TRAF6-dependent signal pathway is essential for the formation of B-cell follicles in secondary lymphoid organs

(TRAF6 依存性シグナル伝達経路は二次リンパ器官の B 細胞濾泡形成に必須である)

秦　俊文

Contents

Abstract

The integrity of B-cell follicles in secondary lymphoid organs is essential for regulation of efficient immune response. It is known that signals triggered by tumor necrosis factor (TNF) and lymphotoxin (Lt) are required for the formation of B-cell follicles in secondary lymphoid organs. However, the global insight of the molecular mechanism involved in the formation of B-cell follicles still remains to be determined. In the present study, I explore the roles of TNF receptor-associated factor 6 (TRAF6), a signal transducer activating NF-κB and AP-1, in the formation of microarchitectures in secondary lymphoid organs using *Traf6*$^{-/-}$ mice. Spleen from *Traf6*$^{-/-}$ mice displayed disruption of B-cell follicles, lack of follicular dendritic cell (FDC) networks and defect of marginal zone (MZ) structures. This phenotype resembles to that of mice defective in Ltβ receptor (LtβR) signaling pathway. Analysis on the formation processes of B-cell follicles revealed that TRAF6 is dispensable for the formation of B-cell clusters, which occurs in early stage of B-cell follicle development, but is essential for the subsequent formation and maintenance of B-cell follicles, whereas LtβR signal pathway is required for the formation of B-cell clusters. Consistent with this finding, I confirmed that TRAF6 is not involved in the LtβR signal pathway in embryonic fibroblast cells. Taken together with the fact that TNF receptor 1 (TNFR1) signal pathway induced by TNF ligation is independent of TRAF6, at least three signal pathways are suggested to be involved in the formation of B-cell follicles: the TNFR1 signal pathway, the LtβR signal pathway, and the TRAF6-dependent signal pathway. Total fetal liver cell transfer and B cell transfer experiments suggested that TRAF6-dependent signal pathway in radiation-resistant stromal cells, most likely follicular dendritic cells, is responsible for the proper formation of B-cell follicles. Interestingly, even if FDCs are present in spleen of *Traf6*$^{-/-}$ mice in the early phase of B-cell follicle development, *Traf6*$^{-/-}$ FDCs failed to express CXCL13, which is a chemokine essential for the formation and maintenance of B-cell follicles. This data suggests that the TRAF6-dependent signal in FDCs is required for the expression of CXCL13, thereby establishing B-cell follicles in secondary lymphoid organs. Overall, TRAF6-dependent signal pathway, which functions independently from the TNFR1 and LtβR signal pathway, is crucial for the formation and maintenance of B-cell follicles in secondary lymphoid organs in developmental stage-dependent manner through inducing the expression of CXCL13 in follicular stromal cells (FDCs).

Introduction

Secondary lymphoid organs, including spleen, Lymph nodes (LN), nasopharyngeal-associated lymphoid tissue (NALT), and mucosal-associated lymphoid tissues (MALT) such as Peyer's patches (PP), provide location for the differentiation, maturation, activation of peripheral lymphocytes, which is necessary for efficient immune responsiveness such as immunosurveillance, self-tolerance and against pathogens. To accomplish these missions, formation and maintenance of highly ordered microarchitectures in secondary lymphoid organs have been demonstrated to be essential (Zinkernagel RM et al., 1997; Cyster JG., 1999; Fu YX et al., 1999). Although the structures of secondary lymphoid organs have been well defined, the detailed molecular mechanism for formation of the microarchitectures in secondary lymphoid organs still remains to be determined.

Microarchitectures of secondary lymphoid organs

Spleen, the largest secondary lymphoid organ, is composed of three major compartments: the red pulp (RP), the white pulp (WP) and the marginal zone, each with its own specific cell types and microarchitectures. The red pulp is a reticuloendothelial compartment, responsible for removal of blood particulate matters such as bacteria and effete red blood cells and also for an exit route for recirculating lymphoid cells, whereas the white pulp is the lymphoid cell compartment in the spleen. The white pulp is mainly composed of B-cell follicles and T-cell zone. The T-cell zone contains T cells, dendritic cells (DC) and resident stromal cells. T cells encircle the central arteriole and form the periarteriolar lymphoid sheath (PALS). B cells are distributed around the T-cell zone in packed follicles: B-cell follicles. B-cell follicles consist of follicular B cells and follicular stromal cells, including FDCs, which are a long-lived radiation-resistant cell population (Humphrey JH et al., 1984; MacLennan IC et al., 2003). The region between the red pulp and the white pulp is called the marginal zone. The marginal zone is separated from the white pulp by marginal sinus, which located on the outer of the white pulp. The marginal zone contains fibroblastic reticular cell (RC) network, marginal zone macrophage population (MZM), marginal metallophilic macrophage population (MMM), marginal zone dendritic cells and marginal zone B cells (Kraal G., 1992; Martin F et al., 2002; Karlsson MC et al., 2003). MMM resides in the inner region of the marginal zone adjacent to the white pulp, whereas MZM is located on the outer region of the marginal zone adjacent to the red pulp (Figure 1).

Peyer's patches (PP) play crucial role in the mucosal immune responsiveness. PP

are composed of a follicle-associated epithelium (FAE), a subepithelial dome (SED) overlying each B-cell follicle which contains B cells, follicular dendritic cells and interfollicular regions (IFRs), which contain high endothelial venules (HEVs) and efferent lymphatics. The T cell aggregation region is located between the B-cell follicles.

Functions of secondary lymphoid organs

Secondary lymphoid organs are essential for the development, selection and survival of lymphocytes and play important roles in immune responsiveness. Spleen is the location for terminal differentiation for developing B cells after they leave the bone marrow. Within the spleen, the white pulp provides an environment for the efficient initiation of Ag-specific immune responsiveness. T-cell zone contains numerous DC that display MHC-peptide complexes to the migrating naïve T cells and induce these cells to differentiate into effecter T cells. In B-cell follicles, the interaction of B cells and FDCs are necessary for formation and maintenance of primary B-cell follicles (Tumanov AV et al., 2003; Victoratos P et al., 2006). Follicular B cells are highly motile, moving through the follicle to ensure that they efficiently contact with FDCs for recognizing surface-displayed antigens (Miller MJ et al., 2002). FDCs are restricted in B-cell follicle field. FDCs express adhesion molecules, including intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and play important roles for B cell proliferation, survival, and differentiation in both Ag-dependent and Ag-independent manners (Liu YJ et al., 1989; Kosco-Vilbois MH et al., 1992; Burton GF et al., 1993; Koopman G et al., 1994; Imal Y et al., 1996; Szabo MC et al., 1997; Tew JG et al., 1997). FDCs capture and display antigen on their surface for recognition by B cells, which is believed to be crucial for the development of high affinity, isotype-switched, memory B cell responses and contribute to the generation of high-affinity recall responses (Burton GF et al., 1993; Liu YJ et al., 1997; Tew JG et al., 1997; Kapasi ZF et al., 1998; Tew JG et al., 2001). In addition to delivery of Ags, FDCs also seem to supply numerous nonspecific stimuli during germinal centre (GC) responses for the generation of an optimal B cell response (Kosco-Vilbois MH et al., 2003). The formation and maintenance of GC depend on the dynamic interactions among B cells, T cells, and FDCs. GCs are formed around the FDCs in the follicles, which provide a crucial microenvironment for T cell-dependent humoral immune responses. Within GC, Ag-specific B cells efficiently undergo clonal expansion, isotype switching, somatic mutation, affinity maturation, and the generation of plasma and memory cells in response to T cell-dependent Ags (MacLennan IC., 1994;

Kosco-Vilbois MH et al., 1997; Fu YX et al., 1999). On the other hand, it has been demonstrated that signals provided by both T and B cells play a role in supporting maturation and survival of FDCs in LNs (Kapasi ZF et al., 1993; Fu YX et al., 1999).

Marginal sinus separates marginal zone from the white pulp. Blood-borne lymphocytes are able to enter into the white pulp through marginal sinus of lining cells expressing mucosal addressin cellular adhesion molecule-1 (Madcam-1) (Kraal G et al., 1995). Madcam-1 expressing endothelium of the marginal sinus has also been suggested to have a role in retention of MMM in the MZ (Wang CC et al., 2000). Marginal zone forms a distinct border between the red pulp and the white pulp and is the location where lymphocytes enter into the white pulp (Kraal G., 1992). The MZ has various roles, including the capture of blood-borne pathogens, the regulation of antigen presentation cells (APC), the trafficking of lymphocytes into the white pulp and the generation of Ag-specific B cell responses to T cells-independent and T cell-dependent Ags (Humphrey JH et al., 1981; Humphrey JH., 1985; Kraal G., 1992; Lyons AB et al., 1995; Martin F et al., 2002). MZM are large cells that facilitate a close association with MZ B cells (Kraal G., 1992). In some cases, mice lacking MZM have been shown to be significantly more susceptible to infection and have altered T cell immune responses (Seiler P et al., 1997; Aichele P et al., 2003). MMM are considered to be essential for the initiation of immune responsiveness to T cell-dependent particulate Ags (Delemarre FG et al., 1990; Buiting AM et al., 1996) and T cell-independent Ags (Kraal G et al., 1989). MMM are also capable of migrating from the MZ into a developing GC during the course of an immune responsiveness, suggesting that MMM are involved in the transportation of unprocessed Ags from the MZ into the B-cell follicles (Groeneveld PH et al., 1986; Mueller CG et al., 2001). In contrast to most cell types, marginal zone B cells are continuously exposed to blood, that is a property ensuring rapid exposure to blood-borne Ags. MZ B cells are confined to the spleen, participate mainly in the T cell-independent antibody responses, and show critical humoral responses against blood-borne pathogens (Martin F et al, 2002; Tanigaki K et al., 2002).

Based on the location along the intestinal tract and their contribution to IgA production, PPs form the first front of mucosal immunity and play an important role in gastrointestinal immune defenses (Griebel PJ et al., 1996; Debard N et al., 1999).

Molecular mechanisms for the formation of the microarchitectures in secondary lymphoid organs

The precise organization of microarchitectures in secondary lymphoid organs is essential for the generation of efficient immune responsiveness and is controlled by

TNFR1 and LtβR signal pathways (Figure 2), and the interaction between chemokines and chemokine receptors. LtβR signal pathway activates alternative NF-κB pathway, which is composed of NF-κB inducing kinase (NIK), IκB kinase α (IKKα), NF-κB2 (p100), and RelB. It was demonstrated that LtβR signal pathway is crucial for the formation of normal splenic microarchitectures, as well as LN, PP, and NALT organogenesis because mice defective in NIK, IKKα, NF-κB2, and RelB as well as Lt and LtβR shows abnormal development of these lymphoid organs (Fu YX et al., 1999; Matsumoto M., 1999; Pasparakis M et al., 2000; Gommerman JL et al., 2003; Mebius RE et al., 2003; Weih F et al., 2003). These mutant mice show nearly complete loss of segregation of B-cell follicles and T-cell zone. As a result, both B cells and T cells appear scattered throughout the white pulp. The B-cell follicle structure is lost, and the network of FDCs is disappeared. The marginal sinus appears to be absent. Similarly, the marginal metallophilic macrophage layer and the marginal zone macrophage layer are undetectable in those mice except for *NF-κB2*$^{-/-}$ mice, which retain almost normal MZM (De Togni P et al., 1994; Fu YX et al., 1997; Franzoso G et al., 1998; Debra S et al., 2001; Senftleben U et al., 2001; Weih DS et al., 2001; Xiao G et al., 2001; Yilmaz ZB et al., 2003).

Homing of B cells to B-cell follicles depends on the expression of CXCR5 in B cells and CXCL13 in radiation-resistant follicular stromal cells (FDCs) (Förster R et al., 1996; Ansel KM et al., 2000; Ansel KM et al., 2002), and the interaction of CXCR5 and CXCL13 plays an essential role for the formation and maintenance of B-cell follicles in secondary lymphoid organs (Forster R et al., 1996; Legler DF et al., 1998; Gunn MD et al., 1998; Ansel KM et al., 2000; Ansel KM et al., 2002; Okada T et al., 2002). CXCR5 is upregulated during B cell maturation and is expressed by all mature B cells, including recirculating follicular B cells (Forster R et al., 1994; Cyster JG., 1999; Bowman EP et al., 2000) and is known to regulate trafficking and retention of B cells in B-cell follicles (Cyster JG et al., 2002). Deficiency of CXCR5 in B cells strongly reduces accumulation of B cells within B-cell follicles, resulting in the defect of B-cell follicles (Förster R et al., 1996; Förster R et al., 1999) as that observed in *CXCL13*$^{-/-}$ mice (Ansel KM et al., 2000). Expression of CXCL13 by follicular stromal cells (FDCs) is largely dependent on the cytokine Lt signaling via LtβR (Ngo VN et al., 1999; Ansel KM et al., 2000; Kuprash DV et al., 2002; Lo JC et al., 2003). Since B cells provide a major source of Lt in spleen, CXCL13 are poorly induced in mice lacking B cells (Ansel KM et al., 2000; Ngo VN et al., 2001). LtβR is expressed by follicular stromal cells (FDCs). The follicle-specific chemokine CXCL13 expressed by follicular stromal cells (FDCs) can be regulated by Lt-engaged LtβR signal pathway via the alternative NF-κB signal

pathway: Lt→LtβR→NIK→IKKα→NF-κB2→RelB signal pathway (Fagarasan S et al., 2000; Dejardin E et al., 2002; Gommerman JL et al., 2003). In addition to Lt-induced CXCL13 expression in follicular stromal cells (FDCs), CXCL13 augments the expression of Lt in B cells, generating a positive feed-back loop (Figure 3). This is of importance for the development of FDC networks and the formation and maintenance of B-cell follicles (Ansel KM et al., 2000).

TNFR1 signal pathway, activating the classical NF-κB signal transduction appears to be dispensable for LN, PP, and NALT organogenesis but is essential for proper organization of B-cell follicles and the development of FDC networks in secondary lymphoid organs (Figure 2). B and T lymphocytes segregate into distinct B and T cell compartments in the mice with targeted deficiency of the members of TNFR1 signal pathway such as TNFα and TNFR1. However, B-cell follicles and FDC networks fail to be formed in these mice (Matsumoto et al., 1996; Pasparakis et al., 1996; Pasparakis et al., 1997; Endres et al., 1999). TNFR1 signal pathway also regulates the expression of CXCL13 with much less potential, compared with that of LtβR signal pathway (Ngo VN et al., 1999; Kuprash DV et al., 2002).

Requirement for TRAF6 in development of lymphoid organs

TRAF6 participates in signal transduction through various cell surface receptors including TNF-related activation-induced cytokine/receptor activator of NF-κB (RANK), CD40, and interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) family, to activate the transcription factors NF-κB and AP-1, and plays important roles for adaptive immunity, innate immunity and bone homeostasis (Cao Z et al., 1996; Lomaga MA et al., 1999; Naito A et al., 1999; Chung JY et al., 2002; Ye H et al., 2002; Kobayashi T et al., 2003; Mackey MF et al., 2003; Wu H et al., 2003; Akiyama T et al., 2005).

Targeted disruption of TRAF6 in mice (*Traf6*$^{-/-}$ mice) has revealed that TRAF6 is essential for organogenesis and the formation of proper structure of the primary and the secondary lymphoid organs. In primary lymphoid organs, *Traf6*$^{-/-}$ mice display the defect of osteoclast formation resulting in severe osteopetrosis (abnormal thickening of the bone), which is similar to that of *RANKL*$^{-/-}$ or *RANK*$^{-/-}$ mice (Naito A et al., 1999; Kim D et al., 2000; Walsh MC et al., 2003). *Traf6*$^{-/-}$ mice also show thymic atrophy. Further studies have demonstrated that TRAF6 is essential for the formation of the thymic microenvironments. Importantly, the *Traf6*$^{-/-}$ thymic stroma is sufficient for induction of autoimmunity, suggesting that the formation of microarchitectures of thymus directed by TRAF6 is essential for preventing the autoimmune diseases

(Akiyama T et al., 2005).

Traf6$^{-/-}$ mice lack mesenteric, mandibular, inguinal, axillary and para-aortic lymph nodes, suggesting a critical role of TRAF6 in the formation of secondary lymphoid organs (Naito A et al., 1999). The spleen and PPs are present in *Traf6*$^{-/-}$ mice (Naito A et al., 1999; Yoshida H et al., 2002). However, it remains to be determined whether or not TRAF6 plays a role in the formation of microarchitectures in secondary lymphoid organs.

In this context, this study is designed to investigate the function of TRAF6 in the formation of the microarchitectures, especially, the formation and maintenance of B-cell follicles, in secondary lymphoid organs. The present data reveal that TRAF6-dependent signal pathway, which functions independently from the well-known TNFR1 and LtβR signal pathway, is essential for the formation and maintenance of B-cell follicles in secondary lymphoid organs, and TRAF6-dependent signal pathway regulates the formation and maintenance of B-cell follicles through inducing the expression of CXCL13 in follicular stromal cells (FDCs).

Materials and methods

Mice

Traf6$^{-/-}$ mice (C57BL/6 and BALB/C background) were generated in my laboratory as previously described (Naito A et al., 1999; Akiyama T et al., 2005). *Aly*/+, *aly/aly* mice were purchased from CLEA Japan (Tokyo, Japan). *RelB*$^{+/-}$ mice, CD45.1 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). *RAG2*$^{-/-}$ mice were kindly provided by Dr. F. W. Alt (Harvard University Medical School, Boston, MA), and *Lt*$\alpha^{-/-}$ mice were from Dr. D. D. Chaplin (University of Alabama, Birmingham, AL). These mice were maintained under specific pathogen-free conditions.

The analyses of *Traf6*$^{-/-}$, *aly/aly*, *RelB*$^{-/-}$ and *Lt*$\alpha^{-/-}$ mice were performed at postnatal day 3, day 5, day 8, day 11 and day 14 through comparing with that of their wild-type littermate mice. 6~10-week-old CD45.1 mice, *RAG2*$^{-/-}$ mice were used in the present experiments. All mice were handled in accordance with the Guidelines for Animal Experiments of the Institute of Medical Science, the University of Tokyo.

Reagents

The following reagents were used in the present studies: biotin-conjugated

anti-mouse B220 (Biolegend, San Diego, CA), biotin-conjugated anti-mouse IgM (clone R6-60.2; BD Phamingen, San Diego, CA), biotin-conjugated rabbit anti-mouse IgM, immunopure rabbit anti-mouse IgM (Pierce, Rockford, IL), biotin-conjugated anti-mouse CD3ε (clone 145-2C11; Pharmingen), monoclonal anti-mouse FDC-M1 (Pharmingen), anti-mouse Madcam-1 (Pharmingen), monoclonal anti-mouse MOMA1 (BMA Biomedical, Augst, Switzerland), monoclonal anti-mouse ER-TR9 (clone ER-TR9; Hycult biotechnology, Uden, Netherlands), biotin-conjugated anti-mouse CD35 (CR1, clone 8C12; Pharmingen), biotinylated anti-mouse FDC-M2 (clone FDC-M2; ImmunoKontact), anti-mouse CXCL13 antibody (R&D Systems, Minneapolis, MN), alkaline phosphatase (AP) conjugated streptavidin (Zymed, San Francisco, CA), horse radish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), alexa fluor 488 donkey anti-goat IgG (H+L), alexa fluor 594 donkey anti-rat IgG, Alexa 488-conjugated streptavidin, Alexa 568-conjugated streptavidin, Alexa 488-conjugated anti-rabbit IgG, Alexa 546-conjugated anti-rabbit IgG, Alexa 546-conjugated anti-rat IgG (All from Molecular Probes, Eugene, OR), anti-Fcγ III/II receptor antibody (clone 2.4G2; Pharmingen), phycoerythrin (PE)-conjugated anti-mouse CD3ε (clone 145-2c11; Pharmingen), PE-conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen), FITC-conjugated anti-mouse CD4 (clone GK1.5; Pharmingen), FITC-conjugated anti-mouse IgM (clone 11/41; eBioscience, San Diego, CA), FITC-conjugated anti-mouse IgD (clone 11-26; eBioscience), FITC-conjugated anti-mouse CD45.1 (clone A20; Pharmingen), PE-conjugated anti-mouse CXCR5 (Pharmingen), PE-Cy5 conjugated anti-mouse CD11c antibody (clone N418; eBioscience), streptavidin-Cy5-chrome (Pharmingen), monoclonal anti-mouse LtβR antibody (clone 4H8 WH2; Alexis, San Diego, CA), Phosphor-IκBα antibody (ser 32/36) (clone 5A5; Cell Signaling, Danvers, MA), monoclonal anti-mouse NF-κB p52 antibody (clone C-5; Santa Cruz), Phosphor-SAPK/JNK antibody (Thr183/Thr185) (Cell Signaling), JNK antibody (clone C-17; Santa Cruz), TOTO3 (Molecular Probes).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

Total RNA were extracted from spleen, Peyer's patches and/or indicated cells using TRIzol (Invitrogen Life Technologies, Gaithersburg, MD), and reverse transcription were performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. Primer sets used in the present studies and PCR conditions were summarized in table 1.

Histological and immunohistochemical analyses

Spleens and Peyer's patches were harvested at defined ages and frozen quickly in OCT compound (Sakura Finetech, Tokyo, Japan) and/or fixed with 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin sections (5-μm thick) were stained with hematoxylin-eosin (HE) and examined by light microscopy. For immunohistochemical analyses, frozen sections (5-μm thick) were fixed in acetone for 20 minutes at 4°C and air dried. Endogenous peroxidase was quenched with 0.2% H_2O_2 in methanol. After blocking with blocking solution containing 10% normal goat serum (Zymed) and/or 10% normal donkey serum (Sigma, Saint Luios, Missouri) in phosphate-buffered saline (PBS, pH 7.4) for 30 minutes at room temperature, sections were incubated with specific primary antibodies diluted in blocking solution for 60 minutes at room temperature and/or overnight at 4°C. Sections were washed with PBS and incubated with defined secondary antibodies for 50 minutes at room temperature. Color development was carried out with AP reaction kit (Vector Laboratories, Burlingame, CA) and/or diaminobenzidine (DAB)-peroxidase reaction (KPL, Gaithersburg, MD). The color images were obtained with a light microscope and/or with a Radiance 2000 Confocal Laser Microscope (Bio-Rad Laboratories, Hercules, CA).

Western blotting analyses

Total spleen was homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Nonident P-40) containing protease inhibitor (1 mM PMSF). Cells were directly solubilized in lysis buffer and/or 1xsample buffer. Samples were boiled and separated by polyacrylamide/SDS gel electrophoresis and then transferred to PVDF membrane (Millipore, Bedford, MA). After blocking with 5% skim milk, membrane was immunoblotted with specific primary antibodies diluted in 5% skim milk for 60 minutes at room temperature and/or overnight at 4°C, followed by treatment with defined HRP-conjugated secondary antibody for 60 minutes at room temperature, the immunocomplexes were visualized with the ECL and/or ECL plus Western Blotting System (Amersham Biosciences, Buckinghamshire, England).

Flow cytometric analyses

For the analyses of $CD4^+CD3^-$ cells, spleen were treated with RPMI medium containing 2.5 mg/ml collagenase/disperse (Roche, Penzberg, Germany), 1% DNase I

(Takara, Otsu, Shiga, Japan) and 2% fetal bovine serum (FBS). After erythrocytes were lysed with ammonium chloride buffer (ACK buffer), the dendritic cells were depleted by MACS system with CD11c microbeads (Mitenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The preparation of other cells was described elsewhere. Single-cell suspensions were treated with anti-Fcγ III/II receptor antibody to block Fc receptors, followed by staining with specific antibodies for cell specific markers. Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) (Epics XL, Beckmann Coulter, Fullerton, CA). Dead cells were excluded from these analyses by staining with 7-aminoactinomycin D (7-AAD; Wako, Osaka, Japan).

Isolation of FDC-enriched cells, IgM positive B cells and total B cells

For isolation of FDC-enriched cells, spleen was digested with RPMI medium containing 2.5 mg/ml collagenase/disperse, 1% DNase I and 2% FBS. For isolation of total B cells and IgM positive B cells, spleen was teased between two frosted slide glasses (Matunami, Asaka, Japan) to obtain single-cell suspensions. After lysing the erythrocytes by ACK buffer, single-cell suspensions were labeled with FDC-M1 antibody, biotin-conjugated FDC-M2 antibody, or biotin-conjugated IgM antibody. FDC-enriched cells and IgM positive B cells were positively selected by MACS system with anti-rat microbeads and/or anti-biotin microbeads (Mitenyi Biotec) according to the manufacturer's instructions. For isolation of total B cells from spleen, B cells were negatively selected from single-cell suspensions of spleen by MACS system with B Cell Isolation Kit (Mitenyi Biotec) according to the manufacturer's instructions. Degree of purity of FDCs was 50~70%, IgM positive B cells and total B cells were 70~90%, 85~95% as analyzed by FACS, respectively.

Cells transfer analyses

Total fetal liver cells were prepared from embryonic 14-day-old *Traf6*$^{-/-}$ and wild-type littermate mice and injected intravenously to the sublethally X-ray irradiated (950 Rad) *RAG2*$^{-/-}$ mice. 6~8 weeks after transplantation, the spleen of these transferred chimera mice were harvested for immunostaining analyses.

1.5~2.5x10^7 total B cells isolated from spleen of 12-day-old wild-type and/or *Traf6*$^{-/-}$ mice were injected intravenously to *RAG2*$^{-/-}$ mice, 2 weeks later, spleen of transferred chimera mice were collected to analyze the reconstitution of B-cell follicles.

0.5~1x10^8 total B cells sorted magnetically or 1~2.5x10^8 total splenocytes derived from spleen of adult CD45.1 mice were injected intraperitoneally into *Traf6*$^{-/-}$ mice at

postnatal day 7 and analyses of the spleen of these chimera mice were performed at postnatal day 14. The restored B cells in number were determined as ~2.0 x 10^7 cells by FACS analyses through counting the CD45.1 and B220 double positive B cells.

Treatment of mouse embryonic fibroblast cells (MEFs) with agonistic anti-lymphotoxin β receptor mAb

Embryonic 14-day-old mice from wild-type, *Traf6*$^{-/-}$ and *aly/aly* mice were used to generate MEFs using standard method. MEFs were expanded for two passages before assay. MEFs were cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 100U/ml penicillin and 100μg/ml streptomycin.

For the processing of p100 to p52 analysis, MEFs were stimulated with 2μg/ml Ltβ receptor mAb for 8 hours and 24 hours. To detect the phosphorylation levels of IκBα and JNK, MEFs were treated with 2μg/ml Ltβ receptor mAb for 15 minutes, 30 minutes and 60 minutes.

Results

Disrupted splenic microarchitectures in *Traf6*$^{-/-}$ mice

Using hematoxylin-eosin (HE) staining, splenic microarchitectures in *Traf6*$^{-/-}$ mice were examined at postnatal day 14, compared with that of wild-type littermate mice. In the spleen of *Traf6*$^{-/-}$ mice, red pulp displayed a proper formation, whereas white pulp showed abnormality with reduction in number of lymphocytes as detected by HE staining (Figure 4A, right). The white pulp and marginal zone microarchitectures were further investigated by immunostaining. Staining with specific antibodies against CD3ε (for T cells) and IgM (for B cells) showed abnormal B/T cell segregation, reduced numbers of IgM-positive B cells, and scattered distribution of IgM-positive B cells at the entire region of the white pulp in spleen of *Traf6*$^{-/-}$ mice. In T-cell zone of the spleen from *Traf6*$^{-/-}$ mice, T cells displayed disordered arrangement as compared with that of wild-type littermate mice, although T cell compartment existed (Figure 4B, upper row, right). Complete disruption of B-cell follicles and lack of follicular dendritic cells were observed in spleen of *Traf6*$^{-/-}$ mice as judged by immunostaining with specific antibodies against IgM and FDC-M1, CR1 (for FDC cells) (Figure 4B, middle and lower row, right). These data strongly suggested that TRAF6 is essential for the formation of B-cell follicles and FDC networks in spleen.

Next, the microarchitectures of marginal zone in spleen of *Traf6*$^{-/-}$ mice were

examined. Staining of Madcam-1, a marker of marginal sinus, revealed reduced numbers of marginal sinus-lineage cells in spleen of *Traf6*$^{-/-}$ mice (Figure 4C, upper row, right). In marginal zone, staining of MOMA1, a marginal metallophilic macrophage marker, ER-TR9, a marginal zone macrophage marker and ER-TR7, marginal reticular cell marker, indicated that MOMA1-, ER-TR9-, and ER-TR7-positive cells scattered throughout the spleen of *Traf6*$^{-/-}$ mice. Thus, the marginal metallophilic macrophage layer, marginal zone macrophage layer and reticular cell layer were not found in the outer region of the white pulp in spleen of *Traf6*$^{-/-}$ mice (Figure 4C), indicating that TRAF6 is also required for the formation of marginal zone structures. Interestingly, the splenic microarchitectures in *Traf6*$^{-/-}$ mice are strikingly similar to those observed in mice lacking molecules involved in the LtβR signal pathway such as Ltα, NIK, and RelB mutant mice (Fu YX et al., 1999; Matsumoto M., 1999; Debra S et al., 2001; and data not shown).

TRAF6 is dispensable for the formation of B-cell clusters, but essential for the subsequent formation and maintenance of B-cell follicles

In the next, the formation processes of B-cell follicle in spleen of *Traf6*$^{-/-}$ mice was investigated. The formation of B-cell follicles is a progressive process including two stages: the formation stage of B-cell clusters before postnatal day 8 and the formation and maintenance stage of B-cell follicles beginning at postnatal day 8 (Friedberg SH et al., 1974; Loder F et al., 1999; Balogh P et al., 2001). In the formation stage of B-cell clusters, ring-like structure with FDCs around the central artery was observed at postnatal day 3. These clusters were gradually enlarged in the size until postnatal day 8. The follicle-like structure with FDCs was initially formed at postnatal day 8, and subsequently, these follicles increased in size gradually (Figure 5, upper row; Friedberg SH et al., 1974; Loder F et al., 1999; Balogh P et al., 2001). In order to determine the role of TRAF6 in the formation processes of B-cell follicles, immunostaining analyses were performed in spleen of *Traf6*$^{-/-}$ mice from postnatal day 3 to day 11. Immunostaining with IgM and FDC-M1 antibodies showed that, until postnatal day 8, B-cell clusters with FDCs was observed in spleen of *Traf6*$^{-/-}$ mice, which is comparable with that of wild-type littermate mice. Although follicle-like structures appeared in spleen of *Traf6*$^{-/-}$ mice at postnatal day 8, slightly disordered arrangement and decreased numbers of IgM-positive B cells were observed. By postnatal day 11, B-cell follicles were almost completely disappeared. On the other hand, the normal FDC-M1-positive FDCs were observed in spleen of *Traf6*$^{-/-}$ mice at postnatal day 8 and persisted until postnatal day 11. However, the number of FDCs was

decreased in spleen of *Traf6*$^{-/-}$ mice compared with that in wild-type control at postnatal day 11 (Figure 5, the second row). By postnatal day 14, neither B-cell follicles nor FDCs were detected in spleen of *Traf6*$^{-/-}$ mice (Figure 4B). These results indicated that TRAF6 is dispensable for the formation stage of B-cell clusters before postnatal day 8, but essential for the subsequent formation and maintenance stage of B-cell follicles beginning at postnatal day 8.

Defect of the formation of B-cell clusters and subsequent formation and maintenance of B-cell follicles in mice lacking molecules involved in LtβR signal pathway

At postnatal day 14, the phenotypes of B-cell follicles in *Traf6*$^{-/-}$ mice (Figure 4B) are similar to those observed in mice lacking molecules involved in LtβR signal pathway, such as Ltα, NIK and RelB (Fu YX et al., 1999; Pasparakis M et al., 2000; Gommerman JL et al., 2003). However, the analysis on the developmental processes of B-cell follicles in spleen of *Traf6*$^{-/-}$ mice revealed that TRAF6 is only required for the late phase in the B-cell follicle development. Therefore, I investigated the role of LtβR signal pathway in the formation processes of B-cell follicles and addressed a potential crosstalk between TRAF6 and LtβR signal pathway. Immunostaining analyses in spleen of mutant mice lacking molecules involved in LtβR signal pathway: *Ltα*$^{-/-}$ mice, *aly/aly* mice, which have a point mutation in NIK gene, and *RelB*$^{-/-}$ mice were carried out to compare with the phenotype of *Traf6*$^{-/-}$ mice. As a result, neither the formation of B-cell clusters in the early postnatal periods nor the subsequent formation of B-cell follicles was observed in these mutant mice. Additionally, FDCs were never detected in these mutant mice (Figure 5). These phenotypes were obviously different from those in *Traf6*$^{-/-}$ mice, which showed normal formation of B-cell clusters during the early postnatal periods, but failure of the subsequent formation and maintenance of B-cell follicles. These results indicate that LtβR signal pathway is essential for the formation stage of B-cell clusters, whereas TRAF6 is indispensable for the formation and maintenance stage of B-cell follicles (Figure 13). These results also suggested that TRAF6 is not involved in the LtβR signal pathway for the formation of B-cell follicles.

TRAF6 is not involved in the LtβR signal pathway

I then determined whether TRAF6 is involved in the LtβR signal pathway or not. The LtβR-engaged signaling activates the classical NF-κB signal pathway, the non-classical NF-κB signal pathway, and MKK/JNK signal pathway (You LR et al., 1999; Dejardin E et al., 2002; Bonizzi G et al., 2004). To elucidate whether TRAF6 is

involved in the LtβR signal pathway, the phosphorylation of IκBα, the processing of p100 to p52 , and the phosphorylation of Jun N-terminal kinase (JNK) , which are hallmark of classical NF-κB signaling pathway, non-classical NF-κB signal pathway, and MKK/JNK signal pathway, respectively, were examined by western blot analyses using primary *Traf6*$^{-/-}$ embryonic fibroblasts (MEFs). LtβR antibody ligation induced phosphorylation of IκBα in *Traf6*$^{-/-}$ MEFs as well as in wild-type MEFs (Figure 6A). Furthremore, LtβR antibody ligation induced the processing of p100 to p52 in *Traf6*$^{-/-}$ MEF, whereas this processing was not detected in MEFs derived from *aly/aly* mice (Figure 6B), which were previously reported to have a defect in the processing of p100 to p52 induced by LtβR signaling in the non-classical NF-κB signal pathway (Dejardin E et al., 2002). Moreover, the extent of JNK phosphorylation in *Traf6*$^{-/-}$ MEFs induced by the ligation of LtβR was comparable to that in wild-type MEFs (Figure 6C). These data revealed that TRAF6 is not involved in the LtβR signal pathway, suggesting that the TRAF6-dependent signal pathway contributes to the control of the formation and maintenance of B-cell follicles in secondary lymphoid organs independent of LtβR signal pathway.

Comparable expression of TNFα, TNFR1, and Ltα, Ltβ, LtβR in spleen from *Traf6*$^{-/-}$ mice

Molecules involved in the TNFR1 and LtβR signal pathway, such as TNFα, TNFR1, Ltα, Ltβ, LtβR, are indispensable for the formation of B-cell follicles and the development of FDC networks (Fu YX et al., 1999; Matsumoto M., 1999; Weih F et al., 2003). Although TRAF6 is not involved in TNFR1 signal pathway (Aggarwal BB., 2003) and LtβR signal pathway, it remained to be elucidated whether or not the deficiency of TRAF6 induced abnormal expression of these molecules in spleen of *Traf6*$^{-/-}$ mice. To address this question, semi-quantitative RT-PCR was performed to detect the expression of these molecules in spleen of *Traf6*$^{-/-}$ mice at postnatal day 14. RT-PCR analyses showed comparable expression of TNFα, TNFR1 in spleen of *Traf6*$^{-/-}$ mice and wild-type mice. No significantly decreased expression of Ltα, Ltβ and LtβR were detected in spleen of *Traf6*$^{-/-}$ mice compared with that of wild-type littermate mice (Figure 7A).

The interaction of B cells and FDCs is important for the formation and maintenance of B-cell follicles. In this event, the expression of TNFα, Ltα, Ltβ in B cells and TNFR1, LtβR in FDCs is required (Tumanov AV et al., 2003; Victoratos P et al., 2006). In spleen, T cells, B cells and inducer cells express cytokine TNFα, Ltα, Ltβ, while FDCs and fibroblastic stromal cells express TNFR1, LtβR (Kuprash DV et al.,

1999; Hjelmstrom P et al., 2000; Kim MY et al., 2007). Although the expression of TNFα, Ltα, Ltβ, TNFR1 and LtβR in whole spleen of *Traf6*$^{-/-}$ mice are comparable to those of wild-type mice, it remained to be determined whether the expression of these molecules was normal in B cells and FDCs from *Traf6*$^{-/-}$ mice. To address this question, IgM-positive B cells (degree of purity was 70~90%) and FDC-enriched cells (degree of purity was 50~70%) were isolated from spleen of wild-type and *Traf6*$^{-/-}$ mice to subject to RT-PCR analyses. Expression of TNFα, Ltα, Ltβ in IgM-positive B cells and TNFR1, LtβR in FDC-enriched cells from *Traf6*$^{-/-}$ mice was similar to that from wild-type littermate mice (Figure 8A, 8B and data not shown). Thus, TRAF6 is not required for the expression of TNFα, TNFR1, Ltα, Ltβ, LtβR in spleen. TRAF6 is also not involved in the TNFR1 and LtβR signal pathway as described above. These findings demonstrated that TNFR1 and LtβR signal pathway is independent of TRAF6 and can normally function in *Traf6*$^{-/-}$ mice. That is, the TRAF6-dependent signal pathway can contribute to the control of the formation of B-cell follicles, independently. These analyses suggest that: the TNFR1 and LtβR signal pathway, and the novel TRAF6-dependant signal pathway play essential roles for the control of the formation of B-cell follicles, respectively (Figure 12).

Normal expression of CXCR5 in IgM-positive B cells from *Traf6*$^{-/-}$ mice

For the development and maintenance of B-cell follicles, the expression of CXCL13, a B-cell attracting chemokine, derived from follicular stromal cells (FDCs) and its receptor CXCR5 expressed in mature B cells is crucial (Förster R et al., 1996; Ansel KM et al., 2000). Deficiency of CXCR5 in B cells results in the disruption of B-cell follicles (Förster R et al., 1996; Förster R et al., 1999). To examine whether abnormal expression of CXCR5 occurred in *Traf6*$^{-/-}$ mice, RT-PCR analyses were performed using spleen of wild-type control and *Traf6*$^{-/-}$ mice at postnatal day 14. CXCR5 in spleen of *Traf6*$^{-/-}$ mice was shown to be decreased as compared with that of wild-type littermate mice (Figure 7A).

Traf6$^{-/-}$ mice show reduced numbers of B cells in spleen as described above (Figure 4B), leading to the hypothesis that the reduced expression of CXCR5 could be caused by the reduced numbers of B cells in *Traf6*$^{-/-}$ mice. Therefore, IgM-positive B cells (degree of purity was 70~90%) were isolated from spleen of *Traf6*$^{-/-}$ mice and wild-type littermate mice at postnatal day 5, day 8 and day 14 to subject to RT-PCR analyses. No significant difference of the expression of CXCR5 in B cells from *Traf6*$^{-/-}$ were detected at these ages as compared with that from wild-type littermate controls. Flow cytometric analyses also showed normal expression of CXCR5 in *Traf6*$^{-/-}$ B cells,

which is consistent with the results of the RT-PCR analyses (Figure 8A). These results indicate that the defect of B-cell follicles in spleen of *Traf6*$^{-/-}$ mice is not caused by the abnormal expression of CXCR5 in B cells.

Reduced expression of chemokine CXCL13 in spleen and FDCs from *Traf6*$^{-/-}$ mice

CXCL13 derived from follicular stromal cells (most likely from FDCs) is essential for the formation and maintenance of B-cell follicles in secondary lymphoid organs. Deficiency of CXCL13 results in the disruption of B-cell follicles in mice (Ansel KM et al., 2000). In the next, whether or not the abnormal expression of CXCL13 occurred in spleen of *Traf6*$^{-/-}$ mice was investigated. RT-PCR analysis showed significantly decreased expression of CXCL13 in spleen of *Traf6*$^{-/-}$ mice as compared with that of wild-type littermate mice (Figure 7A). Western blotting and immunostaining analyses of CXCL13 in spleen of *Traf6*$^{-/-}$ mice also showed the reduced expression (Figure 7B, 7C), these results is consistent with the results of RT-PCR. The decreased expression of CXCL13 could be responsible for the disruption of B-cell follicles and the defect of FDCs in spleen of *Traf6*$^{-/-}$ mice.

Next, the kinetics of the expression of CXCL13 in the formation processes of B-cell follicles were investigated. RT-PCR analysis showed weakly expression of CXCL13 at postnatal day 5, and subsequently, the expression of CXCL13 increased gradually, and the maximum expression of CXCL13 was detected at postnatal day 11 in spleen of wild-type mice. On the other hand, the expression level of CXCL13 in spleen of *Traf6*$^{-/-}$ mice was comparable to that of wild-type mice at postnatal day 5, but decreased expression of CXCL13 was observed at postnatal day 8 and significantly decreased expression of CXCL13 was detected at postnatal day 11. Immunostaining with FDC-M1 and CXCL13 antibodies supported the results of the RT-PCR analyses. Expression of CXCL13 was observed to be distributed in a radiatial pattern in B-cell follicle area in spleen of wild-type mice at postnatal day 8 and day 11, whereas such kind of expression pattern was not observed in spleen of *Traf6*$^{-/-}$ mice at these time-points (Figure 9A). However, FDCs, the major source of CXCL13, are still present in spleen of *Traf6*$^{-/-}$ mice at postnatal day 8 and day 11 (Figure 5, Figure 9A), implying that these FDCs may lack the capacity to express CXCL13 because of the deficiency of TRAF6.

FDCs are well-known as the major source of CXCL13 which is essential for the formation, development and maintenance of B-cell follicles (Fagarasan S et al., 2000; Dejardin E et al., 2002; Gommerman JL et al., 2003; Victoratos P et al., 2006). As mentioned above, *Traf6*$^{-/-}$ mice had normal FDCs in number at postnatal day 3, 5, 8,

however, the number of FDCs was decreased at postnatal day 11. In these cases, whether or not the decreased expression of CXCL13 in spleen of *Traf6*$^{-/-}$ mice was caused by the impaired express of Traf6$^{-/-}$ in FDCs was addressed. Therefore, FDCs were enriched to subject to RT-PCR analysis (degree of purity was 50~70%). RT-PCR assay showed comparable expression of CXCL13 in FDC-enriched cells between wild-type control mice and *Traf6*$^{-/-}$ mice at postnatal day 5, however, significantly decreased expression of CXCL13 was seen in FDC-enriched cells derived from spleen of *Traf6*$^{-/-}$ mice at postnatal day 8 and day 11 (Figure 9B). This is consistent with the results that disorganization of B-cell follicles in *Traf6*$^{-/-}$ mice begins at postnatal day 8, indicating that the induced expression of CXCL13 in FDCs by TRAF6-dependent signal pathway is required for the formation and maintenance stage of B-cell follicles. Taken together, these results suggested that TRAF6-dependent signal pathway is required for the expression of CXCL13 to control the formation and maintenance of B-cell follicles beginning at postnatal day 8.

Splenic stromal cells are responsible for the defect of B-cell follicles in *Traf6*$^{-/-}$ mice

The formation of B-cell follicles depends on the interaction between the B cells and the follicular stromal cells. As described above, *Traf6*$^{-/-}$ mice lack B-cell follicles. However, it remained to be determined whether the defect of B-cell follicles is derived from hematopoietic derived cells, stromal cells or a combination of these cells in *Traf6*$^{-/-}$ mice. To address these questions, *Traf6*$^{-/-}$ fetal liver cells containing the hematopoietic stem cells were transferred into sublethally X-ray irradiated *RAG2*$^{-/-}$ mice, which bear no B and T cells of their own. The spleen of these chimera mice was analyzed at 6~8 weeks after transfer. B-cell follicles were successfully reconstituted in spleen of chimera mice transferred with *Traf6*$^{-/-}$ total fetal liver cells as determined by the immunostaining with IgM and FDC-M1 antibodies. Normal T/B cell segregation, marginal sinus and marginal zone structures were also observed in these mice (Figure 10A). These data suggested that the defect of B-cell follicles in *Traf6*$^{-/-}$ mice is not caused by the deficiency of TRAF6 in hematopoietic stem cells.

As reported previously, *Traf6*$^{-/-}$ mice showed impaired development of bone marrow cavity and severe osteopetrosis (Naito A et al., 1999). B-cells developed from such abnormal environment of bone marrow may have defect in inducing B-cell follicle development. To rule out this possibility, B cells were isolated from the spleen of 12-day-old *Traf6*$^{-/-}$ mice (degree of purity was 85~95%), in which abnormal B-cell follicles were seen at this age, and were transferred into *RAG2*$^{-/-}$ mice. Two-weeks

after the transfer, the spleen of these chimera mice was analyzed by immunostaining. It was found that *RAG2*$^{-/-}$ recipient transferred with *Traf6*$^{-/-}$ B cells as well as wild-type B cells possessed complete B-cell follicles with FDCs (Figure 10B). These results indicated that the failure of the formation and maintenance of B-cell follicles in *Traf6*$^{-/-}$ mice is not caused by the deficiency of TRAF6 in B cells.

It should be noted that *Traf6*$^{-/-}$ mice exhibit reduced numbers of B cells in spleen. Therefore, it is also possible that decreased numbers of B cells was responsible for the failure of the formation and maintenance of B-cell follicles in *Traf6*$^{-/-}$ mice. To address this question, wild-type B cells (degree of purity was 90~98%) derived from spleen or total splenocytes of adult wild-type CD45.1 mice were transferred into *Traf6*$^{-/-}$ mice at postnatal day 7 (when the B-cell clusters show normal structure in *Traf6*$^{-/-}$ mice). Seven days later, despite restoration in number of B cells to some extent (the restored B cells in number was ~$2.0x10^7$ cells as detected by FACS analysis), the disrupted B-cell follicles remained disorganization in spleen of the transferred *Traf6*$^{-/-}$ mice as detected by immunostaining with IgM and FDC-M1 antibodies (Figure 10C). Taken together with the results that TRAF6 is not required for the expression of CXCR5, TNFα, Ltα, Ltβ in B cells (as described above) but decreased expression of CXCL13 in both spleen and FDC-enriched cells from *Traf6*$^{-/-}$ mice, suggesting that radiation-resistant stromal cells are responsible for the defect of B-cell follicles rather than the hematopoietic stem cells and/or B cells in *Traf6*$^{-/-}$ mice.

Recent reports have identified a key role of $CD4^+CD3^-$ lymphoid tissue inducer cells in the development of the segregation of B-cell follicles and T-cell zone in secondary lymphoid organs (Kim MY et al., 2007). I then investigated if TRAF6 is required for the development of the lymphoid tissue inducer cells. Flow cytometric analysis showed $CD4^+CD3^-$ cells are present in spleen of *Traf6*$^{-/-}$ mice in similar ratio with that in wild-type littermate mice (Figure 10D), suggesting that TRAF6-dependent signal is not required for the development of inducer cells in spleen.

Impaired maintenance of B-cell follicles and reduced expression of CXCL13 in Peyer's patchs of *Traf6*$^{-/-}$ mice

TRAF6-dependent signal pathway is essential for the formation and maintenance of B-cell follicles in spleen by induction of the expression of CXCL13 in follicular stromal cells (FDCs) as described above. How about in the other secondary lymphoid organs such as PPs? To elucidate this question, the development of B-cell follicles in PPs was examined from postnatal day 8 to day 14. Also, the expression of CXCL13 at postnatal day 12 between *Traf6*$^{-/-}$ mice and wild-type littermate mice was analyzed.

Using immunostaining analyses with IgM and FDC-M1 antibodies, B-cell follicle-like structure with FDCs was detected in PP of *Traf6*$^{-/-}$ mice as compared with that of wild-type littermate mice at postnatal day 8. However, by postnatal day 14, the B-cell follicles were disrupted, but small populations of FDCs were still retained in B-cell follicle area in PP of *Traf6*$^{-/-}$ mice. The decreased numbers of B cells were also observed in PP of *Traf6*$^{-/-}$ mice at this age (Figure 11A). In PP of *Traf6*$^{-/-}$ mice, the expression of CXCL13 was markedly decreased as determined by both RT-PCR and immunostaining analyses (Figure 11B).

Discussion

TRAF6 is essential for the formation of microarchitectures in secondary lymphoid organs

It has been reported that molecules involved in TNFR1 and LtβR signal pathway such as TNFα, Ltα, Ltβ, TNFR1, and LtβR are required for the formation of microarchitectures in secondary lymphoid organs, based on the results obtained from the phenotypic analyses of mice carrying targeted deficiency of genes (Fu YX et al., 1999; Matsumoto M et al., 1996; Matsumoto M., 1999; Kuprash DV et al., 2002; Mebius RE., 2003; Weih F et al., 2003). In the present study, I newly identified a crucial role of TRAF6 in the formation of microarchitectures in secondary lymphoid organs. *Traf6*$^{-/-}$ mice showed disruption of B-cell follicles, loss of follicular dendritic cell networks (as discussed below), disorganization of marginal zone structures, and decreased numbers of marginal sinus lineage cells in the spleen, suggesting that TRAF6 is essential for the formation of splenic microarchitectures.

The splenic MZ is the major entry route for antigens, APCs and lymphocytes into the white pulp, and the location for antigen specific B cell responses to T cell-independent Ags (Humphrey JH et al., 1981; Humphrey JH., 1985; Lyons AB et al., 1995). To carry out these functions, the ordered structures of MZ are required. However, the MZ structures are not well established in *Traf6*$^{-/-}$ mice; the marginal metallophilic macrophages, marginal zone macrophages and reticular cells, which normally reside in MZ and separate red pulp and white pulp in spleen, are distributed throughout the red pulp and the white pulp. Presence of scattered distribution of MMM and RC in entire spleen and absence of MZ microarchitectures in spleen of *Traf6*$^{-/-}$ mice were observed during the formation periods of MZ (data not shown), indicating that TRAF6 is not essential for the development of MMM, MZM and RC itself but rather for their proper

localization within the marginal zone of spleen.

B cells and the MZ macrophages are indispensable for the integrity of MZ (Dingjan GM et al., 1998; Nolte MA et al., 2004). However, it remains uncertain whether or not the defect of MZ structures in *Traf6*$^{-/-}$ mice is caused by improper MZ macrophages, B cells or stromal cells. At least, adoptive transfer of *Traf6*$^{-/-}$ fetal liver cells or B cells into *RAG2*$^{-/-}$ mice restore MZ structures (Figure 10A and data not shown), suggesting that expression of TRAF6 in B cells is not necessary for the proper formation of MZ structures. In contrast, transfer of wild-type B cells into *Traf6*$^{-/-}$ mice fail to restore MZ structures (data not shown), suggesting that expression of TRAF6 in stromal cells may be responsible for the formation of MZ structures.

Madcam-1 expressing cells appear dispersed throughout the spleen in wild-type mice at the postnatal day 0, the marginal sinus starts to be formed at postnatal day 3. Then, a more clear structure of MS is formed at postnatal day 8 (Sasaki K et al., 1988; and data not shown). In *Traf6*$^{-/-}$ mice, cells expressing Madcam-1 and the formation of marginal sinus structure are observed until postnatal day 8. However, decreased numbers of cells expressing Madcam-1 started at postnatal day 11 (data not shown), suggesting that TRAF6 is required for the maintenance of the lineage of Madcam-1 positive cells. Madcam-1 is one of the crucial homing factors for the correct organization of MZ (Pabst O et al., 2000) and the Madcam-1 expressing marginal sinus has been suggested to have a role in retention of MMM in the MZ (Wang CC et al., 2000). Decreased numbers of Madcam-1 positive cells from postnatal day 11 may result in the disorganization of marginal zone in *Traf6*$^{-/-}$ mice.

TRAF6 is indispensable for the formation and maintenance of B-cell follicles in secondary lymphoid organs

The integrity of B-cell follicles in secondary lymphoid organs is essential for efficient immune responsiveness (MacLennan IC., 1994; Steinman RM et al., 1997). The formation, development and maintenance of B-cell follicles are controlled by TNFR1 and LtβR signal pathway, and the interaction between chemokine CXCL13 and its receptor CXCR5 (Fu YX et al., 1999; Matsumoto M., 1999; Pasparakis M et al., 2000; Gommerman JL et al., 2003; Mebius RE., 2003; Weih F et al., 2003). The present study also reveal that TRAF6-dependent signal pathway (as discussed below) plays essential roles for the formation and maintenance of B-cell follicles in secondary lymphoid organs.

The spleen of *Traf6*$^{-/-}$ mice shows disorganization of B-cell follicles and lack of follicular dendritic cell networks, indicating that TRAF6 is required for the formation of

B-cell follicles. However, further investigations revealed that, in *Traf6*$^{-/-}$ mice, the B-cell clusters with FDCs appeared before postnatal day 8, whereas the subsequent formation and maintenance of B-cell follicles are prevented. This fact indicates TRAF6 is dispensable for the formation of B-cell clusters. Therefore, TRAF6 might be specifically required for the progression stage of B-cell follicle formation after postnatal day 8, and/or for the maintenance of B-cell follicles in the secondary lymphoid organs of adult mice. On the other hand, LtβR signal pathway is essenital for the formation stage of B-cell clusters in the early postnatal periods. These findings indicate that the LtβR signal pathway and the TRAF6-dependent signal pathway regulate the different developmental step during the formation of B-cell follicles. However, in addition to the defective formation of B-cell clusters, mutant mice lacking molecules involved in the LtβR signal pathway also displayed defect of the subsequent formation of B-cell follicles. This might be the result of the failure formation of B-cell clusters. Further experiments are required to reveal whether LtβR signal pathway direct the formation of B-cell follicles beginning at postnatal day 8.

Expressions of TNFα, Ltα, Ltβ and CXCR5 in B cells and TNFR1 and LtβR in FDCs are required for the formation of B-cell follicles (Fu YX et al., 1999; Matsumoto M., 1999; Ansel KM et al., 2000; Weih F et al., 2003). Interestingly, the expression of these molecules in B cells and/or FDCs were not affected by the deficiency of TRAF6 (Figure 8A, 8B, and data not shown), indicating that the disruption of B-cell follicles in *Traf6*$^{-/-}$ mice is not due to the impaired expression of these molecules. These data also suggest that the defect of B-cell follicles in *Traf6*$^{-/-}$ mice might not be ascribed to the deficiency of TRAF6 in B cells. This is confirmed by the cell transfer experiments, in which adaptive transfer of *Traf6*$^{-/-}$ fetal liver cells into sublethally irradiated *Rag2*$^{-/-}$ mice show normally reconstituted both B-cell follicles and FDC networks in spleen. Moreover, transfer of *Traf6*$^{-/-}$ B cells into *RAG2*$^{-/-}$ mice also reconstitutes B-cell follicles with FDCs in spleen. On the contrary, transplantation of wild-type B cells into *Traf6*$^{-/-}$ mice fails to restore the disorganized B-cell follicles in spleen. These results suggest that TRAF6 signal in radiation-resistant cells is responsible for the development of B-cell follicles in secondary lymphoid organs.

The formation of B-cell follicles depends on the expression of CXCL13 in radiation-resistant follicular stromal cells (FDCs), because deficiency of CXCL13 in follicular stromal cells (FDCs) results in the disruption of B-cell follicles (Ansel KM et al., 2000). The expression of CXCL13 in follicular stromal cells (FDCs) has been reported to be controlled by TNFR1 and LtβR signal pathways (Ansel KM et al., 2000; Ansel KM et al., 2002). However, treatment of adult mice with antagonistic LtβR

antibody only partially decreases the expression of CXCL13. Conditional knockout of Ltα and/or Ltβ in B cells also revealed the expression of CXCL13 is partially dependent on Lt signaling (Kuprash DV et al., 1999; Hjelmstrom P et al., 2000). Thus, LtβR signal pathway is not an exclusive pathway for the induction and maintenance of the expression of CXCL13. On the other hand, TNFR1 signal pathway has been demonstrated to induce the expression of CXCL13 weakly (Kuprash DV et al., 1999; Hjelmstrom P et al., 2000). These findings suggest that the other pathways may contribute to the induction of the CXCL13 expression for the formation of B-cell follicles in secondary lymphoid organs. Results presented in this study demonstrate that TRAF6 can regulate the expression of CXCL13 in secondary lymphoid organs. Because TRAF6 is not involved in the signaling from TNFR1 and LtβR, indicating that another signal dependent on TRAF6 controls the induction of CXCL13 in FDCs.

Splenic cells labeled by FDC markers express CXCL13, indicating that FDCs produce CXCL13, despite not all cells do (Cyster JG et al., 2000). Expression of CXCL13 in FDC-enriched cell pools from *Traf6*$^{-/-}$ mice is normal at postnatal day 5, whereas markedly decreased expression of CXCL13 can be detected at postnatal day 8 and day11. This result strongly suggests that TRAF6-dependent signal pathway start function to express CXCL13 at postnatal day 8, and such stage-dependent regulation of CXCL13 expression in FDCs would be important for the formation and maintenance of B-cell follicles. Taken together, these findings suggest that TRAF6-dependent signal pathway regulates the formation and maintenance of B-cell follicles in secondary lymphoid organs through inducing the expression of CXCL13 in follicular stromal cells (FDCs).

The formation and maintenance of B-cell follicles and the development of FDC networks depend on so-called B-cell homing positive feed-back loop. In the B-cell homing positive feed-back loop, expression of CXCL13 is regulated by the signal from LtβR. Then, the generated CXCL13 attract B-cells around the FDCs and induces the expression of surface Ltα and Ltβ, which in turn stimulates the FDC to express CXCL13 (Fu YX et al., 1999; Ansel KM et al., 2000). The fact that B-cell clusters and FDCs are present in the spleen of *Traf6*$^{-/-}$ mice until postnatal day 8 suggests that B-cell homing positive feed-back loop functions normally in spleen of *Traf6*$^{-/-}$ mice until postnatal day 8. This idea was supported by the findings that *Traf6*$^{-/-}$ mice show normal expression of CXCR5, Ltα, Ltβ in B cells and LtβR in FDCs, and that TRAF6 is not required for the LtβR signal pathway. However, the number of FDCs gradually decreased and disorganization of B-cell follicles occur after postnatal day 8 in spleen of *Traf6*$^{-/-}$ mice. A plausible hypothesis is that TRAF6-dependent signal pathway start to

be required for the expression of CXCL13 in FDCs from postnatal day 8 in addition to LtβR signal. The abolishment of CXCL3 expression by the deficiency of TRAF6 in FDCs terminates the attracting B cells around FDCs at postnatal day 8, which breaks the B-cell homing positive feed-back loop. The defect of B-cell homing positive feed-back loop then impairs the survival and differentiation of FDCs.

Integrins αLβ2 and α4β1 expressed in B cells, cell adhesion molecules ICAM-1 and VCAM-1 expressed in FDCs have been reported to be involved in B cells migration into white-pulp and retention of B cells in B-cell follicles (Lo CG et al., 2003; Victoratos P et al., 2006). From this, it is possible that TRAF6-dependent signaling may not directly induce the expression of CXCL13 but instead play roles in promoting B cells to located around FDCs through regulating the expression of integrins αLβ2 and α4β1 in B cells, cell adhesion molecules ICAM-1 and VCAM-1 in FDCs. The expression of integrins αLβ2 and α4β1 can be determined in IgM-positive B cells isolated from spleen of *Traf6*$^{-/-}$ mice (data not shown), which is consistent with that the disruption of B-cell follicles is independent of the deficiency of TRAF6 in B cells. On the other hand, decreased expression of ICAM-1 and VCAM-1 are found in FDCs derived from spleen of *Traf6*$^{-/-}$ mice (data not shown), suggesting that the down-regulated expression of ICAM-1 and VCAM-1 in FDCs may partially be responsible for the disruption of B-cell follicles in *Traf6*$^{-/-}$ mice. Therefore, the defect of the expression of ICAM-1 and VCAM-1 may also indirectly reduce the expression of CXCL13. Further experiments are required to determine whether CXCL13 is a direct target molecule engaged by the TRAF6-dependent signal pathway.

Traf6$^{-/-}$ mice appear disruption of T and B cell segregation. $CD4^+CD3^-$ cells have been reported to be involved in the segregation between B-cell follicles and T-cell zone (Kim MY et al., 2007). Although the ratio of $CD4^+CD3^-$ cells in spleen of *Traf6*$^{-/-}$ mice is similar to that of wild-type mice, it is still possible that, TRAF6-dependent signal pathway regulates the function of these cells to induce the segregation of B-cell follicles and T-cell zone.

TRAF6-dependent signal pathway functions independently from the TNFR1 and LtβR signal pathway, to contribute to the formation and maintenance of B-cell follicles in secondary lymphoid organs

NF-κB activation can be induced by two different signal pathways, the classical and non-classical pathways. The classical NF-κB pathway activated by the TNFR1 signal pathway and the non-classical NF-κB pathway activated via the LtβR signal pathway (Figure 2), are known to be necessary for the formation and maintenance of

B-cell follicles in secondary lymphoid organs (Fu YX et al., 1999; Ngo VN et al., 1999; Fagarasan S et al., 2000; Dejardin E et al., 2002; Kuprash DV et al., 2002; Gommerman JL et al., 2003). It is known that TRAF6 activates NF-κB in various cells (Inoue J et al., 2000; Inoue J et al., 2007). In this study, I found that TRAF6 is required for the formation of B-cell follicles in secondary lymphoid organs. Disorganization of B-cell follicles and lack of FDC networks in *Traf6*$^{-/-}$ mice are found to be similar to that of mice lacking molecules involved in the TNFR1 signal pathway and the LtβR signal pathway (Fu YX et al., 1999; Victoratos P et al., 2006). However, I confirmed that TRAF6 is not involved in the LtβR signal pathway by using *Traf6*$^{-/-}$ cells. Previous studies revealed that TRAF6 is not required for the activation of TNFR1 signal pathway (Aggarwal BB., 2003). These data strongly suggested that TRAF6 signaling is not involved in TNFR1 and LtβR signal pathways in B-cell follicle development. I therefore hypothesize that at least three signal pathways may be involved in the control of the formation and maintenance of B-cell follicles: the TRAF6-dependent signal pathway, the TNFR1 signal pathway and the LtβR signal pathway (Figure 12). However, the molecules involved in the downstream of TNFR1 signal pathway are not well understood in the formation of B-cell follicles (Franzoso G et al., 1998; Weih DS et al., 2001). Since TRAF6 activates the classical NF-κB signal pathway (Inoue J et al., 2000; Inoue J et al., 2007), it is still possible that TRAF6 is involved in the TNFR1 signal pathway for the formation of B-cell follicles in secondary lymphoid organs.

Although the present data suggest that TRAF6 is involved in a novel signal transduction pathway to contribute to the formation and maintenance of B-cell follicles in secondary lymphoid organs, the specific ligands, receptors, and intracellular signals utilizing TRAF6 for the signal transduction to contribute to the formation and maintenance of B-cell follicles remains to be determined. TRAF6 participates in signal transduction from the receptors of RANK and CD40 (Cao Z et al., 1996; Chung JY et al., 2002; Ye H et al., 2002; Inoue J et al., 2007). It is of interest that the expression of CD40 and RANK can be detected in FDCs (Ogata T et al., 1996; and data not shown), since FDCs are indispensable for the establishment of B-cell follicles. CD40 interacts with CD40L, and patients with a congenital deficiency of CD40L have lymph nodes with severe depletion of FDC networks (Facchetti F et al., 1995). Thus, CD40 signal pathway may be essential for the development of FDC networks and the formation of B-cell follicles. However, *CD40*$^{-/-}$ mice show normal B-cell follicles with FDCs (data not shown), indicating that the CD40-TRAF6 signal pathway is not responsible for the defect of B-cell follicles in *Traf6*$^{-/-}$ mice. RANK is known to interact with RANKL. It has been reported that *RANKL*$^{-/-}$ mice display visibly enlarged spleen and altered

splenic B-cell follicles (Kim D et al., 2000), which are similar to those observed in *Traf6*$^{-/-}$ mice. However, *RANKL*$^{-/-}$ mice show normal formation of B-cell follicles at postnatal day 14 (data not shown). This phenotype is obviously different from that observed in *Traf6*$^{-/-}$ mice, indicating that RANK-TRAF6 signal pathway is not required for the formation and maintenance of B-cell follicles. TRAF6, acting as an adaptor factor, may simultaneously transduce plural signal such as that from CD40 and RANK to contribute to the formation and maintenance of B-cell follicles. However, the double deficient mice: *RANKL*$^{-/-}$*CD40*$^{-/-}$ mice do not show any disruption of B-cell follicles (data not shown). These results suggest that the formation and/or maintenance of B-cell follicles engaged by TRAF6-dependent signal pathway should be independent from the CD40L/CD40 and RANKL/RANK signal pathway.

Expression of the other molecules in FDCs (at least in mRNA level; data not shown), such as the TLR/IL-1R family (Cao Z et al., 1996; Lomaga MA et al., 1999), p75NGFR (Khursigara G et al., 1999), BCMA (Shu HB et al., 2000), IL-17R (Schwandner R et al., 2000), XEADR, TROY (Naito A et al., 2002), IL-18 receptor (Ito H et al., 2006), IL-25 receptor (Maezawa Y et al., 2006), which use TRAF6 as a signaling transducer, have been reported to be dispensable for the formation of B-cell follicles alone. However, as mentioned above, TRAF6, acting as an adaptor factor, may simultaneously transduce plural signal to contribute to the formation of B-cell follicles. Further experiments are required to determine the receptors and ligands in the upstream of TRAF6 for the formation of B-cell follicles.

Although TRAF6 has been reported to be involved in the classical NF-κB signal pathway (Inoue J et al., 2000; Inoue J et al., 2007) but not in the LtβR-engaged non-classical NF-κB signal pathway (as described above), TRAF6 is known to interact with NIK, at least *in vitro* (Song HY et al., 1997). Therefore, TRAF6 may transduce signals through the non-classical NF-κB signal pathway. TRAF6 regulates the expression of RelB, a factor of the non-classical NF-κB signal pathway (Akiyama T et al., 2005), which is required for the establishment of B-cell follicles (Debra S et al., 2001). TRAF6 can also activate the classical NF-κB signal pathway to induce the expression of B cell lymphoma 3 (Bcl-3) (Ge B et al., 2003). Lack of Bcl-3 in mice results in the disruption of B-cell follicles in secondary lymphoid organs (Poljak L et al., 1999). These findings suggest that TRAF6 may contribute to activate the classical NF-κB signal pathway and/or non-classical NF-κB signal pathway to control the formation of B-cell follicles in secondary lymphoid organs. Further experiments are required to elucidate these questions.

In conclusion, the present study indicate that TRAF6-dependent signal pathway

functions independently from the TNFR1 and LtβR signal pathway, and contribute to the formation and maintenance of B-cell follicles in secondary lymphoid organs through inducing the expression of CXCL13 in follicular stromal cells (FDCs).

Abbreviations

APC, antigen presentation cell;
DC, dendritic cell;
FAE, follicle-associated epithelium;
FBS, fetal bovine serum;
FDC, follicular dendritic cell;
GC, germinal centre;
HEV, high endothelial venule;
ICAM-1, intracellular adhesion molecule 1;
IFR, interfollicular region;
IL-1R, interleukin-1 receptor;
LN, lymph node;
Lt, lymphotoxin;
LtβR, Ltβ receptor;
Madcam-1, mucosal addressin cellular adhesion molecule-1;
MALT, mucosal-associated lymphoid tissue;
MEF, mouse embryonic fibroblast cells;
MMM, marginal metallophilic macrophage;
MZ, marginal zone;
MZM, marginal zone macrophage;
NALT, nasopharyngeal-associated lymphoid tissue;
NIK, NF-κB-inducing kinase;
PALS, periarteriolar lymphoid sheath;
PP, Peyer s patchs;
RANK, receptor activator of NF-κB;
RC, reticular cell;
RP, red pulp;
SED, subepithelial dome;
TLR, Toll-like receptor;
TNF, tumor necrosis factor;

TNFR1, TNF receptor 1;
TRAF6, TNF receptor-associated factor 6;
VCAM-1, vascular cell adhesion molecule 1;
WP, white pulp.

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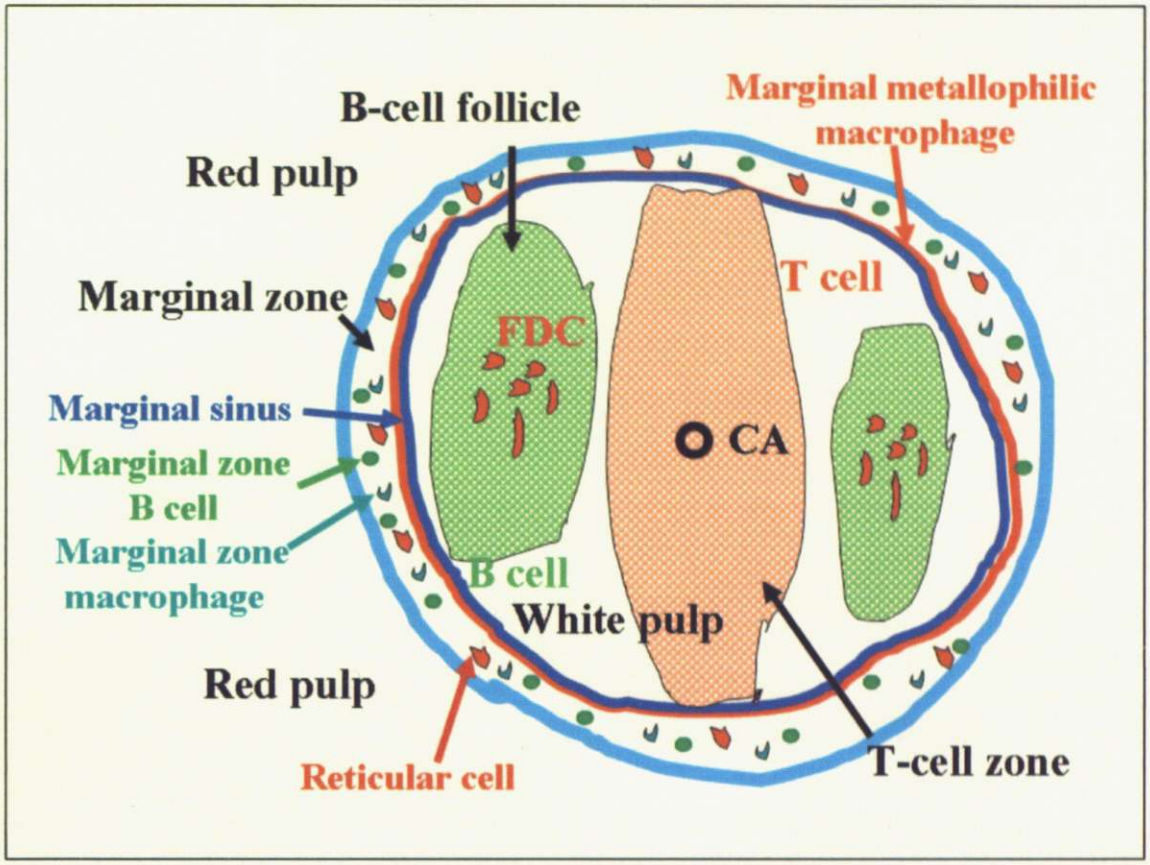

Figure 1. Splenic microarchitectures.

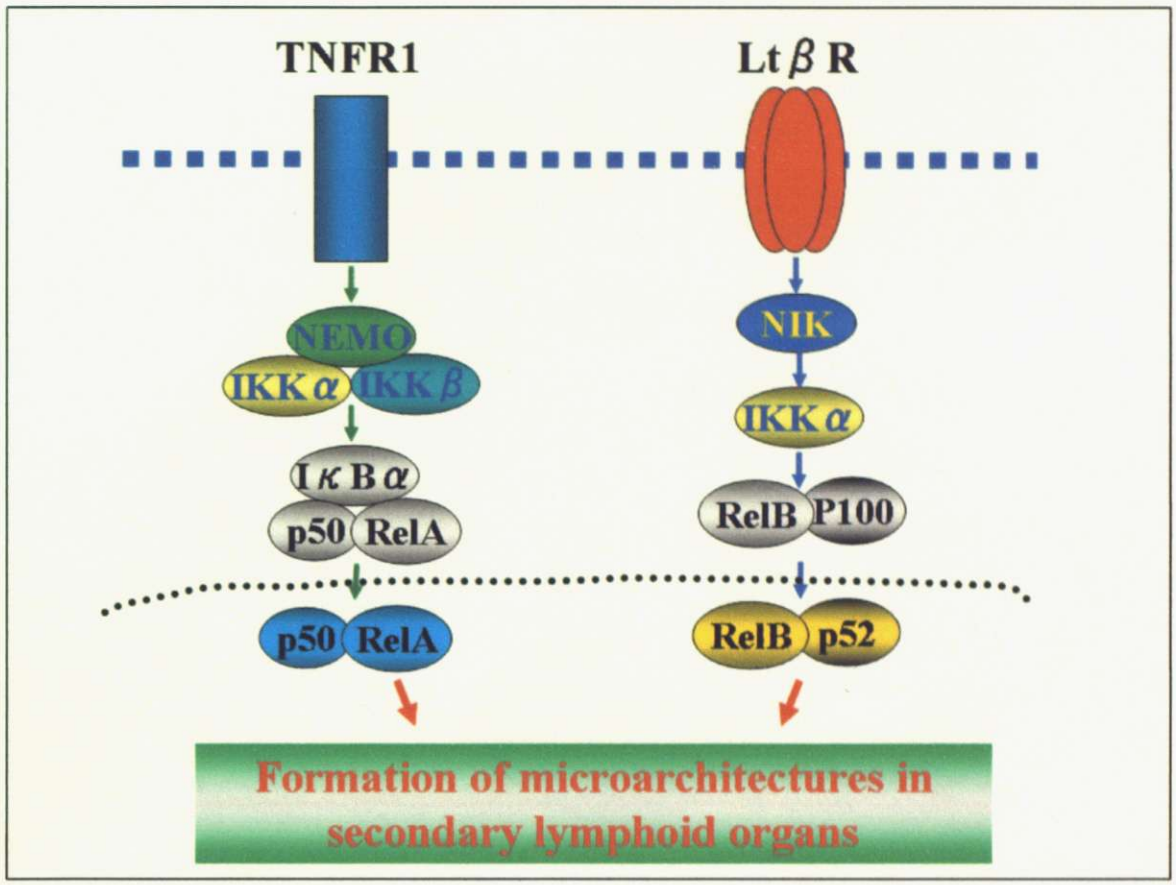

Figure 2. TNFR1 and LtβR signal pathway are required for the establishment of microarchitectures in secondary lymphoid organs.

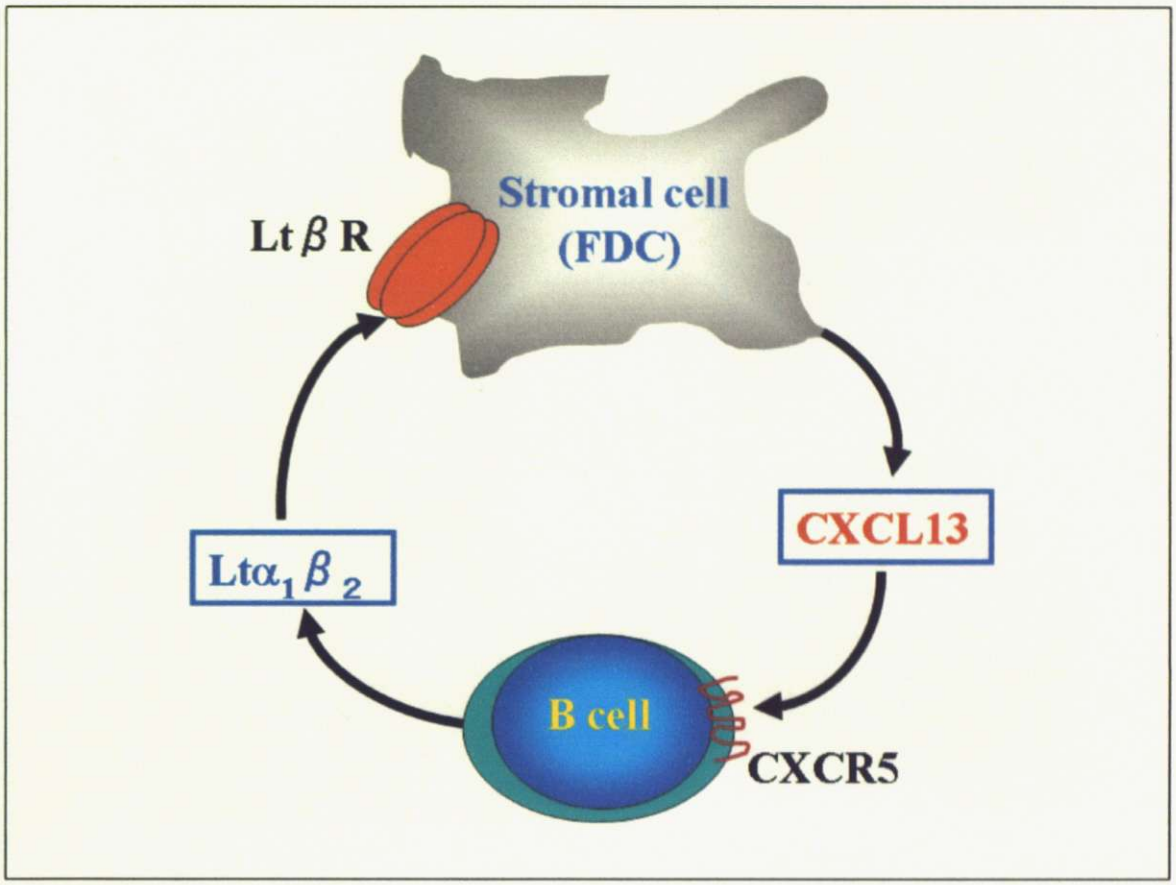

Figure 3. B cell homing positive feed-back loop.
CXCL13 is regulated by the signal from LtβR, the generated CXCL13 attracts B cells around the stromal cells (FDCs) and induces the expression of surface Ltα and Ltβ in B cells, which in turn stimulates the stromal cells (FDCs) to express CXCL13, generating a positive feed-back loop.

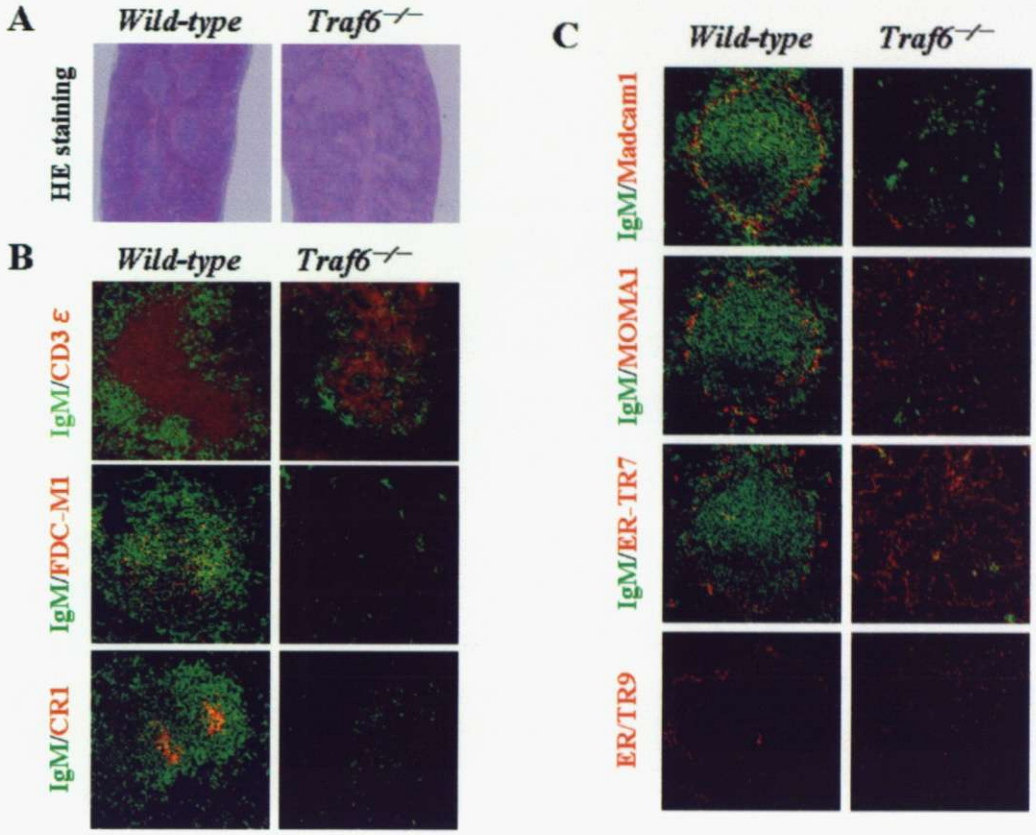

Figure 4. Disruption of splenic microarchitectures in *Traf6*$^{-/-}$ mice.

A. Abnormality of white pulp in spleen of *Traf6*$^{-/-}$ mice. Sections of paraffin-embedded spleen were stained with hematoxylin-eosin (HE) staining. Original magnification, x40.

B. Presence of T-cell zone; disorganization of B-cell follicles, lack of FDCs; reduction in number of B cells in spleen of *Traf6*$^{-/-}$ mice. Cryosections of spleen were stained with specific antibodies: anti-IgM antibody for B cells (green), anti-CD3ε antibody for T cells (red); FDC-M1 and anti-CR1 antibodies for FDC cells (red). Original magnification, x200.

C. Decreased presence of MS; disorganization of MZ in spleen of *Traf6*$^{-/-}$ mice. Cryosections of spleen were stained with specific antibodies: anti-IgM antibody for B cells (green); anti-Madcam-1 antibody for MS (red); MOMA1 antibody for MMM (red); ER-TR7 antibody for RC (red) and ER-TR9 antibody for MZM (red). Original magnification, x200.

Data are representative of 3 independent analyses.

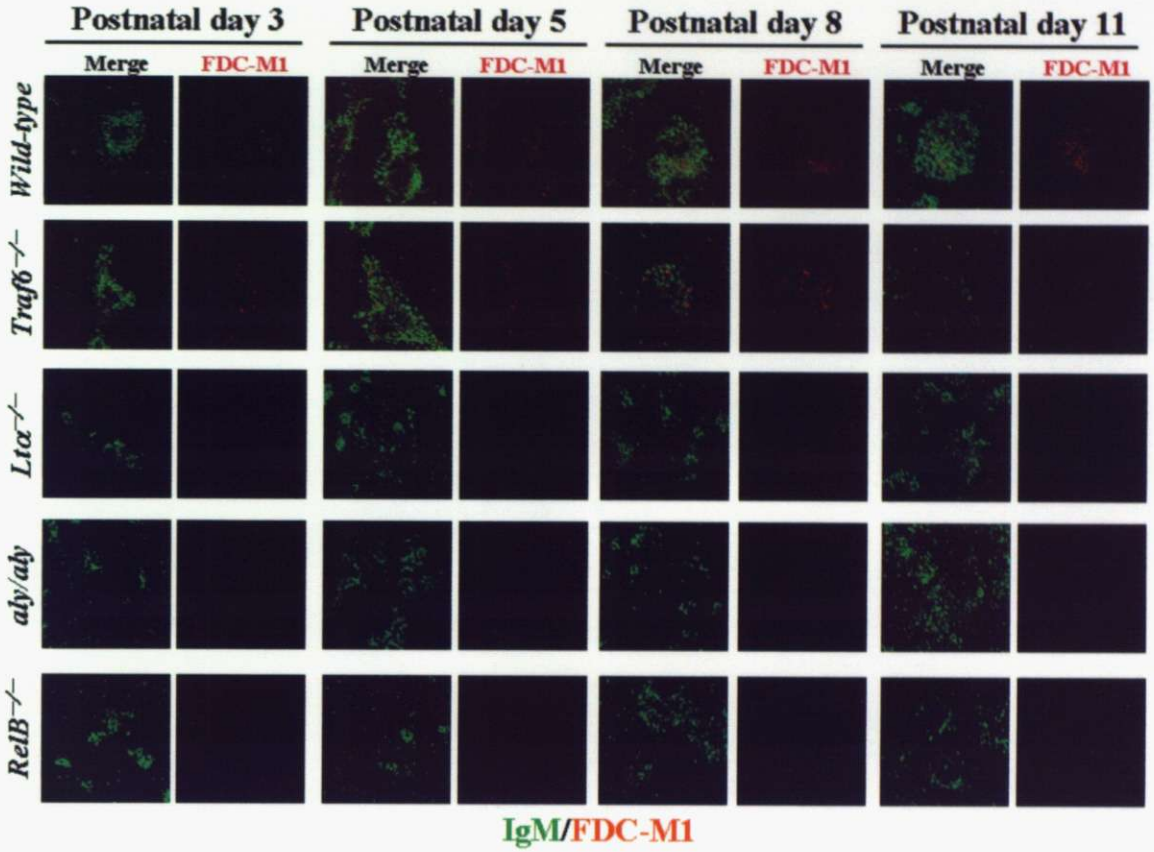

Figure 5. TRAF6 is indispensable for the formation and maintenance stage of B-cell follicles rather than for the formation stage of B-cell clusters, whereas LtβR signal pathway is essential for the formation stage of B-cell clusters.

In spleen of wild-type mice, B-cell clusters with FDCs were detected from postnatal day 3 and the B-cell follicles were initially formed at postnatal day 8. In spleen of *Traf6*$^{-/-}$ mice, the formation of B-cell clusters with FDCs was similar to that of the wild-type mice until postnatal day 8. At postnatal day 8, the slightly disturbed B-cell follicle-like structures were observed, whereas the B-cell follicles almost completely disappeared at postnatal day 11. In this formation processes of B-cell follicles, B-cell clusters and B-cell follicle-like structures with FDCs were never detected in spleen of *Lta*$^{-/-}$ mice, *aly/aly* mice and *RelB*$^{-/-}$ mice. Cryosections of spleen from wild-type mice, *Traf6*$^{-/-}$ mice, *Lta*$^{-/-}$ mice, *aly/aly* mice, and *RelB*$^{-/-}$ mice at postnatal day 3, 5, 8 and day 11 were stained with anti-IgM (green) and FDC-M1 (red) antibodies. Original magnification, x200.

Data are representative of 3 independent analyses.

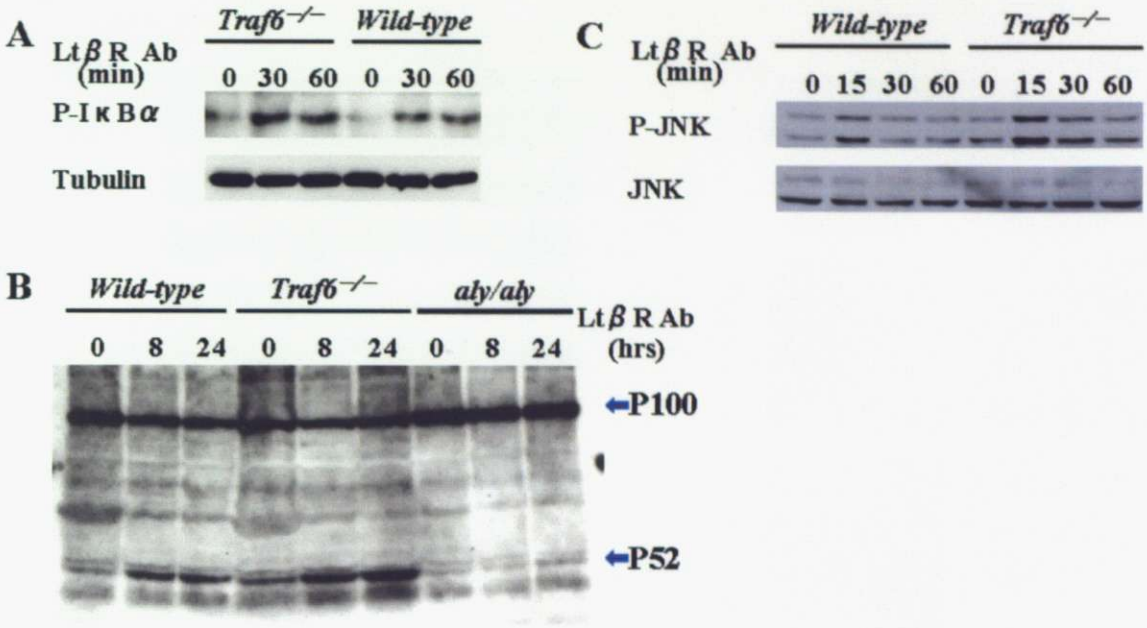

Figure 6. TRAF6 is not involved in the LtβR signal pathway.
Comparable expression of phosphorylation of IκBα (A), processing of p100 to p52 (B) and phosphorylation of JNK (C) induced by LtβR antibody ligation were detected in *Traf6*$^{-/-}$ MEF cells. MEFs derived from wild-type, *Traf6*$^{-/-}$ and *aly/aly* mice were treated with agonistic LtβR antibody for defined times. Total cell lysates were analyzed by western blotting with a phosphor-IκBα specific antibody, p100/p52 specific antibody and phosphor-JNK specific antibody. *Aly/aly* MEFs as a negative control.

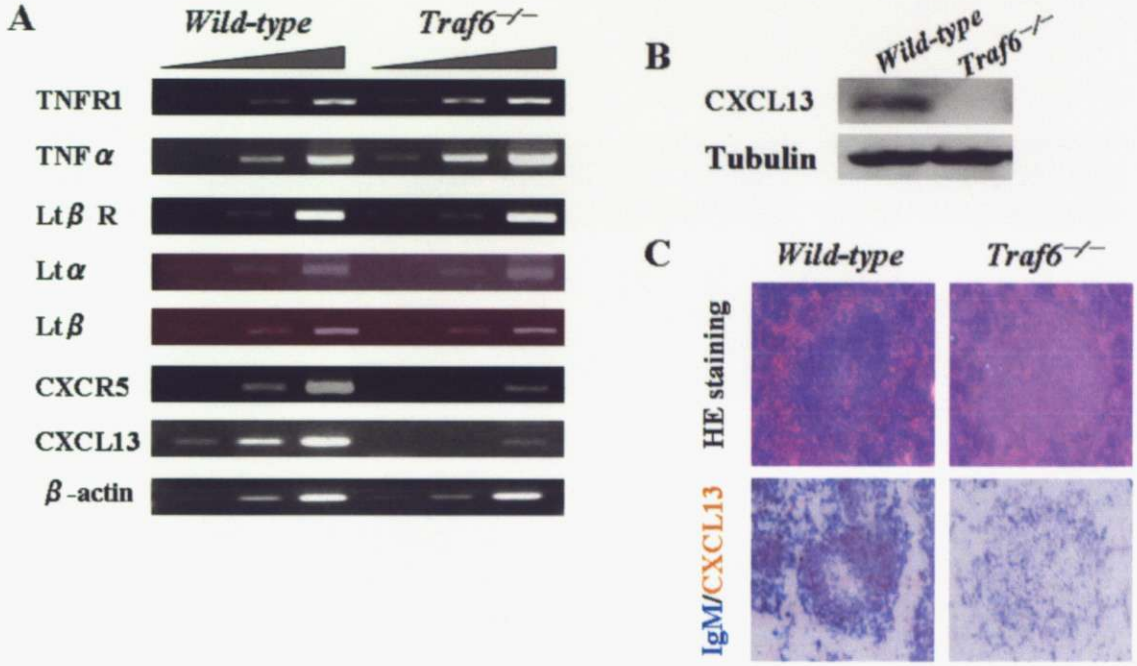

Figure 7. Comparable expression of TNFα and its receptor TNFR1, Lt and its receptor LtβR, and significantly reduced expression of CXCL13 and its receptor CXCR5 in spleen from *Traf6*$^{-/-}$ mice.

A. Total RNA was extracted from spleen of wild-type mice and *Traf6*$^{-/-}$ mice at postnatal day 14 and semi-quantitative RT-PCR analyses (10-fold serially diluted cDNA template) were performed to determine the expression of TNFα, TNFR1, Ltα, Ltβ, LtβR, CXCL13 and CXCR5 in spleen. β-actin as an internal control.

B. Total spleen lysates were obtained from wild-type and *Traf6*$^{-/-}$ mice at postnatal day 12 and western blotting analysis were performed to determine the expression of CXCL13 in spleen. Tubulin as an internal control.

C. Immunohistochemical analyses were performed to detect the expression of CXCL13 in splenic white pulp of wild-type and *Traf6*$^{-/-}$ mice with anti-IgM (blue) and anti-CXCL13 (brown) antibodies (lower row). Consecutive cryosections of spleen were also stained with HE staining (upper row). Original magnification, x100.

Data are representative of 3 independent analyses.

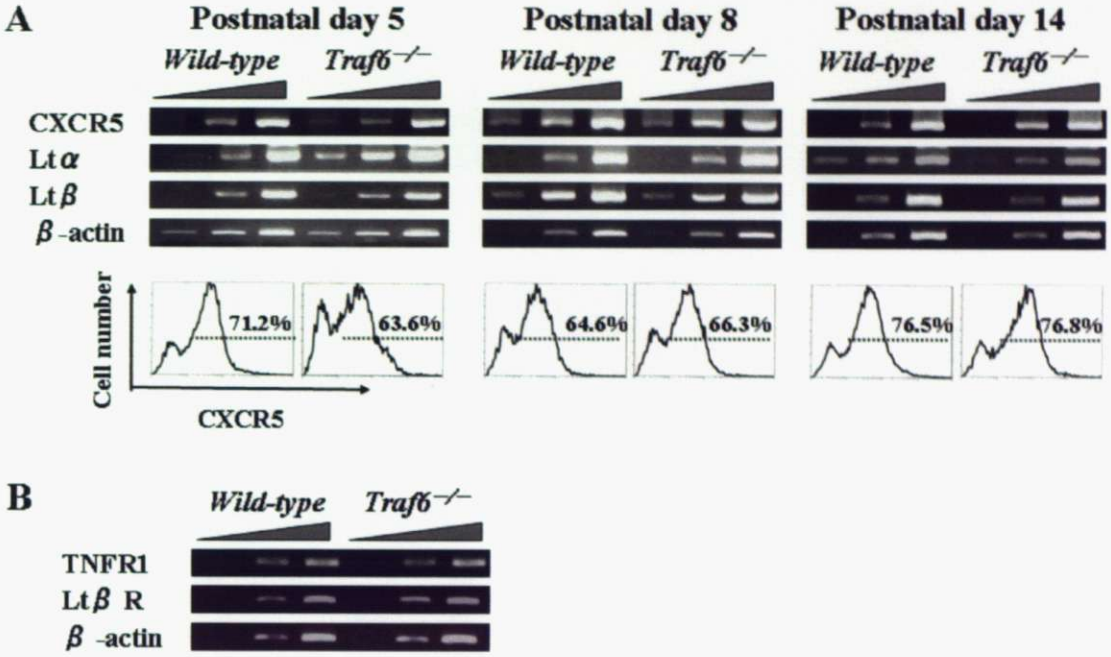

Figure 8. Comparable expression of CXCR5, Ltα, Ltβ in B cells and TNFR1, LtβR in FDC-enriched cells from *Traf6*$^{-/-}$ mice.

A. Total RNA was extracted from IgM-positive B cells (degree of purity were 70~90%) sorted magnetically from spleen of wild-type mice and *Traf6*$^{-/-}$ mice at postnatal day 5, 8, 14 and semi-quantitative RT-PCR analyses (10-fold serially diluted cDNA template) were performed to analyze the expression of CXCR5, Ltα and Ltβ in IgM-positive B cells. β-actin as an internal control. Flow cytometric analyses were performed to detect the expression of CXCR5 in IgM-positive B cells.

B. FDC-enriched cells (degree of purity were 50~70%) were sorted magnetically from the spleen of wild-type and *Traf6*$^{-/-}$ mice at postnatal day 11 and RT-PCR analysis (10-fold serially diluted cDNA template) was performed to determine the expression of TNFR1 and LtβR in FDC-enriched cells. β-actin as an internal control.

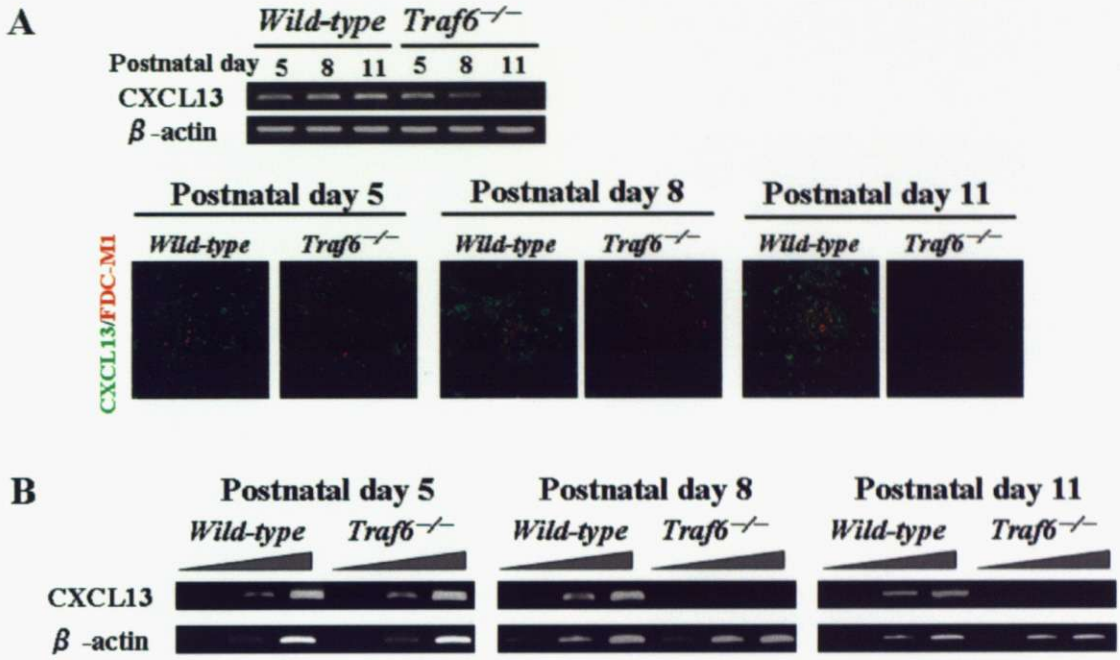

Figure 9. Developmentally decreased expression of CXCL13 in spleen and FDC-enriched cells from *Traf6*$^{-/-}$ mice.

A. Kinetics expression of CXCL13 in spleen of wild-type and *Traf6*$^{-/-}$ mice during the formation periods of B-cell follicles. Total RNA were extracted from the spleen of wild-type and *Traf6*$^{-/-}$ mice at postnatal day 5, 8, 11 and RT-PCR analysis (10-fold serially diluted cDNA template) was performed to determine the expression of CXCL13 in spleen. β-actin as an internal control. Immunostaining were performed to detect the expression of CXCL13 in spleen of wild-type and *Traf6*$^{-/-}$ mice at indicated ages with FDC-M1 (red) and anti-CXCL13 (green) antibodies. Original magnification, x200.

B. Developmentally reduced expression of CXCL13 in FDC-enriched cells (degree of purity were 50~70%) from *Traf6*$^{-/-}$ mice. FDC-enriched cells were sorted magnetically from the spleen of wild-type and *Traf6*$^{-/-}$ mice at postnatal day 5, 8, 11 and RT-PCR analyses (10-fold serially diluted cDNA template) were performed to determine the expression of CXCL13 in FDC-enriched cells. β-actin as an internal control.

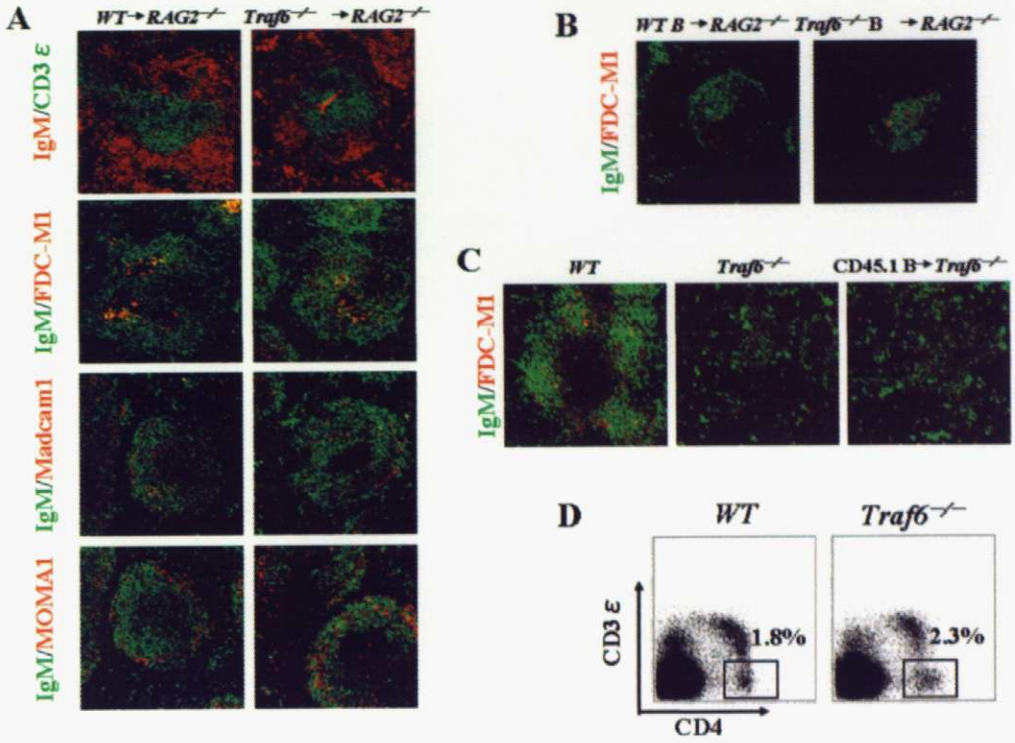

Figure 10. Stromal cells are responsible for the defect of B-cell follicles in *Traf6*$^{-/-}$ mice.

A. Reconstitution of splenic structures with fetal liver cells. Total fetal liver cells of wild-type (WT) and *Traf6*$^{-/-}$ mice at embryonic day 14 were transferred to sublethally irradiated *RAG2*$^{-/-}$ mice. 6~8 weeks later, the sections of spleen from these chimera mice were stained with anti-CD3ε (green), anti-IgM (red, upper row; and/or green), FDC-M1 (red), Madcam-1 (red) and MOMA1 (red) antibodies. Original magnification, x100.

B. Reconstitution of B-cell follicles with B cells. Total B cells (degree of purity was 85~95%) sorted magnetically from spleen of wild-type (WT) and *Traf6*$^{-/-}$ mice at postnatal day 12 were transferred intravenously to *RAG2*$^{-/-}$ mice, 2 weeks later, the sections of spleen from these chimera mice were stained with anti-IgM (green) and FDC-M1 (red) antibodies. Original magnification, x100.

C. Restoration in number of B cells did not regain the disrupted B-cell follicles in spleen of *Traf6*$^{-/-}$ mice. Wild-type (WT) B cells (degree of purity was 90~98%) sorted magnetically from spleen of adult CD45.1 mice were transferred into *Traf6*$^{-/-}$ mice at postnatal day 7, seven days later, the sections of spleen of these chimera mice were stained with anti-IgM (green) and FDC-M1 (red) antibodies. The sections of spleen from wild-type and *Traf6*$^{-/-}$ mice at postnatal day 14 were also stained. Original magnification, x200.

D. Presence of $CD4^+CD3^-$ cells in spleen of *Traf6*$^{-/-}$ mice. Flow cytometric analyses were performed to determine the ratio of $CD4^+CD3^-$ cells in spleen of *Traf6*$^{-/-}$ mice. Dendritic cells were excluded from the analysis.

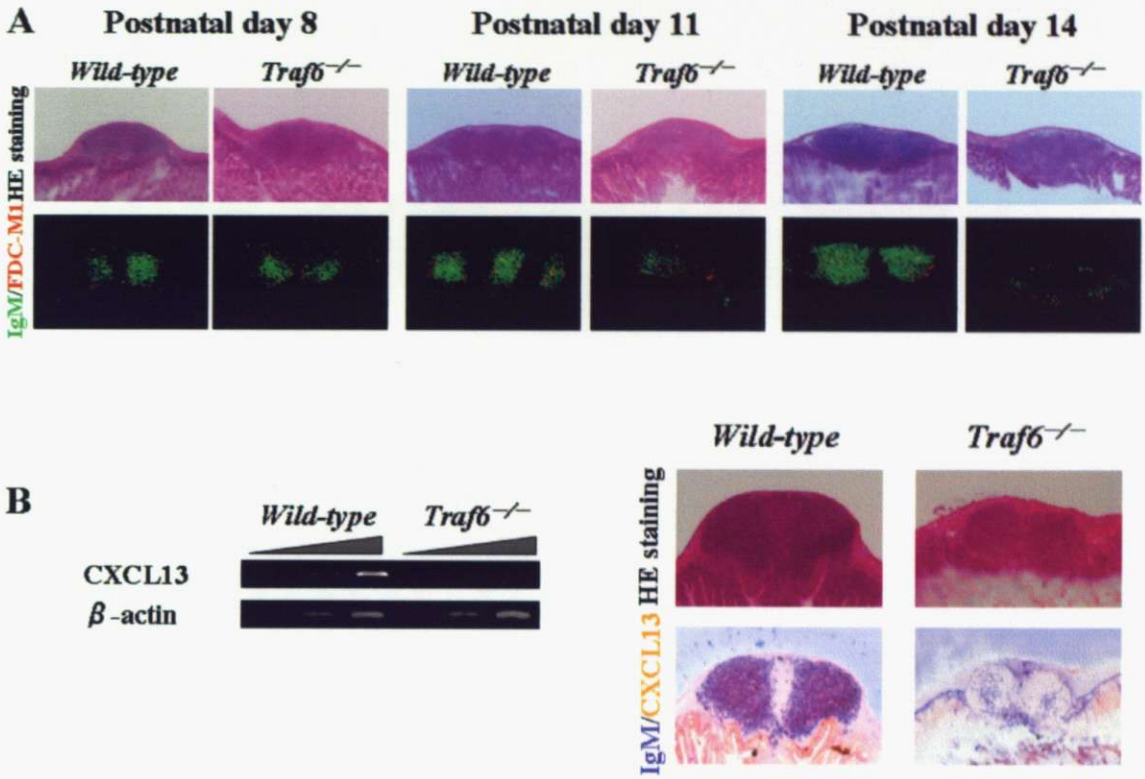

Figure 11. Impaired maintenance of B-cell follicles and decreased expression of CXCL13 in PPs of *Traf6*$^{-/-}$ mice.

A. The development of B-cell follicles in PP of wild-type and *Traf6*$^{-/-}$ mice. PPs were collected as indicated ages. HE staining (upper row) and immunostaining with anti-IgM (green) and FDC-M1 (red) antibodies (lower row) were performed to detect the structure of B-cell follicles. Original magnification, x100.

B. Total RNA were extracted from PPs of wild-type and *Traf6*$^{-/-}$ mice at postnatal day 12 and semi-quantitative RT-PCR analyses (10-fold serially diluted cDNA templates) were performed to detect the expression of CXCL13 in PPs. β-actin as an internal control. Immunostaining was performed to determine the expression of CXCL13 in PPs of wild-type and *Traf6*$^{-/-}$ mice at postnatal day 14 with anti-IgM (blue) and anti-CXCL13 antibodies (brown). Consecutive cryosections of PPs were also stained with HE staining. Original magnification, x100.

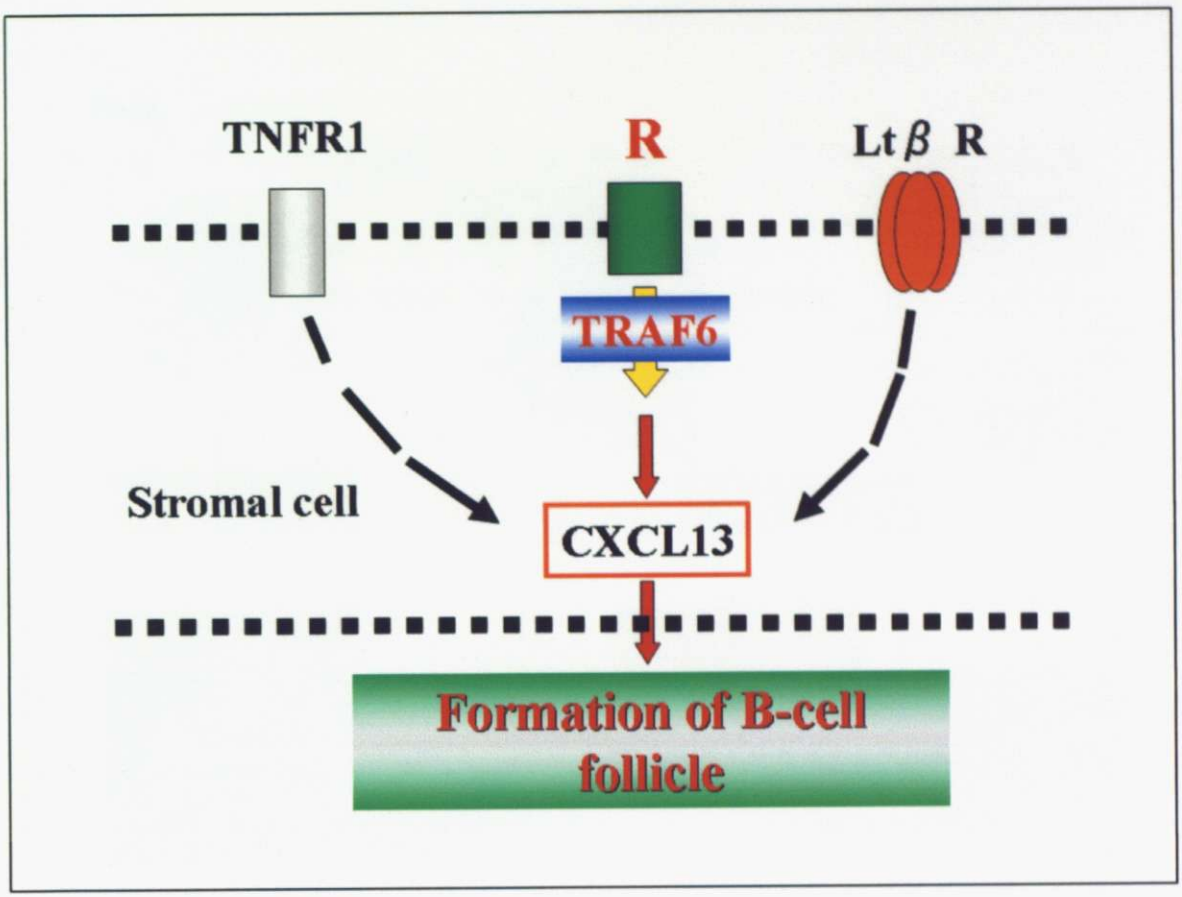

Figure 12. Model for the formation of B-cell follicles in secondary lymphoid organs.

The TNFR1 signal pathway, the LtβR signal pathway, and the TRAF6-dependent signal pathway contribute to the formation of B-cell follicles.

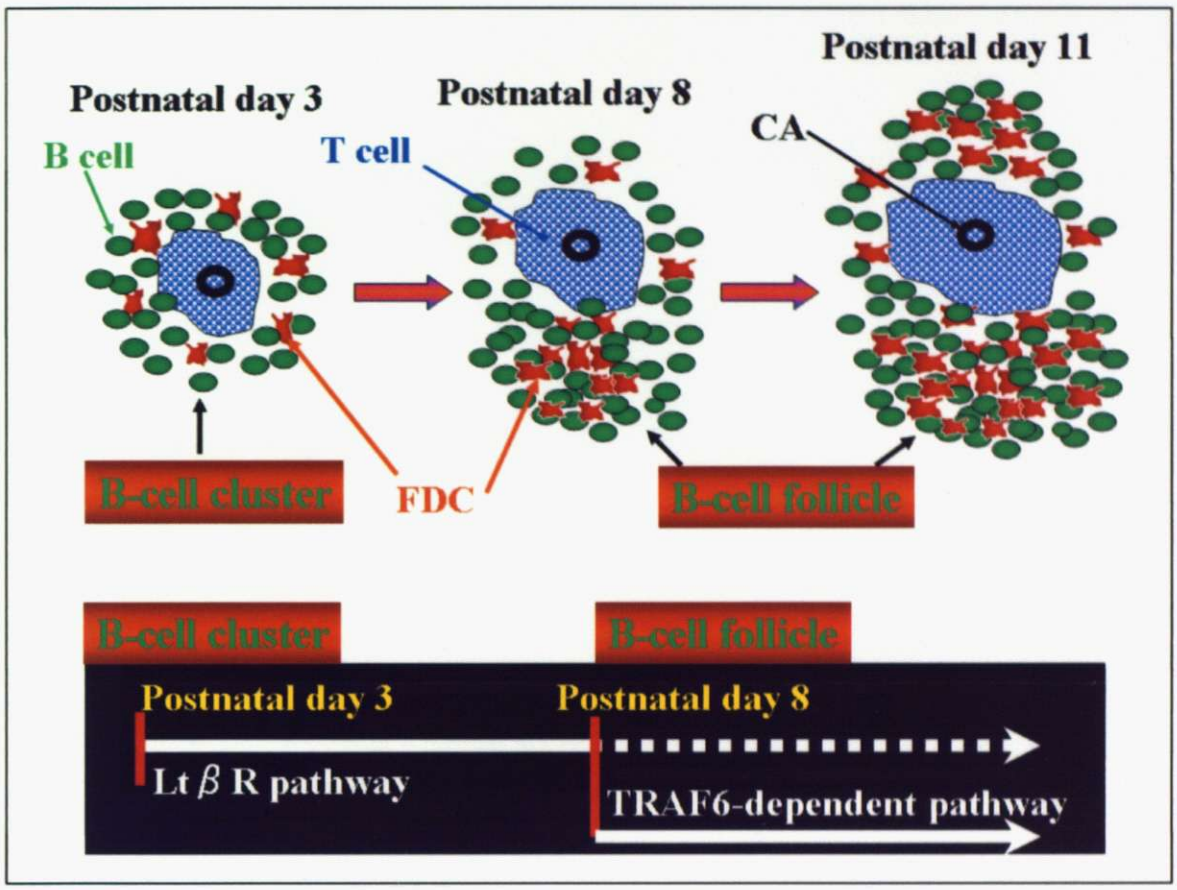

Figure 13. The formation processes of B-cell follicles.
TRAF6-dependent signal pathway is indispensable for the formation and maintenance of B-cell follicles, whereas LtβR signal pathway is required for the formation of B-cell clusters.

Table 1 Primer sequences, melting temperatures, and predicted product lengths

Genes	Primer sequence		Length (base pairs)	Tm (^{0}C)
	Forward primer	Reverse primer		
CXCR5	actacccactaaccctggac	aggtgatgtggatggagaggag	500	57
CXCL13	cagaatgaggctcagcacag	gcatctgaagtgggttgtcac	627	57
Ltα	atgacactgctcggccgtct	ctacagtgcaaaggctccaaa	609	55
Ltβ	ttgttggcagtgcctatcactgtcc	ctcgtgtaccataacgacccgtac	701	55
LtβR	ttatcgcatagaaaaccagacttgc	tcaaagcccagcacaatgtc	228	54
TNFα	atgagcacagaaagcatgatc	ccaaagtagacctgcccggac	691	60
TNFR1	gaacctacttggtgagtgac	cacaacttcatacactcctc	391	54
β-actin	tggaatcctgtggcatccatgaaac	taaaacgcagctcagtaacagtccg	349	54