Brief Definitive Report

Defective Peyer's Patch Organogenesis in Mice Lacking the 55-kD Receptor for Tumor Necrosis Factor

By Brigitte Neumann,* Arne Luz,‡ Klaus Pfeffer,*§ and Bernhard Holzmann*

*From *Institute for Medical Microbiology and Hygiene, Technical University of Munich, D-81675 Munich; ‡GSF-Institut für Pathologie Neuherberg, and §Gesellshaft für Strahlenforschung-Institut für Säugetiergenetik 85764 Oberschleißheim, Germany*

Summary

Lymphotoxin α (LT-α) may form secreted homotrimers binding to p55 and p75 tumor necrosis factor (TNF) receptors or cell surface–bound heterotrimers with LT-β that interact with the LT-β receptor. Genetic ablation of LT-α revealed that mutant mice have no detectable lymph nodes or Peyer's patches and that the organization of the splenic white pulp in T and B cell areas is disturbed. In this report we describe a novel function for the p55 TNF receptor during ontogeny and demonstrate that mice deficient for p55 completely lack organized Peyer's patches. In contrast, lymph nodes and spleen are present in p55-deficient mice and lymphocytes segregate normally into B and T cell areas in these organs. Lamina propria and intraepithelial lymphocytes of the small intestine were detected in normal number and distribution in p55 mutant mice. Lymphocytes and endothelial cells from p55-deficient mice express normal levels of adhesion molecules considered important for lymphocyte migration to mucosal organs; this indicates that the lack of Peyer's patches does not result from a defect in lymphocyte homing. In summary, the p55 receptor for TNF selectively mediates organogenesis of Peyer's patches throughout ontogeny, suggesting that the effects of LT-α on the development of lymphoid organs may be mediated by distinct receptors, each functioning in an organ-specific context.

TNF-α and lymphotoxin α (LT-α, also designated TNF-β) affect the growth, differentiation, and function of multiple cell types. Both cytokines are important mediators of inflammation and cellular immune responses (1–4). The multiple biological activities of TNF-α and LT-α homotrimers are mediated by two distinct cell surface receptors of 55 (TNFRp55) and 75 kD (TNFRp75). LT-α can also associate with LT-β to form a type II cell membrane–bound heterotrimer that may engage the LT-β receptor, originally described as a TNF receptor–related protein (5).

Via gene-targeted mice, a crucial role of LT-α for the development of peripheral lymphoid organs has been demonstrated. LT-$\alpha^{-/-}$ mice have no detectable lymph nodes or Peyer's patches and the organization of the splenic white pulp in T and B cell areas is disturbed (6, 7). Elevated numbers of IgM^+ B cells were seen in the spleen and periphery of these mice. In some of these mutant mice, abnormal lymph node–like structures were observed, the majority of which were located in the mesenteric fat (7). Although these studies demonstrated a fundamental role of LT-α for the development of lymphoid organs, the cellular receptors mediating these effects were not identified. For TNF-α, a putative role in lymphatic tissue development has been suggested by observations demonstrating TNF-α mRNA expression in mouse embryos as well as fetal thymus and spleen (8, 9). However, the fact that LT-$\alpha^{-/-}$ mice exhibit defective lymphoid organ development despite normal TNF-α expression suggests that the function of LT-α in development cannot be compensated for by TNF-α.

Targeted disruption of the gene for TNFRp55 revealed a critical function of this receptor for generating a protective immune response against the facultative intracellular bacterium *Listeria monocytogenes* (10, 11). In addition, loss of TNFRp55 renders mice resistant to the toxic effects of LPS (10, 11) or *Staphylococcus aureus* enterotoxin B (10) after sensitization with D-galactosamine. Thymocyte development and absolute lymphocyte numbers in spleen and lymph nodes are normal in TNFRp55$^{-/-}$ mice, and clonal deletion of potentially self-reactive T cells is not impaired (10). Mice deficient for TNFRp75 show normal T cell development and activity and a normal phenotype of lymphoid organs (12). Challenge with sublethal doses of *L. monocytogenes* revealed increased mortality of TNFRp75$^{-/-}$ mice, although sensitivity to infection appeared less severe than in TNFRp55-deficient mice (12). In contrast to

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TNFRp55 deficiency, genetic ablation of TNFRp75 does not result in resistance to LPS-induced toxicity, but susceptibility to TNF-induced death is reduced (12).

In this report we describe a novel function of TNFRp55 in lymphoid organogenesis and show that TNFRp55$^{-/-}$ mice exhibit a selective defect in the development of Peyer's patches. In contrast, other secondary lymphatic organs, including peripheral lymph nodes, mesenteric lymph nodes, and spleen, were present in TNFRp55$^{-/-}$ mice and appeared morphologically normal. Expression of lymphocyte and endothelial adhesion molecules considered to regulate lymphocyte traffic to mucosal lymphoid organs was not altered in TNFRp55$^{-/-}$ mice. These findings indicate that TNFRp55 selectively mediates organogenesis of Peyer's patches throughout ontogeny.

Materials and Methods

Antibodies. mAbs used included rat anti–murine integrin α4, PS/2 (American Type Culture Collection, [ATCC], Rockville, MD), rat anti–murine LFA-1 α subunit, FD441.8 (ATCC), rat anti–murine integrin β7, Fib30 (13), rat anti–murine mucosal addressin cellular adhesion molecule 1 (MAdCAM-1), R3-3 cl2C7 (14), and rat anti–murine L-selectin, MEL-14 (ATCC). Rat or hamster mAbs against CD45R/B220 (RA3-6B2), CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), or IgA (R5-140) were purchased from PharMingen (San Diego, CA).

Immunohistochemistry. The tissue samples were snap-frozen in 2-methylbutane prechilled by liquid nitrogen. Cryostat sections were prepared at 8 μm, fixed in cold acetone for 10 min, dried, and stored at −80°C. Endogenous peroxidase activity was blocked by preincubation of the sections with methanol and H_2O_2. The sections were incubated for 30 min with 100 μl of the mAbs. After three washes in PBS, 100 μl of secondary mouse anti–rat IgG or mouse anti–hamster IgG antibody labeled with peroxidase (Dianova, Hamburg, Germany) was added for 30 min. All incubations were carried out in a moist, light-protected chamber at room temperature. Nonspecific background staining was reduced by preincubation of the peroxidase-conjugated antiserum with normal mouse serum. The sections were rinsed again in PBS, fixed in 0.1% glutardialdehyde for 5 min, and stained for 10 min in 50 mM acetate buffer containing 0.01% H_2O_2 and 5 mg/ml 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO), which was dissolved in *N,N'*-dimethylformamide. After extensive washing in PBS, the slides were counterstained with Mayer's hematoxylin for 10 min and mounted with glycerol-gelatin.

Flow Cytometry Analysis. Indirect immunofluorescence flow cytometric assays for α4, β7, LFA-1, and L-selectin surface expression on popliteal lymph node cells of TNFRp55$^{-/-}$ and syngenic C57Bl/6 mice were performed. Lymphocytes were incubated with saturating amounts of rat mAbs for 30 min at 4°C, washed two times with PBS containing 1% BSA, and then stained with FITC-conjugated mouse anti-rat IgG F(ab')$_2$ fragments (Dianova) for 30 min at 4°C. After washing with PBS, cells were fixed in 1% paraformaldehyde, and fluorescence was analyzed on an EPICS XL cytometer (Coulter Corp., Hialeah, FL).

Results and Discussion

Development of lymphoid organs was investigated in mice rendered deficient for TNFRp55 by gene targeting (10). Gross inspection as well as histologic examination of animals at 8–12 wk of age revealed that TNFRp55-deficient mice completely lacked organized Peyer's patches (Table 1). In wild-type C57Bl/6 mice, the total number of Peyer's patches varied from 5 to 8 with a mean of 6.5 ± 0.6. Interestingly, in 6 out of 18 TNFRp55$^{-/-}$ mice examined, a single, small lymphoid aggregate located in the terminal ileum was detected histologically (Fig. 1, *b* and *c*). Morphologically, these aggregates were clearly distinct from Peyer's patches, as they were not organized into follicular structures, did not contain germinal centers, and were devoid of a dome area (Fig. 1, *a–c*). Immunohistochemical analysis demonstrated that these unusual lymphoid structures consisted primarily of B cells (Fig. 1 *e*), but also contained some CD4 T lymphocytes (Fig. 1 *f*) and only few CD8 T cells (not shown).

Intraepithelial lymphocytes were present in the small intestine of mutant mice with apparently normal distribution and frequency as demonstrated by immunohistochemical staining using mAbs directed against CD8 (Fig. 1 *g*) or CD3 and integrin αIELβ7 (not shown). In the lamina propria of TNFRp55$^{-/-}$ mice, IgA immunoblasts were detected at a normal frequency (Fig. 1 *h*). Consistent with these observations, IgA$^+$ B cells were also shown to be present in mesenteric lymph nodes of mutant mice (data not shown). Despite profound defects in the development of lymphoid organs, gut intraepithelial lymphocytes were also observed in LT-α$^{-/-}$ mice (6).

In contrast to Peyer's patches, other secondary lymphatic organs, including peripheral lymph nodes (axillary, brachial, popliteal, inguinal, and cervical nodes), mesenteric

Table 1. *TNFRp55 Deficiency Results in a Selective Defect in Lymphoid Organogenesis*

Organs	C57B1/6	TNFRp55$^{-/-}$
Peyer's patches	6.5 (4)*	0 (18)‡
Peripheral lymph nodes	4/4§	18/18
Mesenteric lymph nodes	4/4	18/18
Spleen	4/4	18/18

Development of secondary lymphatic organs was investigated in 18 TNFRp55$^{-/-}$ mice (10) and 4 wild-type C57B1/6 controls at 8–12 wk of age. Presence of lymphatic organs was monitored by gross inspection and confirmed by histopathological analysis. Peripheral lymph nodes consisted of inguinal, popliteal brachial, axillary, and cervical nodes. Immunohistochemistry of lymph nodes and spleen of TNFRp55$^{-/-}$ mice revealed a normal segregation into T and B cell areas (see Fig. 2).

*Average number of organized Peyer's patches detected (number of animals examined).

‡In 6 out of 18 TNFRp55$^{-/-}$ mice examined, a single, small lymphoid aggregate located in the terminal ileum was detected histologically. However, these aggregates were clearly distinct from Peyer's patches, as they were not organized into follicular structures, did not contain germinal centers, and were devoid of a dome area.

§Number of animals with lymphoid organs present/number of mice examined.

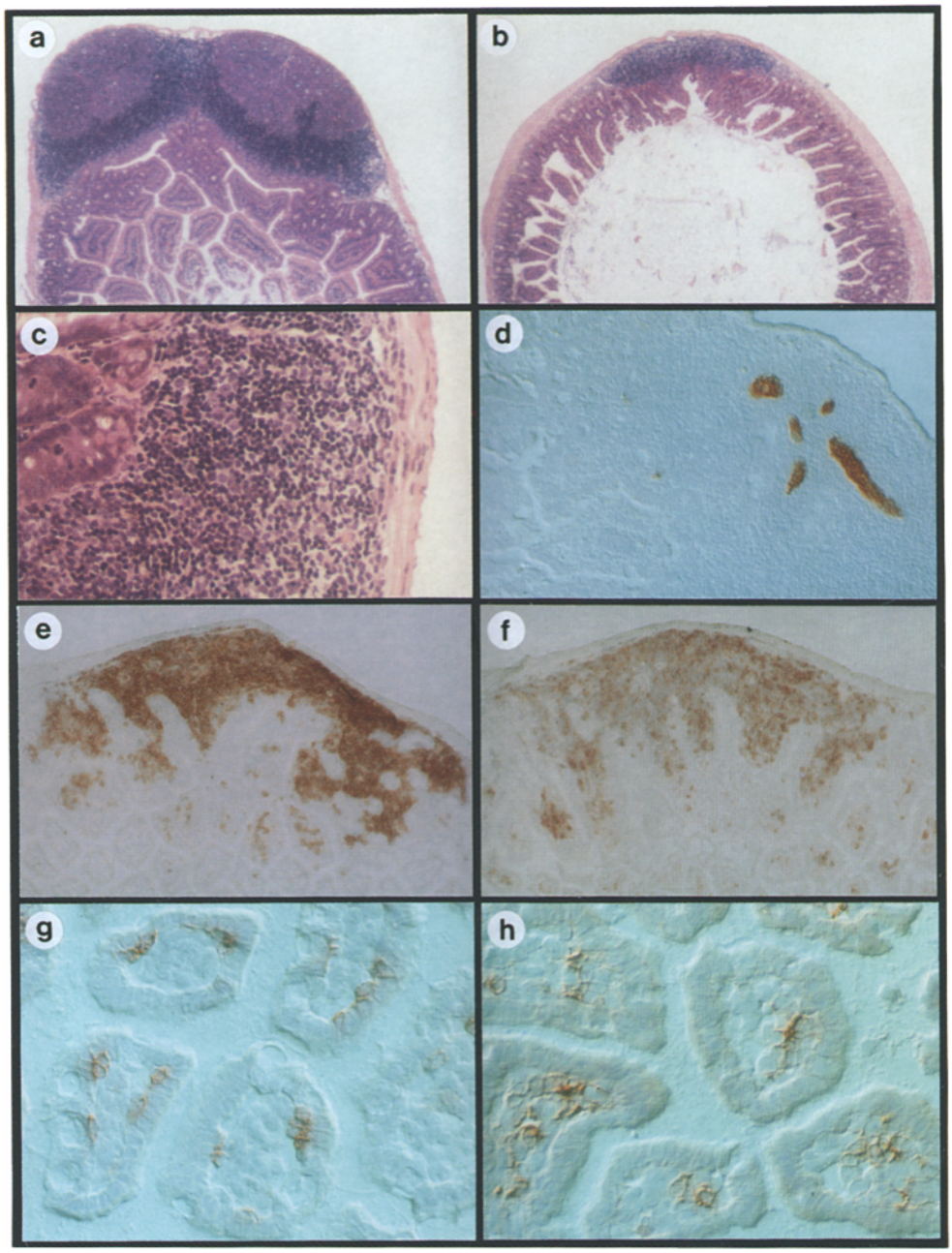

Figure 1. Histological and immunohistochemical analysis of the gut-associated immune system in TNFRp55-deficient mice. For histopathological examination, tissues were fixed in 10% buffered formalin, embedded in paraffin, and tissue sections were stained with hematoxylin and eosin. Representative sections of Peyer's patches derived from wild-type mice (*a*) or lymphoid aggregates present in the small intestine of TNFRp55$^{-/-}$ mice (*b* and *c*) are shown. For immunoperoxidase staining, sections of snap-frozen tissue samples derived from TNFRp55$^{-/-}$ mice were incubated with anti-MAdCAM-1 (*d*), anti-CD45R/B220 (*e*), anti-CD4 (*f*), anti-CD8 (*g*), or anti-IgA (*h*) mAbs. Original magnifications were at 2.5 (*a* and *b*), 20 (*c* and *d*), 10 (*e* and *f*), and 40 (*g* and *h*).

lymph nodes, and spleen were present in TNFRp55$^{-/-}$ mice and appeared morphologically normal. Histologic and immunohistochemical examination revealed that the spleen of mutant mice exhibited a normal organization of the white pulp with T cells clustered in the periarteriolar region and B cells peripheral to the T cell zone (Fig. 2). Mesenteric and peripheral lymph nodes also displayed a normal segregation of B and T lymphocyte areas as compared with wild-type C57/Bl6 mice (data not shown). Previously, it was shown that MAdCAM-1 is expressed on sinus-lining cells in the spleen of wild-type (15) but not of TNFRp55$^{-/-}$ mice (14). However, the functional role of MAdCAM-1 expression on splenic sinus-lining cells is unclear, because antibodies to MAdCAM-1 or $\alpha 4\beta 7$ integrin do not inhibit lymphocyte migration to the spleen (15). Together with previous findings showing a normal development of primary lymphoid organs in TNFRp55$^{-/-}$ mice (10), the data presented here indicate a selective function of TNFR p55 for Peyer's patch organogenesis.

We next examined whether lymphocyte recruitment to gut-associated lymphatic tissues may be impaired in TNFR p55$^{-/-}$ mice. Flow cytometry analysis, however, clearly demonstrated that TNFRp55$^{-/-}$ lymphocytes expressed adhesion molecules such as L-selectin, or integrins $\alpha 4\beta 7$ and LFA-1 at normal levels as compared with wild-type C57Bl/6 mice (Fig. 3). Moreover, immunohistochemical analysis re-

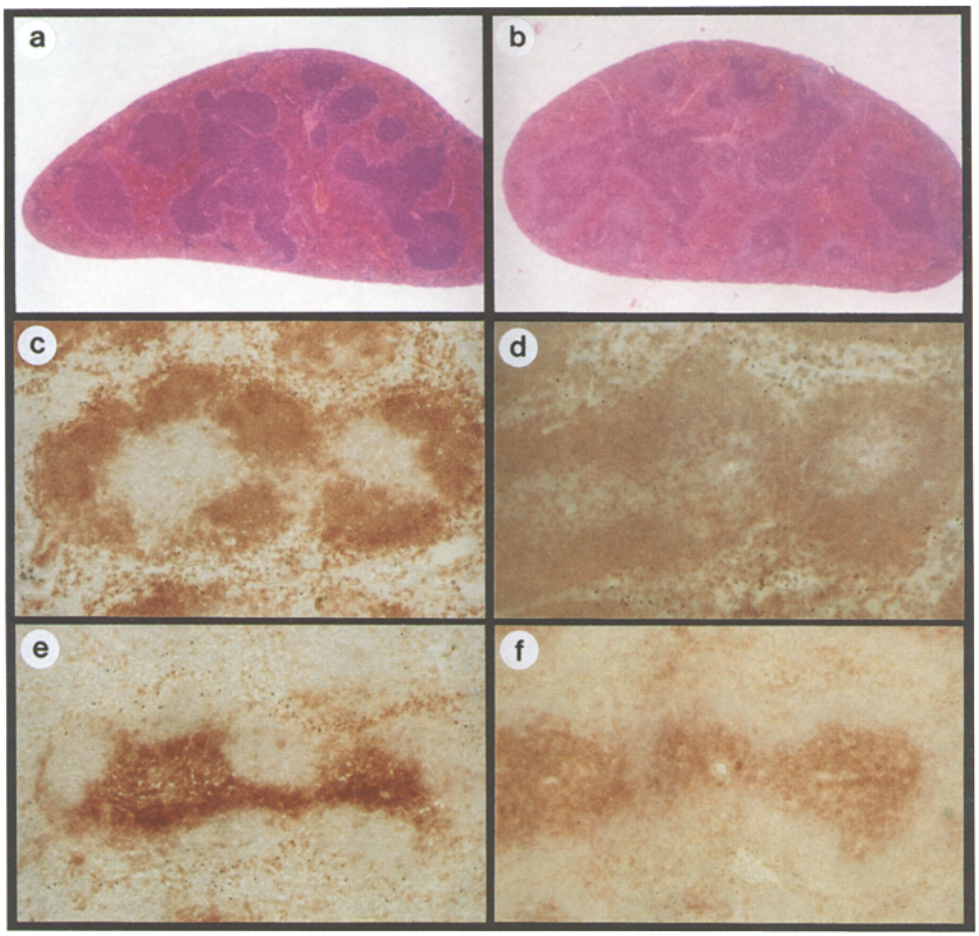

Figure 2. Splenic architecture of TNFRp55$^{-/-}$ and C57/Bl6 mice. For histopathological examination, tissues were fixed in 10% buffered formalin, embedded in paraffin, and tissue sections were stained with hematoxylin and eosin. Representative spleen sections of wild-type C57/Bl6 (*a*) or mice TNFRp55$^{-/-}$ (*b*) are shown. For immunoperoxidase staining, sections of snap-frozen spleen samples derived from C57/Bl6 (*c* and *e*) or TNFRp55$^{-/-}$ mice (*d* and *f*) were incubated with anti-CD45R/B220 (*c* and *d*) or anti-CD3 (*e* and *f*) mAbs. Original magnifications were at 2.5 (*a* and *b*) or 10 (*c* and *f*).

vealed that in TNFRp55$^{-/-}$ mice, MAdCAM-1 was expressed on endothelial cells of the small intestine and on high endothelial cells in mesenteric lymph nodes (data not shown). MAdCAM-1 was also present in high density on endothelial cells of vessels of abnormal lymphoid aggregates located in the terminal ileum of some mutant mice (Fig. 1 *d*).

Previous studies have shown that the integrin α4β7, but not α4β1, mediates lymphocyte adhesion to Peyer's patch high endothelial venules and to MAdCAM-1 (16, 17), as well as in vivo migration of lymphocytes to mucosal sites (13, 18). Consistent with these findings, gut-afferent memory lymphocytes display an α4 high, β1 integrin low phenotype, suggesting that they express high levels of integrin α4β7 (19). In addition, LFA-1 and L-selectin contribute to lymphocyte migration to Peyer's patches (20, 21). Taken together, these results therefore indicate that the lack of Peyer's patches in TNFRp55$^{-/-}$ mice does not result from a defect in lymphocyte recruitment to mucosal sites. This conclusion is also supported by our finding that intraepithelial lymphocytes and lamina propria IgA immunoblasts were present in the small intestine of mutant mice (Fig. 1, *g* and *h*).

LT-α may form secreted homotrimers binding to TNFRp55 and TNFRp75 or cell surface–bound heterotrimers with LT-β that can interact with the LT-β receptor (5, 22–25). Therefore, developmental defects in lymphoid organogenesis associated with LT-α deficiency may result from the lack of signals mediated by several independent cytokine receptors. Recently, it was reported that LT-α$^{-/-}$ mice have no detectable lymph nodes or Peyer's patches and that the organization of the splenic white pulp in T and B cell areas is disturbed (6, 7). The results presented in this study demonstrating that the development of Peyer's patches is selectively controlled by the TNFRp55 therefore suggest that the effects of LT-α on the development of lymphoid organs may be mediated by at least two distinct receptors, each functioning in an organ-specific context. Thus, the data presented in this report partly refute the hypothesis that the effects of LT-α in lymphoid organ development are mediated solely by the LT-β receptor (4). Since lymphatic organs develop normally in TNFRp75$^{-/-}$ mice (12), it is tempting to speculate on a role of the LT-β receptor for lymph node development and formation of a normal splenic architecture.

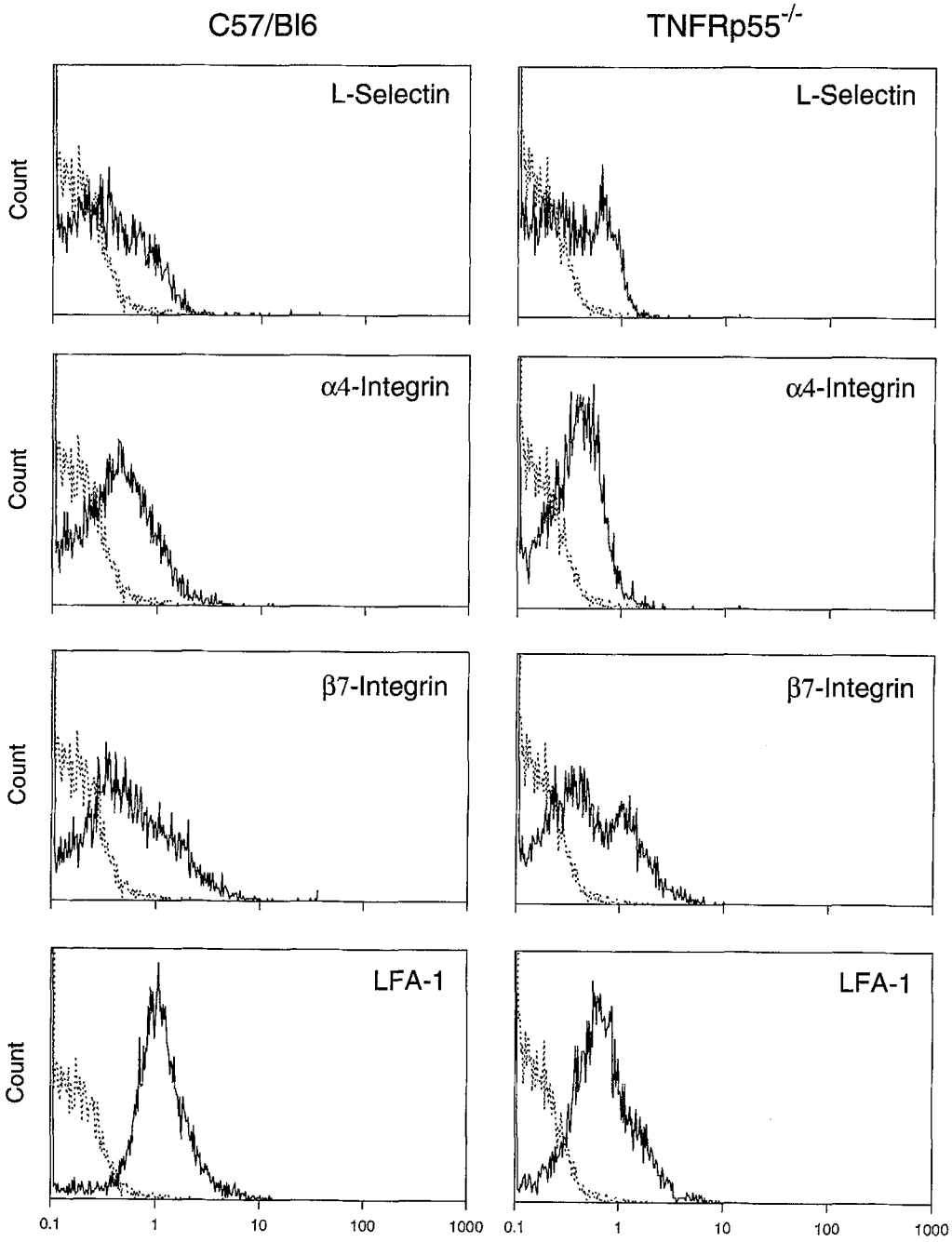

Figure 3. Expression of adhesion molecules on lymphocytes derived from TNFRp55$^{-/-}$ or wild-type C57Bl/6 mice. Lymphocytes freshly isolated from lymph nodes of TNFRp55$^{-/-}$ or C57Bl/6 mice were incubated with saturating amounts of rat mAbs to L-selectin (MEL-14), α4-integrin (PS/2), β7-integrin (Fib 30), or LFA-1 (FD441.8), washed, and stained with FITC-conjugated mouse anti-rat IgG F(ab')$_2$ fragments.

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Address correspondence to Dr. Bernhard Holzmann, Institute for Medical Biology and Hygiene, Technical University of Munich, Trogerstrasse 9, D-81675 Munich, Germany.

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