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*

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.
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 c2C7 (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 H2O2. 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. After incubation, 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 polypeptide lymph node cells of TNFRp55−/− and syngenic C57BI/6 mice were performed. Lymphocytes were incubated with saturating amounts of rat mAbs for 30 min at 4°C, washed twice 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, the 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 C57BI/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 lymph nodes, and spleen, were present in TNFRp55−/− mice (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 C57BI/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).

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

| Organs            | C57BI/6 | TNFRp55−/− |
|-------------------|---------|------------|
| Peyer’s patches   | 6.5 (4) | 0 (18)     |
| Peripheral lymph nodes | 4/45 | 18/18 |
| Mesenteric lymph nodes | 18/18 | 18/18 |
| Spleen            | 4/4    | 18/18      |

Development of secondary lymphatic organs was investigated in 18 TNFRp55−/− mice (10) and 4 wild-type C57BI/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.

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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 α4β7 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 TNFRp55−/− mice. Flow cytometry analysis, however, clearly demonstrated that TNFRp55−/− lymphocytes expressed adhesion molecules such as L-selectin, or integrins α4β7 and LFA-1 at normal levels as compared with wild-type C57Bl/6 mice (Fig. 3). Moreover, immunohistochemical analysis re-
revealed 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 2. Splenic architecture of TNFRp55−/− and C57/B16 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/B16 (a) or mice TNFRp55−/− (b) are shown. For immunoperoxidase staining, sections of snap-frozen spleen samples derived from C57/B16 (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).
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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References

1. Beutler, B., and A. Cerami. 1988. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. *Ann. Rev. Biochem.* 57:505-518.

2. Turetskaya, R.L., S.J. Fashenea, N.L. Paul, and N.H. Ruddle. 1992. Genomic structure, induction, and production of TNF-B. In *Tumor Necrosis Factors: Structure, Function, and Mechanism of Action*, B.B. Aggarwal and J. Vilcek, editors. Marcel Dekker, Inc., New York. 35-60.

3. Vassalli, P. 1992. The pathophysiology of tumor necrosis factor. *Ann. Rev. Immunol.* 10:411-452.

4. Beutler, B., and C. van Huffel. 1994. Unraveling function in the TNF ligand and receptor families. *Science (Wash. DC).* 264:667-668.

5. Crowe, P.D., T.I. VanArsdale, B.N. Walter, C.F. Ware, C. Hession, B. Ehrenfels, J.L. Browning, S.W. Din, R.G. Goodwin, and C.A. Smith. 1994. A lymphphotoxin-β-specific receptor. *Science (Wash. DC).* 264:707-710.

6. De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariathasan, S.C. Smith, R. Carlson, L.P. Shornick, J. Strauss-Schoenberger et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoksin-α and -β. *Science (Wash. DC).* 264:703-707.

7. Banks, T.A., B.T. Rouse, M.K. Kerley, M.P. Blair, V.L. Godfrey, N.A. Kuklin, D.M. Bouley, J. Thomas, S. Kanangat, and M.L. Mucenski. 1995. Lymphotoxin-α-deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685-1693.

8. Giroir, B.P., T. Brown, and B. Beutler. 1992. Constitutive synthesis of tumor necrosis factor in the thymus. *Proc. Natl. Acad. Sci. USA.* 89:4864-4868.

9. De Kossodo, S., G.E. Grau, T. Daneva, P. Pointaire, L. Fosatti, C. Ody, J. Zapf, P.-F. Piguet, R.C. Gaillard, and P. Vassalli. 1992. Tumor necrosis factor α is involved in mouse growth and lymphoid tissue development. *J. Exp. Med.* 176:1259-1264.

10. Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Krönke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *Listeria monocytogenes* infection. *Cell.* 73:457-467.

11. Rothe, J., W. Leslauer, H. Löttscher, Y. Lang, P. Koebel, F. König, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *listeria monocytogenes*. *Nature (Lond.)*. 364:798-802.

12. Erickson, S.L., F.J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillette, K.C.F. Sheehan, R.D. Schreiber, D.V. Goeddel, and M.W. Moore. 1994. Decreased sensitivity to tumour-necrosis factor but normal T cell development in TNF receptor-2-deficient mice. *Nature (Lond.)*. 372:560-563.

13. Andrew, D.P., C. Berlin, S.Honda, T. Yoshino, A. Hamann, B. Holzmann, P.J. Kilshaw, and E.C. Butcher. 1994. Distinct but overlapping epitopes are involved in α4β7-mediated adhesion to vascular cell adhesion molecule-1, mucosal adhesion-1, fibronectin, and lymphocyte aggregation. *J. Immunol.* 153:3847-3861.

14. Neumann, B., T. Machleidt, A. Lifska, K. Pfeffer, D. Vestweber, T.W. Mak, B. Holzmann, and M. Krönke. 1996. Crucial role of 55 kd TNF-receptor in TNF-induced adhesion molecule expression and leukocyte organ infiltration. *J. Immunol.* 156:1587-1593.

15. Kral, G., K. Schornagel, P.R. Streeter, B. Holzmann, and E.C. Butcher. 1995. Expression of the mucosal vascular addressin, MadCAM-1, on sinus-lining cells in the spleen. *Am. J. Pathol.* 147:763-771.

16. Hu, M.C., D.T. Crowe, I.L. Weissman, and B. Holzmann. 1992. Cloning and expression of mouse integrin ββ7(7): a functional role in Peyer's patch-specific lymphocyte homing. *Proc. Natl. Acad. Sci. USA.* 89:8254-8258.

17. Berlin, C., E.L. Berg, M.J. Brisikin, D.P. Andrew, P.J. Kilshaw, B. Holzmann, I.L. Weissman, A. Hamann, and E.C. Butcher. 1993. α4β7 integrin mediates lymphocyte binding to the mucosal vascular addressin MadCAM-1. *Cell.* 74:185-195.

18. Hamann, A., D.P. Andrew, D. Jablonski Westrich, B. Holzmann, and E.C. Butcher. 1994. Role of α4-integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol.* 152:3282-3293.

19. Mackay, C.R., W.L. Marston, L. Dudler, O. Sperti, T.F. Tedder, and W.R. Hein. 1992. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. *Eur. J. Immunol.* 22:887-895.

20. Hamann, A., D. Jablonski Westrich, A. Duijvestijn, E.C. Butcher, H. Baisch, R. Harder, and H.G. Thiele. 1988. Evidence for an accessory role of LFA-1 in lymphocyte–high endothelium interaction during homing. *J. Immunol.* 140:693-699.

21. Hamann, A., D. Jablonski Westrich, P. Jonas, and H.G. Thiele. 1991. Homing receptors reexamined: mouse LECAM-1 (MEL-14 antigen) is involved in lymphocyte migration into gut-associated lymphoid tissue. *Eur. J. Immunol.* 21:2925-2929.

22. Smith, C.A., T. Davis, D. Anderson, L. Solam, M.P. Beckmann, R. Jerzy, S.K. Dower, D. Cosman, and R.G. Goodwin. 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science (Wash. DC)*. 248:1019-1023.

23. Heller, R.A., K. Song, M.A. Onasch, W.H. Fischer, D. Chang, and G.M. Ringold. 1990. Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor. *Proc. Natl. Acad. Sci. USA.* 87:6151-6155.

24. Loetscher, H., Y.C. Pan, H.W. Lahn, R. Gcntz, M. Brockhaus, H. Tabuchi, and W. Leslauer. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell.* 61:351-359.

25. Schall, T.J., M. Lewis, K.J. Koller, A. Lee, G.C. Rice, G.H. Wong, T. Gatanaga, G.A. Granger, R. Lentz, H. Raab et al. 1990. Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell.* 61:361-370.