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Expression of *rel*B transcripts during lymphoid organ development: specific expression in dendritic antigen-presenting cells

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SUMMARY

We have studied the expression of the *rel*B gene during mouse development using in situ hybridization and immunocytochemical analysis. The results show that the expression of the *rel*B gene is highly restricted to a subpopulation of cells that colonize the lymphoid tissues and that appear very late during the process of hemapoietic diversification. RNA transcripts of *rel*B are very low or undetectable in early and late embryos. Low *rel*B expression is observed in the thymus at late stages of embryogenesis but rapidly increases after birth. In adult lymphoid tissues, *rel*B is detected in the medullary region of the thymus, the periarterial lymphatic sheaths of the spleen, and the deep cortex of the lymph nodes, which correspond to the regions where T cells of mature phenotype and interdigitating dendritic cells are present. Using double immunofluorescent labeling of thymic cell suspensions, we have identified the interdigitating dendritic cells as the target of RelB expression. These cells are part of a system of antigen-presenting cells that function in the induction of several immune responses, such as, tolerance, sensitization of MHC-restricted T cells, rejection of organ transplants and formation of T-dependent antibodies. Our observations indicate that RelB may play a particular role in the signal transduction pathways that regulate dendritic cell differentiation and its cellular responses.

Key words: *rel*B expression, lymphoid organ, dendritic, antigen-presenting cell, mouse development

INTRODUCTION

The family of Rel proteins consists of a number of transcription factors defined by a conserved region of approximately 300 amino acids, the Rel homology domain (RHD), which includes sequences important for dimerization, DNA-binding and nuclear localization (for reviews see Gilmore, 1990, 1991, 1992; Baeuerle, 1991; Baeuerle and Baltimore, 1991; Blank et al., 1992; Grilli et al., 1992; Nolan and Baltimore, 1992). The first identified members of this family were the viral oncogene v-*rel*, isolated from the avian reticuloendotheliosis virus strain T (Rev T), which causes lymphoid leukemia (Theilen et al., 1966; Stephens et al., 1983), and its cellular counterpart, the c-*rel* proto-oncogene (Chen et al., 1983; Wilhelmsen et al., 1984). The third member was the *dorsal* protein of *Drosophila melanogaster* involved in establishing the dorsal-ventral polarity in the developing embryo (Steward, 1987). The other recently described members of this family include NFKB1 (p105/p50-NF-κB, also known as KBF1; Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990; Meyer et al., 1991), NFKB2 (p100/p52-NF-κB, also known as p50B, p49 and *lyt*-10; Neri et al., 1991; Schmid et al., 1991; Bours et al., 1992), *rel*A (p65-NF-κB; Nolan et al., 1991; Ruben et al., 1991) and *rel*B (Ryseck et al., 1992).

Additionally to the RHD, the proteins of the Rel family show a regulated movement from the cytoplasm to the nucleus. In particular, the prototype NF-κB complex, i. e., the heterodimer between p50-and p65-NF-κB subunits, is trapped in the cytoplasm by the inhibitory activity of IκB (Baeuerle and Baltimore, 1988a,b, 1989). After stimulating the cell with a proper signal, IκB is inactivated, most likely by phosphorylation/dephosphorylation, allowing the NF-κB complex to translocate into the nucleus where it binds to κB-binding sites (Sen and Baltimore, 1986a; Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Kerr et al., 1991; Link et al., 1992). Many of the NF-κB responsive genes are involved in immune responses and/or acute phase reactions, such as the regulated expression of cytokines like interleukin-2, interleukin-6, and tumor necrosis factor α, as well as the interleukin-2 receptor α, and the immunoglobulin κ light chain. In addition, some viral enhancers, including human immunodeficiency virus (HIV) and cytomegalovirus, contain κB sequences (reviewed in Lenardo and Baltimore, 1989; Baeuerle, 1991).

With the exception of c-*rel*, little is known about the tissue-specific expression of the *rel* family genes in vertebrates. The mammalian c-*rel* transcript is expressed at highest levels in B and T lymphoid cells in mouse and human (Brownell et al., 1987, 1988). The cytoplasmic NF-κB DNA-binding activity was found in many tissues and cells suggesting a widespread expression of *rel*A (p65-NF-

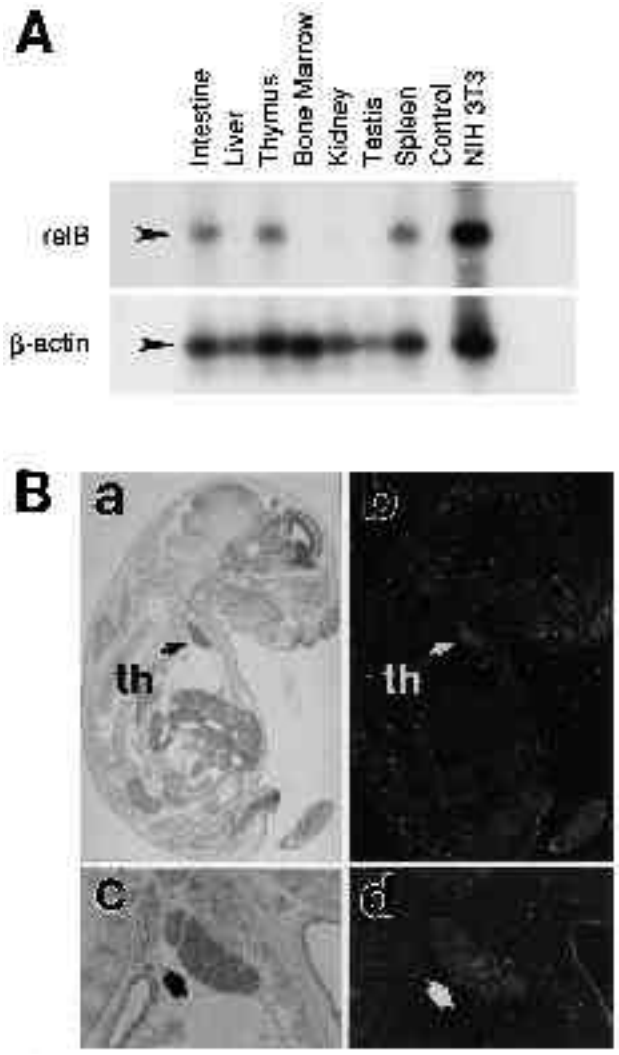

Fig. 1. Expression of *rel*B in mouse lymphoid organs. (A) Analysis of *rel*B gene expression by PCR-mediated cDNA amplification in different adult mouse tissues. Control, no cDNA was added to the PCR reaction. NIH 3T3, quiescent cells stimulated with 20% FCS for 4 hours in the presence of cycloheximide (5 μg/ml). (B) In situ hybridization analysis of *rel*B transcripts in 15 day mouse embryos. A parasagittal section hybridized with specific antisense riboprobe for *rel*B photographed under dark-field illumination is shown in b and d. After hematoxylin staining, the section was photographed under bright-field illumination (a,c). Higher magnification photographs of the thymic region from a and b are shown in c and d, respectively. th, thymus. Magnification, 5× (Ba,b) and 25× (Bc,d).

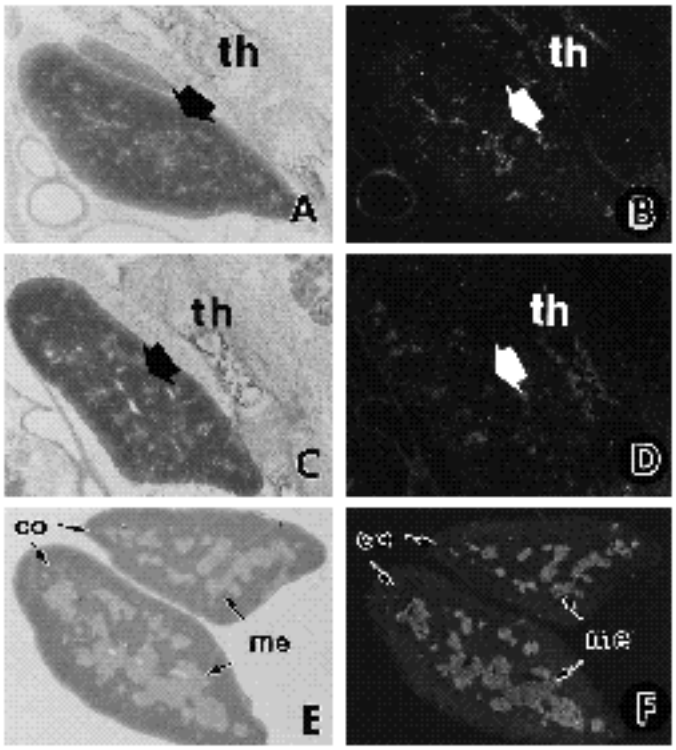

Fig. 2. *rel*B gene expression is restricted to the medulla of the thymus and increases after birth. Sections obtained from a 17 day embryo (A,B), newborn mouse (C,D) and 6 days old mouse thymus (E,F), were hybridized with specific antisense riboprobes for *rel*B and photographed under dark-field (B,D,F) and bright-field illumination (A,C,E). In A-D only the thymic region from the original section is shown. co, cortex; me, medulla. Magnification, 31× (A,B), 26× (C,D) and 12× (E,F).

κB) and NFKB1 (p105/p50-NF-κB), although the composition of the complexes containing NF-κB activity has still to be determined.

In addition to differential tissue expression, many *rel* genes are induced after fibroblast stimulation with mitogens. For example, the expression of mouse c-*rel* (Bull et al., 1989; Grumont and Gerondakis, 1990), NFKB1 (p105/p50-NF-κB) and *rel*B (Ryseck et al., 1992) is rapidly induced upon serum stimulation of 3T3 cells. Similarly, activation of T cells induces different members of the family (Bours et al., 1990, 1992; Grumont and Gerondakis, 1990; Ruben et al., 1992).

Why do cells express different Rel family members? Recent evidence indicates that, although Rel proteins recognize similar DNA-binding sites, the various homodimers and heterodimers vary on their activities (Schmid et al., 1991; Schmitz and Baeuerle, 1991; Urban et al., 1991; Zabel et al., 1991; Fujita et al., 1992; Hansen et al., 1992; Kretzschmar et al., 1992; Kunsch et al., 1992; Ryseck et al., 1992). Therefore, depending on their time of expression during development or their selective activation in particular tissues, they could play differential roles.

In an attempt to address this question and to understand better the biological role of RelB, we studied the expression of *rel*B during mouse development by in situ hybridization. The results show that the expression of *rel*B is mainly restricted to certain regions of lymphoid tissues like spleen, thymus and lymph node. Moreover, we have been able to show that the thymic cells expressing RelB correspond to dendritic antigen-presenting cells.

Our studies show that expression of *rel*B is restricted to a subpopulation of the antigen-presenting cells, the interdigitating dendritic cell. *rel*B expression first appears at significant levels after birth and is restricted to tissues engaged in immune response such as the thymus and the spleen. The appearence of *rel*B transcripts coincides with the timing of the immune system maturation.

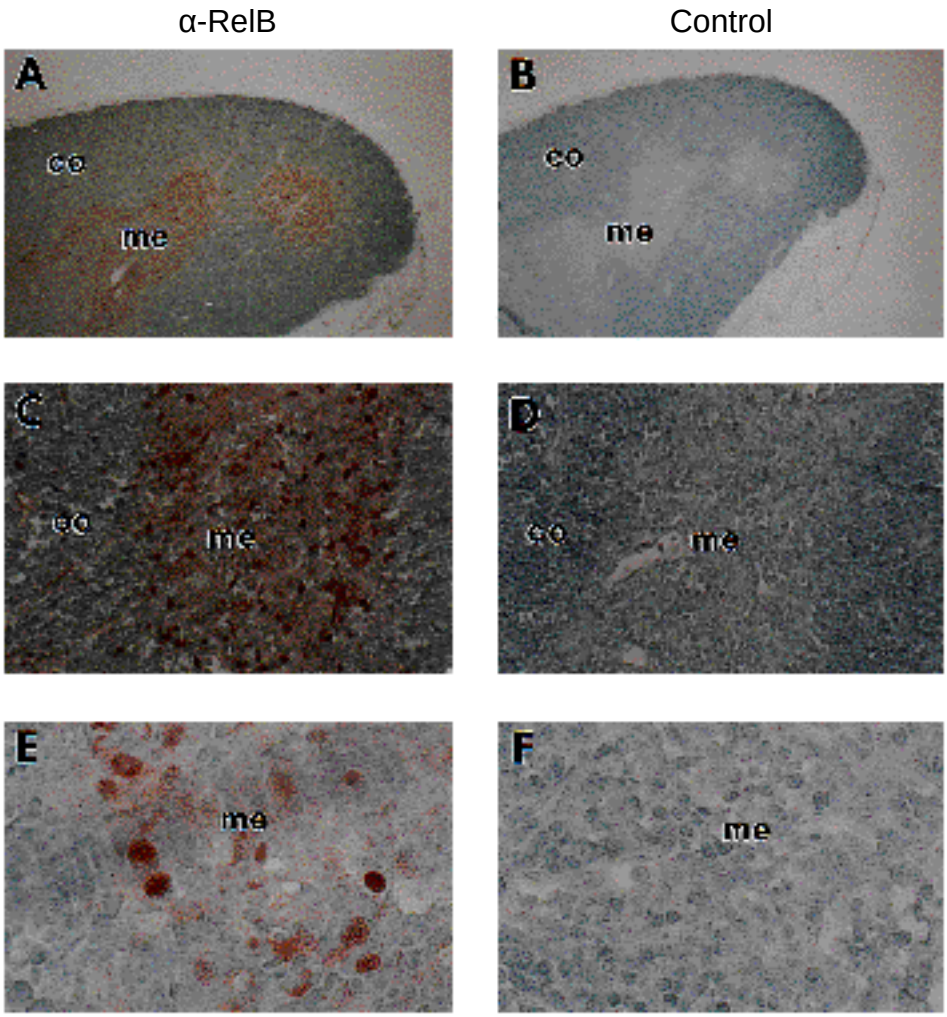

Fig. 3. Immunohistochemical localization of RelB protein in the thymic medulla. Immunoperoxidase staining of thymus sections from a 6-week-old mouse incubated with affinity-purified anti-RelB antibody (α-RelB) or with pre-immune rabbit IgG (Control). Staining is seen only in cells localized in the medulla of the thymus. co, cortex; me, medulla. Magnification, 25× (A,B), 100× (C,D) and 250× (E,F).

MATERIALS AND METHODS

PCR analysis

Total RNA was prepared from different tissues using the guanidine hydrochloride procedure (Chirgwin et al., 1979). After cDNA synthesis, one tenth of the reaction mixture was amplified using Taq polymerase and sequence-specific primers. The two oligonucleotide primers used in all PCR experiments had the sequence 5′-TGCTTCCATATCCCTTCCTGGGCTG-3′ complementary to nucleotides 1382-1406, of *rel*B cDNA (Ryseck et al., 1992), and 5′-CCCAGTGCCTCCTCTGTTACCTCAG-3′, complementary to nucleotides 1731-1746. Primer pairs for mouse β-actin cDNA were included as internal controls (Telford et al., 1990). One tenth of the amplified products were separated by electrophoresis on 3% Nusieve Agarose (FMC) (2:1) gels. After alkaline denaturation, gels were blotted overnight onto nylon filters (Schleicher & Schuell) and then hybridized with specific probes.

Tissue preparation

Embryos were obtained from natural matings of B6C3F1 mice. The time of pregnancy was established by the presence of the vaginal plug and was regarded as day 1 of gestation.

(i) For in situ hybridization

After dissection, the embryos or tissues were transferred to O.C.T. embedding medium (Miles Laboratories), mounted on a cryostat specimen holder and frozen by immersing in liquid Nitrogen. Cryostat sections of 8 to 10 μm thickness were collected on subbed slides (Fisher Scientific Co.), air dried at room temperature for 30 minutes and then stored at −70°C prior to use.

(ii) For immunocytochemistry

Following dissection, tissues were fixed overnight in modified Bouin's solution (0.9% picric acid, 5% acetic acid and 9% [v/v] formaldehyde), dehydrated in increasing concentrations of ethanol and embedded in paraffin wax (Fisher Scientific Co.). Floating sections of 5 to 7 μm were air dried for 30 minutes on subbed slides at room temperature and then stored at 4°C prior to use.

In situ hybridization

A murine cDNA fragment of *rel*B, positions 1 to 390 (Ryseck et al., 1992), was cloned into Bluescript KS+. For in situ hybridization, [^{35}S]UTP-labeled sense and antisense probes were prepared from linearized template DNAs, using T3 or T7 RNA polymerases (Promega). Probes were degraded to an average size of 200 nucleotides by limited alkaline hydrolysis and the unincorporated nucleotide was removed using a Sephadex G-50 column (Pharmacia).

In vitro hybridization was performed essentially as previously described (Dony and Gruss, 1987) with minor modifications.

Antibody generation and immunopurification

The generation and characterization of the anti-RelB antibody was previously described (Ryseck et al., 1992). The antibody was immunopurified according to Kovary and Bravo (1991).

The hamster monoclonal antibody M342-specific for dendritic cells has been previously described (Agger et al., 1992).

Immunohistochemistry

The procedure used was a modified version of the method reported by Heine et al. (1987). Prior to immunostaining, sections were deparaffinized and then submerged in a solution of methanol:hydrogen peroxide 9:1 for 10 minutes. After washing the slides with PBS for 10 minutes, they were treated with saponin (0.5 mg/ml in PBS) for 30 minutes and subsequently washed three times with PBS for 5 minutes. Tissue sections were incubated with immunoaffinity-purified primary antibodies at a concentration of 5 μg/ml (diluted in 10% non-immune goat serum) overnight at 4°C. Control slides were incubated with either an IgG fraction of pre-immune rabbit serum at 5 μg/ml (diluted in 10% non-immune goat serum) or without primary antibody. Slides were then extensively washed in PBS and incubated for 60 minutes at room temperature with biotinylated goat anti-rabbit antibody (1:50, DAKO). After washing in PBS, the sections were exposed to streptavidin-peroxidase complex (Zymed Laboratories Inc.) for 10 minutes at room temperature and washed again in PBS. Samples were then treated with aminoethylcorbazole with 0.03% v/v hydrogen peroxide for 5 to 10 minutes and counterstained in hematoxylin.

Immunofluorescent staining

Cytospin samples of cell suspensions were prepared in medium containing 10% FCS using a Shandon Cytocentrifuge (Astmoor, UK). Cytospins were fixed at room temperature for 2 minutes in acetone:methanol (1:1), air dried and stained. For double immunofluorescence, cytospins were incubated at room temperature for 1 hour with either anti-RelB and M342 (Agger et al., 1992) antibody; anti-RelB and anti-CD4 antibody (Pharmingen); or anti-RelB and anti-CD8 antibody (Pharmingen). M342 antibody was visualized with a goat polyclonal anti-hamster immunoglobulin antibody conjugated with FITC (Boehringer),anti-RelB antibody with a donkey polyclonal anti-rabbit immunoglobulin antibody conjugated with Texas red (TR, Amersham), and anti-CD4 and anti-CD8 antibodies with a goat anti-rat immunoglobulin antibody conjugated with FITC (Boehringer).

RESULTS

Differential expression of the *rel*B gene

To investigate the *rel*B gene expression, PCR analyses of adult mouse tissues and embryos at different stages of development were performed using an appropriate pair of oligonucleotide primers. In adult tissues, *rel*B transcripts are only detected at high levels in spleen, thymus and intestine, and at very low or undetectable levels in bone marrow, liver, testis and kidney (Fig. 1A). In contrast, PCR analysis of mouse embryos from days 7 to 17 revealed that *rel*B expression is very low or undetectable in day 7 embryos but starts to increase at day 15 (not shown). These data show that the *rel*B gene is differentially expressed in adult tissues and during mouse development. Furthermore, it also indicates that *rel*B is preferentially expressed in primary and secondary lymphoid tissues. These results prompted us to investigate the localization of the *rel*B gene transcripts during mouse fetal development and in postnatal animals by in situ hybridization.

In agreement with our PCR results, no *rel*B-specific labeling is detected during gastrulation stages by in situ hybridization (not shown). A very weak *rel*B hybridization, restricted to the emerging thymus, is first detected in sagittal sections of day 14 embryos and is homogeneously distributed throughout the organ (not shown). By day 15 of embryonic life, when the mouse thymus begins to show the most rapid histogenetic and organogenetic changes, *rel*B transcripts are heterogeneously distributed throughout the thymus. This pattern is depicted in Fig. 1Ba and Bb, where a sagittal section through a whole 15 day embryo shows a weak labeling in the thymus. Other tissues, including the liver, which displays the major lymphopoietic activity during this period, are negative for *rel*B expression. Observation of day 15 embryonic thymus at higher magnification clearly shows that *rel*B transcripts are heterogeneously distributed (Fig.1Bc,Bd). This pattern is possibly related to changes in cellular composition, since different hematopoietic cells are colonizing the thymus at this time. After day 17 of embryonic life, the fetal thymus begins to show cortical and medullar differentiation. Interestingly, hybridization of 17-day-old embryos with a *rel*B probe clearly demonstrates that the expression of the *rel*B gene is confined to the thymic medulla (Fig. 2A,B). No signal is detected in the cortex. In all cases, consecutive sections of embryos were hybridized in situ with sense and antisense probes. The sense probe did not give a specific signal above background in any of the sections examined.

*rel*B gene expression in the thymus increases after birth

The observation that *rel*B transcripts are present at significant levels in adult thymus but are weakly detected in the thymus of day 15 embryos indicates that *rel*B expression possibly increases during late development or after birth. To investigate this, mid-sagittal sections from newborn animals and thymic sections prepared from older animals were hybridized with a *rel*B probe. The levels of *rel*B transcripts in the thymus of newborn animals are comparable to those observed in the thymus of 17-day-old embryos (Fig. 2C,D). However, *rel*B expression rapidly increases thereafter reaching maximum levels around 1 week after birth and remains constant throughout adulthood. A sagittal section of the thymus from a 1-week-old animal hybridized with *rel*B is shown in Fig. 2E. As observed in late embryos, *rel*B transcripts are heterogeneously distributed throughout the thymus, with the highest expression levels restricted to the medullary region (Fig. 2E,F). This pattern of expression does not significantly change in adult thymus (6 weeks) compared to that observed in the thymus of newborn and young animals (not shown).

To confirm further that the expression of *rel*B is mainly in the medulla and not in the cortex of the thymus, immunohistochemical staining was performed using an immunoaffinity-purified rabbit anti-RelB antibody. As illustrated in Fig. 3, RelB staining is mainly confined to the medullary region. In agreement with the in situ hybridization, the anti-RelB antibody does not stain cortical structures. The thymic medulla consists predominantly of epithelial cells, with interspersed lymphocytes of more mature phenotype, single positive T cells ($CD4^+CD8^-$ or $CD4^-CD8^+$), interdigitating dendritic antigen-presenting cells and occasional macrophages. B cells and plasma cells are rarely present in this region. Immunostaining with anti-IgM anti-

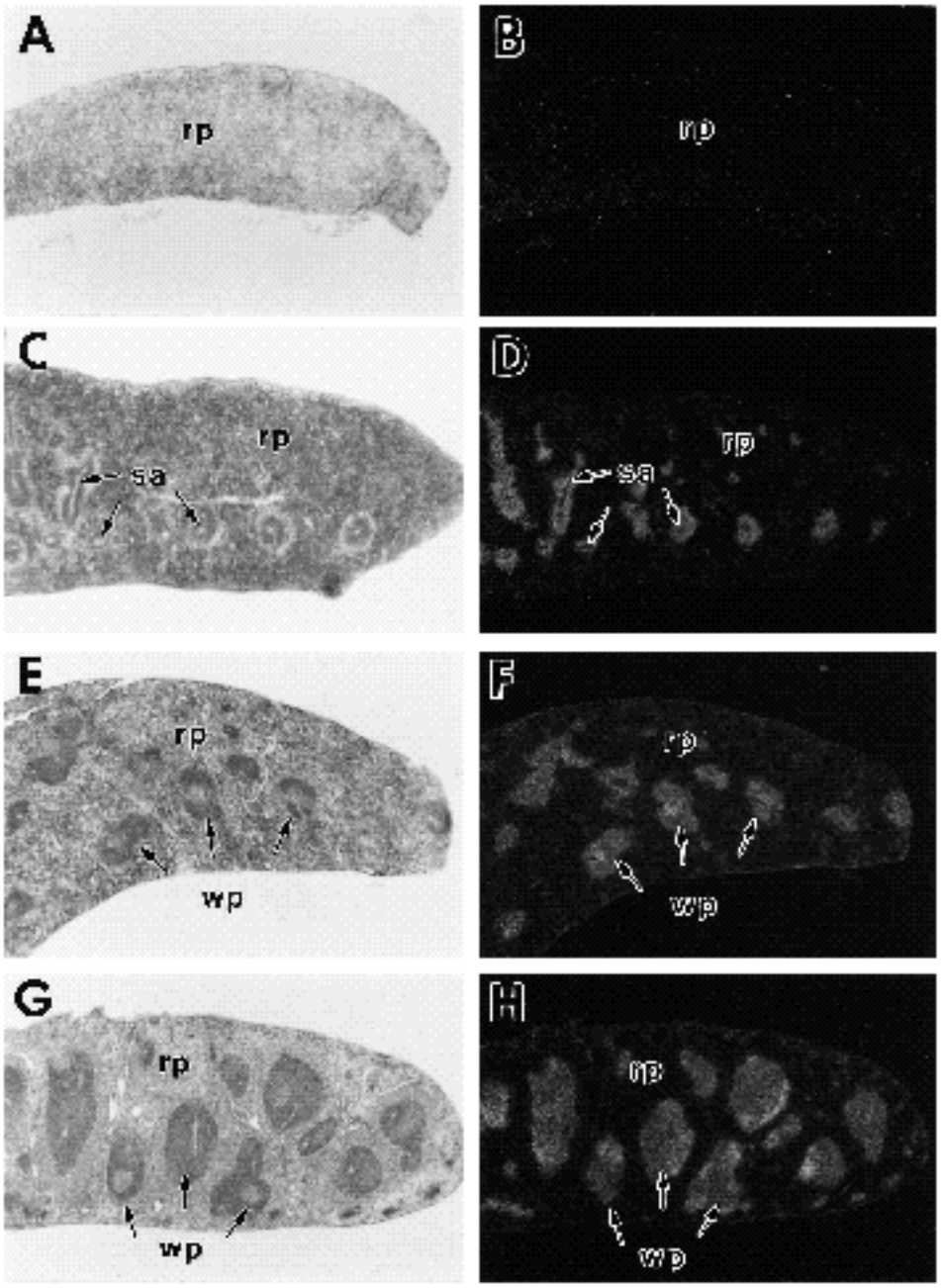

Fig. 4. In situ hybridization analyses of *rel*B in mouse spleen at different times after birth. Serial spleen sections of newborn (A,B), 4 day (C,D), 1 week (E,F) and 4 week (G,H) animals were hybridized with *rel*B antisense riboprobes and photographed under bright-field (A,C,E,G) or dark-field illumination (B,D,F,H). rp, red pulp; sa, sheath artery; wp, white pulp. Magnification, 32× (A,B), 25× (C,D), and 20× (E-H).

bodies (not shown) demonstrates that B and plasma cells are localized mainly in the cortex and corticomedullary region and are much too rare in the medulla to account for the staining depicted by RelB. Thymic macrophages are mainly distributed in the cortex (Morris et al., 1991) and around the perivascular space, a distribution that significantly differs from that observed for RelB-positive cells. In contrast, medullary epithelial cell staining should give a confluent pattern (Rouse et al., 1979), which is not the case for RelB. In addition, RelB staining is not observed in the Hassall bodies, a medullary structure made of epithelial cells. Therefore, the most likely cell candidates expressing RelB within the medulla are the medullary thymic lymphocytes and the interdigitating dendritic cells.

Differential expression of *rel*B in secondary lymphatic organs

The peripheral (secondary) lymphatic organs are very organized structures composed of different anatomical compartments, which are populated by phenotypically and functionally distinct cells. With this advantage, and in order to gain additional information to identify the cells expressing *rel*B, we further analyzed two secondary lymphatic organs, the spleen and the lymph nodes.

Spleen

The spleen displays a complex pattern of hemapoietic activity in which myelopoiesis and erythropoiesis occur during late embryogenesis whereas lymphopoiesis takes

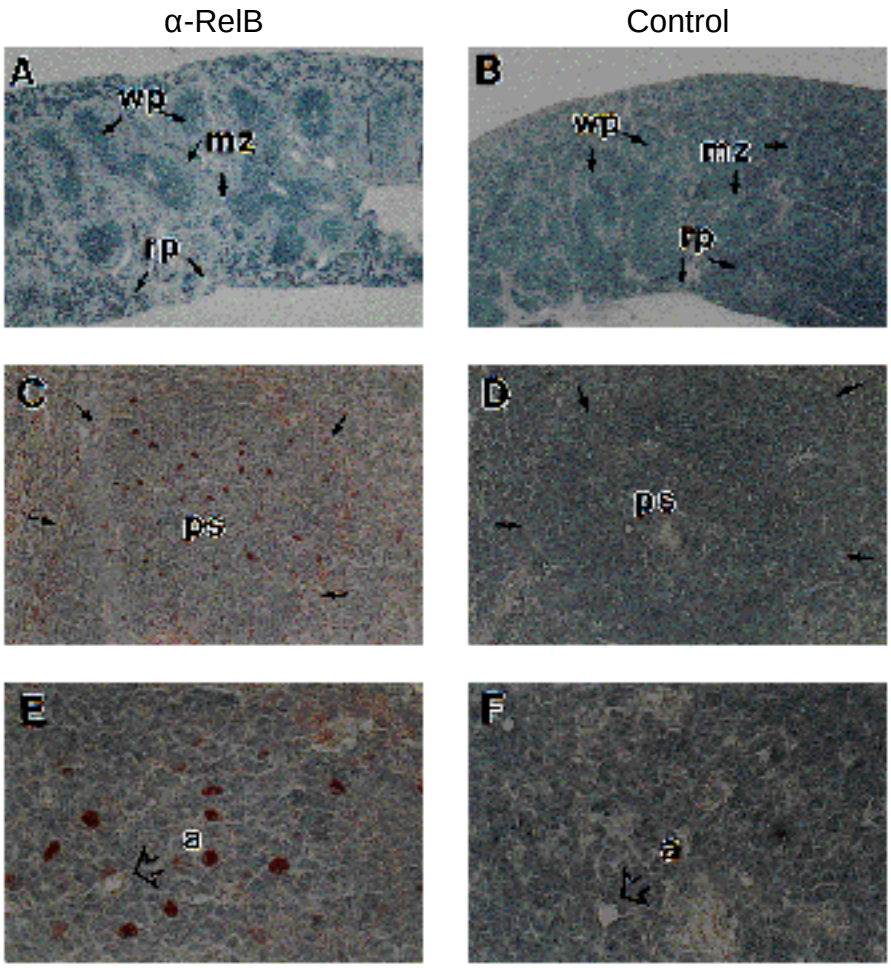

Fig. 5. Immunohistochemical localization of RelB in the periarterial lymphatic sheath of the spleen. Cross sections through the spleen of a 6-week-old mouse incubated with affinity-purified anti-RelB antibodies (α-RelB) or with pre-immune rabbit IgG (Control). Staining is seen in cells localized in the periarterial lymphatic sheath of the white pulp. wp, white pulp; rp, red pulp; mz, marginal zone; ps, periarterial lymphatic sheath; a, artery. The slide incubated with anti-RelB antibody was first photographed under high magnification (C,E) and then overstained to show the gross morphology of the tissue at lower magnification (A). Magnification, 25× (A,B), 100× (C,D), and 250× (E,F).

place after birth (Metcalf and Moore, 1971). In situ hybridization analysis performed with spleen sections prepared from animals at different times after birth indicates that *rel*B is expressed in the cells that colonize the spleen after birth. *rel*B expression is undetected in the spleen of newborn animals (Fig. 4A,B) but rapidly increases thereafter. A significant level of *rel*B transcripts is detected in the emerging white pulp around the central (sheathed) and follicular arteries in the spleen of 4-day-old animals (Fig. 4D,F). As shown in Fig. 4, in older animals, the size of the white pulp increases dramatically and changes in shape with the growth of the spleen. In a detailed analysis of several spleen sections from 4- to 6-week-old mice, we always found that *rel*B expression is confined to the white pulp and that the level of *rel*B transcripts is significantly higher in the periarterial lymphatic region than in the lymphatic follicle (Fig. 6A,B). *rel*B transcripts were not detected in the red pulp or in the marginal zone.

The expression of *rel*B was further investigated by immunohistochemistry using affinity-purified rabbit antibodies against RelB. As shown in Fig. 5, RelB-positive cells are present in the periarterial lymphatic sheaths but absent in lymphatic follicles and the marginal zone, in agreement with the in situ hybridization results. Analysis at higher magnification reveals that the RelB-expressing cells constitute a subpopulation of the cells present in the periarterial lymphatic sheath (Fig. 5E). Interestingly, the periarterial lymphatic region is rich in single positive T cells and is the site to which splenic interdigitating dendritic cells are restricted (Veldman and Kaiserling, 1980).

Lymph nodes

In lymph nodes, the residing cells have different functional capabilities compared to the cells in the spleen. Most of the cells in the lymph node have already encountered an antigen and a significant number of the lymphatic follicles contain a germinal center. Hence, it was of interest to determine the expression of *rel*B in this organ. As shown in Fig. 6, expression of *rel*B is mainly restricted to the paracortical area and is weakly detected in lymphatic follicles containing a germinal center. Therefore, in agreement with what was observed in thymus and spleen, *rel*B in the lymph node is highly expressed in areas where T cells and interdigitating dendritic cells are largely confined, and low levels of expression are detected in the B-cell-rich lymphatic follicles.

Specific expression of *rel*B in thymic dendritic cells

The pattern depicted in our in situ hybridization studies strongly suggests three potential cellular candidates for *rel*B expression: T cells of mature phenotype, both $CD4^+CD8^-$

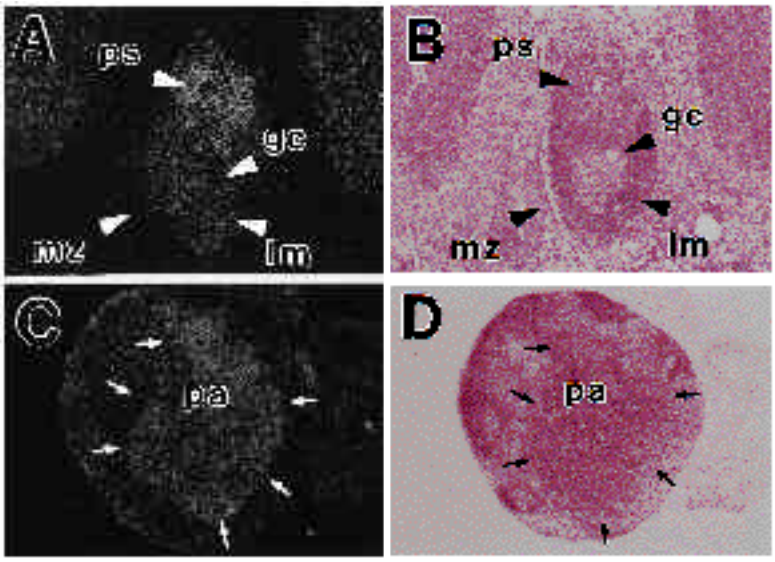

Fig. 6. In situ hybridization analysis of *rel*B gene expression in secondary lymphoid tissues. Frozen sections from the spleen (A,B) and lymph nodes (C,D) from a 6-week-old adult mouse were hybridized with *rel*B antisense riboprobes and photographed under dark-field (A,C) and bright-field (B,D) illumination. ps, periarterial lymphatic sheath; mz, marginal zone; lm, lymphatic mantle; gc, germinal center; pa, paracortical area. Magnification, 60× (A,B) and 38× (C,D).

and $CD4^-CD8^+$, and interdigitating dendritic cells. Using thymic cell suspensions and double immunofluorescence, we evaluated the expression of RelB in these three cellular populations.

Thymic lymphocytes sorted by flow cytometry were spun onto polylysine-coated slides and then tested with anti-RelB polyclonal antibodies. Consistent with the results obtained by in situ hybridization and immunohistochemistry, double positive cortical thymocytes do not display RelB staining (not shown). In addition, RelB staining was also negative in the slightly larger single positive medullary thymocytes $CD4^+CD8^-$ and $CD4^-CD8^+$ (not shown). This prompted us to prepare thymic cells by conventional procedures. A detailed analysis of several cytospin preparations demonstrates that the RelB-positive cells are a small fraction of the total thymic population. More interestingly, morphological evaluation of these cells reveals a striking resemblance to the dendritic cells isolated by Steinman and Cohn (1973). These cells are larger than thymic lymphocytes and the cytoplasm is arranged in pseudopods of varying length, width, form and number, resulting in a variety of cell shapes ranging from stellate (Fig. 7B) to round (Fig. 7C). The cytoplasm contains many large circular structures (Fig. 7B,C), presumably representing the vesicular bodies. In most of the cells, RelB staining is observed in the cytoplasm and in the nucleus, but the strongest labeling is always detected in the nucleus.

To prove further the identity of the RelB-expressing cells, a low density subpopulation of collagenase-treated thymic cells was prepared (Crowley et al., 1989) and used for double immunofluorescent staining. In agreement with the previous observations, the cells displaying RelB staining are CD4 negative (Fig. 7D,E) and, in this particular case, RelB staining is restricted to the nucleus. As shown in Fig. 7, when the dendritic-enriched cell preparations are incubated with a mixture of anti-RelB and M342, a monoclonal antibody that reacts with intracellular granules of dendritic cells (Agger et al., 1992), as primary antibodies, the cells stained for RelB (Fig. 7H) also present the typical perinuclear staining for M342 (Fig. 7G) observed in dendritic cells (Agger et al., 1992).

DISCUSSION

The expression pattern and cell-specific localization of the *rel*B gene product reported in this study indicate that *rel*B may play a physiological role during the development of dendritic cells and in some of its cellular activities. Dendritic cells are a system of Ia-bearing antigen-presenting cells that initiate several immune responses, such as, T cell activation within secondary lymphoid tissues and clonal deletion at the level of thymic medulla (for review see Austyn, 1987; Steinman, 1991).

Restricted and differential expression of *rel*B to lymphoid tissues

The results presented here show that *rel*B expression is restricted to lymphoid tissues. A similar pattern in adult mouse tissues has been previously described for the c-*rel* proto-oncogene (Brownell et al., 1987, 1988). With the exception of bone marrow, the highest levels of *rel*B and c-*rel* mRNA are detected in spleen, thymus and intestine (D. Carrasco, unpublished). Apart from these similarities, our studies indicate that *rel*B and c-*rel* are expressed in different cell types. c-*rel* expression has been shown in thymic medullary thymocytes (Brownell et al., 1988), while we have demonstrated that *rel*B expression is restricted to dendritic cells within the thymus.

The expression of the other members of the Rel family of transcriptional factors such as NFKB1 (p100/p50-NF-κB) and *rel*A (p65-NF-κB) has been shown in a number of murine lymphoid cell lines (Ghosh et al., 1990; Nolan et al., 1991). NF-κB activity has also been demonstrated in non-lymphoid cells (Sen and Baltimore, 1986b).

The differences in the patterns of expression suggest that members of the *rel* family of genes have distinct biological roles and that they have independent mechanisms controlling their expression.

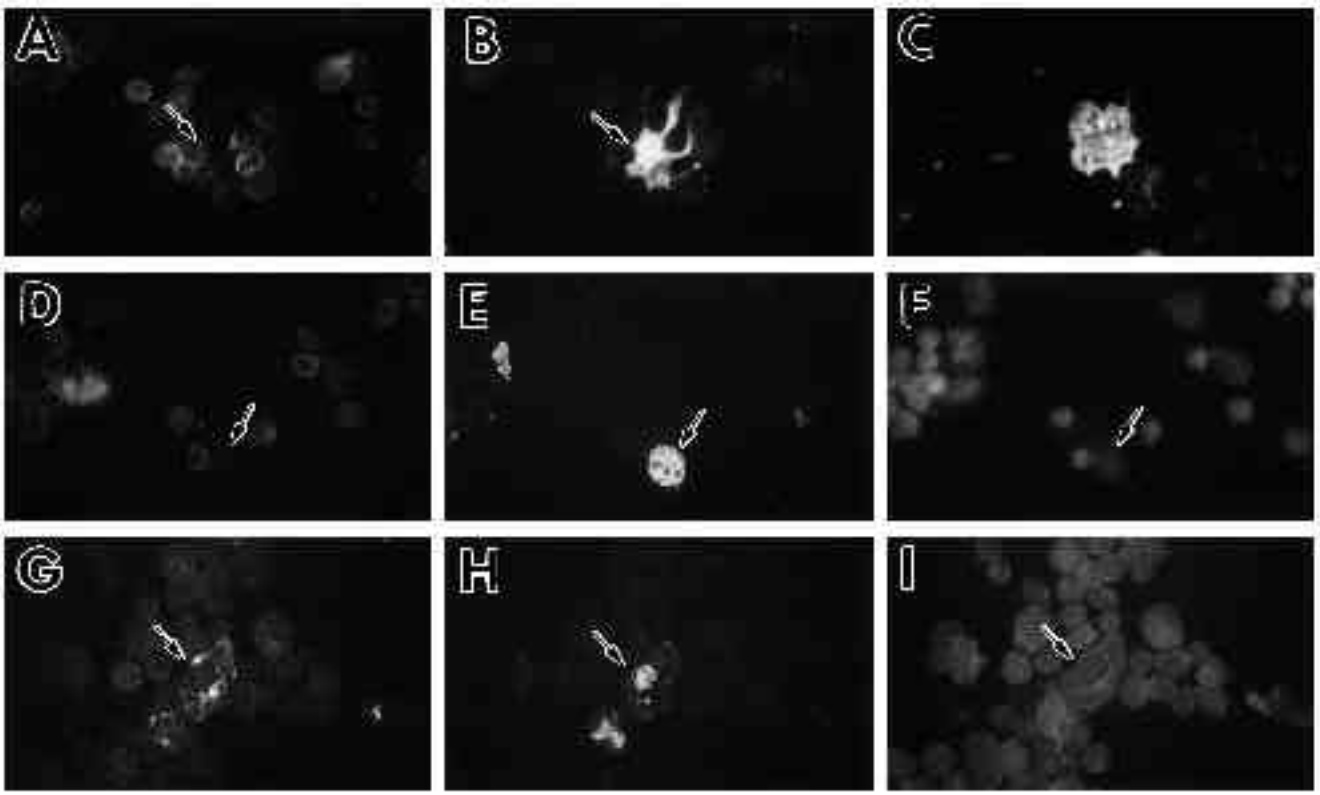

Fig. 7. RelB is expressed in dendritic cells. Top panel: Thymic cell suspensions prepared according to normal procedures were spun onto slides and double stained with a mixture of anti-CD4 and anti-RelB antibodies. C represents another example of a RelB-expressing cell, the corresponding CD4 staining is not shown. Middle and bottom panels: Samples of dendritic-enriched cell thymic suspensions, prepared as described in Materials and Methods, were spun onto slides, fixed and stained with a mixture of either anti-CD4 and anti-RelB antibodies or M342 and anti-RelB antibodies. The anti-CD4 (A,D), anti-RelB (B,C,E,H) and M342 (G) antibodies were visualized as described in Materials and Methods. The corresponding Hoechst staining of D and E, and of G and H are shown in F and I respectively. Magnification, 250×.

Ontogeny of *rel*B expression

The earliest *rel*B transcripts were detected in the thymus at embryonic day 14, which coincides with the time of appearance of Ia-bearing dendritic precursors that initially colonize the embryonic thymic rudiment (Robinson, 1984). After day 15 of thymic development, the amount of *rel*B mRNA increases slowly to reach adult levels 6 days after birth. This observation is particularly interesting in light of the information that thymic clonal deletion does not occur until 1 week after birth, probably due to the functional immaturity of dendritic cells (Schneider et al., 1989a,b; Mazda et al., 1991). This functional immaturity has been found to correlate with the expression of Ia antigens and is reflected in a reduced antigen presentation activity (Lu et al., 1980).

In contrast to the thymus, *rel*B expression in the spleen appears later during ontogeny. *rel*B transcripts are not detected in the embryonic spleen, although significant levels appear 1 week after birth. Lu et al. (1980) detected few Ia-bearing cells in the spleen of 6- and 12-day-old mice and there is minimal antigen-presenting function. Between 2 and 4 weeks of age, a rapid increase in the Ia content and antigen-presenting function of splenic cells occurs (Lu et al., 1980). Taken as a whole, this information indicates that the appearance of *rel*B transcripts in dendritic cells occurs before the appearance of Ia antigen and the antigen-presenting ability of these cells, suggesting that RelB may be directing Ia antigen expression and antigen-presenting abilities.

Specific expression of *rel*B in dendritic cells

One of the most striking observations of our in situ studies is the primary localization of *rel*B-expressing cells to the T cell area of lymphoid organs, i. e., the periarterial lymphatic sheath of the spleen, the paracortex and deep cortex of lymph nodes, and the thymic medulla. These are all sites to which dendritic cells are restricted (Agger et al., 1992). In agreement with their functional role, the particular distribution of dendritic cells correlates with the areas where T cell clonal activation and deletion takes place in lymphoid organs.

Interdigitating dendritic cells have been mainly characterized by three monoclonal antibodies, NLDC-145, which stains a surface protein on dendritic cells as well as thymic epithelium (Kraal et al., 1986), MIDC-8, which stains granules within dendritic cells (Breel et al., 1987), and M342, which recognizes a perinuclear antigen in dendritic cells and some B cells (Agger et al., 1992). Using double immunofluorescence and the M342 monoclonal antibody as a positive marker for dendritic cells, we identified these as the target

cells for RelB expression. RelB-positive cells display the same morphological characteristics of dendritic cells isolated from the thymus by Steinman and Cohn (1973).

Prior studies with the NLCD-145 and M342 monoclonal antibodies (Metlay et al., 1990; Agger et al., 1992) have revealed two populations of dendritic cells in the spleen: a more abundant population at the periphery of the T cell area and a more central population in the periarterial lymphatic sheaths. The restricted expression of RelB to dendritic cells from the periarterial lymphatic sheaths suggests different functional capabilities between these two dendritic cell populations.

The hematopoietic origin of dendritic cells has been demostrated in radiation chimeras in which dendritic cells were derived from reconstituted bone marrow inoculum (Steinman et al., 1974; Barclay and Mayrhofer, 1981; Metlay et al., 1989). Mature dendritic cells could not be identified in the bone marrow either by morphology (Steinman and Cohn, 1973), or by expression of the 33DI antigen (Nussenzweig et al., 1982), or by mixed leukocyte reaction stimulating activity (Steinman and Witmer, 1978), and there is no evidence that mature dendritic cells are normally formed in the bone marrow. Thus, the absence of *rel*B transcripts in our PCR analysis indicates that *rel*B is not expressed in bone marrow dendritic precursors. This suggests that *rel*B activity is required during later stages of dendritic cell differentiation which takes place in the medulla of the thymus and secondary lymphoid organs.

The RelB protein in dendritic cells is detected in two different cellular localizations. In some cells, RelB is distributed in the cytoplasm and nucleus but, in others, it is only detected in the nucleus. As other Rel proteins (Baeuerle and Baltimore, 1988a), RelB is probably regulated at least in part by subcellular localization. The signals that regulate this differential distribution of RelB remains to be determined.

RelB function

One of the roles of RelB would be to regulate the expression of genes that encode surface molecules essential for antigen presentation, dendritic cell-T cell clustering and T cell activation. These molecules include, among others, the MHC gene products, adhesins and cytokine receptors. Consistent with their strong antigen-presenting cell function, dendritic cells express high levels of class II MHC (I-A and I-E). These cells also express interleukin-2 receptor when activated and this receptor seems to be involved in the acquisition of T cell responsiveness during dendritic cell-T cell interactions. The possible involvement of RelB in the expression of these surface molecules is suggested by the observation that both class II MHC and interleukin-2 receptor genes contain a functional κB-binding site in their respective promoters (reviewed in Lenardo and Baltimore, 1989; Baeuerle, 1991).

Members of the Rel family of transcriptional factors have also been implicated in lymphoid malignancies, suggesting a possible role in the control of cell growth and differentiation. For example, the oncogene v-*rel* was identified as the transforming gene of the avian reticuloendotheliosis virus that causes B cell lymphomas in birds (Theilen et al., 1966; Stephens et al., 1983), and the NFKB2 gene (p100/p52), also known as *lyt*-10, was cloned from a B cell lymphoma-associated chromosomal translocation (Neri et al., 1991). Because of the specific expression of RelB in dendritic cells, one might speculate that altered *rel*B gene expression or its conversion to an oncogenic form may be involved in the pathogenesis of some immune disease or other malignancies for which an etiologic agent has not yet been identified.

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REFERENCES

Agger, R., Witmer-Pack, M., Romani, N., Stossel, H., Swiggard, W. J., Metlay, J. P., Storozynsky, E., Freimuth, P. and Steinman, R. M. (1992). Two populations of splenic dendritic cells detected with M342, a new monoclonal to an intracellular antigen of interdigitating dendritic cells and some B lymphocytes. *J. Leukocyte Biol.* **52**, 34-42.

Austyn, J. M. (1987). Lymphoid dendritic cells. *Immunology* **62**, 161-170.

Baeuerle, P. A. (1991). The inducible transcription activator NF-κB: regulation by distinct protein subunits. *Biochim. Biophys. Acta* **1072**, 63-80.

Baeuerle, P. A. and Baltimore, D. (1988a). Activation of DNA-binding activity is an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell* **53**, 211-217.

Baeuerle, P. A. and Baltimore, D. (1988b). IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242**, 540-546.

Baeuerle, P. A. and Baltimore, D. (1989). A 65-κB subunit of active NF-κB is required for inhibition of NF-κB by IκB. *Genes Dev.* **3**, 1689-1698.

Baeuerle, P. A. and Baltimore, D. (1991). The physiology of the NF-κB transcription factor. In *The Hormonal Control Regulation of Gene Transcription, volume* **6** (ed. P. Cohen, and J. G. Foulkes), pp. 409-432. Amsterdam: Elsevier.

Barclay, A. N. and Mayrhofer, G. (1981). Bone marrow origin of Ia-positive cells in the medulla of rat thymus. *J. Immunol.* **153**, 1666-1671.

Blank, V., Kourilsky, P. and Israel, A. (1992). NF-κB and related proteins: Rel/*dorsal* homologies meet ankyrin-like repeats. *Trends Biochem. Sci.* **17**, 135-140.

Bours, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Ryseck, R.-P., Bravo, R., Kelly, K. and Siebenlist, U. (1992). A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. *Mol. Cell. Biol.* **12**, 343-350.

Bours, V., Villalobos, J., Burd, P. R., Kelly, K. and Siebenlist, U. (1990). Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. *Nature* **348**, 76-80.

Breel, M., Mebius, R. E. and Kraal, G. (1987). Dendritic cells of the mouse recognized by two monoclonal antibodies. *Eur. J. Immunol.* **17**, 1555-1559.

Brownell, E., Mathieson, B., Young, H. A., Keller, J., Ihle, J. N. and Rice, N. R. (1987). Detection of c-*rel*-related transcripts in mouse hematopoietic tissues, fractionated lymphocyte populations, and cell lines. *Mol. Cell. Biol.* **7**, 1304-1309.

Brownell, E., Ruscetti, F. W., Smith, R. G. and Rice, N. R. (1988). Detection of *rel*-related RNA and protein in human lymphoid cells. *Oncogene* **3**, 93-98.

Bull, P., Hunter, T. and Verma, I. (1989). Transcriptional induction of the murine c-rel gene with serum and phorbol-12-myristate-13-acetate in fibroblasts. *Mol. Cell. Biol.* **9**, 5239-5243.

Chen, I., Wilhelmsen, K. and Temin, H. M. (1983). Structure and expression of c-*rel*, the cellular homolog to the oncogene of reticuloendotheliosis virus strain T. *J. Virol.* **45**, 104-113.

Chirgwin, J. M., Przylya, A. E., Macdonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.

Crowley, M., Inaba, K., Witmer-Pack, M. and Steiman, R. M. (1989).

The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* **118**, 108-125.

Dony, C. and Gruss, P. (1987). Proto-oncogene c-*fos* expression in growth regions of fetal bone and mesodermal web tissue. *Nature* **328**, 711-714.

Fujita, T., Nolan, G. P., Ghosh, S. and Baltimore, D. (1992). Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. *Genes Dev.* **6**, 775-787.

Ghosh, S. and Baltimore, D. (1990). Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. *Nature* **344**, 678-682.

Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P. and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. *Cell* **62**, 1019-1029.

Gilmore, T. D. (1990). NF-κB, KBF1, *dorsal*, and related matters. *Cell* **62**, 841-843.

Gilmore, T. D. (1991). Malignant transformation by mutant Rel proteins. *Trends Genet* **7**, 318-322.

Gilmore, T. D. (1992). Role of *rel* family genes in normal and malignant lymphoid cell growth. *Cancer Surveys* **15**, 69-87.

Grilli, M., Chiu, J.-S. and Lenardo, M. J. (1992). NF-κB and Rel-participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.* (*in press).*

Grumont, R. J. and Gerondakis, S. (1990). Murine c-*rel* transcription is rapidly induced in T-cells and fibroblasts by mitogenic agents and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. *Cell Growth Differ.* **1**, 345-350.

Hansen, S. K., Nerlov, C., Zabel, U., Verde, P., Johnsen, M., Baeuerle, P. A. and Blasi, F. (1992). A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *EMBO J.* **11**, 205-213.

Heine, U. I., Munoz, E. F., Flankers, K. C., Ellingsworth, L. R., Lam, H.-Y. P., Thompson, N. L., Roberts, A. B. and Sporn, M. B. (1987). Role of transforming growth factor-β in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861-2876.

Kerr, L. D., Inoue, J., Davis, N., Link, E., Baeuerle, P. A., Bose, H. J. and Verma, I. M. (1991). The rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. *Genes Dev.* **5**, 1464-1476.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., LeBail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A. and Israel, A. (1990). The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* **62**, 1007-1018.

Kovary, K., and Bravo, R. (1991). The *jun* and *fos* protein families are both required for cell cycle progression in fibroblasts. *Mol. Cell. Biol.* **11**, 4466-4472.

Kraal, G., Breel, M., Janse, M. and Bruin, G. (1986). Langerhans cells, vieled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* **163**, 981-997.

Kretzschmar, M., Meisterernst, M., Scheidereit, C., Li, G. and Roeder, R. G. (1992). Transcriptional regulation of the HIV-1 promoter by NF-κB in vitro. *Genes Dev.* **6**, 761-774.

Kunsch, C., Ruben, S. M. and Rosen, C. A. (1992). Selection of optimal κB/Rel DNA-binding motifs: Interaction of both subunits of NF-κB with DNA is required for transcriptional activation. *Mol. Cell. Biol.* **12**, 4412-4421.

Lenardo, M. J. and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**, 227-229.

Link, E., Kerr, L. D., Schreck, R., Zabel, U., Verma, I. and Baeuerle, P. A. (1992). Purified IκBβ is inactivated upon dephosphorylation. *J. Biol. Chem.* **267**, 239-46.

Lu, C. Y., Beller, D. I. and Unanue, E. R. (1980). During ontogeny, Ia-bearing accessory cells are found early in the thymus but late in the spleen. *Proc. Natl. Acad. Sci. USA.* **77**, 1597-1601.

Mazda, O., Watanabe, Y., Gyotoku, J.-I. and Katsura, Y. (1991). Requirement of dendritic cells and B cells in the clonal deletion of M1s-reactive T cells in the thymus. *J. Exp. Med.* **173**, 539-547.

Metcalf, D. and Moore, M. A. S. (1971). Embryonic aspects of haemopoiesis. In *Frontiers of Biology* (ed. A. Neuberger and E. L. Tatum). Amsterdam, London: North-Holland Publishing Co.

Metlay, J. P., Pure, E. and Steinman, R. M. (1989). Control of the immune response at the level of antigen-presenting cells: A comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* **47**, 45-116.

Metlay, J. P., Witmer-Pack, M. D., Agger, R., Crowley, M. T., Lawless, D. and Steinman, R. M. (1990). The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* **171**, 1753-1771.

Meyer, R., Hatada, E. N., Hohmann, H. P., Haiker, M., Bartsch, C., Rothlisberger, U., Lahm, H. W., Schlaeger, E. J., van Loon. A. P. and Scheidereit, C. (1991). Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. USA* **88**, 966-970.

Morris, L., Graham, C. F. and Gordon, S. (1991). Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development* **112**, 517-526.

Neri, A., Chang, C. C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A. T., Chaganti, R. S. and Dalla-Favera, R. (1991). B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt*-10, homologous to NF-κB p50. *Cell* **67**, 1075-1087.

Nolan, G. P. and Baltimore, D. (1992). The inhibitory ankyrin and activator Rel proteins. *Curr. Opin. Genet. Develop.* **2**, 211-220.

Nolan, G. P., Ghosh, S., Liou, H. C., Tempst, P. and Baltimore, D. (1991). DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. *Cell* **64**, 961-969.

Nussenzweig, M. C., Steinman, R. M., Witmer, M. D. and Gutchinov, B. (1982). A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA* **79**, 161-165.

Robinson, J. H. (1984). The ontogeny of thymic macrophages: Thymic macrophages express Ia from 15 days gestation onwards in the mouse. *Cell. Immunol.* **84**, 422-426.

Rouse, R. V., van Ewijk, W., Jones, P. P. and Weissman, I. L. (1979). Expression of MHC antigens by mouse thymic dendritic cells. *J. Immunol.* **122**, 2508-2515.

Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A. and Rosen, C. A. (1991). Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. *Science* **251**, 1490-1493.

Ryseck, R.-P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P. and Bravo, R. (1992). RelB, a new Rel family transcription activator that can interact with p50-NF-κB. *Mol. Cell. Biol.* **12**, 674-684.

Schmid, R. M., Perkins, N. D., Duckett, C. S., Andrews, P. C. and Nabel, G. J. (1991). Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. *Nature* **352**, 733-736.

Schmitz, M. L. and Baeuerle, P. A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-κB. *EMBO J.* **10**, 3805-3817.

Schneider, R., Lees, R. M., Pedrazzini, T., Odermat, B., Speiser, D. E., Zinkernagel, R. M., Hengartner, H. and MacDonald, H. R. (1989a). Fate of potentially self-reactive T cells in neonatal mice: analysis of $V\beta6^+$ T cells in M1s mice. *Thymus* **13**, 35-43.

Schneider, R., Lees, R. K., Pedrazzini, T., Zinkernagel, R. M., Hengartner, H. and MacDonald, H. R. (1989b). Postnatal disappearance of self reactive ($V\beta6^+$) cells from the thymus of M1s mice. Implications for T cell development and autoimmunity. *J. Exp. Med.* **169**, 2149-2158.

Sen, R. and Baltimore, D. (1986a). Inducibility of κB by a posttranslational mechanism. *Cell* **47**, 921-928.

Sen, R. and Baltimore, D. (1986b). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **46**, 705-716.

Shirakawa, F. and Mizel, S. B. (1989). In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* **9**, 2424-2430.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271-296.

Steinman, R. M. and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. Morphology, quantitation and tissue distribution. *J. Exp. Med.* **137**, 1142-1162.

Steinman, R. M., Lustig, D. S. and Cohn, Z. A. (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. III. Functional properties in vivo. *J. Exp. Med.* **139**, 1431-1445.

Steinman, R. M. and Witmer, M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. USA* **75**, 5132-5136.

Stephens, R. M., Rice, N. F., Hiebsch, R. R., Bose, H. R., Jr. and Gilden, R. V. (1983). Nucleotide sequence of v-*rel*: the oncogene of reticuloendotheliosis virus. *Proc. Natl. Acad. Sci. USA* **80**, 6229-6233.

Steward, R. (1987). Dorsal, an embryonic polarity gene in Drosophila, is

homologous to the vertebrate proto-oncogene, c-*rel*. *Science* **238**, 692-694.

Telford, N. A., Hogan, A., Franz, C. R. and Schultz, G. A. (1990). Expression of genes for insulin and insulin-like growth factors and receptors in early postimplantation mouse embryos and embryonal carcinoma cells. *Mol. Rep. Dev.* **27**, 81-92.

Theilen, G. H., Zeigel, R. F. and Twiehaus, M. J. (1966). Biological studies with RE virus (strain T) that induces reticuloendotheliosis in turkeys, chickens, and Japanese quails. *J. Natl. Cancer Inst.* **37**, 731-743.

Urban, M. B., Schreck, R. and Baeuerle, P. A. (1991). NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* **10**, 1817-1825.

Veldman, J. E. and Kaiserling, E. (1980). Interdigitating cells. In *The Reticuloendothelial System, Morphology* (ed. I. Carr and W. T. Daems), pp. 381-416. New York: Plenum Publishing.

Wilhelmsen, K. C., Eggleton, K. and Temin, H. M. (1984). Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-*rel*. *J. Virol.* **52**, 172-182.

Zabel, U., Schreck, R. and Baeuerle, P. A. (1991). DNA binding of purified transcription factor NF-κB. Affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. *J. Biol. Chem.* **266**, 252-260.

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