

Requirement for the NF- κ B Family Member RelA in the Development of Secondary Lymphoid Organs

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Abstract

The transcription factor nuclear factor (NF)- κ B has been suggested to be a key mediator of the development of lymph nodes and Peyer's patches. However, targeted deletion of NF- κ B/Rel family members has not yet corroborated such a function. Here we report that when mice lacking the RelA subunit of NF- κ 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 γ , TRAF6, Id2, and the nuclear factor κ 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 β 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 α interaction with TNFR1 and intracellular signaling through I κ B kinase α (IKK α) (18–22). Less is known about the development of splenic structures: of the molecules involved in lymph node development, only

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

NIK, IKK α , LT α β ₂, and to a lesser degree TNF α and RANKL/TRANCE/OPGL have been shown to be required in hematopoietic cells, and NIK, IKK α , LT β 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- κ B/Rel family of transcription factors. RANK, NIK, IKK α , TRAF6, LT β R, and TNFR1 mediate cellular responses to extracellular signals at least in part by activating NF- κ B, and genes encoding the cytokines RANKL/TRANCE/OPGL, TNF α , and LT α are targets of NF- κ B-activated gene transcription. Therefore, it has been hypothesized that NF- κ B might also play a role in the development of secondary lymphoid organs.

NF- κ B transcription factors are homodimeric and heterodimeric complexes of five family members: p50 (NF- κ B1), p52 (NF- κ B2), c-Rel, RelB, and RelA (p65). Most cells contain only the complex p50/p65, held inactive in the cytoplasm by association with I κ B inhibitory proteins. Activation of the cell leads to degradation of I κ B and nuclear translocation of NF- κ 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- κ 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- κ 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 CO₂ 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 μ m thick), stained with hematoxylin and eosin, and examined by light microscopy.

Spleens and LNs were sectioned at 6 μ m, mounted on slides, and allowed to dry at room temperature for 5–10 min before storing at -20° 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% H₂O₂/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 $^{\circ}$ 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 $^{\circ}$ 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- μ 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- μ m strainer. Cells were plated in 1 ml complete RPMI (C10) at a density of 2×10^6 cells per well of a 24-well plate and stimulated for 18 h with 1 μ g/ml anti-CD3e (145-2C11; BD PharMingen). After stimulation, cells were scraped from the plate and washed three times with FACS[®] 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 γ 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 α (B12-1; BD PharMingen), anti-B220-biotin (RA3-6B2; BD PharMingen), mLT β R-IgG1 (gift of Biogen Inc.), anti-LT β (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 α , LT β , RelA, TNFR1, or HPRT with the following primer combinations: (a) LT α : cagcaggtccagctcttttc, agtgcaaaagctccaagaa; (b) LT β : ggagcacaggtcagaaaag, gagctcaggggtgaggtcag; (c) RelA: gagccattggagtccagta, tgggggaaaactcatcaag; (d) TNFR1: accaagtccacaaggaac, cagcactggaagtgtgtct; (e) HPRT cttgtgactctgtggatt, aaccttaaccatttggggc.

Reconstitution of Bone Marrow with Fetal Liver Cells. Radiation chimeras were generated essentially as described (50). Briefly, donor embryos were generated by crossing TNFR^{+/+} RelA^{+/-} males to females and TNFR1^{-/-} RelA^{+/-} 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⁺ hosts were irradiated with two doses, 800 rads and 400 rads separated by 3 h, using a ¹³⁷Cs source. Mice were anesthetized with Avertin (2.5% solution of 2,2,2-tribromoethanol/tert amyl alcohol, 12 μ l/g mouse) immediately after the second irradiation and transplanted with 10⁶ liver cells from wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetuses in 200 μ 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 NP₇CG (75 μ 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 NP₂₃BSA (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⁻, 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⁻, and TNFR1/RelA-deficient mice were immunized with alum-precipitated NP₇-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 (NP₂₃BSA). 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

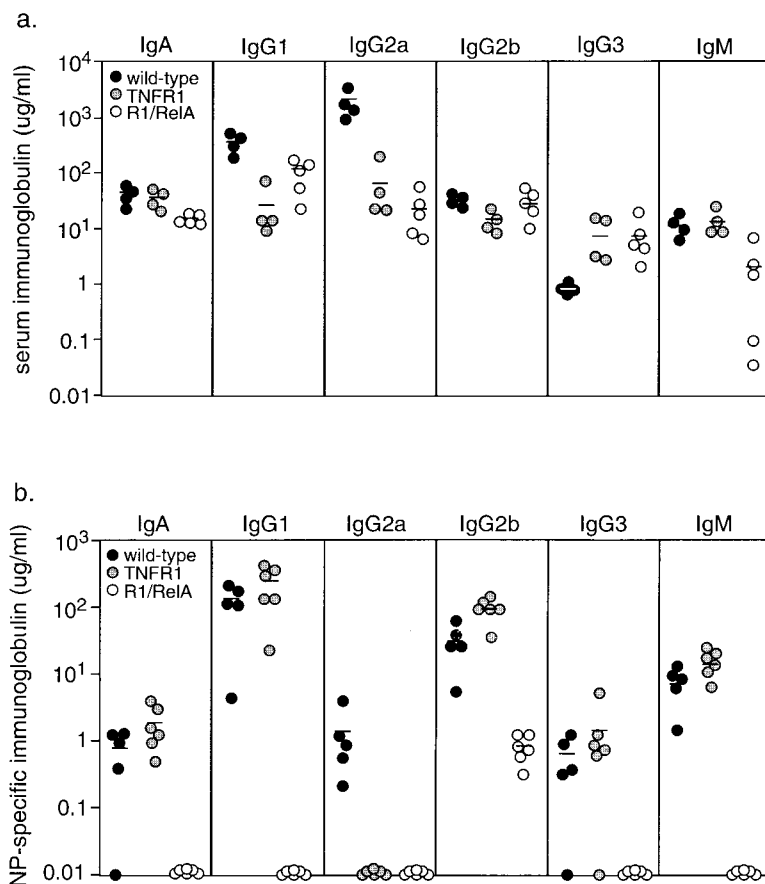


Figure 1. Severely reduced T cell-dependent antibody response in TNFR1/RelA-deficient mice in vivo. (a) Basal immunoglobulin concentrations. Serum was collected from 3-wk-old wild-type (black circle), TNFR1-deficient (gray circle), and TNFR1/RelA-deficient (open circle) mice and immunoglobulin concentrations 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 NP₇-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⁺); 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⁺CD3⁻IL-7Rα⁺ Cells. A population of CD4⁺CD3⁻IL-7Rα⁺ 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⁻; of these, 1.85, 2.34, and 1.65% were CD4⁺IL-7Rα⁺ in wild-type, TNFR1⁻, and TNFR1/RelA-deficient samples, respectively (Fig. 4). Thus, the CD4⁺CD3⁻IL-7Rα⁺ 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 LTα₁β₂. Expression of the membrane-bound cytokine LTα₁β₂ is required for the development of LN, PP, and all splenic structures (for reviews, see references 54 and 55). To determine if LTα₁β₂ 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 LTα₁β₂ or in the level of LTα₁β₂ 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⁺ wild-type hosts were engrafted with CD45.2⁺ 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⁺ 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.

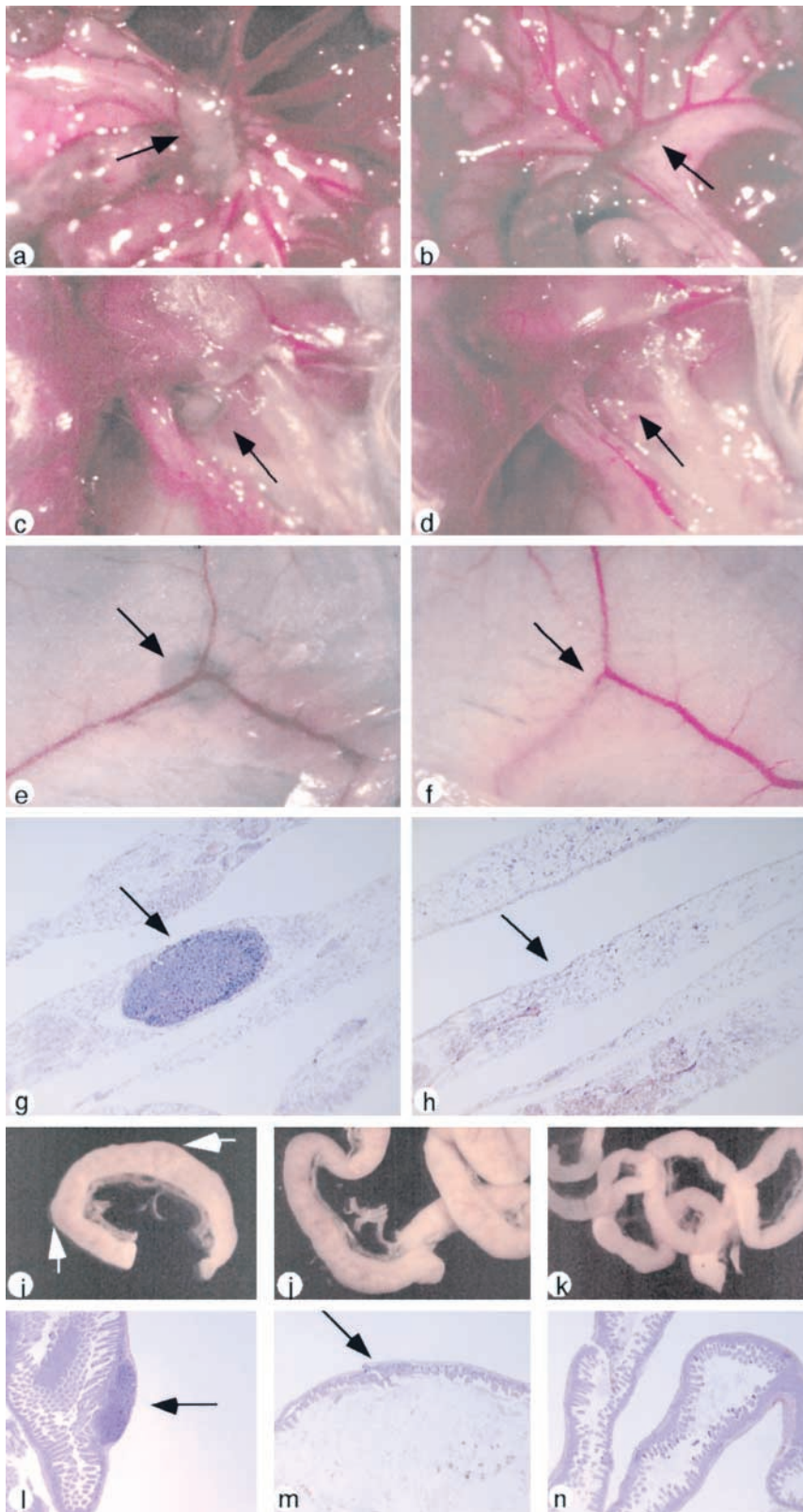


Figure 2. Absence of LNs and PPs in TNFR1/RelA-deficient mice. Five 3-wk-old wild-type (a, c, e, g, i, and l), TNFR1-deficient (j and m) and TNFR1/RelA-deficient (b, d, f, h, k, and n) mice were injected intraperitoneally with Evans Blue (1 mg/ml, 20 μ l per mouse) 18 h before dissection to visualize 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-

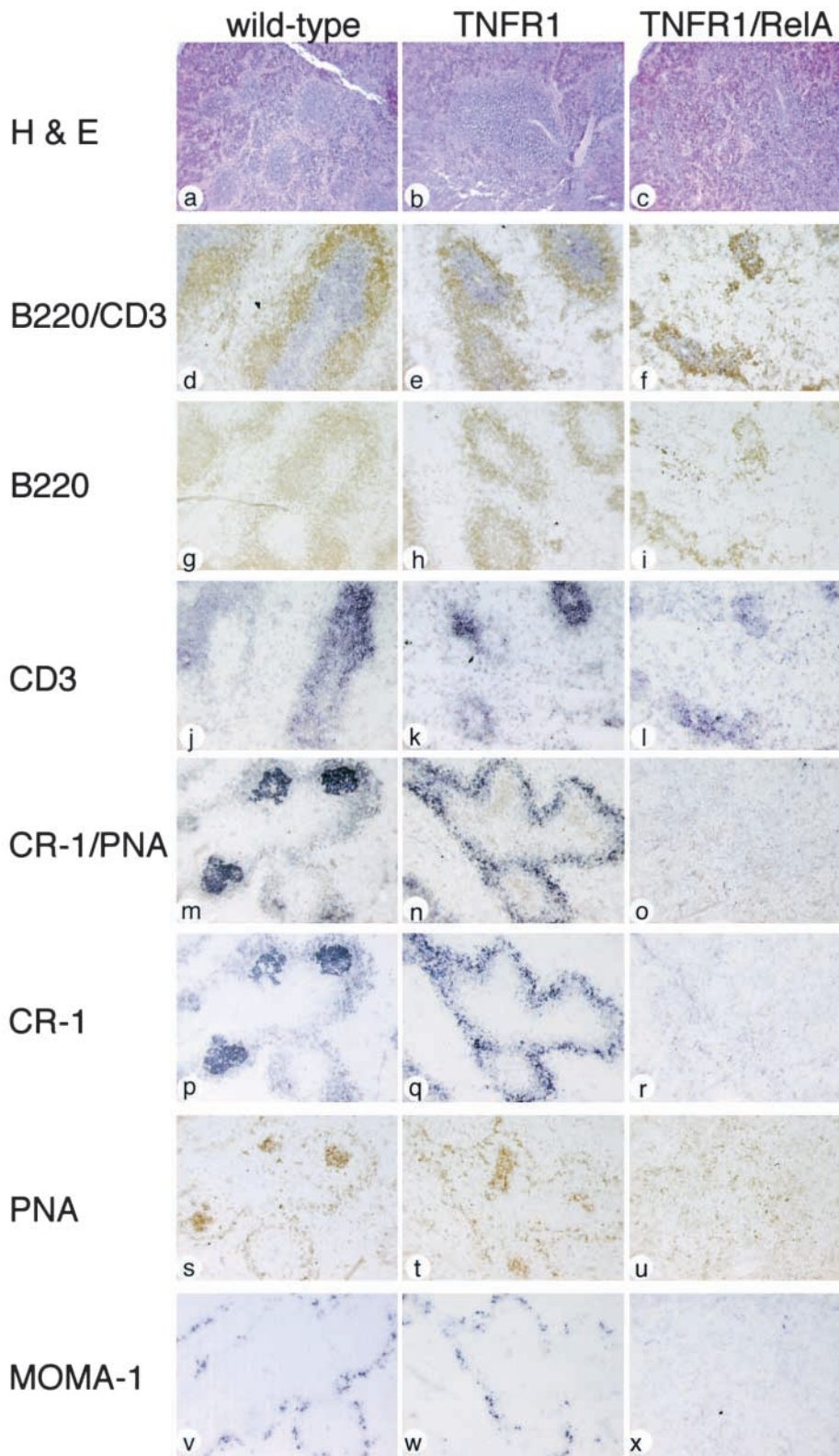


Figure 3. Aberrant organization of lymphocytes and absence of secondary immune response structures in TNFR1/RelA-deficient spleens. 3-wk-old wild-type (a, d, g, j, m, p, s, and v), TNFR1-deficient (b, e, h, k, n, q, t, and w), and TNFR1/RelA-deficient mice (c, f, i, l, o, r, u, and x) were immunized with NP₇-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

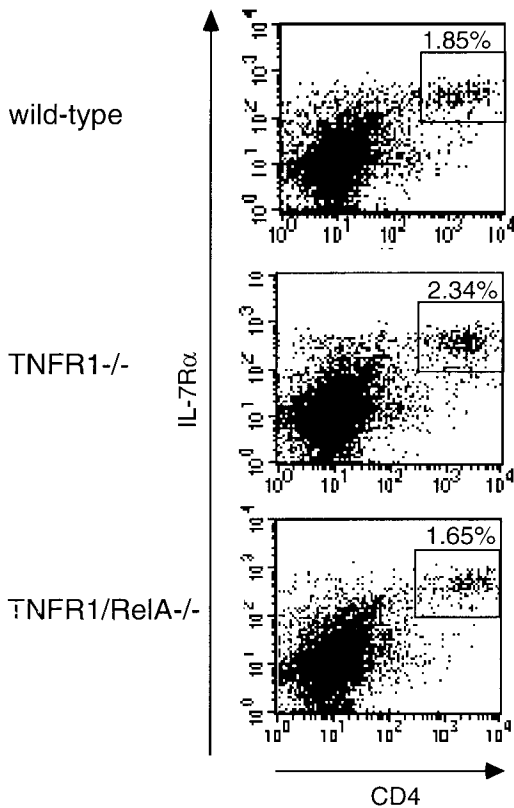


Figure 4. Presence of CD4⁺CD3⁻IL-7R α ⁺ cells in TNFR1/RelA-deficient embryonic intestine. Flow cytometry was used to analyze intestinal cells from E17.5 wild-type, TNFR1-deficient, and TNFR1/RelA fetuses with the indicated cell-surface markers. All events presented are negative for CD3. Each sample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

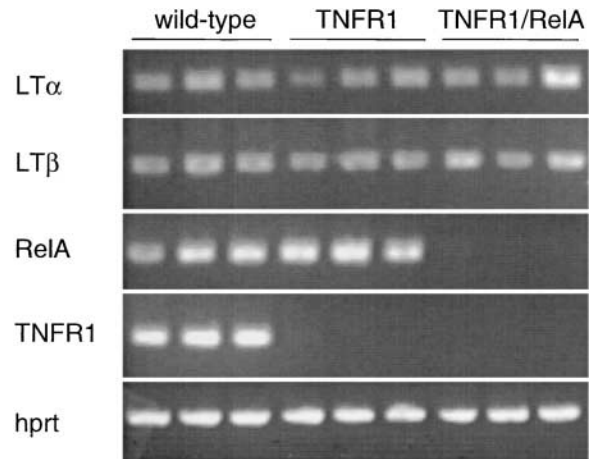


Figure 5. Normal expression of LT α β ₂ in TNFR1/RelA-deficient embryonic intestine. RNA was prepared from E17.5 wild-type, TNFR1-deficient, TNFR1/RelA-deficient intestines and assayed by RT-PCR for expression of LT α and LT β , RelA, TNFR1, and hpri (control).

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 α ⁺ hematopoietic cells were present in TNFR1/RelA-deficient embryonic tissue, and mRNA and protein levels of membrane-bound cytokine LT α β ₂ in embryonic tissue were comparable to those in wild-type tissue. This suggests that, in contrast to the Id2-, LT α -, or LT β -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 non-hematopoietic 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-

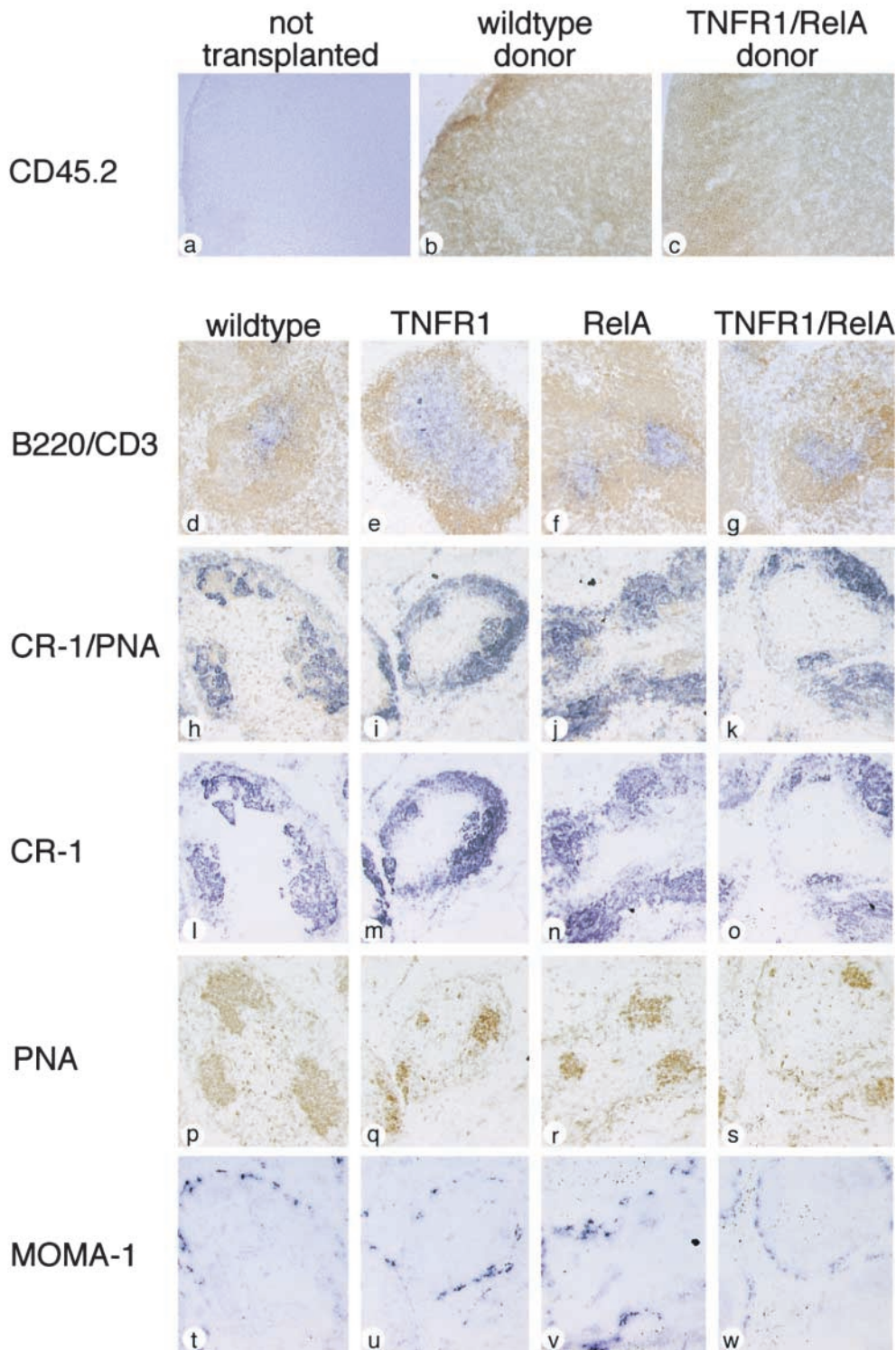


Figure 6. Repopulation of LNs and organization of spleen in adoptive transfer mice reconstituted with TNFR1/RelA-deficient hematopoietic cells. C57BL/6 CD45.1⁺ mice were lethally irradiated and transplanted with wild-type, TNFR1-deficient, RelA-deficient, or TNFR1/RelA-deficient fetal liver cells. 4 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 micro-architecture. 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- κ 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- κ 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⁺CD3⁻IL-7R α ⁺ 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 β R signaling in nonhematopoietic cells. Targeted deletion of LT β R or disruption of signaling by LT β 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 β R in nonhematopoietic cells for the formation of secondary lymphoid organs (64, 65). Finally, LT β 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 β 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 β R is a member of the TNF superfamily of receptors which, like TNFR1, not only activates NF- κ 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 β R and its activation provides protection against the cytotoxic effects of LT β R-mediated signaling; in the absence of RelA, LT β 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 β R stimulation, or the responses of mouse embryonic fibroblasts are not representative of the responses of stromal cells expressing LT β 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- κ 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 β R with mLT $\alpha\beta$ (11). NF- κ 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.

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