

Surface Lymphotoxin α/β Complex Is Required for the Development of Peripheral Lymphoid Organs

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Summary

For more than a decade, the biological roles and the apparent redundancy of the cytokines tumor necrosis factor (TNF) and lymphotoxin (LT) have been debated. LT α exists in its soluble form as a homotrimer, which like TNF only binds the TNF receptors, TNF-R55 or TNF-R75. The cell surface form of LT exists as a heteromer of LT α and LT β subunits and this complex specifically binds the LT β receptor (LT β -R). To discriminate the functions of the LT and TNF systems, soluble LT β -R-immunoglobulin (Ig) or TNF-R-Ig fusion proteins were introduced into embryonic circulation by injecting pregnant mice. Exposure to LT β -R-Ig during gestation disrupted lymph node development and splenic architecture in the progeny indicating that both effects are mediated by the surface LT α/β complex. These data are the first to identify a cell surface ligand involved in immune organ morphogenesis. Moreover, they unambiguously discriminate the functions of the various TNF/LT ligands, provide a unique model to study compartmentalization of immune responses and illustrate the generic utility of receptor-Ig fusion proteins for dissecting/ordering ontogenetic events in the absence of genetic modifications.

TNF, lymphotoxin (LT) α , and LT β are members of the TNF superfamily (1). While TNF is expressed by many cell types, LT α is basically restricted to activated lymphocytes. Both molecules can be secreted as homotrimers, yet TNF also exists as a cell surface homotrimer retained by a transmembrane domain. LT α (also called TNF β) and TNF homotrimers bind and signal via either of two TNF receptors, TNF-R55 or TNF-R75, to mediate host defense processes (1, 2). Unlike LT α , LT β has not been detected in a secreted form nor as a homotrimer, rather, LT β as a type II integral membrane protein complexes with LT α to form membrane anchored heteromers (3, 4). The predominant surface LT α/β complex does not bind the TNF receptors, but does bind to a unique receptor termed the LT β -R (5). Recent reports have shown that recombinant soluble human LT α/β complexes bind LT β -R to mediate cytolysis of a subset of human adenocarcinomas (6) but only inefficiently mediate proinflammatory activities (6a).

In addition to initiating proinflammatory processes, studies suggest that TNF and LT α can regulate immune function directly (7, 8). For example, in vitro TNF signaling can affect thymocyte proliferation and differentiation, B cell

activation and proliferation, and mixed lymphocyte responses. The direct involvement of LT α in the development/organization of peripheral lymphoid organs was demonstrated using the targeted gene disruption approach. LT $\alpha^{-/-}$ mice lack LNs and Peyer's patches, and their spleens have indistinct follicular marginal zones, T/B cell disorganization, and fail to form germinal centers (GC) (9-11). Such effects were not noted in mice lacking TNF-R75 (12-14). TNF-R55-deficient mice express more limited alterations, i.e., the absence of only Peyer's patches (15, 16), the failure to express MAdCAM-1 on splenic marginal zone cells, and the inability to form GC (8). Since the TNF-R do not appear to mediate the development of the peripheral lymphoid system, it was postulated that the membrane LT α/β /LT β -R pathway may regulate LN genesis (9). Recently, it has been suggested that lymphotoxin mediates "neoorganogenesis," a pathologic process mimicking normal lymph node genesis (17). Restricted expression of LT α in the pancreas and kidney of transgenic mice caused inflammation in the target tissue manifested by the induction of LN-like structures within these organs.

To investigate the role of LT α/β and TNF in signaling immune system development, we used a novel strategy. Since human IgG₁ binds the murine Fc receptor FcR_n and crosses the yolk sac (18), it was considered that introduc-

¹Abbreviations used in this paper. GC, germinal centers; LT, lymphotoxin.

tion of receptor decoys, i.e., soluble receptor human IgG1 fusion proteins during gestation might interfere with developmental processes in the absence of permanent genetic alterations.

Materials and Methods

Animals. Timed pregnant Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed under conventional barrier protection, and handled in accordance with institutional guidelines. Receptor-Ig proteins or mAbs were injected i.v. into the tail vein.

Fusion Proteins and Antibodies. Fusion proteins comprised of the extracellular domain of either murine LT β -R or LFA-3 (which does not bind murine CD2) fused to the hinge, C_{H2}, and C_{H3} domains of human IgG₁ were prepared as described (19, 20). The murine TNF-R55-IgG₁ was created in a similar manner (Browning, J., manuscript in preparation). The absolute specificity of the human receptors for their ligands has been demonstrated in cell-based assays (6). In the murine LT system, binding of LT β -R-Ig requires expression of both LT α and LT β proteins (19). Murine LT β -R-Ig fusion protein can inhibit the activity of LT α / β in a mouse LT β -R-based cytotoxicity assay in which murine TNF-R55-Ig is inactive. Likewise, the specificity of the interaction of TNF-R55 with LT α has been well-characterized in human systems and we have shown further that both human and mouse TNF-R55-Ig can block the activity of recombinant murine LT α in a TNF-R55-based cytotoxicity assay (Mackay, F., and J. Browning, manuscript in preparation). A hamster IgG mAb BBF6 specific for murine LT β was prepared by conventional methodology (Browning, J., manuscript in preparation). Anti-LT β mAb BBF6 can block murine LT α / β signaling in an LT β -R-based cytotoxicity assay (Mackay, F., and J. Browning, manuscript in preparation). Polyclonal hamster IgG (PharMingen, San Diego, CA) was used as a control.

ELISAs. Analyses for receptor-Ig in plasma used mAbs specific for murine LT β -R (Browning, J., I. Sizing, P. Lawton, P. Bourdon, P. Rennert, G. Majeau, C. Ambrose, C. Hession, K. Miatkowski, D. Griffiths et al., manuscript in preparation), LFA-3 (20), or the C_{H3} domain of human IgG₁ (CDG5, prepared at Biogen, Cambridge, MA) directly immobilized (10 μ g/ml) on 96-well microtiter plates for capture, and donkey anti-human IgG₁-horseradish peroxidase for detection (1:4,000 dilutions; Jackson ImmunoResearch Laboratories, West Grove, PA). ELISA for hamster mAbs in plasma used immobilized goat anti-hamster IgG (10 μ g/ml; Southern Biotechnology Associates, Birmingham, AL) for capture, and goat anti-hamster IgG-horseradish peroxidase for detection (1:6,000; Southern Biotechnology Associates).

Immunohistochemistry. Tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned for hematoxylin/eosin staining. For immunofluorescence staining, frozen spleen sections were acetone fixed, air dried, and preblocked with 5 μ g/ml anti-CD16/CD32 Fc block (PharMingen) in Tris-buffered saline with 0.25% BSA, 0.05% Tween 20, and 10% heat-aggregated rabbit serum. Cells were stained with 5 μ g/ml biotinylated anti-B220 mAb (PharMingen) in the same buffer, followed by 10 μ g/ml fluorescein conjugated-neutralite (FITC-avidin; Southern Biotechnology Associates) and 5 μ g/ml PE-conjugated anti-CD4 mAb (PharMingen). Slides were viewed under $\times 100$ optics and separate red and green images, digitized. Each of the images was analyzed with Adobe PhotoshopTM in a consistent manner followed by overlaying of the green and red images in the screen

mode. The final images are representative of these sections. Staining of sections for MAdCAM-1 expression used mAb MECA 367 (PharMingen) followed by PE-conjugated goat anti-rat IgG (PharMingen).

Results and Discussion

Gestational Blockade of LT α / β Disrupts LN Genesis. The loss of TNF signaling as revealed by the genetic ablation of murine TNF-R55 leads to defects in the initiation of acute inflammatory cascades, an inability to defend against intracellular parasites, failure to generate GC, and the absence of Peyer's patches (8, 12–14, 16). In contrast, mice with a disrupted LT α gene lack LNs and Peyer's patches, and possess spleens with indistinct follicular marginal zones and T/B cell disorganization (9–11). These genetic experiments suggest that disruption of cell surface LT α / β heterotrimers mediates most aspects of the defects in the LT α knockout mice. To evaluate the role of murine LT α / β complexes in the development of the secondary lymphoid organs, the actions of surface LT ligand in the fetus and young mice were blocked by introducing either murine LT β -R-Ig or murine TNF-R55-Ig fusion proteins, i.e., soluble receptor decoys, into the fetus via maternal transfer or by direct injection into neonates.

The regimen of fusion protein administration chosen was influenced by (a) the reported time of LN development during rodent embryogenesis (21); (b) in situ hybridization studies showing that LT β mRNA is expressed in thymocytes and lymph nodes (but not spleen) in mouse embryos at day 16.5 of gestation (French, L., J. Tschopp, and J. Browning, manuscript in preparation); and (c) observations that LT β -R message is detected in embryos as early as 7 d after coitus (22), and is expressed in some epithelial tissues and thymus at day 14.5 of gestation (French, L., J. Tschopp, and J. Browning, manuscript in preparation). Thus, mice were injected i.v. with 200 μ g of fusion protein on days 14 and 17 of gestation, and the progeny were visually inspected for the presence of LNs at various ages. All LNs and Peyer's patches were readily detected in the progeny of mice that received either PBS, LFA-3-Ig, or TNF-R55-Ig. In contrast, inguinal and popliteal LNs and Peyer's patches were absent in the progeny of mice treated with LT β -R-Ig (Table 1). Microscopic examination revealed the presence of juxtaposed lymphatic and circulatory vessels but no anlage of lymphoid organs at the sites where these nodes are usually observed (Fig. 1). Interestingly, brachial and mesenteric LNs were present in the progeny of mice that had been treated with LT β -R on days 14 and 17, but the former were apparently smaller than those in progeny of control-treated mice. Since adult mice that had cleared the LT β -R-Ig still lacked LNs even after immunization with antigen in CFA, the interruption of LT signaling during fetal development has irreversible consequences for lymph node formation.

ELISA analyses showed that all three IgG₁ fusion proteins were present but not at equivalent levels in the circulation of the progeny (Table 2). To determine that the effect

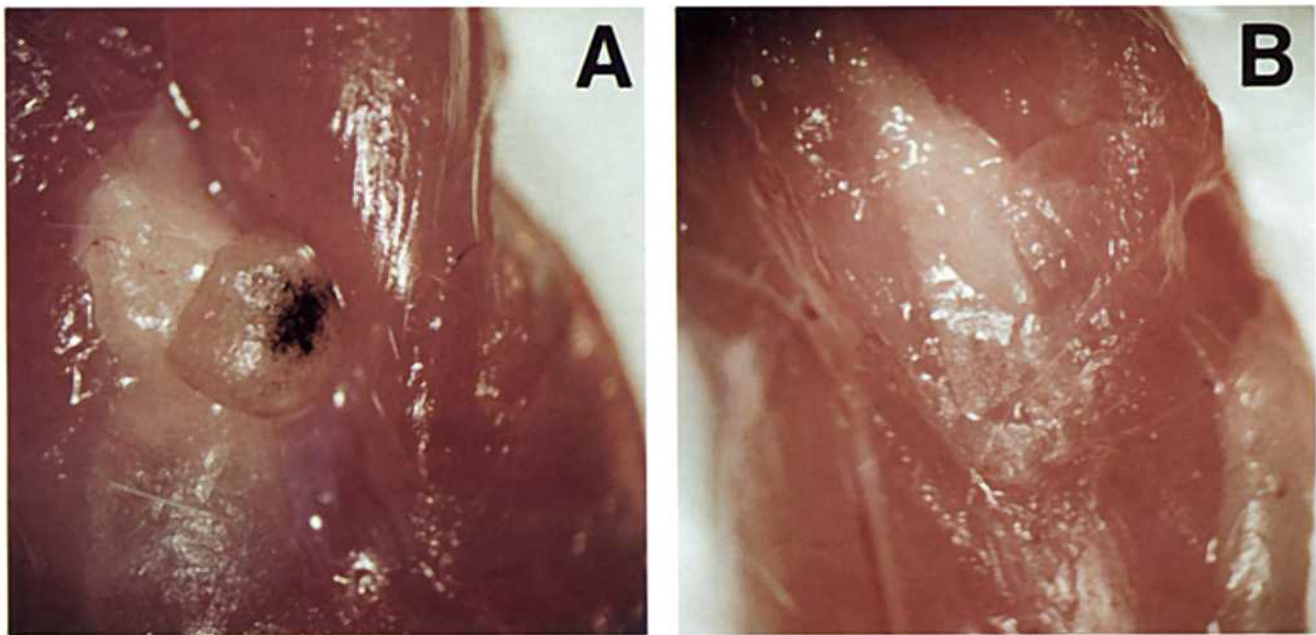


Figure 1. Gestational treatment with LT β -R-Ig ablates the formation of popliteal LNs. Pictures represent the presence or absence of popliteal LNs in the 22-d-old progeny of pregnant mice that were injected on days 14 and 17 of gestation with 200 μ g of either LFA-3-Ig (A) or LT β -R-Ig (B). The popliteal LN is highlighted by the concentration of indigo ink which was injected into the footpad of the mouse 0.5 h before killing.

of LT β -R decoy did not merely reflect better maternal-fetal transport, the dose of LT β -R-Ig given to pregnant mice was titrated. The progeny of mice that received a single i.v. injection of as little as 1 (but not 0.2) μ g of LT β -R-Ig on day 14 of gestation, and had a plasma concentration below the levels of ELISA detection (10 ng/ml) at 2 d of age lacked popliteal and inguinal LNs. Activity of this low dose indicates the effect of the LT β -R-Ig decoy on LN genesis is specific and not simply due to achieving higher levels of the former in the fetus. The presence of Peyer's patches in TNF-R55-Ig treated mice (Table 1) contrasts to their absence in TNF-R55-deficient mice (16). Although repeated administration of the TNF-R55-Ig has *in vivo* effects (see the effects of fusion proteins on splenic MAdCAM-1 expression below), and as the TNF-R55-Ig serum level did not persist comparably to that of LT β -R-Ig fusion protein in the progeny (Table 2), it is possible that TNF-R55-Ig was not present at sufficient levels to disrupt development of Peyer's patches which occurs after birth.

The selective effect of LT β -R-Ig treatment could result from its specific engagement of LT α/β complexes or its binding to an undefined alternative ligand. Although we cannot yet ascertain if the endogenous LT β -R is the mediating receptor, the role of the LT β protein was directly examined. LN development was ablated by gestational treatment with a hamster anti-murine LT β mAb (Table 1) that blocks murine LT α/β signaling of murine LT β -R in an *in vitro* assay (Mackay, F., and J. Browning, manuscript in preparation). The LN deficient phenotype of the LT $\alpha^{-/-}$ mice and of mice treated with either LT β -R-Ig or murine LT β -specific mAb indicate that the LT α/β complex is required to mediate LN genesis during ontogeny. The iden-

tity of the LT α/β ⁺ fetal cell type required for LN genesis is being investigated. Although LT α/β is an activation antigen restricted in expression to T and B lymphocytes and NK cells in adult mice (Browning, J., et al., manuscript submitted for publication), the ligand is apparently expressed

Table 1. *The Development of LNs and Peyer's Patches Is Disrupted in the Progeny of Mice Treated with LT β -specific Reagents*

Treatment regime	Number of litters	Number of progeny	Lymph nodes*	Peyer's patches*	Spleen and Thymus*
Receptor-Ig[‡]					
LT β -R	15	79	–	–	+
TNF-R55	4	15	+	+	+
LFA-3	8	27	+	+	+
Hamster antibodies[§]					
LT β specific	5	21	–	–	+
Control IgG	3	14	+	+	+

*A minus sign denotes the absence and a plus sign the presence of popliteal and inguinal LNs, Peyer's patches, spleens, or thymi as designated. Of the 79 mice treated with LT β -R, none showed any signs of possessing popliteal or inguinal nodes.

Progeny of mice injected i.v. on days 14 and 17 with [‡]200 μ g of soluble receptor -Ig or [§]200 μ g of either hamster anti-LT β BBF6 or polyclonal hamster IgG were examined at various ages for the presence or absence of lymphoid organs. Detection of popliteal and inguinal LNs was aided by injecting 100 μ l of indigo ink into rear footpads 2 h before examination.

Table 2. Concentration of Receptor-Ig or mAbs Present in the Plasma of the Progeny of Treated Mice

Treatment	Days after birth			
	2-4	7-8	14-18	21-24
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Receptor-Ig				
LT β -R	31 \pm 6.3 (7)	9 \pm 8 (6)	3.3 \pm 1.5 (5)	2 \pm 2 (8)
TNF-R55	9 \pm 0.8 (4)	0 (6)	ND	ND
LFA-3	6 \pm 1.7 (7)	0.5 \pm 0.25 (2)	0.01 \pm 0.02 (5)	0 (2)
Hamster antibodies				
Anti-LT β	1.5 \pm 1 (5)	0.5 \pm 0.25 (2)	0 (2)	ND
Control IgG	10 \pm 4 (4)	7 \pm 2 (2)	0.2 (1)	ND

Progeny of mice that had been injected i.v. with either 200 μg of receptor-Ig or 200 μg of hamster antibody on days 14 and 17 of gestation were killed on the days indicated. Mean \pm SD and sample size (*n*) represent that of progeny of multiple mothers from multiple experiments. ND, not done.

in the absence of extrinsic activation on a fetal cell population.

Ordering of LN Development. By varying the gestational day of LT β -R-Ig injection, the genesis of LNs and Peyer's patches was ordered and shown to be as follows: mesenterics; brachials; axillaries; inguinals and popliteals; Peyer's patches (Table 3). This may reflect the extension of the lymphatic and circulatory vasculature during murine ontogeny as peripheral LN development appears to proceed in an anterior-to-posterior order. Interestingly, mesenteric LNs were detected in the progeny of mice that had been treated with LT β -R-Ig as early as gestational day 9, suggesting that an additional LT α -specific pathway may be involved in the genesis of the mesenteric nodes. Alternatively, effective yolk sac transport may not occur early

enough during development to ablate the formation of these nodes. Together with the inability to detect an empty anlage, these data indicate that LT β -R-Ig binds its specific ligand to influence the development of LNs rather than to obliterate LNs already established during embryogenesis. Ongoing studies that do not show the gross deletion of the LN anlage in normal adult mice treated with LT β -R-Ig (Mackay, F., and J. Browning, manuscript in preparation) or in mice expressing an LT β -R-Ig transgene after birth (22a) also suggest that the effect of LT β -R-Ig on LN genesis has a specific temporal requirement corresponding to the time of LN formation during ontogeny.

Blockade of LT α / β Alters Splenic Architecture. The presence of the thymus and spleen in the progeny of LT β -R-Ig-treated mice and in LT α ^{-/-} mice shows that LT α / β

Table 3. LT β -R-Ig Treatment Orders the Development of Peripheral Lymphoid Organs

Gestational day treated [‡]	Lymph nodes*					Peyer's patches*	Spleen*
	Mesenteric	Brachial	Axillary	Inguinal	Popliteal		
9	+	-	-	-	-	ND	+
10	+	-	-	-	-	ND	+
11	+	-	-	-	-	ND	+
12	+	-	-	-	-	ND	+
13	+	+	-	-	-	ND	+
14	+	+	-	-	-	ND	+
15	+	+	+	-	-	ND	+
16	+	+	+	+	-	ND	+
17	+	+	+	+	+	-	+
18	+	+	+	+	+	-	+

*Plus sign indicates that lymphoid organ is present; minus sign indicates its absence.

[‡]Pregnant mice were injected i.v. with 100 μg LT β -R-Ig on indicated day of gestation. Six or more progeny were evaluated for the presence of secondary lymphoid organs.

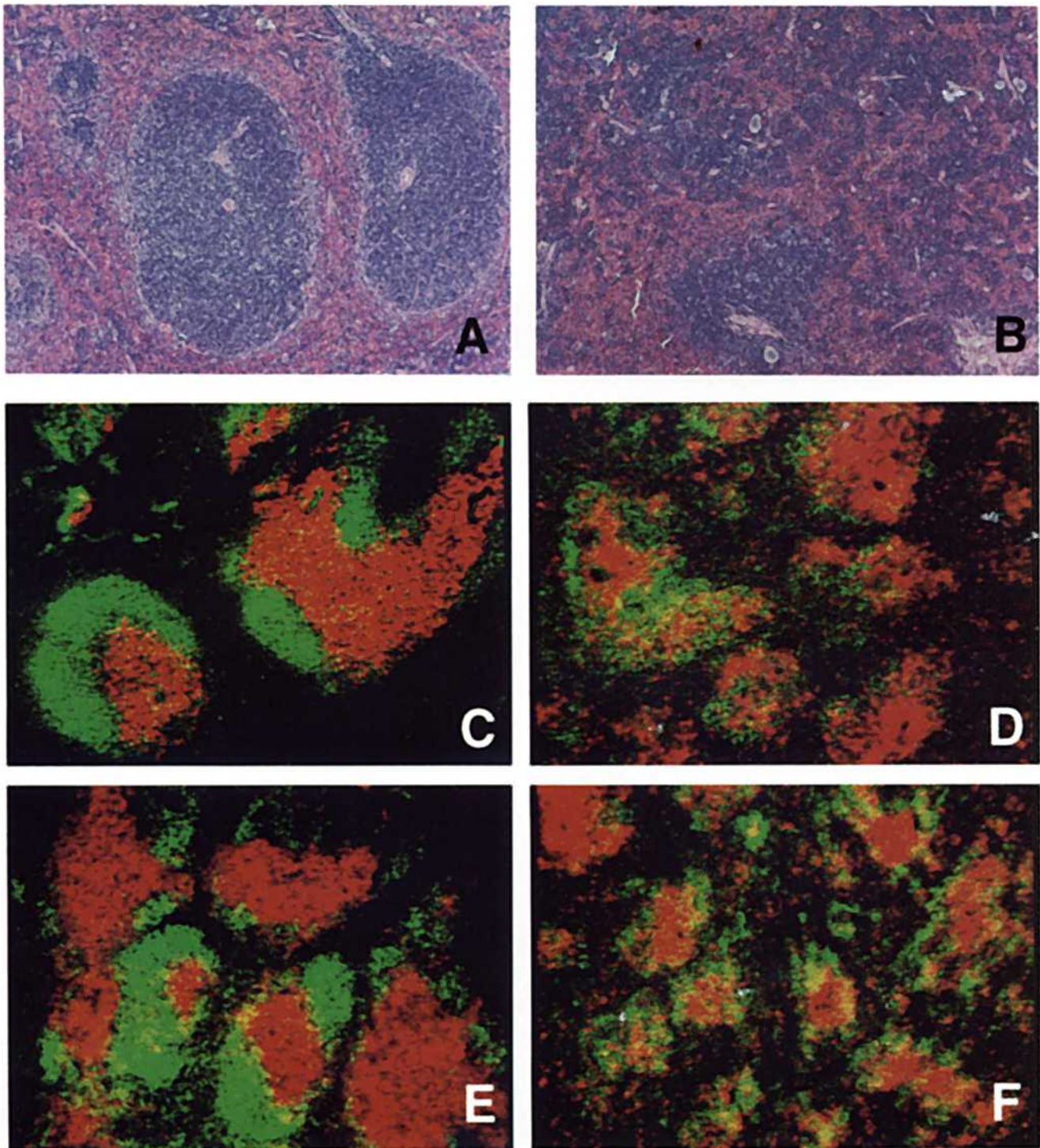


Figure 2. Effects of $LT\alpha/\beta$ inhibition on splenic architecture. (Top) Hematoxylin/eosin staining of spleens of mice treated in utero and postnatally with a (A) control protein LFA-3-Ig or (B) $LT\beta$ -R-Ig. Pregnant mice were injected i.v. with 200 μ g of soluble receptor on days 14 and 18 of gestation. Pups were then injected i.p. on 7, 14, 21, and 28 d after birth and their spleens were analyzed at 5 wk. (Bottom) Comparison of the disruption of the T and B cell spleen zones in the $LT\alpha^{-/-}$ mice with those of the progeny of mice injected with $LT\beta$ -R-Ig. Frozen spleen sections were stained for $CD4^{+}$ T cells (red) or B220⁺ B cells (green). Yellow color shows overlapping staining. Shown are spleen sections from (C) an adult $LT\alpha^{+/+}$ mouse, (D) an $LT\alpha^{-/-}$ mouse, (E) a 5-wk-old mouse whose mother was injected with 200 μ g of $LT\beta$ -R-Ig on days 14 and 17 of gestation, and (F) a 5-wk-old mouse derived as per E but with continued injections i.p. at 7, 14, 21 and 28 d after birth.

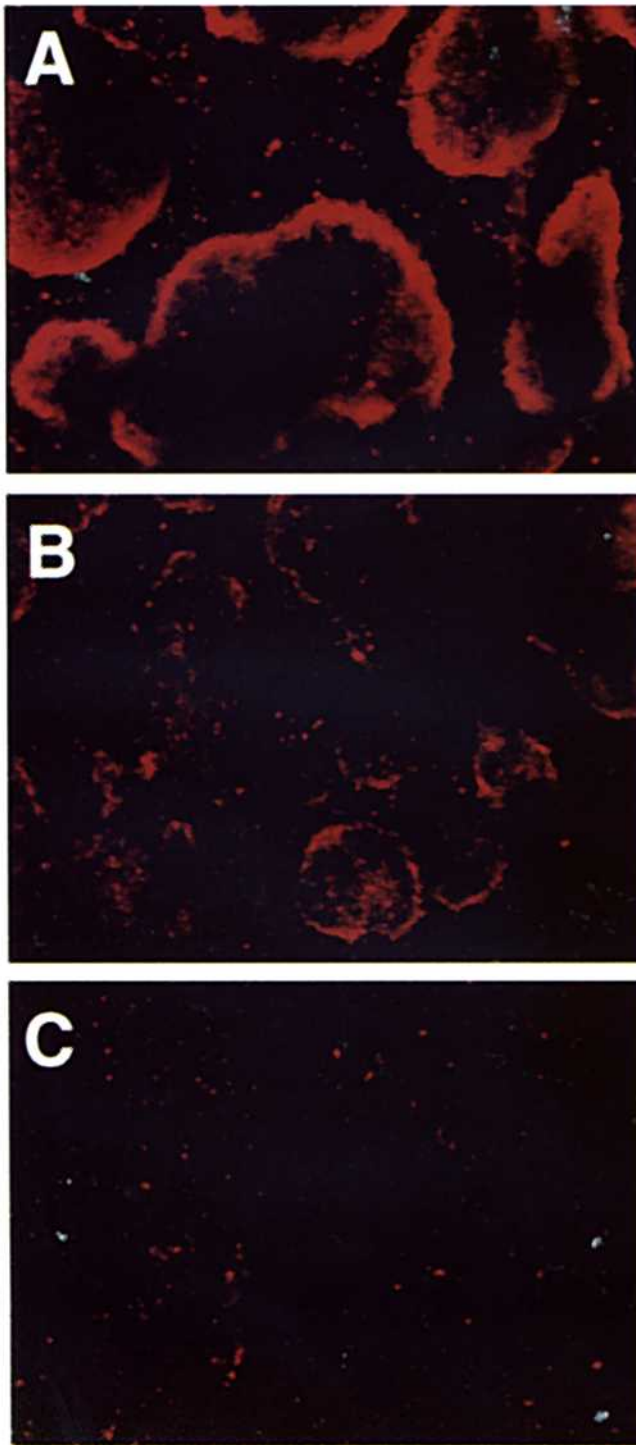


Figure 3. Expression of MAdCAM-1 on splenic marginal zone cells. MAdCAM-1 expression on splenic marginal zone cells of progeny derived from pregnant mice treated i.v. (A) on days 14 and 17 of gestation with 200 μ g of LFA-3-Ig (control), (B) on days 14 and 17 of gestation with 200 μ g of LT β -R-Ig followed by i.p. injections on days 7, 14, 21, and 28 after birth with LFA-3-Ig, and (C) as per B but all injections were with LT β -R-Ig. Each digital image was processed in an identical manner.

engagement is not required for their morphogenesis. To probe for more subtle effects, histological examinations of the spleens and thymi from treated and control mice were performed. As the spleen is populated with lymphocytes in the weeks after birth, injections of soluble LT β -R-Ig-treated progeny were continued postnatally (100 μ g once weekly for 5 wk). Histological analyses showed that thymi had clearly defined cortical and medullary zones (data not shown) and that both red and white pulp components are detected in the spleens of LT β -R-Ig mice treated only during fetal development. However, after multiple LT β -R-Ig injections the red and white pulp became disorganized (Fig. 2, A and B) as in LT α ^{-/-} mice (9, 10). Moreover, immunohistochemical analyses showed alterations in lymphocyte positioning in the white pulp. Although T cells still appeared to concentrate around the central arterioles, these periarteriolar lymphoid sheaths were reduced in size, the B cells follicles had ragged edges, and T and B cells failed to segregate exclusively into these zones (Fig. 2 F) as in spleens of control mice (Fig. 2 C). Such lymphocyte disorganization was observed in LT α ^{-/-} (Fig. 2 D, and 9, 11) but not in TNF-R55^{-/-} mice (8).

Marginal zone cells bounding the T/B regions of the spleens from LT α ^{-/-} mice fail to express a characteristic marker, MOMA-1 (8). In our system, the expression of yet another marginal zone marker, MAdCAM-1, was lacking in spleens of mice treated multiple times with LT β -R-Ig (Fig. 3 C) or TNF-R55-Ig (data not shown). Similar effects of LT β -R-Ig but not TNF-R55-Ig on expression of other marginal zone markers (i.e., sialoadhesin) were also noted (data not shown). When LT β -R-Ig was introduced only during gestation, relatively minor T and B cell disorganization was noted (Fig. 2 E), yet marginal zone MAdCAM-1 expression was diminished (Fig. 3 B) and the size of white pulp islands appears to be reduced (data not shown). This apparent continuum of LT β -R-Ig splenic effects suggests that continuous interaction of LT α / β with LT β -R or an alternative receptor influences the organization of cells in this secondary lymphoid organ.

In this work, we have reproduced most aspects of the phenotype of the LT α ^{-/-} mouse (except the absence of mesenteric LNs) by administering soluble LT β -R-Ig, thus showing that membrane LT α / β and not LT α 3 signals a specific molecular pathway which controls LN genesis, organization of lymphocytes within splenic follicles, and the integrity of the splenic marginal zone. It will be interesting to determine whether LT α / β acts as a master switch directing the expression of cell surface markers (i.e., MAdCAM-1 or MOMA-1) essential to these processes or controlling other activities of as yet undefined organizing cell types. TNF-R55^{-/-} mice express MOMA-1 (8) but not MAdCAM-1 (23) in the splenic marginal zones, suggesting that MAdCAM-1 expression on marginal zone cells does not directly correlate with lymphocyte disorganization in the white pulp. Our data also show reasonable T/B organization when MAdCAM-1 expression is reduced (Figs. 2 E and 3 B) thus questioning the requirement of this adhesion

molecule for splenic lymphocyte organization. Moreover, experiments using toxic liposomes to eliminate MOMA-1⁺ splenic macrophages also did not show splenic disorganization (24). The failure of mutant *aly* mice (25, 26), *relB*-deficient mice (27, 28), and Ikaros knockout mice (29) to develop LNs and the disorganized lymphoid architecture in the spleen of the *aly* (26) and *relB*-deficient mice (27, 28) designates roles for other components in these processes. However, our data show that expression of the LT β gene, like the *Hox11* gene which controls the genesis of the spleen (30), has a central role in the development of the secondary lymphoid organs in the absence of the immunocompromised phenotype noted in the *aly* mice, *relB*, and Ikaros-deficient mice. Moreover, LT α/β is distinct from these transcription factors, representing the first cell surface ligand identified to be critical for such immune morphogenetic processes.

LT β -R is expressed on reticular dendritic cells in the red pulp of human fetal spleen (30a) and has not been found on T or B lymphocytes. Surface ligand in contrast has been observed only on activated human T and B lymphocytes and NK cells (31). Contact between trafficking/activated LT α/β ⁺ lymphocytes and stromal elements such as dendritic cells in peripheral lymphoid organs could facilitate the subsequent positioning of lymphocytes as in GC formation and thus influence the maturation of the immune response. The absence of such a signal would result in the

splenic phenotype and impaired GC formation observed in the LT α knockout mice (8). As the LT α ^{-/-} mice manifest an LT α/β ⁻ phenotype, the role of LT α/β in GC formation is under study using the tools described herein. Such a role for membrane LT is also consistent with reports that TNF superfamily members participate in cognate interactions (31, 32). Impairment of CD40/CD40L interactions causes profound immunodeficiency (33), and disruption of either the CD40/CD40L interaction or the TNF-R55 or LT α genes contributes to the failure to form GC (38). As CD40 ligation induces expression of LT α/β complexes on B cells (35, and Hochman, P., unpublished data), CD40 and surface LT may represent steps in a linear sequence of events. Alternatively, since both CD40 and LT β -R share a cytoplasmic signal transduction element, TRAF-3 (36), these pathways may have interdependent roles in the formation and maintenance of GC.

Finally, this report documents a technique that disrupts a discrete developmental pathway without the need for genetic manipulation. Taken together with the *Hox11* knockout (30), this strategy can generate powerful models to evaluate the compartmentalization of the immune system. Since soluble receptors can be introduced at specific points in ontogeny, the approach shown herein can order developmental events and facilitate the study of multifunctional pathways where genetic disruption has led to a lethal phenotype, as in the case of ablation of the $\alpha 4$ integrin gene (37).

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