, catalog no. L8274a; Sigma Chemical Co.) used in this study contains 5–10% protein, and activated B cells in CBA/N mice (data not shown).

dition, neither oral nor intramuscular administration of lipoprotein-containing LPS could induce hemolytic anemia in the Tg/*xid* F1 male mice whereas the control autoAb Tg mice responded to LPS as described above (Table 1). Since the absence of B-1 cells eliminates autoimmune symptoms induced by either oral or intramuscular administration of LPS in the Tg/*xid* mice, B-1 cells must be responsible for production of autoAbs upon LPS administration. These results suggest that enteric LPS may activate B-1 cells in the peritoneal cavity, and systemically administered LPS may activate B-1 cells in the spleen and bone marrow.

Lamina Propria also Contains B-1 Cells That Escape from Clonal Deletion. To assess how enteric LPS activates peritoneal B-1 cells in the Tg mice, we examined B cells in each compartment of GALT of the Tg mice. Visual examination showed that anti-RBC autoAb-Tg mice have the normal number and normal size of Peyer's patches and mesenteric lymph nodes (data not shown). Immunohistochemical staining of the gut tissues showed that a normal number of IgM⁺ cells were found in the lamina propria of the gut (Fig. 5). These IgM⁺ cells in the lamina propria of the Tg mice were also stained with the S54 Ab which recognizes the idiotype of the anti-RBC Ab (4C8) (data not shown). Although a normal number of T cells was found, only a small number of IgM⁺ cells were present in Peyer's patches. These results indicate that autoreactive B cells in the lamina propria of the gut escape from clonal deletion like peritoneal B cells whereas autoreactive B cells in the Peyer's patches are deleted like spleen B cells.

To characterize IgM⁺ cells of the lamina propria of the gut, we analyzed mononuclear cells from epithelia, lamina propria, Peyer's patches, and mesenteric lymph nodes by flow cytometry. In the Tg mice, B cells (IgM⁺, B220⁺) were almost completely deleted in the Peyer's patches and mesenteric lymph nodes as is the case for the spleen and lymph nodes (Fig. 6). However, the lamina propria contained a normal number of B cells (IgM⁺, B220⁺) in agreement with immunohistochemical analysis. More than 90% of B cells in the lamina propria of the gut were Mac-1⁺ (Fig. 6) and S54⁺ (data not shown), indicating that lamina propria B cells are almost all autoreactive B-1 cells. In normal mice, B cells in Peyer's patches and mesenteric lymph nodes are al-

most all conventional B cells whereas the majority of B cells in the lamina propria as well as the peritoneal cavity are B-1 cells. Intraepithelial lymphocytes of both Tg and normal mice were almost all T cells, and B cells were hardly detected (data not shown), as reported previously (31, 32). In summary, autoreactive B-1 cells survive from clonal deletion in the lamina propria and the peritoneal cavity of the Tg mice. By contrast, almost all the B cells are deleted in Peyer's patches, lymph nodes and spleen of anti-RBC autoAb Tg mice. These results strongly suggest that lamina propria B-1 cells may be generated independently of Peyer's patches and may have a close relationship with peritoneal B-1 cells.

Oral Administration of LPS Appears to Activate B-1 Cells First in the Lamina Propria and then in the Peritoneal Cavity. To assess whether peritoneal B-1 cells are functionally related with lamina propria B-1 cells, we measured the number of IgM-producing cells in each compartment of GALT before and after oral administration of LPS to the Tg and normal mice. In the Tg mice that contain the normal number of B-1 cells and few conventional B cells in the peritoneal cavity and GALT, oral administration of LPS increased the number of IgM-producing cells 8-fold in the lamina propria and 11-fold in the peritoneal cavity whereas IgM-producing cells were not detected in Peyer's patches either before or after administration of LPS (Table 2). Similarly in normal mice, the activation of peritoneal and lamina propria B cells was induced only by the oral administration of LPS but not by intraperitoneal or intramuscular injection of LPS, whereas B cells in Peyer's patches were activated by administration of LPS through any routes. The results again support the above conclusion that

the peritoneal and lamina propria B-1 cells are related and independent from Peyer's patch B cells. Judging from the anatomical position of peritoneal cavity and lamina propria of the gut, enteric LPS may first activate lamina propria B-1 cells and then peritoneal B-1 cells, leading to the induction of the autoimmune disease in anti-RBC autoAb Tg mice.

To confirm that activation of B-1 cells in the lamina propria and peritoneal cavity does not depend on Peyer's patch B cells, we administered LPS orally to the mice with the *aly* mutation, which causes loss of lymph nodes and Peyer's patches (23). 7 d after oral administration of LPS, the number of IgM-producing cells in the peritoneal cavity and the lamina propria increased by eight- to ninefold in *aly/aly* mice (Table 2). The level of stimulation in *aly/aly* mice was not significantly different from that in *aly/+* mice, which contain normal numbers of Peyer's patches. These results clearly demonstrate that peritoneal and lamina propria B cells can be activated by enteric antigens in the absence of Peyer's patches.

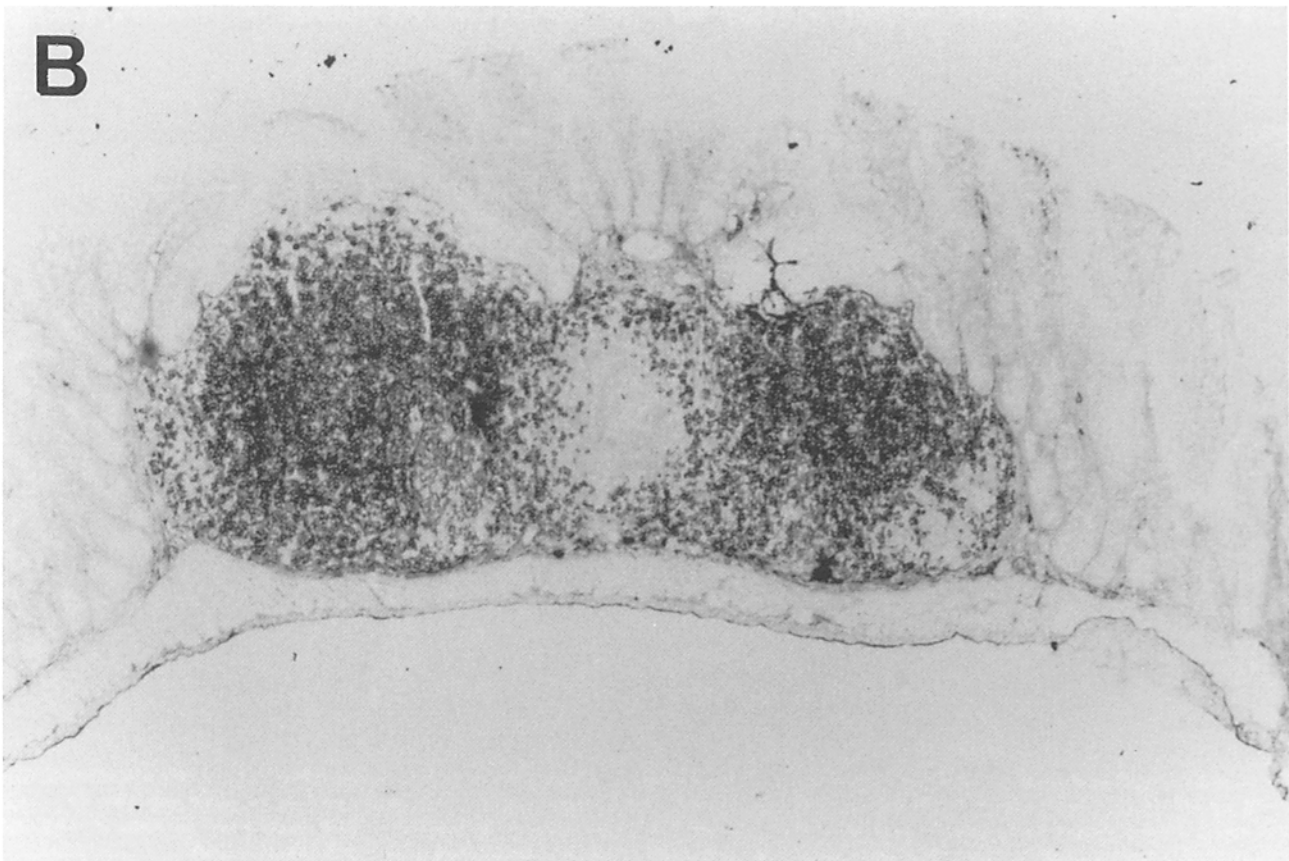
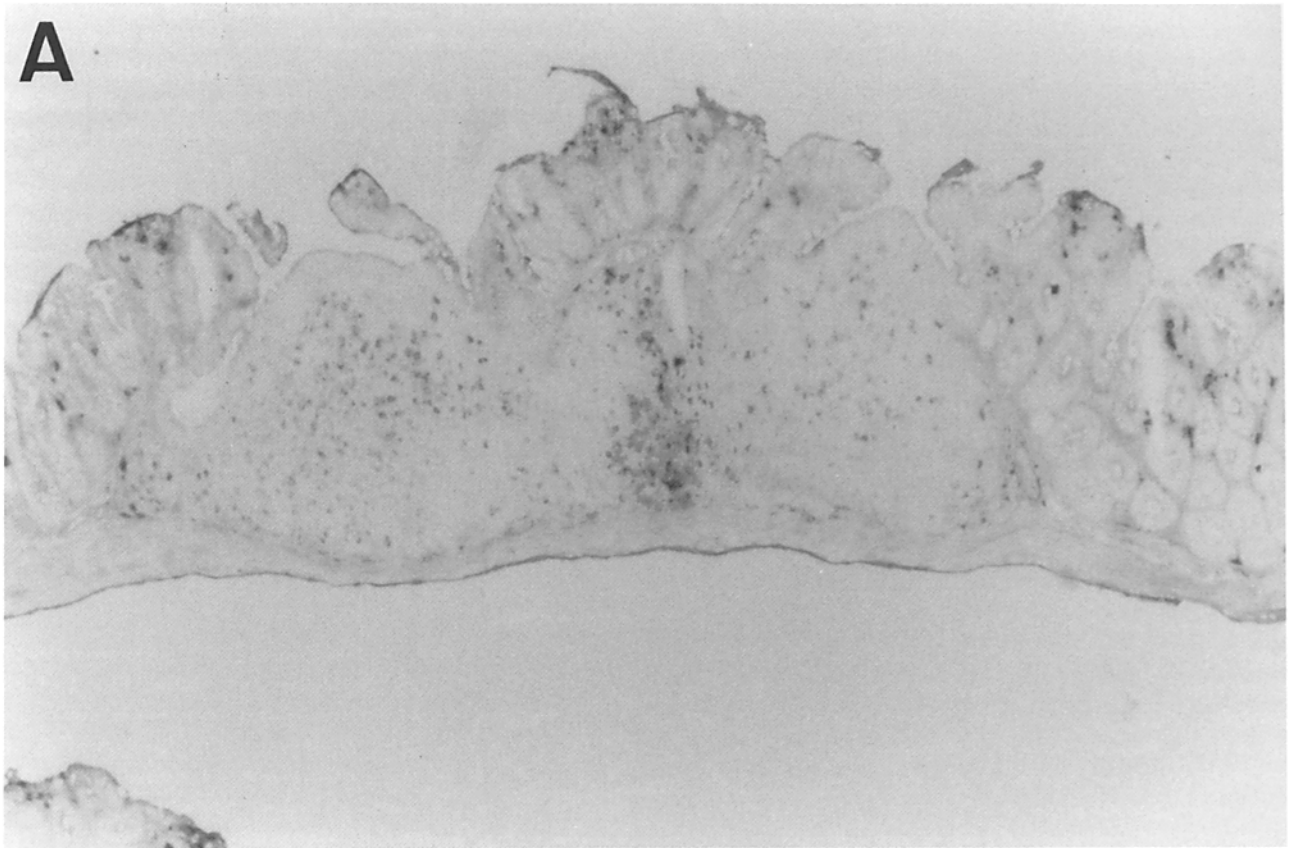
Discussion

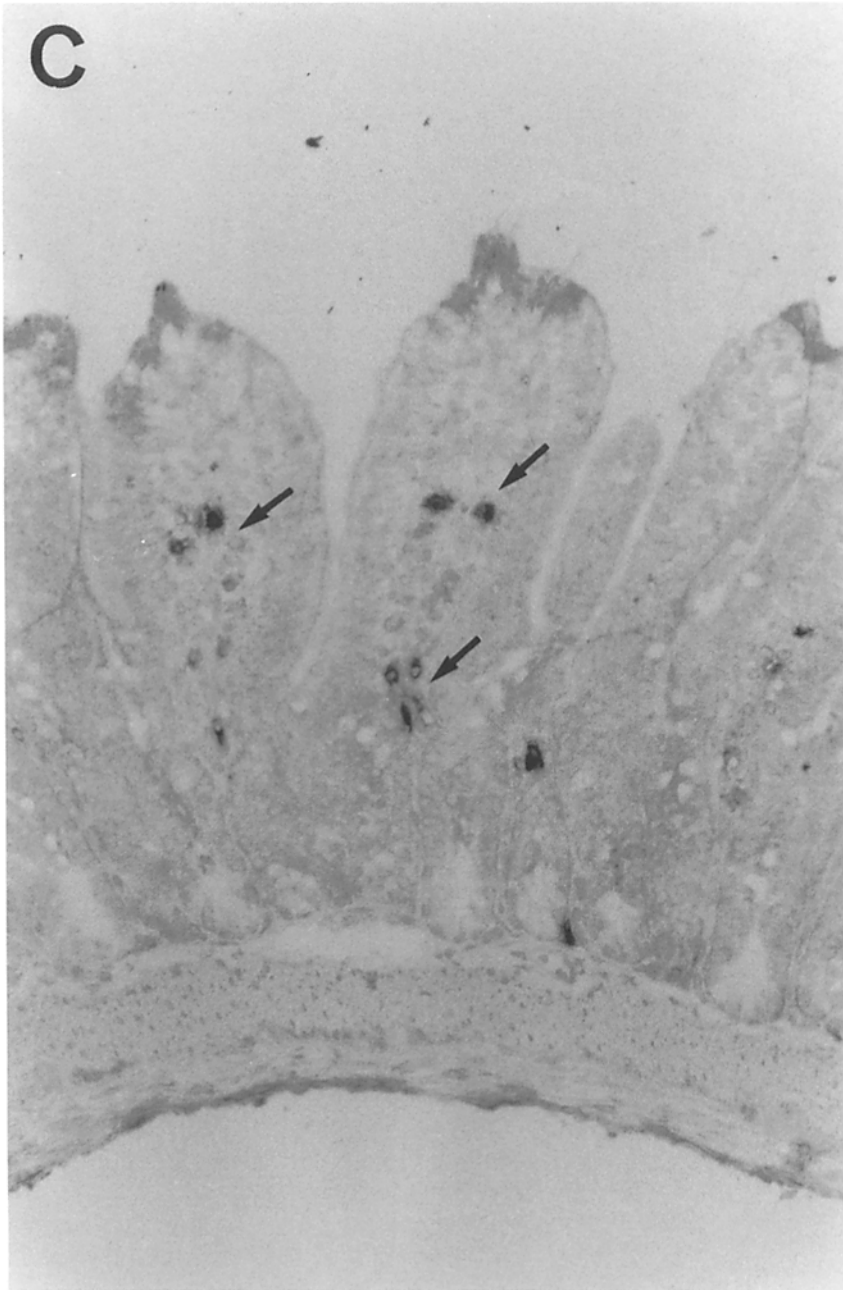
LPS Activates B-1 Cells and Induces the Autoimmune Disease in the Tg Mice. In this study we have shown that LPS administration through either muscle, peritoneal cavity, or mouth induces hemolytic anemia in the Tg mice (Fig. 1), which depends on the presence and activation of B-1 cells because the Tg/*xid* male mice did not become anemic by LPS injection (Fig. 4, Table 1). Intramuscular or intraperitoneal administration of LPS appears to activate a small number of B-1 cells present in the spleen but not B-1 cells in the perito-

Table 2. Increased Number of IgM-producing Cells in GALT by LPS Injection

Mice	Routes of LPS administration	No. of IgM-producing cells		
		PEC	LPL	P.P.
			<i>per 10⁵ cells</i>	
Tg	none	28 ± 6	23 ± 12	<5
	<i>p.o.</i>	312 ± 72	179 ± 30	<5
Normal	none	122 ± 16	48 ± 6	52 ± 12
	<i>i.m.</i>	136 ± 32	62 ± 8	262 ± 24
	<i>i.p.</i>	98 ± 26	58 ± 12	302 ± 31
	<i>p.o.</i>	336 ± 32	132 ± 26	282 ± 48
<i>aly/+</i>	none	92 ± 13	42 ± 14	—
	<i>p.o.</i>	521 ± 89	340 ± 21	—
<i>aly/aly</i>	none	48 ± 18	32 ± 16	—
	<i>p.o.</i>	336 ± 28	282 ± 48	—

The numbers of IgM producing cells in the peritoneal cavity, lamina propria, and Peyer's patch were examined before and 7 d after oral administration of 100 µg of LPS to normal mice (C57/BL6), Tg mice, and mice with heterozygous (*aly/+*) or homozygous *aly* mutation (*aly/aly*). *aly/+* mice have almost normal phenotype but *aly/aly* mice lack Peyer's patches and lymph nodes congenitally (23). Each experiment was done in three mice. Each number indicates mean ± S.D. PEC, peritoneal exuded cells; LPL, lamina propria lymphocytes; P.P., Peyer's patch.





neal cavity (Fig. 2, Table 2). On the other hand, oral administration of LPS activates peritoneal B-1 cells preferentially. Since the activation of peritoneal B-1 cells is responsible for spontaneous autoimmune hemolytic anemia that occurs in a half of the Tg mice (10), oral administration of LPS mimics the physiological onset of the autoimmune disease in the anti-RBC autoAb-Tg mice. The anti-RBC autoAb-Tg mouse thus provides a unique autoimmune model system in which we can induce the disease without an exception by oral administration of LPS (Fig. 1) and cure the disease without an exception by intraperitoneal injection of the self-antigen RBC (10).

Enteric Bacterial Infection May Be Involved in the Occurrence of the Autoimmune Disease in the Tg Mice. The fact that enteric LPS can induce the symptoms similar to spontaneously occurring autoimmune hemolytic anemia in the Tg mice suggests that infection of bacteria through the gut may be responsible for triggering the autoimmune disease that occurs spontaneously in a half of the Tg mice. Indeed, the incidence of the autoimmune hemolytic anemia sharply decreased when we moved the Tg animal colony into cleaner facilities (data not shown). Several lines of evidence indicate that bacterial infection may activate autoreactive lymphocytes in vivo by

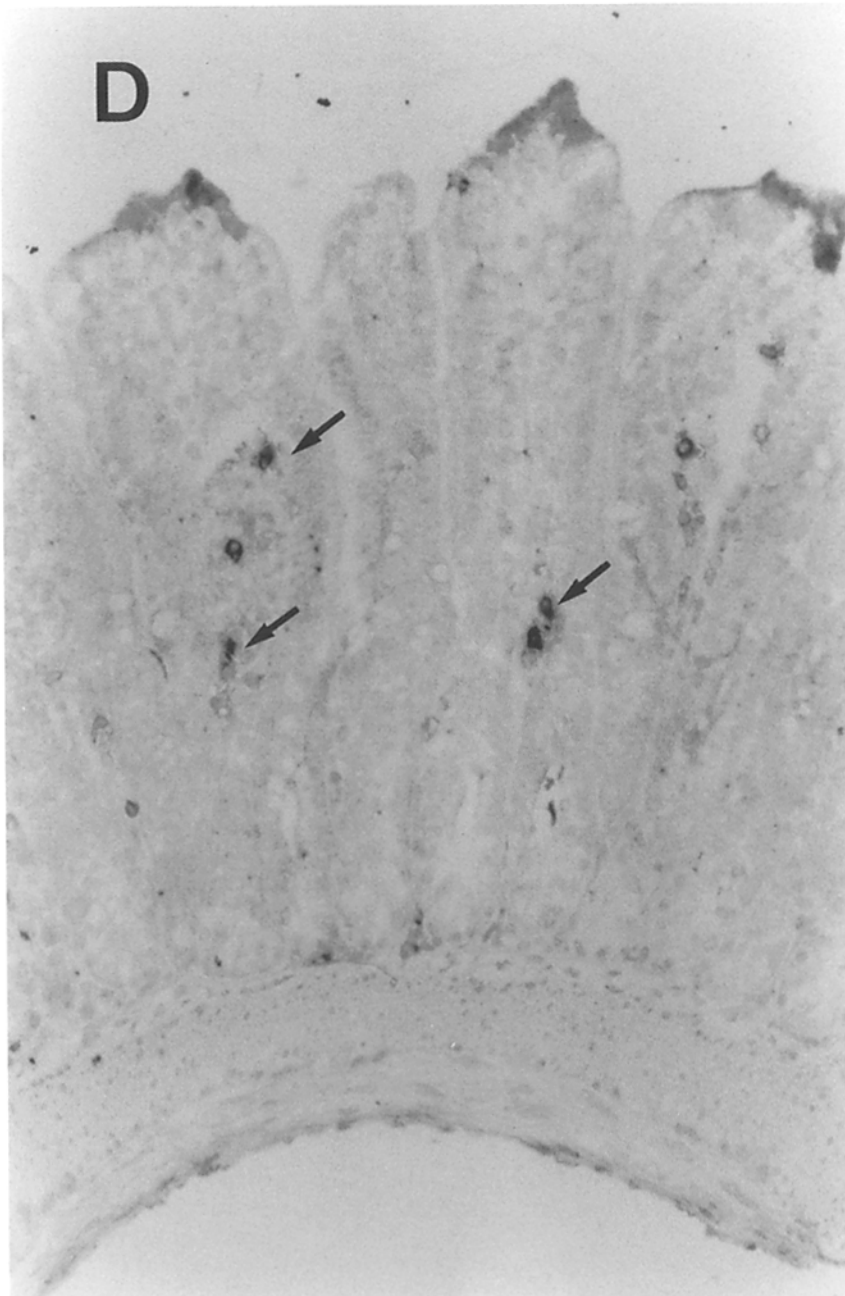


Figure 5. Histochemical analysis of GALT in Tg mice. Axial sections of the gut in the Tg mice (*A* and *C*) and normal littermates (*B* and *D*) were stained with biotin-labeled polyclonal anti-mouse IgM Ab by the avidin/biotin/peroxidase complex technique (26). Arrows indicate lamina propria B cells. Magnification: 40 \times in Peyer's patches (*A* and *B*) and 100 \times in lamina propria lymphocytes (*C* and *D*).

several mechanisms. LPS derived from gram-negative bacteria is well known to activate B cells polyclonally and the administration of LPS induces the production of autoantibodies even in normal mice (33). Enterotoxins derived from bacteria can bind MHC nonspecifically on B cells and a specific T cell receptor on T cells, stimulate and expand a wide population of T cells as superantigens (34). Autoreactive lymphocytes may be activated by the infection of bacteria and viruses, which have certain structural similarities with self-antigens (molecular mimicry) (35).

The induction of autoimmune disease by infection was suggested recently in experimental allergic encephalomyelitis (EAE) Tg model mice, in which all T cells express a myelin basic protein-specific T cell receptors. EAE did not occur in specific pathogen-free conditions, but the frequency of autoimmune symptom increased in conventional conditions (36). However, there exist several arguments against the association of autoimmune disease with infection; the frequency of autoimmune disease is known to increase in nonobese insulin-dependent diabetes mice breeding in specific pathogen-free

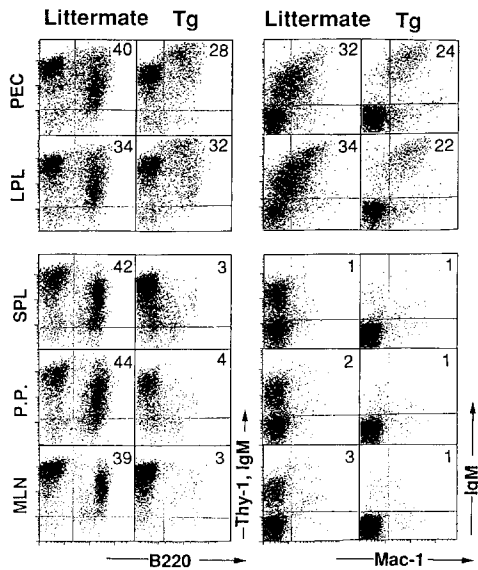


Figure 6. Flow cytometric analysis of GALT of Tg and normal mice. Lymphocytes were collected from intraepithelia (IEL), lamina propria (LPL), Peyer's patches (PP), and mesenteric lymph nodes (MLN) of the small intestine of the Tg mice or normal littermates, and stained with antibodies against Thy-1, IgM, and B220 or antibodies against IgM and Mac-1. Numbers indicate the percentage of B cells (IgM⁺, B220⁺) or B-1 (Mac-1⁺, IgM⁺).

conditions (37) or to remain unchanged in autoimmune gastritis breeding in germ-free conditions (38). In this study, we showed that LPS induced autoimmune disease in anti-RBC autoAb Tg mice, but other mechanisms or molecules associated with bacterial infection may be involved in the occurrence of autoimmune diseases. It also remains to be tested whether the activation of autoreactive B-1 cells by intestinal bacteria is involved in the occurrence of other autoimmune diseases in humans and mice.

Enteric LPS May Activate Lamina Propria B-1 Cells before Peritoneal B-1 Cells. It is an intriguing question how enteric LPS activates peritoneal B-1 cells. We suspected communication between the peritoneal and GALT lymphocytes. Flow cytometric and histochemical analysis of GALT of the Tg mice demonstrated that only B-1 cells existed in the peritoneum and the lamina propria whereas conventional B cells were almost completely deleted from Peyer's patches and mesenteric lymph nodes (Fig. 6) as was the case for spleen B cells. Moreover, both peritoneal and lamina propria B-1 cells were effectively activated by oral administration of LPS but not by systemic injection of LPS (Fig. 2, Table 2). These results suggest a scenario that lamina propria B-1 cells are first activated by enteric LPS, and move into the peritoneal cavity. Such linkage of B cells in the peritoneal cavity and GALT is not unique to the Tg mice as B cells in the peritoneal cavity, lamina propria, and Peyer's patches of normal mice were all activated by enteric LPS (Table 2). The traffic of B-1 cells

from the peritoneal cavity to the lamina propria have already been shown by the experiment that peritoneal B cells from host mice differentiated into Ab-producing cells in the lamina propria of irradiated recipient mice (20). Frequent migration may take place between peritoneal and lamina propria B-1 cells.

GALT Is Composed of Two Different Lineages of Lymphocytes. Peyer's patches are believed to be necessary for the activation of B cells in GALT. M cells, which are located in the luminal side of Peyer's patches, uptake enteric antigens and transport them into Peyer's patches. After stimulation with antigens in Peyer's patches, B cells circulate into the periphery and return to GALT (22). However, so far it is not clear whether B-1 cells are involved in this circulation of B cells in GALT. In this study, we have demonstrated that, in normal mice, Peyer's patches contain few B-1 cells and abundant conventional B cells whereas B-1 cells preferentially exist in the lamina propria of the gut as well as in the peritoneal cavity (Fig. 6). In addition, in anti-RBC autoAb Tg mice, B-1 cells existed in the lamina propria and in the peritoneal cavity in spite of almost total absence of conventional B cells in every lymphoid organs including GALT. These B-1 cells in the two locations were activated by enteric bacterial antigens such as LPS. We have also demonstrated that both peritoneal and lamina propria B-1 cells are activated by oral administration of LPS even in *aly* mice, which lack Peyer's patches (Table 2). These results clearly demonstrate that conventional B cells in Peyer's patches are separate from and not linked with peritoneal and lamina propria B-1 cells, and that GALT contains two separate lineages of B cells; one is conventional B cells whose generation is probably dependent on Peyer's patches; the other is lamina propria B-1 cells which communicate with B-1 cells in the peritoneal cavity and grow independently of Peyer's patches.

Intraepithelial T cells, frequently reactive to enteric bacteria with strong killer activities, partly originate independently of the thymus and partly have γ/δ T cell receptors (31, 32). B-1 cells and γ/δ T cells share many common characteristics; predominant localization outside lymphoid tissues such as the gut and restricted antigen specificity to enteric and self-antigens. These primitive immune systems must have played an important role for the first line of defense against invading pathogens in lower vertebrate until more sophisticated immune systems, i.e., conventional B cells and α/β T cells, arose later in evolution. Upon the invasion of bacteria, antibacterial IgM antibodies, albeit with low affinity, may be immediately produced by B-1 cells in the peritoneal cavity and the lamina propria. Subsequently, after somatic mutation and class switching of conventional B cells in Peyer's patches, IgG or IgA class antibodies with high affinity will be produced. Since the gut mucosa is continuously attacked by numerous microorganisms, GALT may require two layered defense lines, namely the ancestral immune system of γ/δ T and B-1 cells, and the modern immune system of α/β T and conventional B cells.

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