

# Requirement for the NF- $\kappa$ B Family Member RelA in the Development of Secondary Lymphoid Organs

Elizabeth Alcamo,<sup>1</sup> Nir Hacohen,<sup>2</sup> Leah C. Schulte,<sup>2</sup>  
Paul D. Rennert,<sup>3</sup> Richard O. Hynes,<sup>1</sup> and David Baltimore<sup>4</sup>

<sup>1</sup>Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

<sup>2</sup>Whitehead Institute, Cambridge, MA 02142

<sup>3</sup>Biogen Inc., Cambridge, MA 02142

<sup>4</sup>California Institute of Technology, Pasadena, CA 91125

## Abstract

The transcription factor nuclear factor (NF)- $\kappa$ B has been suggested to be a key mediator of the development of lymph nodes and Peyer's patches. However, targeted deletion of NF- $\kappa$ B/Rel family members has not yet corroborated such a function. Here we report that when mice lacking the RelA subunit of NF- $\kappa$ B are brought to term by breeding onto a tumor necrosis factor receptor (TNFR)1-deficient background, the mice that are born lack lymph nodes, Peyer's patches, and an organized splenic microarchitecture, and have a profound defect in T cell-dependent antigen responses. Analyses of TNFR1/RelA-deficient embryonic tissues and of radiation chimeras suggest that the dependence on RelA is manifest not in hematopoietic cells but rather in radioresistant stromal cells needed for the development of secondary lymphoid organs.

Key words: lymph nodes • Peyer's patches • spleen • p65 • TNFR1

## Introduction

The microarchitecture of secondary lymphoid organs plays an important role in facilitating the activation and maturation of antigen-specific lymphocytes and ultimately the mounting of an adaptive immune response (1). T cells are primed by antigen-presenting cells in the T cell zones of the periarteriolar lymphoid sheaths of the spleen, the paracortex of LNs, and the dome area of Peyer's patches (PPs)\* lining the intestine. Primed T cells and antigen-activated B cells then migrate into the primary follicles where they associate with follicular dendritic cells (FDCs), giving rise to germinal centers (GCs). Here, they undergo antigen-driven clonal expansion and differentiation into plasma and memory cells.

Targeted gene deletion and radiation chimera analyses have provided insights into the molecular machinery un-

derlying the development of secondary lymphoid organs, emphasizing the importance of crosstalk between hematopoietic and nonhematopoietic cells. For example, RANK, its ligand RANKL/TRANCE/OPGL, ROR $\gamma$ , TRAF6, Id2, and the nuclear factor  $\kappa$ B-inducing kinase (NIK; as implicated by the spontaneous mouse mutant *aly* [2]) appear to be required to mediate signals important for the survival and/or activation of a hematopoietic cell population involved in LN development (3–10). NIK is also required to mediate signals important for survival and/or activation of a nonhematopoietic cell population at the LN anlage (10, 11). Signaling between the hematopoietic and nonhematopoietic cells relies on the interaction between the membrane-bound cytokine LT $\alpha_1\beta_2$  on hematopoietic cells and its receptor, LT $\beta$ R, on nonhematopoietic cells (11–17). PP development requires all of the same molecules, although RANK and its ligand play a less significant role in the development of these organs than they do in LN development (3, 4, 6, 7, 9–12, 14, 15, 17). In addition, PP development also relies to some extent on intercellular signaling via TNF $\alpha$  interaction with TNFR1 and intracellular signaling through I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ) (18–22). Less is known about the development of splenic structures: of the molecules involved in lymph node development, only

E. Alcamo and N. Hacohen contributed equally to this work.

E. Alcamo's present address is Department of Biological Sciences, Stanford University, Stanford, CA 94305.

Address correspondence to Dr. Nir Hacohen, Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02142. Phone: 617-258-9205; Fax: 617-258-5578; E-mail: hacohen@wi.mit.edu

\*Abbreviations used in this paper: FDC, follicular dendritic cell; GC, germinal center; H & E, hematoxylin and eosin; IKK $\alpha$ , I $\kappa$ B kinase  $\alpha$ ; MZ, marginal zone; NF, nuclear factor; NIK, NF- $\kappa$ B-inducing kinase; PP, Peyer's patch; RT, reverse transcription.

NIK, IKK $\alpha$ , LT $\alpha$  $\beta$ <sub>2</sub>, and to a lesser degree TNF $\alpha$  and RANKL/TRANCE/OPGL have been shown to be required in hematopoietic cells, and NIK, IKK $\alpha$ , LT $\beta$ R, and to a lesser degree TNFR1 and RANK in nonhematopoietic cells (3, 5, 10, 12–15, 17, 19, 22–28).

A number of the aforementioned molecules rely on signaling by members of the NF- $\kappa$ B/Rel family of transcription factors. RANK, NIK, IKK $\alpha$ , TRAF6, LT $\beta$ R, and TNFR1 mediate cellular responses to extracellular signals at least in part by activating NF- $\kappa$ B, and genes encoding the cytokines RANKL/TRANCE/OPGL, TNF $\alpha$ , and LT $\alpha$  are targets of NF- $\kappa$ B-activated gene transcription. Therefore, it has been hypothesized that NF- $\kappa$ B might also play a role in the development of secondary lymphoid organs.

NF- $\kappa$ B transcription factors are homodimeric and heterodimeric complexes of five family members: p50 (NF- $\kappa$ B1), p52 (NF- $\kappa$ B2), c-Rel, RelB, and RelA (p65). Most cells contain only the complex p50/p65, held inactive in the cytoplasm by association with I $\kappa$ B inhibitory proteins. Activation of the cell leads to degradation of I $\kappa$ B and nuclear translocation of NF- $\kappa$ B, resulting in the transcription of multiple target genes (for reviews, see references 29–32). Mice deficient for one or more of the Rel family members have been generated (33–43), revealing redundant and nonredundant roles for Rel proteins in cell survival, hematopoiesis, and innate as well as adaptive immune responses (for reviews, see references 44 and 45).

A role for NF- $\kappa$ B in the development of the splenic microarchitecture has been demonstrated by targeted deletion and radiation chimera studies of p50 and p52: p50 is required in hematopoietic cells for the formation of marginal zone (MZ) cells (46), and p52 is required in nonhematopoietic cells for the formation of FDCs, GCs, and MZ cells (36, 47). In contrast, a role for NF- $\kappa$ B in the development of LNs or PPs has not yet been assigned. However, the importance of the Rel family member RelA in the development of these structures has yet to be determined, because targeted disruption of RelA is lethal at embryonic day 15 (E15) due to the absence of RelA-mediated protection from TNFR1-signaled apoptosis in hepatocytes (33, 48). We and others have generated mice deficient for RelA by breeding the deficiency onto a TNFR1-deficient background (49, 50). We report here that LNs and PPs are absent and the splenic microarchitecture is severely disorganized in TNFR1/RelA-deficient mice, demonstrating a critical role for RelA in secondary lymphoid organ development.

## Materials and Methods

**Histology.** Mice were killed by CO<sub>2</sub> and the intestines and mammary glands excised and fixed overnight in buffered formalin or 70% ethanol/5% formaldehyde/5% acetic acid, respectively. Tissues were embedded in paraffin, sectioned (4–6  $\mu$ m thick), stained with hematoxylin and eosin, and examined by light microscopy.

Spleens and LNs were sectioned at 6  $\mu$ m, mounted on slides, and allowed to dry at room temperature for 5–10 min before storing at  $-20^{\circ}$ C. At the time of staining, sections were fixed in ice-cold acetone for 10 min, washed with PBS/0.1% Tween (PBT), incubated 10 min in 3% H<sub>2</sub>O<sub>2</sub>/methanol if horseradish peroxidase was to be used to visualize staining, and blocked for 15 min in PBT/5% BSA. They were then labeled for 1 h at 37°C with one of the following primary reagents: anti-B220-biotin (1:100; RA3-6B2; BD PharMingen), anti-CD3e-biotin (1:50; 145-2C11; BD PharMingen), anti-CR-1-biotin (8C12; BD PharMingen), PNA-biotin (1:50; Vector Laboratories), MOMA-1 (1:10; Serotec), anti-CD45.2-biotin (1:100; 104; BD PharMingen). Labeling was detected using horseradish peroxidase or alkaline phosphatase Vectastain ABC kits. Sections to be stained for two antigens were washed well in PBT after staining for the first antigen was complete, blocked with avidin and biotin sequentially (Vector), and incubated with the next primary reagent. All sections were photographed at 10 $\times$  magnification using digital imaging technology.

**Flow Cytometry.** Embryonic intestines were harvested from E17.5 fetuses, incubated in Collagenase D (Roche Laboratories) for 1 h at 37°C, then passed twice through a 21-gauge needle, twice through a 23-gauge needle, once through a 26-gauge needle, and filtered through a 70- $\mu$ m strainer to make a single cell suspension for use in flow cytometry.

Adult splenocytes were prepared by crushing spleens between two glass slides and filtering through a 70- $\mu$ m strainer. Cells were plated in 1 ml complete RPMI (C10) at a density of  $2 \times 10^6$  cells per well of a 24-well plate and stimulated for 18 h with 1  $\mu$ g/ml anti-CD3e (145-2C11; BD PharMingen). After stimulation, cells were scraped from the plate and washed three times with FACS<sup>®</sup> buffer to generate a single cell suspension for use in flow cytometry.

For flow cytometric analysis, cells were first incubated with Fc/block (anti-CD32/CD16) (Fc $\gamma$ II/III Receptor, 2.4G2; BD PharMingen) for 5 min. They were then incubated with combinations of the following primary and secondary antibodies: anti-CD4-phycoerythrin (RM4-5; BD PharMingen), anti-CD3e (145-2C11; BD PharMingen), anti-IL-7R $\alpha$  (B12-1; BD PharMingen), anti-B220-biotin (RA3-6B2; BD PharMingen), mLT $\beta$ R-IgG1 (gift of Biogen Inc.), anti-LT $\beta$  (BBF6; Biogen Inc.), streptavidin-FITC (BD PharMingen), streptavidin cyochrome (BD PharMingen), and anti-human IgG-FITC (Jackson ImmunoResearch Laboratories).

**RNA Analysis.** Embryonic intestines were harvested from day E17.5 fetuses and snap frozen in liquid nitrogen. When all tissue samples had been collected and frozen, samples were simultaneously thawed and homogenized with a Polytron homogenizer (Brinkmann) in 1 ml TRIzol reagent (Molecular Research, Inc.). Total RNA was extracted and precipitated as per manufacturer's instructions.

1, 5, or 25 ng of total RNA was used as input for reverse transcription (RT)-PCR (OneStep RT-PCR; QIAGEN) for amplification of LT $\alpha$ , LT $\beta$ , RelA, TNFR1, or HPRT with the following primer combinations: (a) LT $\alpha$ : cagcaggtccagctcttttc, agtgcaaggctccaagaa; (b) LT $\beta$ : ggagcacaggtccagaaaag, gagctcaggggtgaggtcag; (c) RelA: gagccattggagttccagta, tgggggaaaactcatcaag; (d) TNFR1: accaagtccacaaggaac, cagcactggaagtgtgtct; (e) HPRT cttgtgactgctgtggatt, aacctaaccatttggggc.

**Reconstitution of Bone Marrow with Fetal Liver Cells.** Radiation chimeras were generated essentially as described (50). Briefly, donor embryos were generated by crossing TNFR<sup>+/+</sup> RelA<sup>+/-</sup> males to females and TNFR1<sup>-/-</sup> RelA<sup>+/-</sup> males to fe-

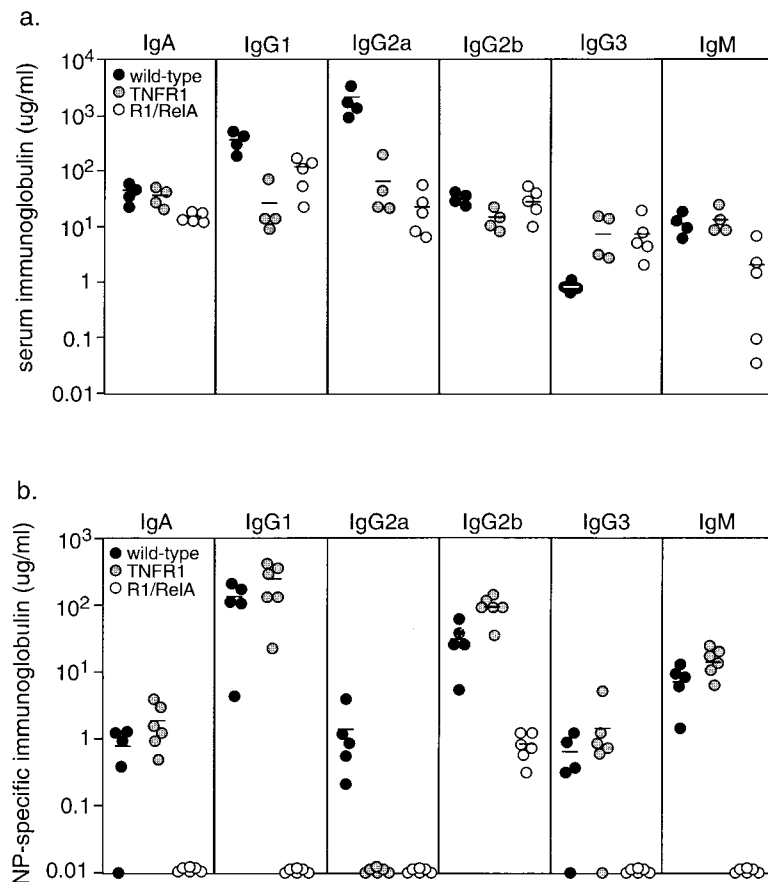
males. Fetal livers were harvested from day 14.5 embryos, and prepared and genotyped as described. Meanwhile, 6–8-wk-old C57BL/6-CD45.1<sup>+</sup> hosts were irradiated with two doses, 800 rads and 400 rads separated by 3 h, using a <sup>137</sup>Cs source. Mice were anesthetized with Avertin (2.5% solution of 2,2,2-tribromoethanol/tert amyl alcohol, 12  $\mu$ l/g mouse) immediately after the second irradiation and transplanted with 10<sup>6</sup> liver cells from wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetuses in 200  $\mu$ l medium by retro-orbital injection. The extent of reconstitution was analyzed 4 mo after transplantation by flow cytometry of one half of the spleen; 90–95% of the immune system of each animal assayed was reconstituted with donor hematopoietic cells (data not shown).

**Immunoglobulin Isotype Analysis.** Sera were prepared from 4-wk-old naive mice or mice that had been intraperitoneally immunized 1 wk before with alum-precipitated NP<sub>7</sub>CG (75  $\mu$ g per mouse). Immunoglobulin concentrations were quantitatively determined using a sandwich ELISA by comparison to isotype standards (Southern Biotechnology Associates, Inc.). Briefly, plates were coated with pan-specific capture antibody (for basal antibody measurements) or NP<sub>23</sub>BSA (for NP-specific response) overnight at 4°C. The next day, they were washed with PBS/0.05% Tween-20 and blocked for 1 h with PBS/1% BSA. Plates were then washed again and incubated overnight at 4°C with sera or isotype standards diluted in series in PBS/1% BSA/0.025% Tween-20. Plates were washed the next morning and the concentration of immunoglobulin measured with isotype-specific antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.).

## Results

**Basal and Specific Antibody Production in TNFR1/RelA-deficient Mice.** Innate immune responses were previously shown to be impaired in TNFR1/RelA-deficient mice (50). To assess the ability of TNFR1/RelA-deficient mice to mount an adaptive immune response, we measured basal and specific antibody production. Basal Ig production was determined by measuring the resting serum immunoglobulin concentrations in unimmunized, age-matched wild-type, TNFR1<sup>-</sup>, and TNFR1/RelA-deficient mice. All classes of Ig were made by all animals but the quantitative data showed that both TNFR1-dependent and TNFR1-independent, RelA-dependent signaling pathways are involved in the production of basal levels of most Ig isotypes (Fig. 1 a).

To assay T cell-dependent response, age-matched wild-type, TNFR1<sup>-</sup>, and TNFR1/RelA-deficient mice were immunized with alum-precipitated NP<sub>7</sub>-CG. 7 d later, the concentration of total (high and low affinity) NP-specific antibodies in sera was measured by binding to densely NP-haptenated bovine serum albumin (NP<sub>23</sub>BSA). Given the sensitivity conferred upon this assay by using a highly haptenated BSA, it was striking to find that no NP-specific IgA, IgG1, IgG2a, IgG3, or IgM antibodies could be detected in sera from TNFR1/RelA-deficient mice relative to wild-type sera (Fig. 1 b); the only NP-specific antibody that could be detected of those tested was IgG2b, and its



**Figure 1.** Severely reduced T cell-dependent antibody response in TNFR1/RelA-deficient mice in vivo. (a) Basal immunoglobulin concentrations. Serum was collected from 3-wk-old wild-type (black circle), TNFR1-deficient (gray circle), and TNFR1/RelA-deficient (open circle) mice and immunoglobulin concentrations determined by isotype-specific ELISA. (b) T cell-dependent antibody production. 3-wk-old mice were immunized with NP-CG adsorbed to alum and the level of NP-specific antibodies determined 1 wk later by ELISA using NP-BSA as capture antibody. Each point represents the mean of duplicate assays from one animal; horizontal bars indicate mean concentration of the group.

concentration was 36-fold less in TNFR1/RelA-deficient sera than in those from wild-type mice (Fig. 1 b). In contrast, the concentration of all NP-specific antibodies was elevated two- to threefold in sera from TNFR1-deficient mice relative to wild-type sera with the exception of IgG2a, which could not be detected in TNFR1-deficient sera (Fig. 1 b). Thus, it appears that TNFR1-independent, RelA-dependent signaling pathways play a critical role in mounting T cell-dependent antibody responses by most isotype classes of immunoglobulin.

*Analysis of Secondary Immune Organs and Structures.* The interactions between the various antigen-presenting cells and lymphocytes that are required to mount a T cell-dependent antibody response are facilitated by the organization of the LNs, PPs, and spleen. Therefore, these structures were inspected in 3-wk-old wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice. Inguinal, iliac, sacral, mesenteric, axillary, lateral axillary, and cervical LNs were readily observed in wild-type and TNFR1-deficient mice by visual inspection. In contrast, although lymphatic vessels were apparent, LNs could not be detected in TNFR1/RelA-deficient mice (Fig. 2, a–f, and data not shown). Inguinal LN or nodal remnants could not be detected in TNFR1/RelA-deficient inguinal fat pads that had been sectioned and hematoxylin and eosin (H & E) stained (Fig. 2, g and h), suggesting that LNs were truly absent rather than reduced in size.

PPs were visible by dissecting microscopy only in wild-type mice (Fig. 2, i–k). However, PPs were observable in TNFR1-deficient mice after sectioning and H & E staining of the intestine (Fig. 2, l and m; references 18 and 20). In contrast, neither PPs nor PP remnants were visible in stained serial sections of the intestines of three TNFR1/RelA-deficient mice (Fig. 2 n), suggesting that TNFR1/RelA-deficient mice lack these organs as well.

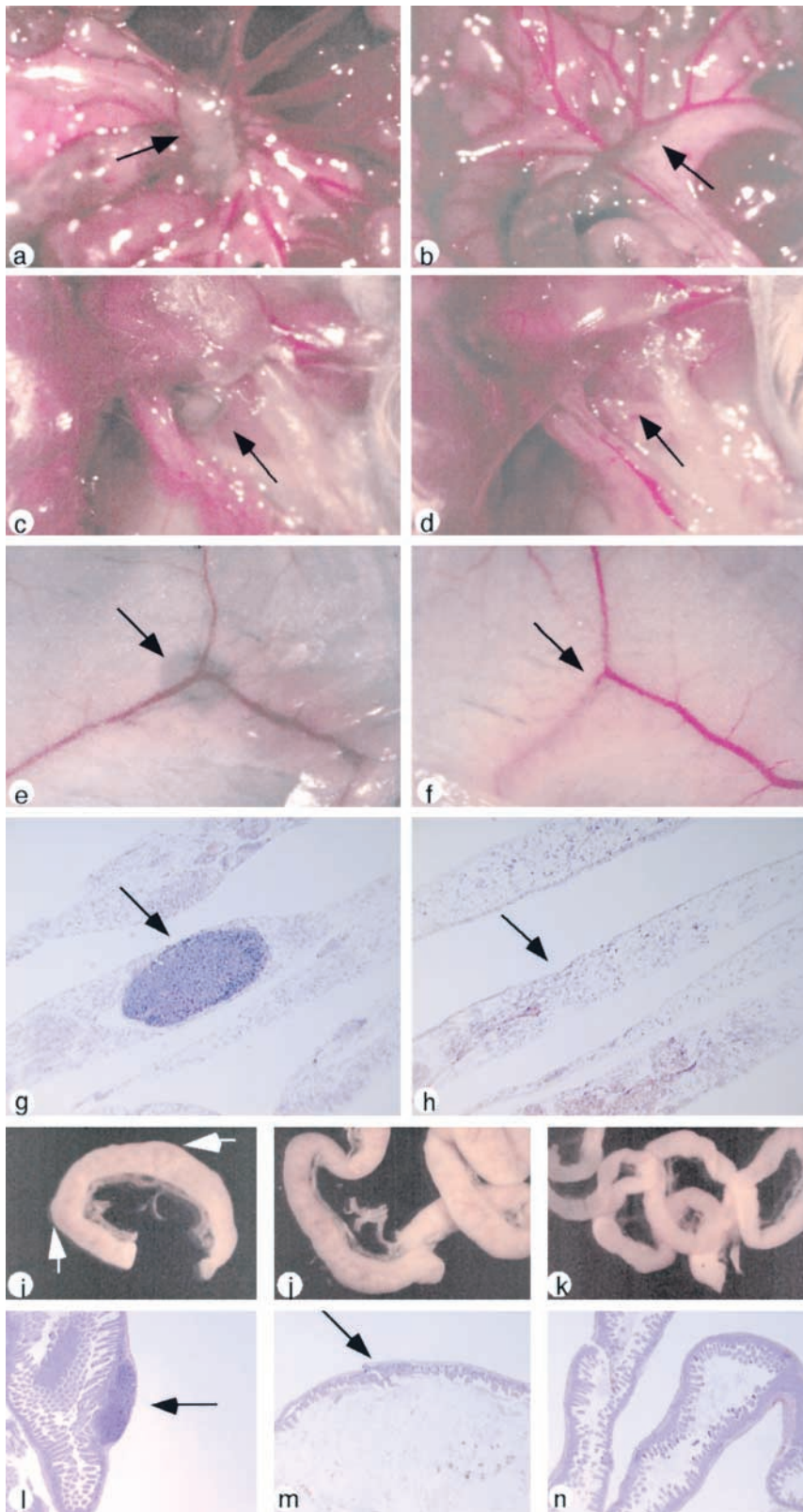
H & E staining of sections of wild-type, TNFR1-deficient, and TNFR1/RelA-deficient spleens revealed that the white pulp of TNFR1/RelA-deficient spleens was markedly disorganized relative to the white pulp of wild-type and TNFR1-deficient spleens (Fig. 3, a–c). To determine the extent to which the splenic microarchitecture was altered in TNFR1/RelA-deficient spleens, 3-wk-old mice were immunized with NP<sub>7</sub>-CG to induce GC formation. Spleens were analyzed 1 wk later by immunohistochemical labeling of T cell zones (anti-CD3) and B cell follicles (anti-B220); FDCs (anti-CR-1) and GCs (PNA<sup>+</sup>); and MZ resident B lymphocytes (anti-CR-1) and metallophilic macrophages (anti-MOMA-1). Consistent with previous results (18, 20, 25, 27, 28), TNFR1-deficient spleens had clearly defined T cell zones (Fig. 3, e and k), B cell follicles (Fig. 3, e and h), and MZs (Fig. 3, q and w). However, B cell follicles appeared mildly reduced (Fig. 3 h), MZ B cell populations were mildly expanded (Fig. 3 q), the GCs were reduced in number and misplaced around the central arteriole (Fig. 3 t), and FDCs were absent (Fig. 3, n and q). Like TNFR1-deficient spleens, TNFR1/RelA-deficient spleens lacked FDCs (Fig. 3, o and r); however, in contrast to TNFR1 mutants, TNFR1/

RelA-deficient spleens also lacked GCs (Fig. 3, o and u) and MZs (Fig. 3, o, r, and x), and B and T cell zones appeared smaller and intermixed around the central arteriole (Fig. 3, f, i, and l).

*Analysis of Embryonic Tissue for CD4<sup>+</sup>CD3<sup>-</sup>IL-7Rα<sup>+</sup> Cells.* A population of CD4<sup>+</sup>CD3<sup>-</sup>IL-7Rα<sup>+</sup> cells is present in embryonic tissues at the sites of LN and PP formation that is believed to induce the development of these secondary lymphoid organs (9, 51–53). To determine if these cells were present in TNFR1/RelA-deficient embryos, embryonic intestinal cells from E17.5 wild-type, TNFR1-, and TNFR1/RelA-deficient mice were analyzed by flow cytometry. From 94 to 96% of these samples were CD3<sup>-</sup>; of these, 1.85, 2.34, and 1.65% were CD4<sup>+</sup>IL-7Rα<sup>+</sup> in wild-type, TNFR1-, and TNFR1/RelA-deficient samples, respectively (Fig. 4). Thus, the CD4<sup>+</sup>CD3<sup>-</sup>IL-7Rα<sup>+</sup> population was represented in embryonic intestines of all genotypes, suggesting that the lack of LNs and PPs in TNFR1/RelA-deficient mice is not due to the failure of this cell population to develop.

*Analysis of Embryonic Tissue for Expression of LTα<sub>1</sub>β<sub>2</sub>.* Expression of the membrane-bound cytokine LTα<sub>1</sub>β<sub>2</sub> is required for the development of LN, PP, and all splenic structures (for reviews, see references 54 and 55). To determine if LTα<sub>1</sub>β<sub>2</sub> is expressed by cells in TNFR1/RelA-deficient embryos, we measured LTα and LTβ expression levels in E17.5 intestinal tissue by RT-PCR. LTα and LTβ levels were comparable in wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice (Fig. 5). Consistent with these results, similar levels of surface LTα/β expression was also observed in all three genotypes by flow cytometry using murine LTβR-Ig (unpublished data) (14, 56). These results suggest that the absence of LNs and PPs in TNFR1/RelA-deficient mice is not due to a decrease in the number of cells expressing LTα<sub>1</sub>β<sub>2</sub> or in the level of LTα<sub>1</sub>β<sub>2</sub> expression by these cells.

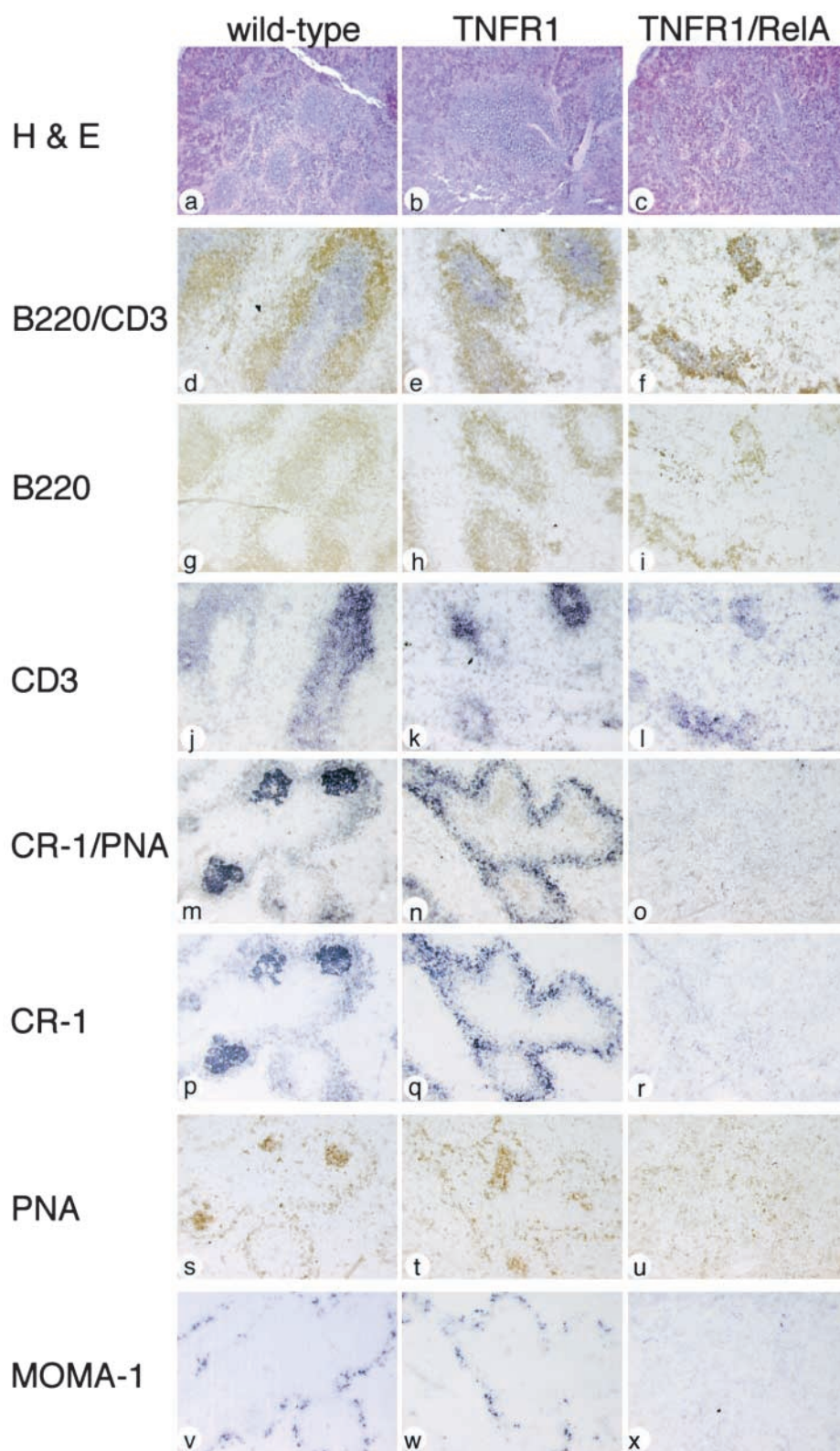
*LN Repopulation and Splenic Architecture in Radiation Chimeras.* An apparent absence of LNs and PPs could be due to an intrinsic defect in the ability of hematopoietic cells to home to or populate stromal structures. To determine if this was true of TNFR1/RelA-deficient hematopoietic cells, lethally irradiated C57BL/6 CD45.1<sup>+</sup> wild-type hosts were engrafted with CD45.2<sup>+</sup> wild-type, TNFR1-, RelA-, or TNFR1/RelA-deficient fetal liver cells and their immune systems allowed to reconstitute for 4 mo. LNs of these radiation chimeras were then assayed for the presence of donor cells by immunohistochemical labeling of cryosections for CD45.2<sup>+</sup> donor populations. Labeling for CD45.2 was comparably extensive in cryosections of LNs harvested from mice reconstituted with all genotypes (Fig. 6, b and c); the absence of background staining was confirmed by labeling sections of LNs from untransplanted hosts (Fig. 6 a). This demonstrates that TNFR1/RelA-deficient hematopoietic cells can repopulate existing LNs as well as wild-type hematopoietic cells, and suggests that the absence of LNs and PPs is not due to an intrinsic defect in the ability of TNFR1/RelA hematopoietic cells to home to these organs.



**Figure 2.** Absence of LNs and PPs in TNFR1/RelA-deficient mice. Five 3-wk-old wild-type (a, c, e, g, i, and l), TNFR1-deficient (j and m) and TNFR1/RelA-deficient (b, d, f, h, k, and n) mice were injected intraperitoneally with Evans Blue (1 mg/ml, 20  $\mu$ l per mouse) 18 h before dissection to visualize LNs and PPs. (a and b) mesenteries; (c and d) lateral axillary fossae; (e and f) inguinal fat pad; (g and h) inguinal fat pad, sectioned and H & E-stained; (i, j, and k) intestine whole mounts; (l, m, and n) intestine, sectioned and H & E-stained. Arrows indicate regions in which LNs and PPs should be found. 15 additional mice of each mutant genotype were inspected for the presence of these organs without prior injection of Evan Blue; similar results were observed.

Similarly, previous studies of radiation chimera models have suggested that the absence of splenic structures could be due to an intrinsic defect in the ability of hematopoietic cells to induce their formation and maintenance (25,

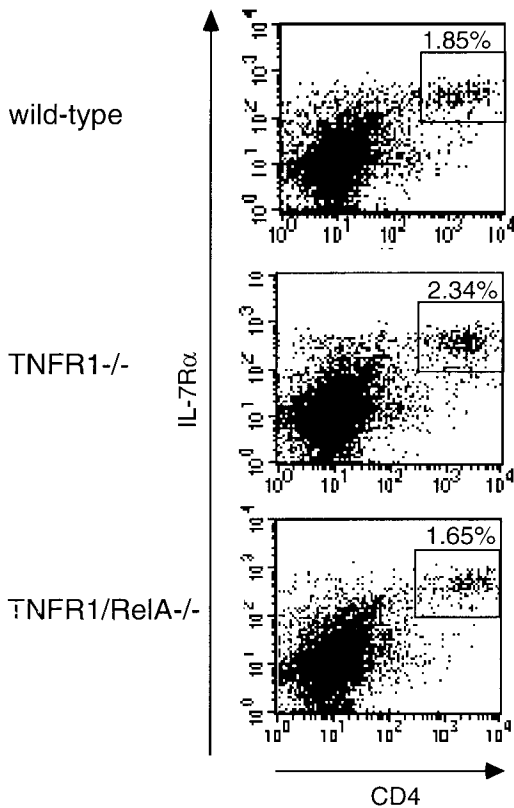
57). To determine if the disrupted microarchitecture in TNFR1/RelA-deficient spleens was due to a hematopoietic cell-intrinsic defect in inducing these structures, the spleens of the aforementioned radiation chimeras were an-



**Figure 3.** Aberrant organization of lymphocytes and absence of secondary immune response structures in TNFR1/RelA-deficient spleens. 3-wk-old wild-type (a, d, g, j, m, p, s, and v), TNFR1-deficient (b, e, h, k, n, q, t, and w), and TNFR1/RelA-deficient mice (c, f, i, l, o, r, u, and x) were immunized with NP<sub>7</sub>-CG and killed 1 wk later for analysis of splenic structures. Two wild-type, two TNFR1-deficient, and five TNFR1/RelA-deficient spleens were assayed. Sequential sections of spleen were stained with (a–c) H & E; (d–l) anti-B220 (brown) and/or anti-CD3 (purple); (m–u) anti-CR-1 (purple) and/or PNA (brown); and (v–x) MOMA-1 (purple).

alyzed by immunohistochemical labeling. T cell zones (Fig. 6, d–g), B cell follicles (Fig. 6, d–g), FDCs (Fig. 6, h–o), GCs (Fig. 6, h–k, p–s), and MZs (Fig. 6, t–w) were evident in spleens from radiation chimeras generated with

any type of donor population, suggesting that the TNFR1/RelA-deficient hematopoietic cells are competent to induce splenic structures and that the absence of splenic structures in TNFR1/RelA-deficient mice is



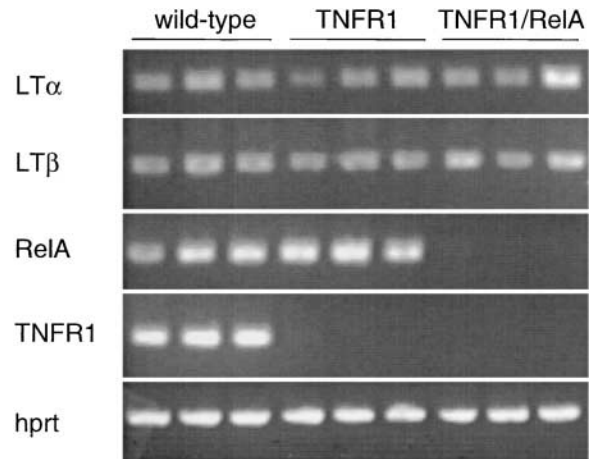
**Figure 4.** Presence of  $CD4^+CD3^-IL-7R\alpha^+$  cells in  $TNFR1/RelA$ -deficient embryonic intestine. Flow cytometry was used to analyze intestinal cells from E17.5 wild-type,  $TNFR1$ -deficient, and  $TNFR1/RelA$  fetuses with the indicated cell-surface markers. All events presented are negative for  $CD3$ . Each sample assayed consisted of tissue from three embryos of the same genotype. Similar results were obtained from two independent FACS<sup>®</sup> experiments.

therefore due to the absence of  $TNFR1$  and/or  $RelA$  in nonhematopoietic cell populations.

## Discussion

We demonstrate here that  $TNFR1/RelA$ -deficient mice lacked LNs, PPs, an organized splenic microarchitecture, and T cell-dependent Ig responses. In contrast,  $TNFR1$ -deficient mice lacked only a subset of splenic structures and had relatively intact Ig responses. The disrupted architecture of the  $TNFR1$ -deficient spleen demonstrates that  $TNFR1$  plays an important role in mediating the stimulation of FDC and GC formation, a role which may or may not rely on  $RelA$  activation. However, the more severe phenotype of the  $TNFR1/RelA$ -deficient mice demonstrates definitively that  $RelA$  plays an essential role in the development of LNs, PPs, and splenic B cell follicles and MZs, and that this role must be independent of signaling through  $TNFR1$ .

Consistent with this, we observed that whereas NP antigen-specific antibody production was largely normal in  $TNFR1$ -deficient mice, it was profoundly reduced or absent in  $TNFR1/RelA$ -deficient mice. In contrast, basal

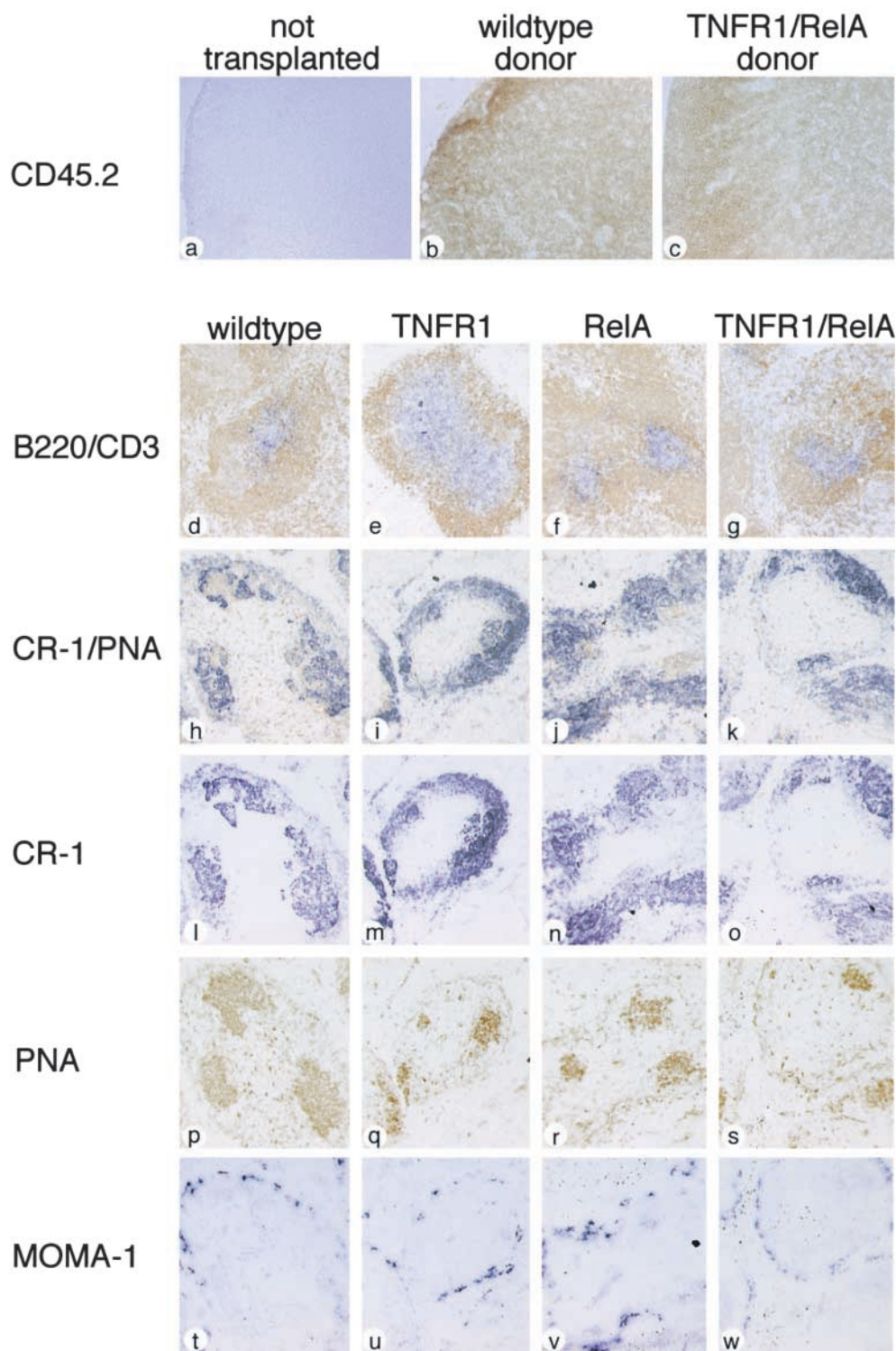


**Figure 5.** Normal expression of  $LT\alpha_1\beta_2$  in  $TNFR1/RelA$ -deficient embryonic intestine. RNA was prepared from E17.5 wild-type,  $TNFR1$ -deficient,  $TNFR1/RelA$ -deficient intestines and assayed by RT-PCR for expression of  $LT\alpha$  and  $LT\beta$ ,  $RelA$ ,  $TNFR1$ , and  $hprt$  (control).

immunoglobulin production was only somewhat reduced in both  $TNFR1$ - and  $TNFR1/RelA$ -deficient mice. This demonstrates a significant defect in the T cell-dependent antibody responses of  $TNFR1/RelA$ -deficient mice, and suggests that this defect is due to the loss of  $RelA$ -dependent transcription that is not induced by  $TNFR1$ . A similar defect in antibody production has been observed in other mutant mice lacking LNs, PPs, and an organized splenic microarchitecture (14, 17, 19, 58–61), thereby providing corroborative evidence that organized lymphoid structures may play a minor role in determining basal levels of Ig but are required to mount an antibody response.

In which cells is  $RelA$  required? In the case of LN and PP formation, this question is best addressed by assaying the putative anlage sites for the presence of cells and proteins known to be important in the development of these organs.  $CD4^+CD3^-IL-7R\alpha^+$  hematopoietic cells were present in  $TNFR1/RelA$ -deficient embryonic tissue, and mRNA and protein levels of membrane-bound cytokine  $LT\alpha_1\beta_2$  in embryonic tissue were comparable to those in wild-type tissue. This suggests that, in contrast to the  $Id2$ -,  $LT\alpha$ -, or  $LT\beta$ -deficient mice, the failure of  $TNFR1/RelA$ -deficient mice to develop LNs and PPs is not due to a defect in the ability of hematopoietic cells to induce nonhematopoietic cells to promote the development of secondary immune organs.

The cellular requirement for  $RelA$  in the development of splenic structures is best assessed by studying radiation chimeras: in contrast to the development of LNs and PPs, which occurs during a fixed window of embryogenesis (13, 62), the development of the splenic microarchitecture is plastic through adulthood. We show here that B cells, T cells, FDCs, GCs, and MZ cells were present and appropriately organized in radiation chimeras generated with  $TNFR1/RelA$ -deficient fetal liver cells, suggesting that  $RelA$  is required in the nonhematopoietic cell compart-



**Figure 6.** Repopulation of LNs and organization of spleen in adoptive transfer mice reconstituted with TNFR1/RelA-deficient hematopoietic cells. C57BL/6 CD45.1<sup>+</sup> mice were lethally irradiated and transplanted with wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetal liver cells. 4 mo after transplant, animals were killed and tissues removed for cryosectioning. LNs from one animal and spleens from two animals of each genotype were assayed. (a–c) Inguinal LNs stained with donor-specific anti-CD45.2 (brown). (d–w) Sequential sections of spleen stained with (d–g) anti-B220 (brown) and/or anti-CD3 (purple); (h–s) anti-CR-1 (purple) and/or PNA (brown); and (t–w) anti-MOMA-1 (purple).

ment for the development of an organized splenic microarchitecture. We attempted to confirm this result by performing reciprocal radiation chimera experiments, in which wild-type hematopoietic cells were used as donors to reconstitute the immune systems of irradiated wild-type, TNFR1-deficient, and TNFR1/RelA-deficient mice.

However, TNFR1/RelA-deficient hosts consistently did not survive more than 2 wk after radiation and transplantation, a time too short for reconstitution to be complete (unpublished data).

While our radiation chimera experiments suggest that RelA is required in nonhematopoietic cells for proper



spleen development, it should be noted that we and others have also observed intrinsic defects in RelA-deficient lymphocytes, i.e., a defect in the ability of TNFR1/RelA- and RelA-deficient T and B cells to be activated by polyclonal stimuli *in vitro* (unpublished data) (35, 63). Such an intrinsic cellular defect could, in principle, contribute to the reduction in the T cell-dependent immune response observed in TNFR1/RelA-deficient mice. However, our analysis of splenic structures in radiation chimeras suggests that these intrinsic defects do not have a qualitative effect on the ability of TNFR1/RelA-deficient cells to organize into structures in the spleen. A more quantitative study will be required to assess whether there is a measurable effect of these intrinsic defects on the size and organization of these structures.

Studies with other NF- $\kappa$ B radiation chimeras have demonstrated a similar nonhematopoietic cell requirement for p52 in the formation of FDCs, GCs, and MZs (36, 47). Given that the defect in secondary lymphoid organ development in p52-deficient mice is limited to the spleen, whereas that of TNFR1/RelA-deficient mice extends to LNs and PPs as well, it is possible that p52/RelA heterodimers are responsible for the formation of splenic structures, but that RelA heterodimerizes with other Rel family members to fulfill a comparable role in stromal cells of the LN and PP anlage.

Given that NF- $\kappa$ B is known to either directly regulate the expression of signaling molecules that have been implicated in secondary lymphoid organ development or to regulate the expression of genes downstream of them, a number of potential roles for RelA can be envisioned in the development of these structures. For example, the phenotype of the TNFR1/RelA-deficient mouse could be a synthetic phenotype due to the deletion of TNFR1 combined with the abrogation of RANK signaling as a result of the absence of RelA activity. However, this is unlikely, as TNFR1/RelA-deficient animals have normal numbers of embryonic CD4<sup>+</sup>CD3<sup>-</sup>IL-7R $\alpha$ <sup>+</sup> cells whereas these numbers are severely reduced in RANKL/TRANCE/OPGL-deficient animals (5); in addition, RANK- and RANKL/TRANCE/OPGL-deficient mice lack teeth (3, 4), which TNFR1/RelA-deficient mice possess.

A more straightforward model is that RelA mediates LT $\beta$ R signaling in nonhematopoietic cells. Targeted deletion of LT $\beta$ R or disruption of signaling by LT $\beta$ R leads to the absence of the same secondary organs and structures that are absent in TNFR1/RelA-deficient mice. In addition, similar to our results, radiation chimera experiments have demonstrated a requirement for LT $\beta$ R in nonhematopoietic cells for the formation of secondary lymphoid organs (64, 65). Finally, LT $\beta$ R mutant mice lack an antibody response to an extent that is comparable to that of the TNFR1/RelA-deficient mice. Together, these results suggest that the developmental phenotype observed in the TNFR1/RelA-deficient mouse occurs because LT $\beta$ R-mediated signaling to activate transcription in nonhemo-

poietic cells is genetically abrogated in the absence of RelA.

What could be the importance of RelA-mediated transcription activity? LT $\beta$ R is a member of the TNF superfamily of receptors which, like TNFR1, not only activates NF- $\kappa$ B during signaling (66) but also mediates cytotoxic responses in some cell lines (67, 68). It is possible that, similar to its role downstream of TNFR1, RelA is acting downstream of LT $\beta$ R and its activation provides protection against the cytotoxic effects of LT $\beta$ R-mediated signaling; in the absence of RelA, LT $\beta$ R-expressing stromal cells would die instead of develop into secondary lymphoid organs. However, MEFs generated from RelA-deficient and TNFR1/RelA-deficient embryos were as viable as wild-type and TNFR1-deficient MEFs after treatment with concentrations of mLT $\alpha\beta$  that have proven cytotoxic to several cell lines (unpublished data). Thus, either RelA does not play an antiapoptotic role after LT $\beta$ R stimulation, or the responses of mouse embryonic fibroblasts are not representative of the responses of stromal cells expressing LT $\beta$ R *in vivo*.

We have observed that stromal cells of the lung require RelA to produce chemokines and cell adhesion molecules in response to LPS (50). Similarly, RelA might play a role in secondary lymphoid development by inducing the expression of chemokines and cell adhesion molecules that are necessary for trafficking hematopoietic cells to anlage sites. For example, NF- $\kappa$ B regulates the expression of vascular cell adhesion molecule (VCAM)-1 (69), which is expressed on clusters of cells that mark embryonic sites for PP development (70) and can be induced in fibroblasts by stimulation of LT $\beta$ R with mLT $\alpha\beta$  (11). NF- $\kappa$ B also regulates the expression of two genes that are transiently expressed in the high endothelial venules of developing LNs: intercellular adhesion molecule (ICAM)-1 (71–73), which promotes adhesion between leukocytes and endothelium, and the mucosal vascular addressin cell adhesion molecule (MadCAM)-1 (74), which interacts with homing receptors on lymphocytes and monocytes (75, 76). More extensive analysis of gene and protein expression during lymphoid organ formation will reveal if the expression of these and other molecules is changed in the absence of RelA, providing further insights into the role of RelA in the development of secondary lymphoid organs.

We wish to thank Jeffrey L. Browning and colleagues at Biogen, Inc. for creating, characterizing, and producing LT-specific reagents; Denise Crowley for advice on immunohistochemistry; Jianzhu Chen for advice on antibody analysis; Paul Majewski for technical assistance; and Stephen Robinson for helpful suggestions and critical review of this work as it progressed.

This work was supported by the National Institutes of Health grants (to D. Baltimore and E. Alcamo), and by grants from the Whitehead Institute Fellows Program, Hascoe Foundation, and Rippel Foundation (to N. Hacohen).

Submitted: 13 November 2001

Accepted: 30 November 2001

## References

1. Karrer, U., A. Althage, B. Odermatt, C.W. Roberts, S.J. Korsmeyer, S. Miyawaki, H. Hengartner, and R.M. Zinkernagel. 1997. On the key role of secondary lymphoid organs in antiviral immune responses studied in alymphoplastic (aly/aly) and spleenless (Hox11(-)/-) mutant mice. *J. Exp. Med.* 185:2157–2170.
2. Shinkura, R., K. Kitada, F. Matsuda, K. Tashiro, K. Ikuta, M. Suzuki, K. Kogishi, T. Serikawa, and T. Honjo. 1999. Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-kappa b-inducing kinase. *Nat. Genet.* 22: 74–77.
3. Dougall, W.C., M. Glaccum, K. Charrier, K. Rohrbach, K. Brasel, T. De Smedt, E. Daro, J. Smith, M.E. Tometsko, C.R. Maliszewski, et al. 1999. RANK is essential for osteoclast and lymph node development. *Genes Dev.* 13:2412–2424.
4. Kong, Y.Y., H. Yoshida, I. Sarosi, H.L. Tan, E. Timms, C. Capparelli, S. Morony, A.J. Oliveira-dos-Santos, G. Van, A. Itie, et al. 1999. OPG is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* 397:315–323.
5. Kim, D., R.E. Mebius, J.D. MacMicking, S. Jung, T. Cupedo, Y. Castellanos, J. Rho, B.R. Wong, R. Josien, N. Kim, et al. 2000. Regulation of peripheral lymph node genesis by the tumor necrosis factor family member TRANCE. *J. Exp. Med.* 192:1467–1478.
6. Sun, Z., D. Unutmaz, Y.R. Zou, M.J. Sunshine, A. Pierani, S. Brenner-Morton, R.E. Mebius, and D.R. Littman. 2000. Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science.* 288:2369–2373.
7. Kurebayashi, S., E. Ueda, M. Sakaue, D.D. Patel, A. Medvedev, F. Zhang, and A.M. Jetten. 2000. Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc. Natl. Acad. Sci. USA.* 97:10132–10137.
8. Naito, A., S. Azuma, S. Tanaka, T. Miyazaki, S. Takaki, K. Takatsu, K. Nakao, K. Nakamura, M. Katsuki, T. Yamamoto, and J. Inoue. 1999. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells.* 4:353–362.
9. Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature.* 397:702–706.
10. Miyawaki, S., Y. Nakamura, H. Suzuka, M. Koba, R. Yasumizu, S. Ikehara, and Y. Shibata. 1994. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* 24:429–434.
11. Matsumoto, M., K. Iwamasa, P.D. Rennert, T. Yamada, R. Suzuki, A. Matsushima, M. Okabe, S. Fujita, and M. Yokoyama. 1999. Involvement of distinct cellular compartments in the abnormal lymphoid organogenesis in lymphotoxin-alpha-deficient mice and alymphoplasia (aly) mice defined by the chimeric analysis. *J. Immunol.* 163:1584–1591.
12. 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 lymphotoxin. *Science.* 264:703–707.
13. Rennert, P.D., J.L. Browning, R. Mebius, F. Mackay, and P.S. Hochman. 1996. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184:1999–2006.
14. Koni, P.A., R. Sacca, P. Lawton, J.L. Browning, N.H. Ruddle, and R.A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity.* 6:491–500.
15. Alimzhanov, M.B., D.V. Kuprash, M.H. Kosco-Vilbois, A. Luz, R.L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S.A. Nedospasov, and K. Pfeffer. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. *Proc. Natl. Acad. Sci. USA.* 94:9302–9307.
16. Rennert, P.D., D. James, F. Mackay, J.L. Browning, and P.S. Hochman. 1998. Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. *Immunity.* 9:71–79.
17. Futterer, A., K. Mink, A. Luz, M.H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity.* 9:59–70.
18. Neumann, B., A. Luz, K. Pfeffer, and B. Holzmann. 1996. Defective Peyer's patch organogenesis in mice lacking the 55-kD receptor for tumor necrosis factor. *J. Exp. Med.* 184: 259–264.
19. Komer, H., M. Cook, D.S. Riminton, F.A. Lemckert, R.M. Hoek, B. Ledermann, F. Kontgen, B. Fazekas de St Groth, and J.D. Sedgwick. 1997. Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue. *Eur. J. Immunol.* 27:2600–2609.
20. Pasparakis, M., L. Alexopoulou, M. Grell, K. Pfizenmaier, H. Bluethmann, and G. Kollias. 1997. Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor [published erratum at 94:9510]. *Proc. Natl. Acad. Sci. USA.* 94:6319–6323.
21. Matsushima, A., T. Kaisho, P.D. Rennert, H. Nakano, K. Kurosawa, D. Uchida, K. Takeda, S. Akira, and M. Matsumoto. 2001. Essential role of nuclear factor (NF)-kappaB-inducing kinase and inhibitor of kappaB (IkappaB) kinase alpha in NF-kappaB activation through lymphotoxin beta receptor, but not through tumor necrosis factor receptor I. *J. Exp. Med.* 193:631–636.
22. Senfleben, U., Y. Cao, G. Xiao, F.R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S.C. Sun, and M. Karin. 2001. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science.* 293:1495–1499.
23. Koike, R., T. Nishimura, R. Yasumizu, H. Tanaka, Y. Hataba, T. Watanabe, S. Miyawaki, and M. Miyasaka. 1996. The splenic marginal zone is absent in alymphoplastic aly mutant mice. *Eur. J. Immunol.* 26:669–675.
24. Yamada, T., T. Mitani, K. Yorita, D. Uchida, A. Matsushima, K. Iwamasa, S. Fujita, and M. Matsumoto. 2000. Abnormal immune function of hemopoietic cells from alymphoplasia (aly) mice, a natural strain with mutant NF-kappa B-inducing kinase. *J. Immunol.* 165:804–812.
25. Matsumoto, M., S. Mariathasan, M.H. Nahm, F. Baranyay, J.J. Peschon, and D.D. Chaplin. 1996. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science.* 271:1289–1291.
26. Endres, R., M.B. Alimzhanov, T. Plitz, A. Futterer, M.H. Kosco-Vilbois, S.A. Nedospasov, K. Rajewsky, and K. Pfeffer.

- fer. 1999. Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J. Exp. Med.* 189:159–168.
27. Le Hir, M., H. Bluethmann, M.H. Kosco-Vilbois, M. Muller, F. di Padova, M. Moore, B. Ryffel, and H.P. Eugster. 1996. Differentiation of follicular dendritic cells and full antibody responses require tumor necrosis factor receptor-1 signaling. *J. Exp. Med.* 183:2367–2372.
  28. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* 184:1397–1411.
  29. Baeuerle, P.A. 1998. Pro-inflammatory signaling: last pieces in the NF-kappaB puzzle? *Curr Biol.* 8:R19–22.
  30. Baeuerle, P.A. 1998. IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell.* 95:729–731.
  31. May, M.J., and S. Ghosh. 1998. Signal transduction through NF-kappa B. *Immunol. Today.* 19:80–88.
  32. Verma, I.M., J.K. Stevenson, E.M. Schwarz, D. Van Antwerp, and S. Miyamoto. 1995. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev.* 9:2723–2735.
  33. Beg, A.A., W.C. Sha, R.T. Bronson, S. Ghosh, and D. Baltimore. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature.* 376:167–170.
  34. Burkly, L., C. Hession, L. Ogata, C. Reilly, L.A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature.* 373:531–536.
  35. Doi, T.S., T. Takahashi, O. Taguchi, T. Azuma, and Y. Obata. 1997. NF-kappa B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. *J. Exp. Med.* 185:953–961.
  36. Franzoso, G., L. Carlson, L. Poljak, E.W. Shores, S. Epstein, A. Leonardi, A. Grinberg, T. Tran, T. Scharton-Kersten, M. Anver, et al. 1998. Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *J. Exp. Med.* 187:147–159.
  37. Grossmann, M., D. Metcalf, J. Merryfull, A. Beg, D. Baltimore, and S. Gerondakis. 1999. The combined absence of the transcription factors rel and RelA leads to multiple hemopoietic cell defects. *Proc. Natl. Acad. Sci. USA.* 96:11848–11853.
  38. Horwitz, B.H., M.L. Scott, S.R. Cherry, R.T. Bronson, and D. Baltimore. 1997. Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. *Immunity.* 6:765–772.
  39. Iotsova, V., J. Caamano, J. Loy, Y. Yang, A. Lewin, and R. Bravo. 1997. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat. Med.* 3:1285–1289.
  40. Kontgen, F., R.J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis. 1995. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev.* 9:1965–1977.
  41. Sha, W.C., H.C. Liou, E.I. Tuomanen, and D. Baltimore. 1995. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell.* 80:321–330.
  42. Weih, F., D. Carrasco, S.K. Durham, D.S. Barton, C.A. Rizzo, R.P. Ryseck, S.A. Lira, and R. Bravo. 1995. Multi-organ inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. *Cell.* 80:331–340.
  43. Weih, F., S.K. Durham, D.S. Barton, W.C. Sha, D. Baltimore, and R. Bravo. 1997. p50-NF-kappaB complexes partially compensate for the absence of RelB: severely increased pathology in p50(-/-)relB(-/-) double-knockout mice. *J. Exp. Med.* 185:1359–1370.
  44. Barnes, P.J., and M. Karin. 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336:1066–1071.
  45. Sha, W.C. 1998. Regulation of immune responses by NF-kappa B/Rel transcription factor [published erratum at 187:661]. *J. Exp. Med.* 187:143–146.
  46. Cariappa, A., H.C. Liou, B.H. Horwitz, and S. Pillai. 2000. Nuclear factor kappaB is required for the development of marginal zone B lymphocytes. *J. Exp. Med.* 192:1175–1182.
  47. Caamano, J.H., C.A. Rizzo, S.K. Durham, D.S. Barton, C. Raventos-Suarez, C.M. Snapper, and R. Bravo. 1998. Nuclear factor (NF)-kappa B2 (p100/p52) is required for normal splenic microarchitecture and B cell-mediated immune responses. *J. Exp. Med.* 187:185–196.
  48. Beg, A.A., and D. Baltimore. 1996. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science.* 274:782–784.
  49. Doi, T.S., M.W. Marino, T. Takahashi, T. Yoshida, T. Sakakura, L.J. Old, and Y. Obata. 1999. Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. *Proc. Natl. Acad. Sci. USA.* 96:2994–2999.
  50. Alcamo, E., J.P. Mizgerd, B.H. Horwitz, R. Bronson, A.A. Beg, M. Scott, C.M. Doerschuk, R.O. Hynes, and D. Baltimore. 2001. Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappaB in leukocyte recruitment. *J. Immunol.* 167:1592–1600.
  51. Mebius, R.E., P. Rennert, and I.L. Weissman. 1997. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity.* 7:493–504.
  52. Adachi, S., H. Yoshida, K. Honda, K. Maki, K. Saijo, K. Ikuta, T. Saito, and S.I. Nishikawa. 1998. Essential role of IL-7 receptor alpha in the formation of Peyer's patch anlage. *Int. Immunol.* 10:1–6.
  53. Yoshida, H., K. Honda, R. Shinkura, S. Adachi, S. Nishikawa, K. Maki, K. Ikuta, and S.I. Nishikawa. 1999. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int. Immunol.* 11:643–655.
  54. Chaplin, D.D., and Y. Fu. 1998. Cytokine regulation of secondary lymphoid organ development. *Curr. Opin. Immunol.* 10:289–297.
  55. von Boehmer, H. 1997. Lymphotoxins: from cytotoxicity to lymphoid organogenesis. *Proc. Natl. Acad. Sci. USA.* 94:8926–8927.
  56. Browning, J.L., I.D. Sizing, P. Lawton, P.R. Bourdon, P.D. Rennert, G.R. Majeau, C.M. Ambrose, C. Hession, K. Miatkowski, D.A. Griffiths, et al. 1997. Characterization of lymphotoxin-alpha beta complexes on the surface of mouse lymphocytes. *J. Immunol.* 159:3288–3298.

57. Mebius, R.E., S. van Tuijl, I.L. Weissman, and T.D. Randall. 1998. Transfer of primitive stem/progenitor bone marrow cells from LT  $\alpha^{-/-}$  donors to wild-type hosts: implications for the generation of architectural events in lymphoid B cell domains. *J. Immunol.* 161:3836–3843.
58. Fu, Y.X., H. Molina, M. Matsumoto, G. Huang, J. Min, and D.D. Chaplin. 1997. Lymphotoxin- $\alpha$  (LT $\alpha$ ) supports development of splenic follicular structure that is required for IgG responses. *J. Exp. Med.* 185:2111–2120.
59. Banks, T.A., B.T. Rouse, M.K. Kerley, P.J. Blair, V.L. Godfrey, N.A. Kuklin, D.M. Bouley, J. Thomas, S. Kanangat, and M.L. Mucenski. 1995. Lymphotoxin- $\alpha$ -deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685–1693.
60. Eugster, H.P., M. Muller, U. Karrer, B.D. Car, B. Schnyder, V.M. Eng, G. Woerly, M. Le Hir, F. di Padova, M. Aguet, et al. 1996. Multiple immune abnormalities in tumor necrosis factor and lymphotoxin- $\alpha$  double-deficient mice. *Int. Immunol.* 8:23–36.
61. Ryffel, B., F. Di Padova, M.H. Schreier, M. Le Hir, H.P. Eugster, and V.F. Quesniaux. 1997. Lack of type 2 T cell-independent B cell responses and defect in isotype switching in TNF-lymphotoxin  $\alpha$ -deficient mice. *J. Immunol.* 158:2126–2133.
62. Ettinger, R., J.L. Browning, S.A. Michie, W. van Ewijk, and H.O. McDevitt. 1996. Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin- $\beta$  receptor-IgG1 fusion protein. *Proc. Natl. Acad. Sci. USA.* 93:13102–13107.
63. Horwitz, B.H., P. Zelazowski, Y. Shen, K.M. Wolcott, M.L. Scott, D. Baltimore, and C.M. Snapper. 1999. The p65 subunit of NF- $\kappa$ B is redundant with p50 during B cell proliferative responses, and is required for germline CH transcription and class switching to IgG3. *J. Immunol.* 162:1941–1946.
64. Matsumoto, M., Y.X. Fu, H. Molina, G. Huang, J. Kim, D.A. Thomas, M.H. Nahm, and D.D. Chaplin. 1997. Distinct roles of lymphotoxin  $\alpha$  and the type I tumor necrosis factor (TNF) receptor in the establishment of follicular dendritic cells from non-bone marrow-derived cells. *J. Exp. Med.* 186:1997–2004.
65. Tkachuk, M., S. Bolliger, B. Ryffel, G. Pluschke, T.A. Banks, S. Herren, R.H. Gisler, and M.H. Kosco-Vilbois. 1998. Crucial role of tumor necrosis factor receptor 1 expression on nonhematopoietic cells for B cell localization within the splenic white pulp. *J. Exp. Med.* 187:469–477.
66. Mackay, F., G.R. Majeau, P.S. Hochman, and J.L. Browning. 1996. Lymphotoxin  $\beta$  receptor triggering induces activation of the nuclear factor  $\kappa$ B transcription factor in some cell types. *J. Biol. Chem.* 271:24934–24938.
67. Browning, J.L., K. Miatkowski, I. Sizing, D. Griffiths, M. Zafari, C.D. Benjamin, W. Meier, and F. Mackay. 1996. Signaling through the lymphotoxin  $\beta$  receptor induces the death of some adenocarcinoma tumor lines. *J. Exp. Med.* 183:867–878.
68. Mackay, F., P.R. Bourdon, D.A. Griffiths, P. Lawton, M. Zafari, I.D. Sizing, K. Miatkowski, A. Ngam-ek, C.D. Benjamin, C. Hession, et al. 1997. Cytotoxic activities of recombinant soluble murine lymphotoxin- $\alpha$  and lymphotoxin- $\alpha$   $\beta$  complexes. *J. Immunol.* 159:3299–3310.
69. Iademarco, M.F., J.J. McQuillan, G.D. Rosen, and D.C. Dean. 1992. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J. Biol. Chem.* 267:16323–16329.
70. Adachi, S., H. Yoshida, H. Kataoka, and S. Nishikawa. 1997. Three distinctive steps in Peyer's patch formation of murine embryo. *Int. Immunol.* 9:507–514.
71. Hou, J., V. Baichwal, and Z. Cao. 1994. Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* 91:11641–11645.
72. Jahnke, A., and J.P. Johnson. 1994. Synergistic activation of intercellular adhesion molecule 1 (ICAM-1) by TNF- $\alpha$  and IFN- $\gamma$  is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor Stat1  $\alpha$  (p91) that can be activated by both IFN- $\gamma$  and IFN- $\alpha$ . *FEBS Lett.* 354:220–226.
73. Parry, G.C., and N. Mackman. 1994. A set of inducible genes expressed by activated human monocytic and endothelial cells contain  $\kappa$ B-like sites that specifically bind c-Rel-p65 heterodimers. *J. Biol. Chem.* 269:20823–20825.
74. Takeuchi, M., and V.R. Baichwal. 1995. Induction of the gene encoding mucosal vascular addressin cell adhesion molecule 1 by tumor necrosis factor  $\alpha$  is mediated by NF- $\kappa$ B proteins. *Proc. Natl. Acad. Sci. USA.* 92:3561–3565.
75. Kratz, A., A. Campos-Neto, M.S. Hanson, and N.H. Ruddle. 1996. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J. Exp. Med.* 183:1461–1472.
76. Mebius, R.E., P.R. Streeter, S. Michie, E.C. Butcher, and I.L. Weissman. 1996. A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4<sup>+</sup> CD3<sup>-</sup> cells to colonize lymph nodes. *Proc. Natl. Acad. Sci. USA.* 93:11019–11024.