

## Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin- $\beta$ receptor-IgG1 fusion protein

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**ABSTRACT** Early in ontogeny, the secondary lymphoid organs become populated with numerous cells of mesodermal origin which forms both the lymphoid and stromal elements. The critical receptor/ligand interactions necessary for lymphoid organogenesis to occur are for the most part unknown. Although lymphotoxin- $\alpha$  (LT $\alpha$ ) has been shown to be required for normal lymph node, Peyer's patch, and splenic development, it is unclear if soluble LT $\alpha$ 3, and/or cell-bound lymphotoxin- $\alpha$  $\beta$  (LT $\alpha$  $\beta$ ) mediate these developmental events. Here we report that blocking LT $\alpha$  $\beta$ /lymphotoxin- $\beta$  receptor (LT $\beta$ R) interaction *in vivo* by generating mice which express a soluble LT $\beta$ R-Fc fusion protein driven by the human cytomegalovirus promoter results in an array of anatomic abnormalities affecting both the spleen and Peyer's patches, but not the lymph nodes. These results demonstrate that surface LT $\alpha$  $\beta$  ligand plays a critical role in normal lymphoid organ development.

Tumor necrosis factor (TNF), lymphotoxin  $\alpha$  (LT $\alpha$ ), and lymphotoxin- $\beta$  (LT $\beta$ ) are related cytokines which belong to the TNF ligand family and are encoded by genes clustered within the major histocompatibility complex gene complex (1). Both TNF and LT $\alpha$  self-associate into structurally related homotrimers that bind the same receptors, the p55–60-kDa receptor (type 1 or CD120a; TNFRp60) and the p75–80-kDa receptor (type 2 or CD120b; TNFRp80) (2). TNF is expressed in both cell-bound and soluble forms, while LT $\alpha$ 3 is only produced as a secreted molecule. LT $\beta$ , on the other hand, exists as a membrane-bound heterotrimeric complex in association with LT $\alpha$  forming two complexes, LT $\alpha$ 1 $\beta$ 2, and LT $\alpha$ 2 $\beta$ 1 (1). LT $\alpha$ 1 $\beta$ 2 is the major cell surface complex expressed only on lymphoid cells, and binds the lymphotoxin- $\beta$  receptor (LT $\beta$ R) expressed on nonlymphoid cells (1, 3, 4).

Human and murine TNF and human LT $\alpha$ 3 have been extensively characterized and have been shown to induce many of the same functions. Thus, it has been suggested that these cytokines are redundant. Little is known about the murine LT $\alpha$ 3 and LT $\alpha$  $\beta$  ligands, largely due to the lack of specific reagents. However, recent reports have demonstrated that mice lacking LT $\alpha$  expression by selective gene targeting have disorganized splenic architecture and lack lymph nodes and Peyer's patches, while the thymus is unaffected (5, 6). Since normal lymph node development occurs in TNFRp60- and TNFRp80-deficient mice, or in mice expressing a soluble TNFRp60-Fc transgene, a crucial role for LT $\alpha$  $\beta$  ligand in these processes is likely (7–10). To directly test if surface LT $\alpha$  $\beta$  is required for normal lymphoid organogenesis to occur, we neutralized LT $\alpha$  $\beta$ /LT $\beta$ R interactions *in vivo* by generating mice which constitutively express a soluble murine LT $\beta$ R-human IgG1 (LT $\beta$ R-Fc) transgene, whose expression is driven

by the human cytomegalovirus (CMV) promoter. The human IgG1 Fc component was specifically mutated to inhibit FcR binding and complement fixation. Our results demonstrate that surface LT $\alpha$  $\beta$  is a crucial ligand for normal splenic and Peyer's patch development.

### MATERIALS AND METHODS

**Plasmid Construction.** To generate soluble LT $\beta$ R-Fc chimeric protein, the extracellular domain of murine LT $\beta$ R and a mutated human IgG1 Fc (kindly provided by C. Ambrose, Biogen) were used (11). To inhibit FcR binding and complement fixation, the human IgG1 Fc was mutated at the CH2 domain (L234A, L235E, G237A, and P331S) (12–14). The extracellular domain of the LT $\beta$ R and the mutated hIgG1 were isolated as a 0.69-kb *NotI/SalI* fragment, and a 0.67-kb *SalI/NotI* fragment, respectively. The two DNA fragments were then ligated into the *NotI* site of the CA116 vector containing the human CMV promoter (–553 to +71) (CA116, a derivative of SAB132 was provided by C. Ambrose) (15). The CMV-LT $\beta$ R-Fc transgene was then isolated as an *EcoRI/HindIII* fragment, and purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA) following manufacturer's instructions.

**Transgenic Mouse Production and Detection.** The purified CMV-LT $\beta$ R-Fc transgene (2–10 ng/ $\mu$ l) was microinjected into BALB/c-fertilized oocytes from FVB mice, and implanted into pseudopregnant (BALB/c  $\times$  129)F<sub>1</sub> mice in a Stanford transgenic mouse facility. All mice were bred and maintained at the Stanford University animal facility under barrier isolation conditions. Genomic DNA was isolated from mice tails biopsied at 3 weeks of age, and offspring containing the integrated transgene were identified by Southern blot analysis using *PstI* digested genomic DNA. Soluble chimeric protein in the sera was quantitated by ELISA: 96-well polyvinyl microtiter dishes were coated with 10  $\mu$ g/ml of purified goat anti-human antibody (Sigma), the sera were then titered onto the treated plates and detected with alkaline phosphate-conjugated goat anti-human Fc polyclonal antibody (Caltag, South San Francisco, CA) and substrate [*p*-nitrophenyl phosphate (PNPP) in DEA buffer]. Optical density was quantified at 420 nm. Transgene positive mice were then crossed to BALB/c mice to establish founder lines, from which heterozygous mice were intercrossed.

**Immunohistology.** All mice, from two separate founder lines (1610 and 201), were weighed and spleens, lymph nodes, Peyer's patches, and thymi were removed from age and sex matched transgenic-positive and -negative mice. When trans-

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Abbreviations: CMV, cytomegalovirus; LT $\alpha$ , lymphotoxin- $\alpha$ ; LT $\alpha$  $\beta$ , lymphotoxin- $\alpha$  $\beta$ ; LT $\beta$ , lymphotoxin- $\beta$ ; LT $\beta$ R, lymphotoxin- $\beta$  receptor; PALS, peri-arteriolar-lymphoid sheath; TNF, tumor necrosis factor; TNFRp60, TNF receptor-p60; TNFRp80, TNF receptor-p80. ||To whom reprint requests should be addressed.

genic-negative littermates were not available, age and sex matched BALB/c mice were used as control mice. Lymphoid organs were excised and either: fixed in formalin, embedded in paraffin, and stained with hematoxylin/eosin or embedded in O.C.T. (Miles) compound and frozen in liquid nitrogen. Frozen tissue sections were fixed in ice-cold acetone for 10 min and double stained for T- and B-cell surface antigens with phycoerythrin-conjugated anti-CD4 (PharMingen) and biotinylated anti-B220 (PharMingen), followed by fluorescein-conjugated avidin (Neutralite, Southern Biotechnology Associates). The combined T- and B-cell images were digitized and processed as described (16). Frozen tissue sections were also stained with antibodies specific for reticular fibroblasts (ER-TR7), marginal zone macrophages (ER-TR9), marginal zone metallophilic macrophages (MOMA-1), or MAdCAM-1 vascular addressin (MECA367) as described (17). The antibodies were detected with anti-rat immunoglobulin-conjugated with horseradish peroxidase (Dako), followed by incubation with diaminobenzidine (Sigma). In data not shown, mesenteric lymph nodes were stained with antibodies to MAdCAM-1 vascular addressin on high endothelial venules (MECA367), and macrophages (ER-TR9, and MOMA-1), and thymi were stained with antibodies specific for cortical epithelium (ER-TR4), medullary epithelium (ER-TR5), fibroblasts (ER-TR7), and dendritic cells (N418) (18–20).

**Specificity and Bioactivity of the LT $\beta$ R–Fc Fusion Protein.** The capacity of the LT $\beta$ R–Fc fusion protein present in the sera to neutralize LT $\alpha\beta$ -mediated cytotoxicity was determined with the murine WEHI-164 fibrosarcoma cycloheximide (CHX) methyl tetrazolium salt (MTT) cytotoxicity assay as described (21). Briefly,  $1 \times 10^4$  cells were incubated overnight in 96-well plates. Titrations of purified LT $\alpha\beta$  were added to the cells in the presence of 10  $\mu$ g/ml CHX and either 1% normal mouse sera (NMS), 1% sera isolated from the transgenic-positive mice, or 10 ng/ml of purified control LT $\beta$ R–Fc fusion protein in the presence of 1% NMS. Cell viability was determined 16 hr later by MTT (Sigma). Relative inhibition was determined by the concentration of LT $\alpha\beta$  ligand which was required for 50% cytolysis of the target population in the presence of 1% serum from transgenic-positive mice relative to the amount of ligand which gave 50% cytolysis in the presence of 1% NMS. Specificity of the LT $\beta$ R–Fc transgene was determined by the inability of 1% sera from transgenic-positive mice to inhibit either TNF or LT $\alpha$ -mediated killing of the WEHI-164 cells. The recombinant forms of murine LT $\alpha$  and murine LT $\alpha\beta$  were prepared as described for the human ligands and shown to signal through the TNFRp60 and LT $\beta$ R, respectively, in this assay system (unpublished data).

## RESULTS

To test the role of surface LT $\alpha\beta$  in normal lymphoid development and function, we blocked LT $\alpha\beta$ /LT $\beta$ R interactions *in*

*vivo* by generating mice which constitutively express a soluble murine LT $\beta$ R–human IgG1 (Fc) transgene driven by the CMV promoter. The Fc portion of the transgene was specifically mutated to inhibit FcR binding and complement fixation to avoid depletion of LT $\alpha\beta$ -expressing cells *in vivo*. The lack of FcR interactions by the mutated IgG1 chimeric protein were previously verified in FcR binding assays (22). The LT $\beta$ R–Fc chimeric construct was put under the control of the CMV promoter to achieve systemic expression of the soluble fusion protein *in vivo*. The CMV promoter has been reported to be transcriptionally active early in ontogeny and expressed in most organs including thymus, spleen, bone marrow, and stomach (23, 24).

The purified LT $\beta$ R–Fc fusion construct was microinjected into BALB/c-fertilized FVB oocytes, and mice with an integrated transgene were identified by Southern blot analysis. Soluble chimeric protein activity in the sera was quantitated by ELISA as described in *Materials and Methods*. From 11 mice which integrated the LT $\beta$ R–Fc transgene, 4 separate founder lines were established, 2 of which expressed high circulating levels of the fusion protein in the sera ( $\approx 1.5$   $\mu$ g/ml), and 2 of which expressed low serum levels ( $\approx 50$ –100 ng/ml). The level of soluble fusion protein in the sera detected by ELISA correlated directly with the ability of the circulating chimeric protein to specifically inhibit LT $\alpha\beta$ - but not LT $\alpha$ 3- or TNF- $\alpha$ -mediated killing of WEHI-164 cells (Table 1). Offspring derived from the founders which expressed low serum levels of the fusion protein, consistently also expressed low circulating levels. Conversely, mice derived from founders which expressed high transgene levels were found to have a large variation in circulating LT $\beta$ R–Fc fusion protein (0–1.5  $\mu$ g/ml). Heterozygous mice expressing the highest levels of the LT $\beta$ R–Fc fusion protein were intercrossed to obtain mice which expressed up to 6.5  $\mu$ g/ml of the chimeric protein in the sera. However, circulating LT $\beta$ R–Fc fusion protein did not reach neutralizing levels (based on *in vitro* assays) until 3 days after birth; newborn pups had undetectable fusion protein in their sera. Circulating LT $\beta$ R–Fc fusion protein dropped from high to almost undetectable levels during pregnancy. Thus, placental transfer of the LT $\beta$ R–Fc protein was presumably not sufficient to neutralize surface LT $\alpha\beta$  ligand in the developing fetus.

Although mice which expressed low levels of the soluble LT $\beta$ R–Fc fusion protein appeared phenotypically and histologically normal, correlating with the low ability of the sera to neutralize LT $\alpha\beta$  activity *in vitro* (Table 1), mice which expressed high levels of the chimeric protein had many immunologic abnormalities. Offspring derived from the same high-expressor founder line had a large variation in transgene product expression, resulting in wide phenotypic variation. Many of the mice expressing high circulating LT $\beta$ R–Fc protein levels had reduced body and spleen weight compared with

Table 1. Specificity and dose-dependency of the LT $\beta$ R–Fc chimeric fusion protein

Mouse*	Tg†	LT $\beta$ R–Fc‡, $\mu$ g/ml	Relative inhibition of cytokine cytotoxicity§			Body, g	Spleen, g	Thymus, g	Peyer's patches	
			LT $\alpha\beta$	LT $\alpha$	TNF				n	cm <sup>2</sup>
1610.20¶	+	0.05	1.1	<1	<1	19.5	0.093	0.128	11	0.35
1610.30	+	0.10	1.6	<1	<1	20.3	0.084	0.097	8	0.31
1610.1	+	0.50	6.7	<1	<1	19.8	0.078	0.121	10	0.15
1610.3	+	0.50	5.8	<1	<1	17.1	0.055	0.089	7	0.16
1610.10	+	1.25	18.3	<1	<1	17.8	0.055	0.084	5	0.09
1610.2	+	1.30	19.2	<1	<1	14.5	0.049	0.113	5	0.07

\*Mice were 6-week-old female littermates born from heterozygous parents.

†Expression of the LT $\beta$ R–Fc transgene was determined by Southern blot analysis.

‡Concentration of the soluble LT $\beta$ R–Fc fusion protein in the sera was determined by ELISA.

§Determined by the ability of 1% sera to inhibit either LT $\alpha\beta$ -, LT $\alpha$ -, or TNF-mediated killing of WEHI-164 cells; 10 ng/ml of purified murine LT $\beta$ R–Fc fusion protein in 1% NMS was able to inhibit LT $\alpha\beta$ -killing by 19.2-fold.

¶Similar values were observed with age- and sex-matched nontransgenic mice.

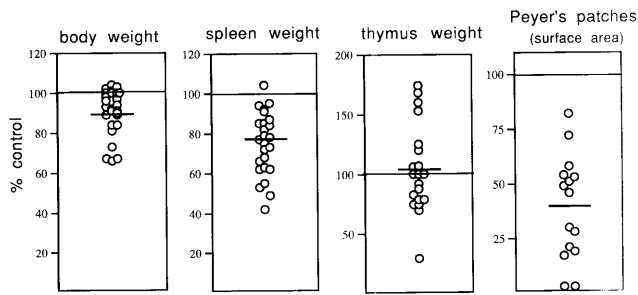


FIG. 1. Reduced size of spleen and Peyer's patches in mice expressing a soluble  $LT\beta R$ -Fc transgene. Three- to 15-week-old mice expressing high levels of  $LT\beta R$ -Fc transgene product in the sera ( $0.5$ – $6.5 \mu\text{g/ml}$ ) from two separate founder lines were weighed and sacrificed, and the weight of the thymi and spleens was determined. Small intestine Peyer's patches were counted, and the serosal surface area was determined. The percent control was determined by directly comparing sex-matched nontransgenic littermates to transgene-positive mice. Horizontal lines represent the group averages, and the mean  $\pm$  the SD are as follows: body weight,  $91\% \pm 11.0$  (40 mice from 10 separate litters); spleen weight,  $75\% \pm 15.7$  (35 mice from 9 separate litters); thymus weight,  $103\% \pm 35.2$  (34 mice from 8 separate litters); and Peyer's patch area,  $39\% \pm 23.9$  (23 mice from 6 separate litters).

their nontransgenic littermates. Peyer's patches were reduced in size or totally absent, and large variations in thymus size were observed (Fig. 1). The reduction of spleen weight and Peyer's patch size correlated directly with circulating  $LT\beta R$ -Fc protein levels in most mice examined. Conversely, thymus size and transgene expression appeared not to be correlated (Table 1). All transgenic mice with low body weight had high serum levels of the  $LT\beta R$ -Fc chimeric protein,

however, not all mice with high circulating  $LT\beta R$ -Fc levels had low body weight.

Unexpectedly, lymph nodes were present in all mice examined, and appeared phenotypically normal with respect to T cell and B cell surface antigens (data not shown). Circulating  $LT\beta R$ -Fc transgene levels were undetectable at birth, and did not reach neutralizing levels until after day 3 of life (data not shown). Rennert *et al.* (16) have recently demonstrated that soluble  $LT\beta R$ -Fc was able to inhibit lymph node development in newborn mice if administered to the pregnant mothers during days 11–16 of gestation; lymph node development occurred normally if the fusion protein was administered after day 16 of gestation. Thus, it appears that lymph node development is occurring in our mice due to late onset expression of the  $LT\beta R$ -Fc transgene.

Expression of the transgene did not appear to affect lymph node or thymic structure. Brachial, axillary and popliteal lymph nodes, stained with hematoxylin/eosin, showed normal architecture in mice expressing both low and high levels of the  $LT\beta R$ -Fc fusion protein (Fig. 2*A* and *B*). Segregation between the T-cell-rich paracortex, and the B-cell-rich primary follicles was also observed in these mice (Fig. 2*C* and *D*). High endothelial venules and macrophages of mesenteric lymph nodes appeared normal, in both their presence and distribution, as determined by immunohistology (data not shown). Even though the Peyer's patches were reduced (or absent) both in size and number in mice expressing high levels of the  $LT\beta R$ -Fc fusion protein, the Peyer's patches which did develop appeared normal, with visible germinal centers (Fig. 2*E* and *F*). Both the medullary and cortical zones of the thymus were unaffected by transgene expression, as demonstrated by hematoxylin/eosin staining and immunohistology using antibodies specific for cortical epithelium, medullary epithelium, dendritic cells, and fibroblasts (data not shown). The ratio of

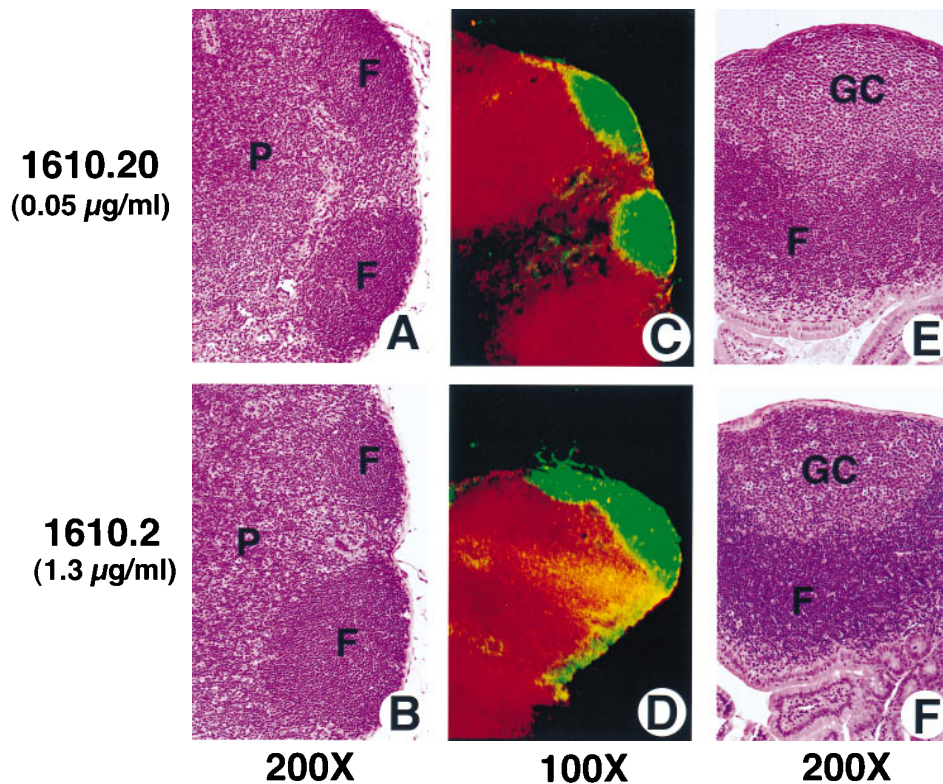


FIG. 2. Normal architecture of lymph nodes and Peyer's patches from  $LT\beta R$ -Fc-expressing mice. Tissue sections of lymph node (*A*–*D*) and Peyer's patch (*E* and *F*) from either low-expressing (*A*, *C*, and *E*), or high-expressing (*B*, *D*, and *F*) 6-week-old female  $LT\beta R$ -Fc transgenic mice (1610.2 and 1610.20 from Table 1). Tissue sections were stained with either hematoxylin/eosin (*A* and *B*, *E* and *F*) ( $\times 200$ ), or with antibodies specific for T-cell (anti-CD4, red) and B-cell (anti-B220, green) antigens (*C* and *D*) ( $\times 100$ ). Yellow staining indicates colocalization of both T and B cells. F, P, and GC indicate the location of the B-cell-rich follicles, the T-cell-rich paracortex, and the germinal centers, respectively.

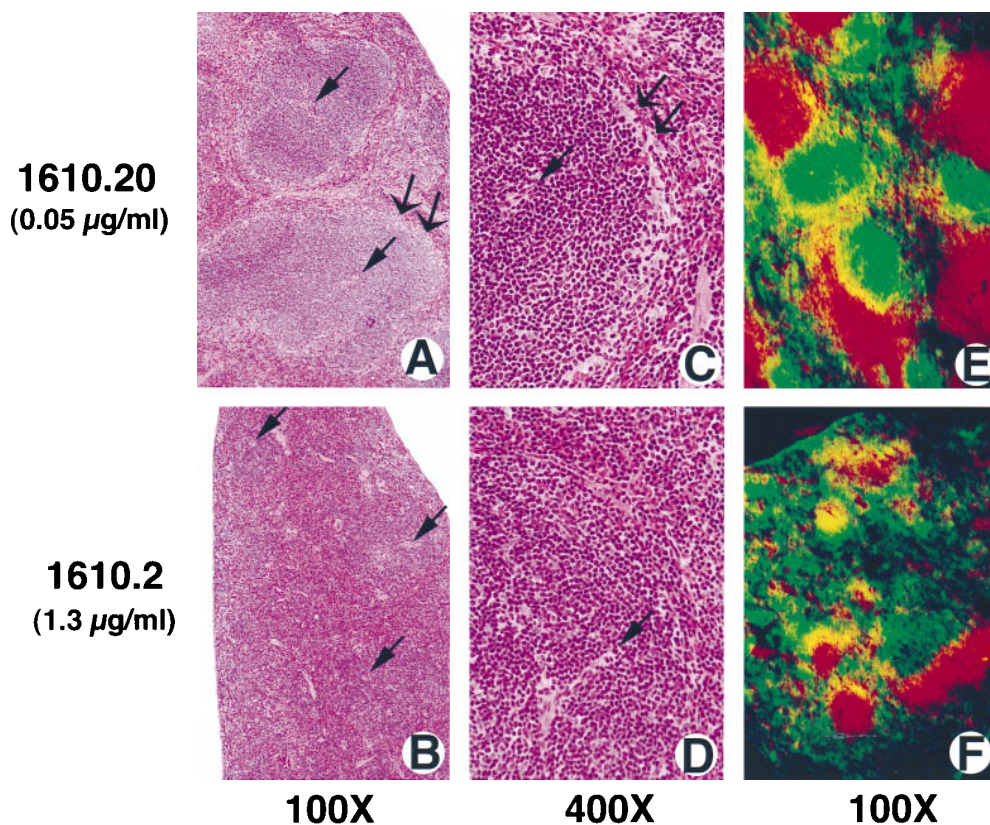


FIG. 3. Blockade of  $LT\alpha\beta/LT\beta R$  interactions results in abnormal splenic architecture. Splenic tissue sections from mice expressing either low (A, C, and E) or high (B, D, and F) levels of the  $LT\beta R$ -Fc transgene, as described in Fig. 2. Formalin-fixed sections were stained with hematoxylin/eosin (A–D). A single arrow represents the location of the central arterioles, and a double arrow defines the marginal zones. Frozen tissue sections were stained with antibodies specific for T-cell (red) and B-cell (green) antigens (E and F), as described in Fig. 2. (A, B, E, and F,  $\times 100$ ; C and D,  $\times 400$ .)

single-positive and double-positive thymocytes isolated from “high-expressing” mice was indistinguishable from that of the transgenic-negative littermates. Blood isolated from these mice was normal with respect to hemoglobin, hematocrit, as well as leukocyte and platelet counts, compared with non-transgenic littermates. Bone marrow also appeared phenotypically normal.

However, histologic and immunohistologic analysis of mice from 13 separate litters, derived from two separate founder lines (1610 and 201), showed a specific and dose-dependent effect of  $LT\beta R$ -Fc transgene expression on splenic development. In all mice expressing high serum levels of the  $LT\beta R$ -Fc fusion protein (0.5–6.5  $\mu\text{g/ml}$ ), a dramatic disruption of splenic architecture was observed. As circulating  $LT\beta R$ -Fc expression increased, a progressive loss of splenic marginal zones, as well as T-cell/B-cell organization were found. Specifically, hematoxylin/eosin stained tissue sections showed that in mice expressing high, but not low, levels of the  $LT\beta R$ -Fc transgene, the total volume of the white pulp was decreased, with condensation of the peri-arteriolar-lymphoid sheath (PALS) (Fig. 3A–D). In frozen tissue sections of mice from three separate litters, T-cell and B-cell areas were disrupted, with no clear separation between the B-cell-rich lymphoid follicles and the T-cell-rich PALS (Fig. 3F). Clear segregation between the T-cell and B-cell areas in spleens from mice expressing low or no circulating  $LT\beta R$ -Fc protein was observed (Fig. 3E, and data not shown). The absence of the marginal zone surrounding the white pulp was most striking, especially seen at high magnification (Fig. 3D). Moreover, immunohistochemistry with antibodies specific for reticular fibroblasts (Fig. 4A and B), marginal zone macrophages (Fig. 4C and D), marginal zone metallophilic macrophages (Fig. 4E and F), and MAdCAM-1 vascular addressin (MECA367:

data not shown) confirmed the lack of the marginal zones. The absence of a reticular fibroblast network demonstrates that even the stromal elements of the marginal zone have not developed normally. However, the spleen was found to be phenotypically normal with respect to the percentage of cells expressing B- and T-cell surface antigens (data not shown).

## DISCUSSION

$LT\alpha$  has clearly been shown to be required for peripheral lymphoid development (5, 6). Furthermore, it has been demonstrated that surface  $LT\alpha\beta$  interactions during days 11–16 of gestation are critical for normal lymph node development. Splenic T- and B-cell organization and Peyer’s patch development seem to require  $LT\alpha\beta$  interactions after day 17 of gestation (16). Consistent with this observation, our data demonstrate that high expression of the  $LT\beta R$ -Fc fusion protein after 3 days of life resulted in normal lymph node development; however, splenic and Peyer’s patch development were dramatically affected. Thus, this suggests that different secondary lymphoid organs require  $LT\alpha\beta/LT\beta R$  interactions at different times during development. Alternatively, different lymphoid organs may require distinct combinations of  $LT\alpha\beta$ - and  $LT\beta R$ -expressing lymphoid and stromal elements for organogenesis to occur.

The splenic marginal zone is composed of a network of reticular cells, predominantly containing marginal zone macrophages and resident  $IgM^+$ ,  $IgD^-$  B lymphocytes, surrounding the white pulp, and separating it from the red pulp. Presumably, many activated  $LT\alpha\beta$ -expressing T and B cells pass through the marginal zone, trafficking to the PALS, and the follicles, respectively. The mechanism of this migration is unknown; however, it is believed that various types of marginal

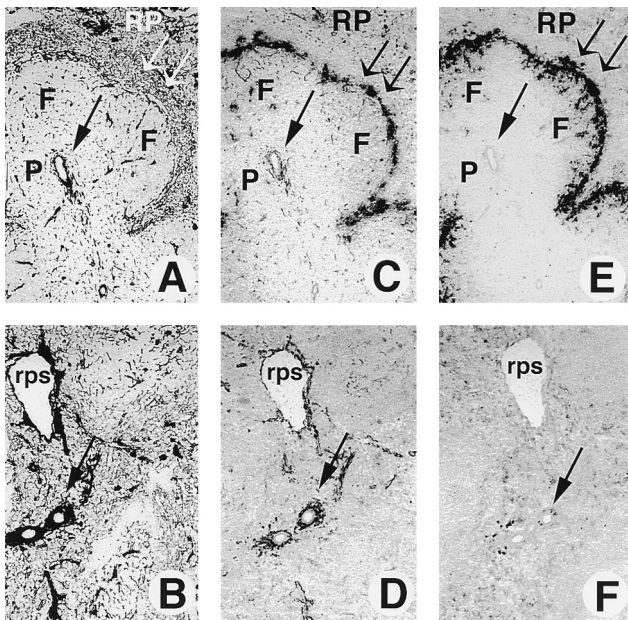


FIG. 4. Absence of splenic marginal zones in mice expressing a soluble  $LT\beta R$ -Fc chimeric fusion protein. Frozen tissue sections of spleen from 10-week-old female mice expressing high levels of the  $LT\beta R$ -Fc protein (2.5  $\mu\text{g}/\text{ml}$ , from founder 201) (B, D, and F) or transgenic-negative mice (A, C, and E) were stained with antibodies specific for reticular fibroblasts with ER-TR7 (A and B), marginal zone macrophages with ER-TR9 (C and D), or marginal zone metallophilic macrophages with MOMA-1 (E and F), as described. A single arrow represents the location of the central arterioles, and a double arrow defines the marginal zones. F, P, RP, and rps shows the location of the B-cell-rich follicles, the T-cell-rich PALS, the red pulp, and the red pulp sinus, respectively. ( $\times 125$ .)

zone macrophages play a role (19). A specific dose-dependent effect of the  $LT\beta R$ -Fc chimeric protein was observed on splenic marginal zone development and T-cell/B-cell organization. Moreover, immunohistochemistry using monoclonal antibodies specific for marginal zone macrophages and reticular fibroblasts in conjunction with routine histology clearly demonstrated the absence of distinct marginal zones in spleens from mice expressing high levels of the  $LT\beta R$ -Fc fusion protein. Thus, blockade of  $LT\alpha\beta/LT\beta R$  interactions does not simply result in a down regulation of adhesion molecules by marginal zone cells, but results in the absence of the distinct populations of cells which make up the marginal zone. It is unclear if the lack of splenic marginal zones is directly responsible for the loss of splenic T-cell/B-cell organization in the mice expressing the  $LT\beta R$ -Fc chimeric protein. Alternatively,

Table 2. Effects of TNF,  $LT\alpha$ , and  $LT\beta$  on lymphoid development

	Mice		
	$LT\alpha^{-/-}$	$TNFRp60^{-/-}$	$LT\beta R$ -Fc Tg
Thymus	Normal	Normal	Normal
Lymph nodes	-	+	+*
Peyer's patches	-	-	↓ or -
Spleen			
T-cell/B-cell areas	Disrupted	Normal	Disrupted
Marginal zones	-	+	-
MOMA-1	-	+	-
ER-TR7	ND	ND	-
ER-TR9	ND	ND	-
MAdCAM-1	-	-	-
Germinal centers	-	-	ND

If absent, -; if present, +; ND, not determined.

\*no lymph node development if  $LT\alpha\beta$  is neutralized during days 11-16 of gestation (16).

splenic stromal development and T-cell/B-cell organization may occur through mechanisms which are mutually exclusive.

Recently, a number of reports have suggested that both  $TNFRp60$  and  $LT\beta R$  are able to signal crucial events required for normal lymphoid development and function (5-9, 16, 25, 26). It is becoming increasingly clear which receptor/ligand interactions are pivotal to these processes (Table 2). Although early reports demonstrated that  $LT\alpha$ -deficient mice, but not  $TNFRp60$ - or  $p80$ -deficient mice, have a complete lack of lymph node development, closer evaluation has revealed that  $TNFRp60$ -deficient mice have splenic and Peyer's patch abnormalities. Both  $TNFRp60$ - and  $LT\alpha$ -deficient mice do not develop Peyer's patches, have a lack of splenic marginal zone MAdCAM-1 expression, and do not form germinal centers following immunization (25, 26). In addition to a reduction of Peyer's patches and an absence of splenic MAdCAM-1 expression, mice expressing a soluble  $LT\beta R$ -Fc fusion protein also lack splenic marginal zone MOMA-1, and ER-TR9 expression (Table 2). Lymph node development is also disrupted if surface  $LT\alpha\beta$  ligand is neutralized early in ontogeny (16). Expression of the  $LT\beta R$ -Fc transgene product did not appear to effect germinal center formation in Peyer's patches. Germinal centers were present in all Peyer's patches isolated from mice expressing the  $LT\beta R$ -Fc fusion protein. Germinal centers persist chronically in Peyer's patches; however, in spleen and lymph nodes, germinal center formation follows antigen stimulation (27). The ability to form germinal centers in spleens of  $LT\beta R$ -Fc transgenic mice following antigen challenge is currently being evaluated.

Thus, if  $LT\alpha\beta$  only binds  $LT\beta R$ , and not other yet undetected receptor(s), then it appears that while  $TNFRp60$  and  $LT\beta R$  are both essential for Peyer's patch organogenesis, lymph node development requires only  $LT\beta R$  engagement (excluding mesenteric lymph nodes). The specific interactions necessary for splenic marginal zone formation, and T-cell/B-cell organization, also requires  $LT\beta R$ , although splenic MAdCAM-1 expression seems to be dependent on both  $TNFRp60$  and  $LT\beta R$  engagement. Germinal center formation requires  $TNFRp60$  and  $LT\alpha$ ; however, the requirement of surface  $LT\alpha\beta$  ligand in germinal center formation is still unclear. Blockade of, or a lack of,  $LT\alpha\beta/LT\beta R$ ,  $TNF/TNFRp60$  or  $LT\alpha3/TNFRp60$  interactions may also affect the expression of other receptor/ligand pairs required for these process to occur. Our results, together with the results of Rennert *et al.* (16) demonstrate that surface  $LT\alpha\beta$  (presumably signaling through the  $LT\beta R$ ) is required for normal lymph node, splenic and Peyer's patch development.

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