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RelB Regulation of Chemokine Expression Modulates Local Inflammation

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The resolution of acute inflammation is incompletely understood but presumably requires the elimination of both inflammatory cells and production of inflammatory cytokines. In the case of recruited bone-marrow-derived inflammatory cells such as granulocytes and macrophages, their short life span helps eliminate these cells and the cytokines they produce. By contrast, resident permanent cells such as fibroblasts require other mechanisms to stop the production of chemokines generated in response to inflammatory triggers such as lipopolysaccharide. Here we demonstrate that RelB is an important regulator of chemokine expression in fibroblasts, thereby playing a key role in the resolution of acute inflammation. Activation of normal fibroblasts by lipopolysaccharide induced a transient production of chemokines, closely followed by induction of RelB expression. However, stimulated RelB$^{-/-}$ fibroblasts exhibited dramatic persistent induction of seven chemokines (RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, JE/MCP-1, and KC/CINC). The persistent overexpression of chemokines correlated with increased NF-κB binding as well as with increased p50, p65/RelA, and IκBα expression. Transfection of RelB cDNA into RelB-deficient fibroblasts reversed the lipopolysaccharide-induced chemokine overexpression. *In vivo*, activated RelB$^{-/-}$ fibroblasts dramatically increased recruitment of granulocytes into tissues. In view of the apparent role of RelB in the resolution of acute inflammation in tissues and previous work showing a requirement for RelB in the initiation of immune responses through the differentiation of antigen-presenting cells, RelB may be an important factor regulating the transition from innate to adaptive immunity. *(Am J Pathol 1997, 151:375–387)*

Attraction of leukocytes to areas of infection is essential for host defense. Chemokines play a fundamental role in establishing sites of inflammation and are produced in response to various danger stimuli such as lipopolysaccharide (LPS), interleukin (IL)-1, or tumor necrosis factor (TNF)-α. They participate in the creation of inflammatory sites by 1) evoking transient increases in integrin activity on neutrophils and monocytes leading to endothelial cell adherence and transmigration,[1,2] 2) providing a chemotactic gradient to guide leukocyte migration to the site of inflammation,[3] and 3) activating granulocyte and monocyte effector functions.[4,5] Yet, while much is known about the activation of inflammatory events such as chemokine production, less is understood about the mechanisms that control inflammation. Serum antibodies and the Duffy erythrocyte cell surface antigen have been identified as mechanisms useful in inhibiting chemokine activity within blood vessels.[6,7] These may limit the extent of inflammation but cannot serve to resolve inflammation at the primary interstitial site. *In vitro* studies have also implicated IL-4 and IL-10 as possible chemokine inhibitors, but the *in vivo* translation of these observations is unclear.[8]

The expression of chemokines is thought to be regulated in part by the action of members of the NF-κB/Rel family of transcription factors. Members of the Rel family appear to have diverse functions, being implicated in growth control,[9] apoptosis,[10] adaptive immune responses,[11] and inflammation.[12,13] In the present study, we explore the role of RelB as it relates to the control of chemokine responses and inflammation. Five members currently make up the NF-κB/Rel family in vertebrates: NF-κB1 (p50), NF-κB2 (p52, p49, p50B), RelA (p65), c-rel, and RelB.[14] Although all Rel family members bind DNA at consensus decameric sequences, κB sites, as homo- or heterodimers, Rel B is unusual in that it apparently does not homodimerize but instead requires heterodimerization with p50 or p52.[15] Whereas most Rel family members are involved in inducible transactivation, Lernbecher and colleagues have suggested that RelB is associated with constitutive activity in lymphoid cells.[16] This constitutive activity may in part be due to a reduced affinity of the inhibitor IκBα for p50/RelB complexes.[17]

Three observations suggest a link between NF-κB/Rel proteins and the regulation of inflammation. First, the promoter sequences of several chemokine genes contain κB motifs.[18,19] Second, stimuli known to induce NF-κB activity include common signals associated with the on-

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set of inflammation, such as bacterial LPS, proinflammatory cytokines, viruses, and changes in osmolarity.[11,17] Third, RelB and IκBα knock-out mice exhibit severe inflammatory syndromes.[12,13,20] Studies of RelB-deficient mice have shown that RelB is important for the development of medullary epithelium, mature dendritic cell function, secondary lymphoid tissue organization, and thymic negative selection.[12,13] In addition, multi-organ inflammation contributes significantly to premature mortality in these mice. Although the immune system lesions observed in RelB-deficient mice are consistent with the established role of NF-κB regulation of gene expression in hematopoietic tissues, the position RelB plays in the inflammatory process remains obscure.

In this study, we demonstrate that RelB functions to limit the inflammatory process at interstitial sites through the control of chemokine expression by stimulated tissue fibroblasts. Chemokine and RelB expression is transiently increased after LPS stimulation of normal fibroblasts. In contrast, $RelB^{-/-}$ fibroblasts exhibited dysregulated chemokine production after LPS stimulation *in vitro*. The overproduction of seven chemokines by $RelB^{-/-}$ fibroblasts correlated with increased NF-κB (p50/p65) activity, suggesting that RelB may influence chemokine expression by regulating NF-κB activity. Importantly, the dysregulated chemokine expression is corrected with the transfection of RelB cDNA into $RelB^{-/-}$ fibroblasts. *In vivo* data also support a role for RelB in regulating inflammation as both intraperitoneal and subcutaneous injections of stimulated RelB-deficient fibroblasts result in significantly greater inflammation than injections of wild-type fibroblasts.

Materials and Methods

Mice

RelB-deficient mice were generated and initially characterized as previously described.[21] They were generated on an inbred C57BL/6J background and also bred onto a B10.D2 background, and control mice were all of B10.D2 origin. Bone marrow chimeras were made by treating C57BL/6J mice with 1100 rad and then reconstituting with 7×10^6 bone marrow cells from C57BL/6J control or RelB mutant mice. All mice (RelB mutant, B10.D2, and SCID) were housed in specific-pathogen-free facilities. All experimental procedures were carried out according to the guidelines laid out in the National Institutes of Health Guide to the Care and Use of Laboratory Animals.

Cells

Murine fibroblasts isolated from kidneys of wild-type or RelB-deficient mice as previously described[22] were cultured for at least 15 passages to obtain homogeneous cell populations before being used in the experiments described. Cultures were maintained at 37°C, 5% CO_2, in Dulbecco's minimal essential medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Murine peritoneal macrophages were collected as described.[23] Briefly, 4-week-old mice were injected intraperitoneally with 0.8 ml of thioglycollate broth (Life Technologies, Grand Island, NY). Four days later, the mice were sacrificed by suffocation with CO_2. Peritoneal lavages were performed using 8 ml of ice-cold phosphate-buffered saline (PBS, pH 7.3) containing heparin (10 U/ml). After 1 minute of light, rapid massage, the lavage fluid was slowly aspirated. Cells were centrifuged at $250 \times g$ for 10 minutes at 4°C and washed twice with minimal essential medium. Macrophages were allowed to rest for 24 hours in complete medium before LPS stimulation. Due to the limited numbers of macrophages available, a macrophage cell line (RAW 246.7) was employed for some assays. The RAW 246.7 cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in complete medium at 37°C/5% CO_2.

Stimulation of all cells was performed using a final concentration of 1 μg/ml LPS (List Biological Laboratories, Campbell, CA) or 1 to 100 ng/ml TNF-α in complete medium. Cells were removed from culture using trypsin/EDTA (Life Technologies) immediately before use. Cells were prepared for *in vivo* studies as described below.

RNAse Protection Assays

Total RNA was isolated using a single-step method.[24] Mouse probes for RANTES (regulated on activation, normal T cell expressed, and secreted; nucleotides 368 to 618 as defined in GeneBank sequence accession number U02298), macrophage inflammatory protein (MIP)-1β (111 to 341, accession number M35590), MIP-1α (139 to 349, accession number X12531), MIP-2 (131 to 311, accession number X53798), γ-interferon-inducible cytokine (IP-10; 100 to 260, accession number 33266), JE/monocyte chemoattractant protein (MCP)-1 (395 to 535, accession number J04467), cytokine-induced neutrophil chemoattractant (KC/CINC; 127 to 259, accession number J04596), L-32 (93 bp), p65 (220 bp), p50 (190 bp), and IκBα (245 bp) were generated by polymerase chain reaction (PCR) using cDNA templates. Amplified PCR products were cloned into pGEM4Z (Promega, Madison, WI). All plasmids were linearized by *Bam*HI digestion and transcribed with T7 RNA polymerase using [α-^{32}P]UTP. RNAse protection assays were performed as previously described.[25]

Transfection

Stable transfection of $RelB^{-/-}$ fibroblasts was performed using Lipofectamine (Life Technologies) as described by the manufacturer. Murine RelB cDNA was inserted into the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA) using the *Eco*RI (5′ end) and *Xho*I (3′ end) sites. Stable transfectants were selected using complete medium containing 0.5 mg/ml G418. Transfectant RelB expression was confirmed by reverse transcription PCR and RNAse protection assays. Expression levels of

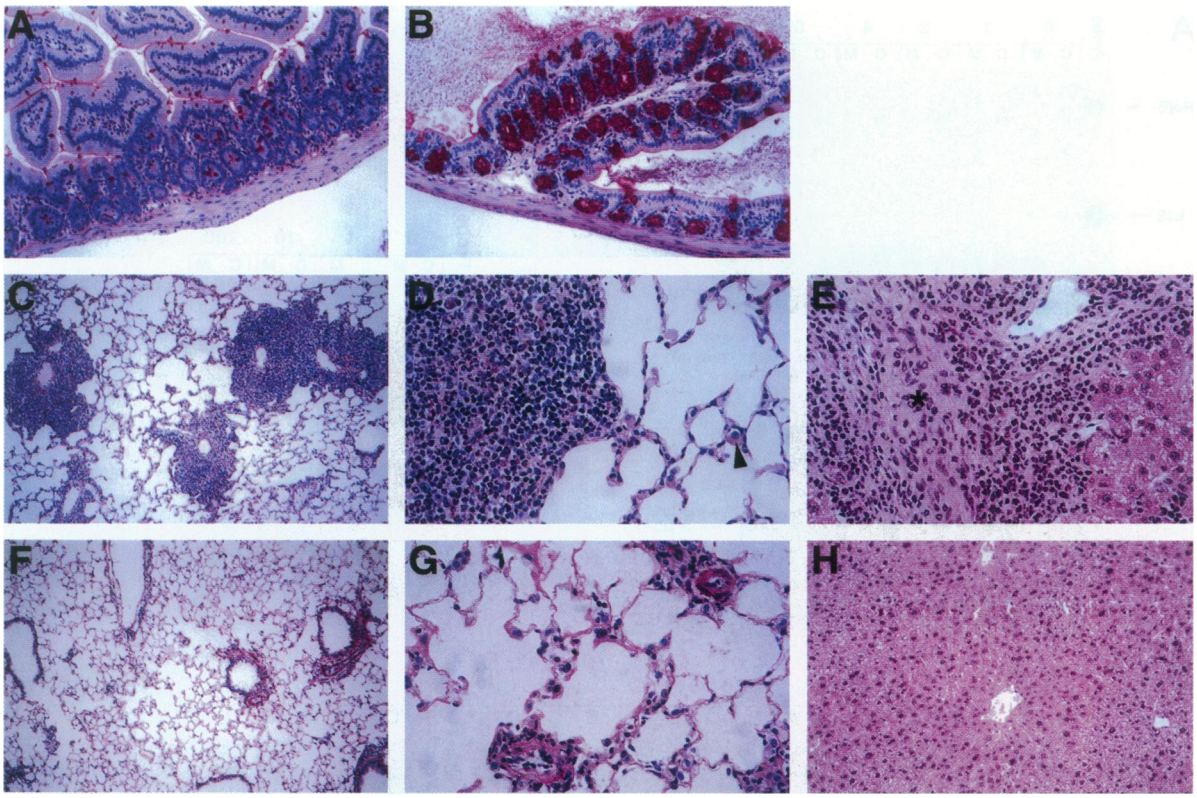


Figure 1. Histology of RelB-deficient mice and bone marrow chimeras. Despite multi-organ tissue inflammation, intestinal epithelium was free of evidence of infection. **A:** Small intestine (PAS; magnification, ×200). **B:** Large intestine (PAS; magnification, ×200). **C** and **D:** Lung tissue showed only interstitial infiltrates with no exudates or inflammation in airways or alveolar spaces except for occasional alveolar macrophages (**arrowhead**). **C:** PAS; magnification, ×100. **D:** PAS; magnification, ×400. **E:** Liver infiltrates were not isolated foci but instead accumulated around areas of fibrosis (**asterisk**). H&E; magnification, ×400. **F** to **H:** By contrast, RelB mutant bone marrow reconstitution of wild-type mice resulted in little or no inflammation in lung (**F** (PAS; magnification, ×100) and **G** (PAS; magnification, ×400)) or liver (**H** (H&E; magnification, ×200)).

RelB in transfectants was slightly lower than nonstimulated wild-type fibroblasts (data not shown).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described by Dignam and colleagues[26] with slight modifications. Cells (10^7) were washed with PBS, resuspended in 500 μl of buffer A (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin), pelleted and resuspended in 400 μl of buffer A, and put on ice for 15 minutes. After adding 25 μl of 10% Nonidet P-40, samples were vortexed for 10 seconds and centrifuged at 12,000 × *g* for 30 seconds. The cytoplasmic supernatant was frozen at −70°C. The pellet was resuspended in 50 μl of buffer C (420 mmol/L NaCl, 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% glycerol, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin) and placed on ice for 20 minutes. The extract was centrifuged at 10,000 × *g*, and the supernatant containing the nuclear fraction was frozen at −70°C. The oligonucleotide sequences (Santa Cruz Biotechnology, Santa Cruz, CA) were as follows: murine intronic κ-chain κB site (underlined),[2] 5′-AGTTGAGGGGACTTTCCCAGG-3′ (NF-κB consensus); mutant κB site with the G to C substitution (underlined) in the NF-κB DNA motif, 5′-AGTTGAGGCGACTTTCCCAGG-3′. Oligonucleotides were labeled with [γ-^{32}P]ATP and a T4 polynucleotide kinase kit (Promega). EMSAs were performed in 12 μl of reaction buffer containing 0.2 ng of DNA probe, 2 μg of nuclear extract, 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 5% glycerol, and 1 μg of poly(dI-dC). In supershift assays, the nuclear extracts were isolated as described above and preincubated with 1 μg of the indicated rabbit antisera (Santa Cruz Biotechnology) or normal rabbit serum for 20 minutes at 25°C before addition of other reaction components.

Immunoprecipitation

Fibroblasts were stimulated with LPS in the presence of 0.2 mCi Tran^{35}S (ICN, Costa Mesa, CA) for 4 hours at 37°C, 5% CO_2. Cell were lysed in a solution of 150 mmol/L NaCl, 10% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mmol/L Tris (pH 8.0). Protein concentrations were determined by Bradford analysis using bovine serum albumin as a standard. Two 50-μl sets of 10% Pansorbin (Calbiochem, La Jolla, CA) were prewashed with TETN250 buffer (25 mmol/L Tris/HCl (pH 7.5), 5 mmol/L EDTA (pH 7.5), 250 mmol/L NaCl, 1% Triton X-100). After centrifugation, the pellets were resuspended in 50 μl of TETN250 buffer.

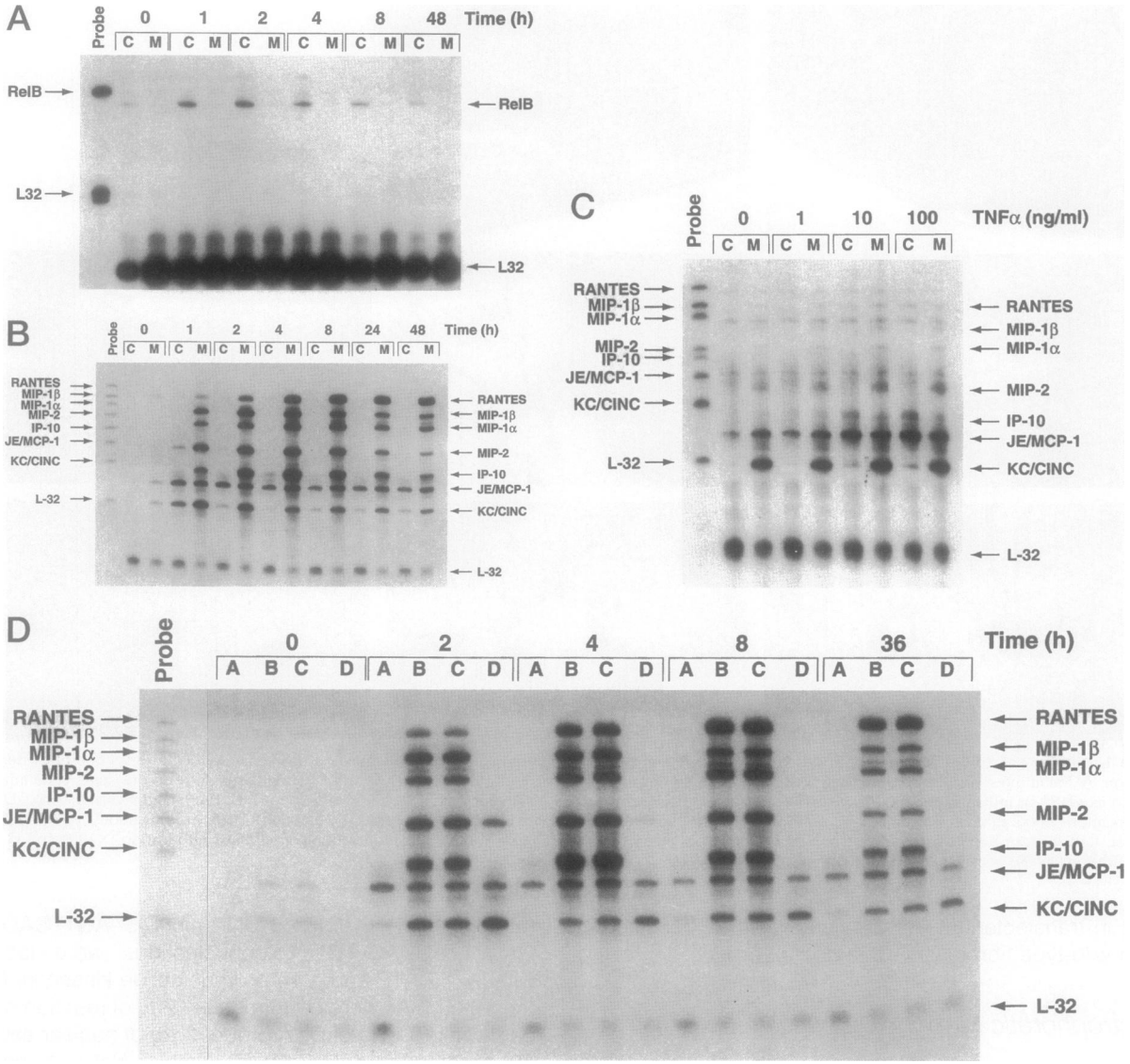


Figure 2. Chemokine expression is dysregulated in RelB-deficient fibroblasts after LPS stimulation. RNAse protection assays of LPS-stimulated wild-type (C, control) and RelB-deficient (M, mutant) fibroblasts as described in Materials and Methods. Total RNA was isolated at various times after stimulation as indicated, and 1 μg of total RNA was used for each time point. The protected bands are smaller than the probes (far left lane) because polylinker regions present in the probes are not protected when annealed to cellular RNA. A probe for L32 that detects a housekeeping gene product was used as an internal reference. **A:** RelB mRNA is expressed in control but not in mutant fibroblasts. **B:** Superinduction of chemokine expression by LPS is evident in mutant but not in control fibroblasts. **C:** Similar patterns of inducible chemokine expression also occurred after stimulation of fibroblasts with 1, 10, or 100 ng/ml TNF-α. **D:** Dysregulated chemokine expression is reversed by RelB transfection of RelB-deficient fibroblasts. After selection, transfected cells were stimulated with LPS, and isolated total RNA was assayed as described above. Wild-type fibroblasts were transfected with the pcDNA3 plasmid only (**lane A**). Mutant fibroblasts were transfected with either pcDNA3 plasmid alone (**lane C**) or recombinant pcDNA3 containing RelB cDNA (**lane D**). Results from nontransfected mutant fibroblasts are shown in **lane B**.

Each sample (200 μg of protein) was preabsorbed with one set of prewashed Pansorbin. The preabsorbed sample was then incubated with of 1 μg of rabbit anti-p50 or anti-RelB serum (Santa Cruz Biotechnology) in 100 μl of TETN250 containing 5% bovine serum albumin. After 5 hours of incubation, 50 μl of prewashed Pansorbin was added and incubated for 10 minutes at 25°C with occasional vortexing. The precipitable immune complexes were washed once with TETN250 and then twice with 10 mmol/L Tris/HCl (pH 7.5), 5 mmol/L EDTA (pH 7.5). The immunoprecipitated samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot

Immune complexes were precipitated using rabbit anti-RelB antiserum (Santa Cruz Biotechnology), subjected to SDS-PAGE using 7 to 20% gradient gels under reducing conditions, and transferred to polyvinylidene difluoride membranes. Nonspecific protein interactions were blocked by incubation of the membrane in Tris-buffered saline (TBS; 20 mmol/L Tris/HCL, pH 7.5, 100 mmol/L NaCl) containing 5% nonfat dried milk for 2 hours at 25°C with shaking. Immunoblotting was performed by incubating the membrane with 5 μg/ml rabbit anti-RelB in TBS

plus 5% milk overnight at 4°C. The blot was washed four times in TBS and 0.5% Triton X-100 before incubating with alkaline-phosphatase-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN).

Histology

SCID mice were injected subcutaneously in the ear with PBS, LPS-treated wild-type fibroblasts, or LPS-treated RelB mutant fibroblasts (10^6 cells/ml). Ear tissue was harvested 6 hours after injection. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Cryostat sections were fixed in 1% paraformaldehyde and counterstained with hematoxylin before fluorescence microscopy.

In Vivo *Assays and Flow Cytometry*

Fibroblasts were stimulated 4 hours with 1 μg/ml LPS, washed five times with PBS, and allowed to rest overnight in complete medium. Monolayers were incubated with 5 μmol/L green 5-chloromethylfluorescein diacetate cell tracker probe (Molecular Probes, Eugene, OR) for 15 to 20 minutes at 37°C, 5% CO_2 and then washed twice with PBS. Cells were trypsinized and washed twice in PBS to provide a single-cell suspension for injection. Labeled fibroblasts were injected subcutaneously into the ears of or intraperitoneally into SCID mice. Cells were recovered by peritoneal lavage using 2 ml of PBS from CB.17 SCID/SCID mice (TSRI breeding colony) 6 hours after intraperitoneal injection of PBS, 0.5×10^6 LPS-treated wild-type fibroblasts, or 0.5×10^6 LPS-treated RelB mutant fibroblasts.

Cells obtained from peritoneal lavage fluid were stained with biotinylated anti-GR-1 (Pharmingen, San Diego, CA) followed by streptavidin-phycoerythrin (Pharmingen). Stained cells were fixed in 1% paraformaldehyde before analysis on the FACScan (Becton Dickinson, Rutherford, NJ) using Cell Quest software (Becton Dickinson). Analyses included all cells. Ratios were calculated for cells analyzed from each injected mouse: percent GR-1 positive/percent green 5-chloromethylfluorescein diacetate cell tracker positive. Statistical analyses of ratios were performed using the Wilcoxon rank sum test. Cell concentrations obtained from each lavage were also determined. Mean values for each experiment were calculated, and statistical analyses were performed using the Student's *t*-test.

Results

Persistent Inflammation in RelB-Deficient Mice Is Due to Non-Bone-Marrow-Derived Cells

Our initial studies of RelB-deficient mice described a syndrome of multi-organ inflammation.[21] Infiltrating cells consisted primarily of neutrophils, with some macrophages and lymphocytes. This dominance of neutrophils is characteristic of acute inflammation and suggests an

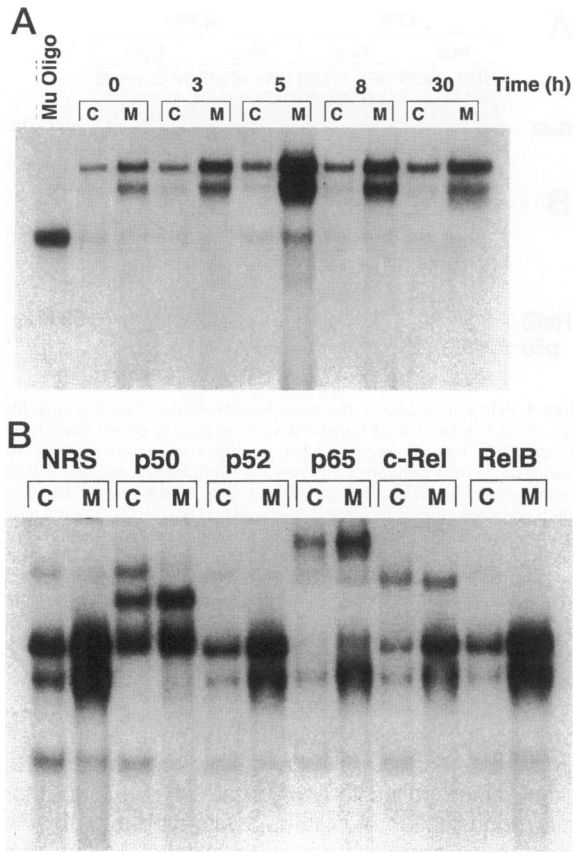


Figure 3. κB binding activity persists in RelB-deficient fibroblasts after LPS stimulation, as shown by EMSA with an NF-κB-specific DNA probe and nuclear extracts from RelB-deficient (M) and control (C) fibroblasts. **A:** Persistent activation of NF-κB is induced by LPS in RelB-deficient but not wild-type fibroblasts. A mutant oligonucleotide (Mu Oligo) was used as a control (far left lane). **B:** Fibroblast κB-specific complexes contain p50, p65, and c-rel. Extracts were obtained after 5 hours of stimulation with LPS and were incubated before EMSA with antisera as indicated at the top of the lanes.

infectious origin. However, all mice were housed under specific-pathogen-free conditions with sentinel animals remaining free of common murine viral pathogens making viral infection of the mutants unlikely. Additional observations suggest a noninfectious process; for example, if RelB-deficient mice were unusually susceptible to infection, then it should be evident in tissues such as the intestine and lung. Curiously, the intestinal epithelium was notably free of obvious infection or inflammation (Figure 1, A and B). In the lung, extensive interstitial infiltrates were seen, but no inflammatory cells could be found in alveolar spaces (as would be seen in bacterial pneumonia) aside from the occasional alveolar macrophage (Figure 1, C and D). In the liver, inflammatory infiltrates showed a curious accumulation around areas of fibrosis (Figure 1E). Taken together, these observations and those of others[13] suggest that this inflammation is not the result of a viral infectious agent, but a bacterial origin has not been formally ruled out.

Additional clues to the source of the inflammation came from adoptive transfer and bone marrow chimera studies.[12,21,27] If the inflammatory syndrome was intrinsic to the RelB-deficient bone marrow, then inflammation

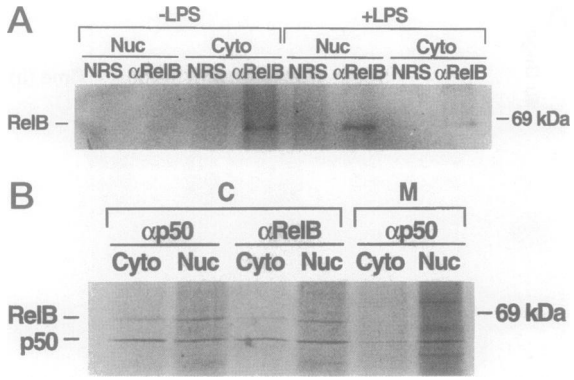


Figure 4. RelB translocates to the nucleus upon stimulation of normal fibroblasts. **A:** LPS induces RelB translocation from the cytoplasm (Cyto) to the nucleus (Nuc). Cytoplasmic or nuclear extracts were prepared from either LPS-stimulated or nonstimulated fibroblasts. Immunoprecipitation followed by immunoblotting was performed with extracts from wild-type fibroblasts. **B:** RelB co-precipitates with p50 in LPS-stimulated control (C) but not mutant (M) fibroblast extracts. Cytoplasmic (Cyto) or nuclear (Nuc) extracts from LPS-stimulated ^{35}S metabolically labeled fibroblasts were immunoprecipitated with either anti-p50 (αp50) or anti-RelB (αRelB) antiserum before fractionation of immune complexes by SDS-PAGE. Mutant fibroblast extracts (M) were used as a negative control.

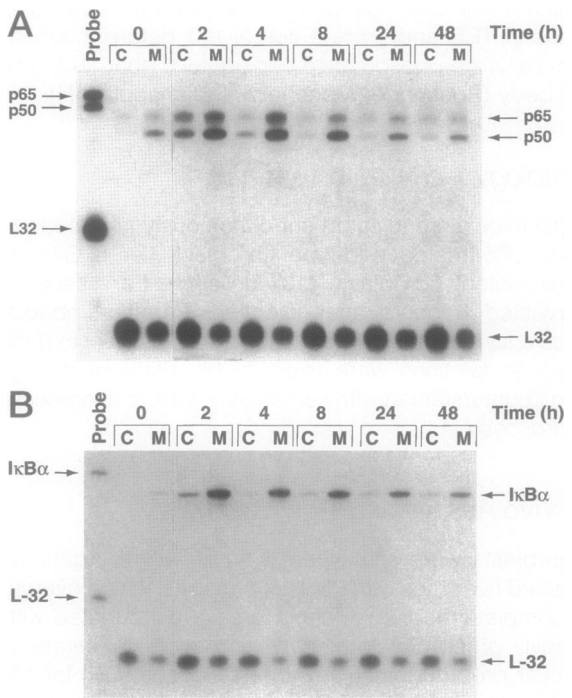


Figure 5. p65, p50, and IκBα expression is prolonged after stimulation of RelB $^{-/-}$ fibroblasts, as shown by RNAse protection assays of LPS-stimulated wild-type (C) and RelB-deficient (M) fibroblasts using probes specific for p65, p50, and IκBα. **A:** Time course analyses indicate induction of p65 and p50 mRNA expression is extended in LPS-treated RelB $^{-/-}$ fibroblasts as compared with wild type. **B:** IκBα mRNA expression in LPS-stimulated mutant fibroblasts is also increased compared with control fibroblasts.

should correlate with the presence of RelB-deficient bone marrow. Surprisingly, this was not the case, as transfer of normal spleen cells[21] or bone marrow into irradiated RelB mutant mice[27] failed to correct the inflammation. Moreover, reconstitution of irradiated wild-type mice by RelB mutant bone marrow resulted in minimal, if any, tissue inflammation[27] (Figure 1, F–H).

Together, the above observations suggested that the tissue inflammation in RelB-deficient mice was due to cells that were radioresistant and not bone marrow derived. As reported previously, RelB-deficient mice have defects in antigen-presenting cell development and function that were shown to be intrinsic to bone marrow,[12,27,28] so it appeared that the inflammatory syndrome was a distinct consequence of RelB deficiency. Our studies therefore focused next on the apparent separate function of RelB in nonhemopoietic cells.

Chemokine Expression Is Regulated by RelB

As numerous chemokines provoke the chemotaxis of granulocytes and monocytes facilitating inflammation at specific sites,[29] it seemed plausible that chemokine gene regulation might be adversely affected in RelB-deficient mice. Although some pro-inflammatory chemokine genes have κB or κB-like promoter elements, RelB has not been previously associated with the regulation of these genes.[18,19] RelB was originally identified as an activation-dependent gene in fibroblasts, so RelB expression was monitored after stimulation of wild-type fibroblasts with LPS. Fibroblasts maintained a low basal level of RelB mRNA that substantially increased within 1 hour after LPS stimulation (Figure 2A). Peak expression occurred at 2 hours after stimulation, with a return to basal levels within 48 hours. These data are consistent with the previously described inducible expression of RelB after serum stimulation of murine 3T3 cells.[15]

Exploration of chemokine expression using RNAse protection assays revealed striking differences between normal fibroblasts and those deficient in RelB (Figure 2B). Typically, LPS stimulation of wild-type fibroblasts caused only a transient induction of MIP-2 and IP-10, both returning to basal levels within 8 hours. A more sustained induction of JE/MCP-1 and KC was observed, although the levels of these mRNAs were always notably less than seen in the RelB $^{-/-}$ fibroblasts. The LPS-stimulated RelB $^{-/-}$ fibroblasts exhibited dramatic increases in mRNA levels of seven chemokines: RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, JE/MCP-1, and KC/CINC. The up-regulation of chemokine expression continued for at least 48 hours after stimulation. A representative promoter-reporter (luciferase) assay, using the MIP-2 promoter, showed significant up-regulation of reporter gene expression in cells that lacked RelB, suggesting that the up-regulation of chemokine mRNA levels is indeed due to increased transcriptional activation (data not shown). In addition, RelB-deficient fibroblasts stimulated with various concentrations of TNF-α also demonstrated dysregulated chemokine expression profiles; however, the expression of C-X-C chemokines (particularly MIP-2 and KC/CINC) appears to be affected to a greater extent than the C-C chemokines (Figure 2C).

The striking phenotype of fibroblasts from RelB-deficient mice might have been a secondary consequence of the *in vivo* inflammatory syndrome or some other influence, so to demonstrate a direct relationship between

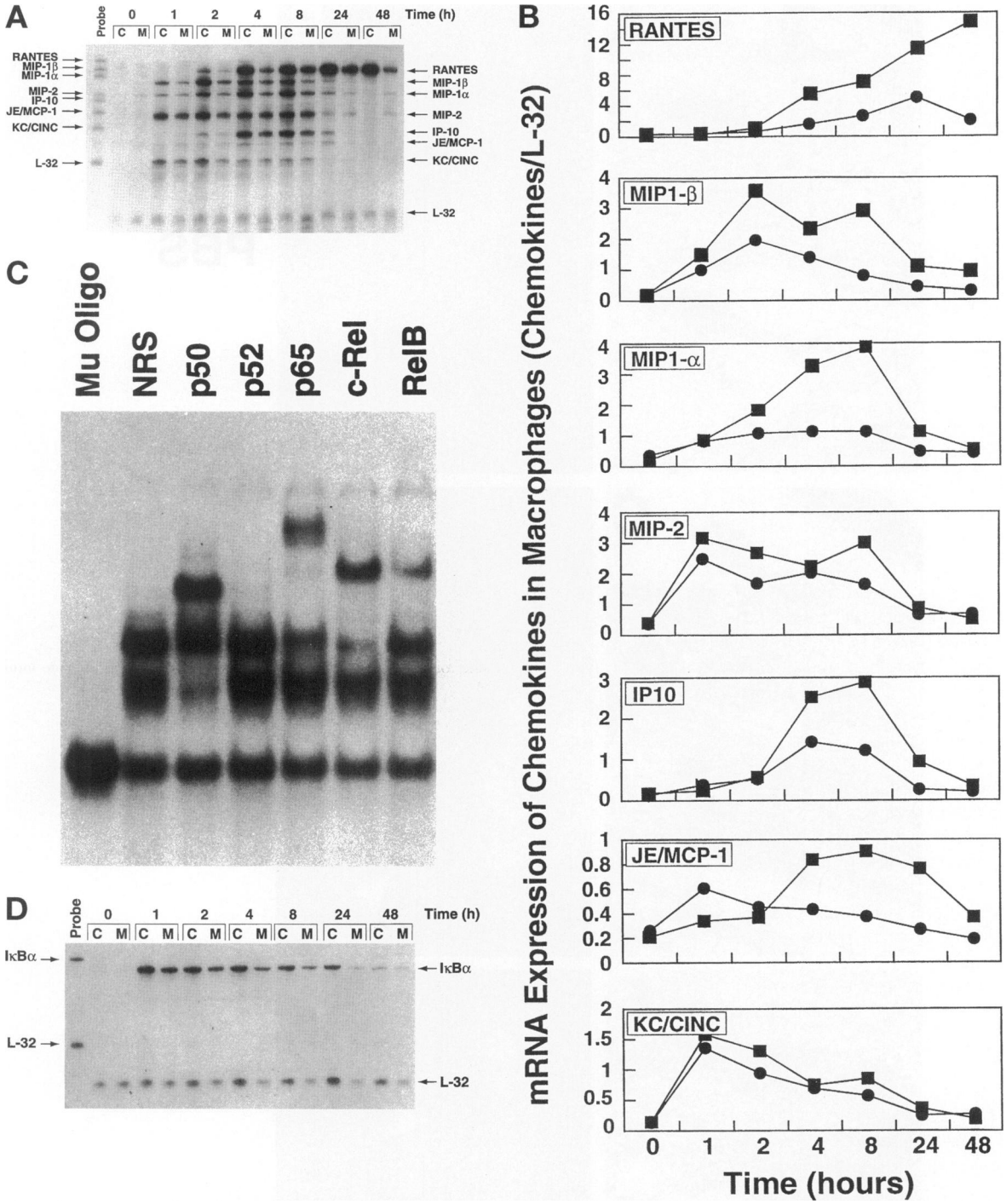


Figure 6. RelB function is cell specific. **A:** RNAse protection assays were performed using mRNA from LPS-induced peritoneal macrophages isolated from control (C) and mutant (M) mice. **B:** The absence of RelB correlates with reduced chemokine expression after LPS treatment of peritoneal macrophages. The expression levels of chemokine mRNA in control (■) and mutant (●) macrophages were quantitated on the AMBIS radioanalytic imaging system (AMBIS System, San Diego, CA). The final values are expressed relative to the mRNA levels of L-32. **C:** Characterization of the polypeptide composition of the κB-specific complexes in the macrophage cell line RAW 246.7 shows p50, p65, c-rel, and RelB all participate in binding. Cells were stimulated with LPS for 3 hours, and nuclear extracts were then preincubated with the indicated antisera before performing EMSA using an NF-κB-specific probe as well as a mutant probe (Mu Oligo). **D:** IκBα mRNA expression is slightly reduced in LPS-stimulated mutant (M) peritoneal macrophages as compared with control (C) as determined in RNAse protection assays.

chemokine regulation and RelB, the RelB-deficient fibroblasts were transfected with a construct driving constitutive expression of a RelB cDNA. When compared with wild-type, mutant, and control transfected cells, the RelB-transfected cells displayed an LPS-inducible chemokine expression phenotype similar to wild-type fibroblasts (Figure 2D). Although some differences were observed in the kinetics of chemokine expression in transfected compared with wild-type cells, this was likely due to the constitutive expression of the transfected RelB gene. To-

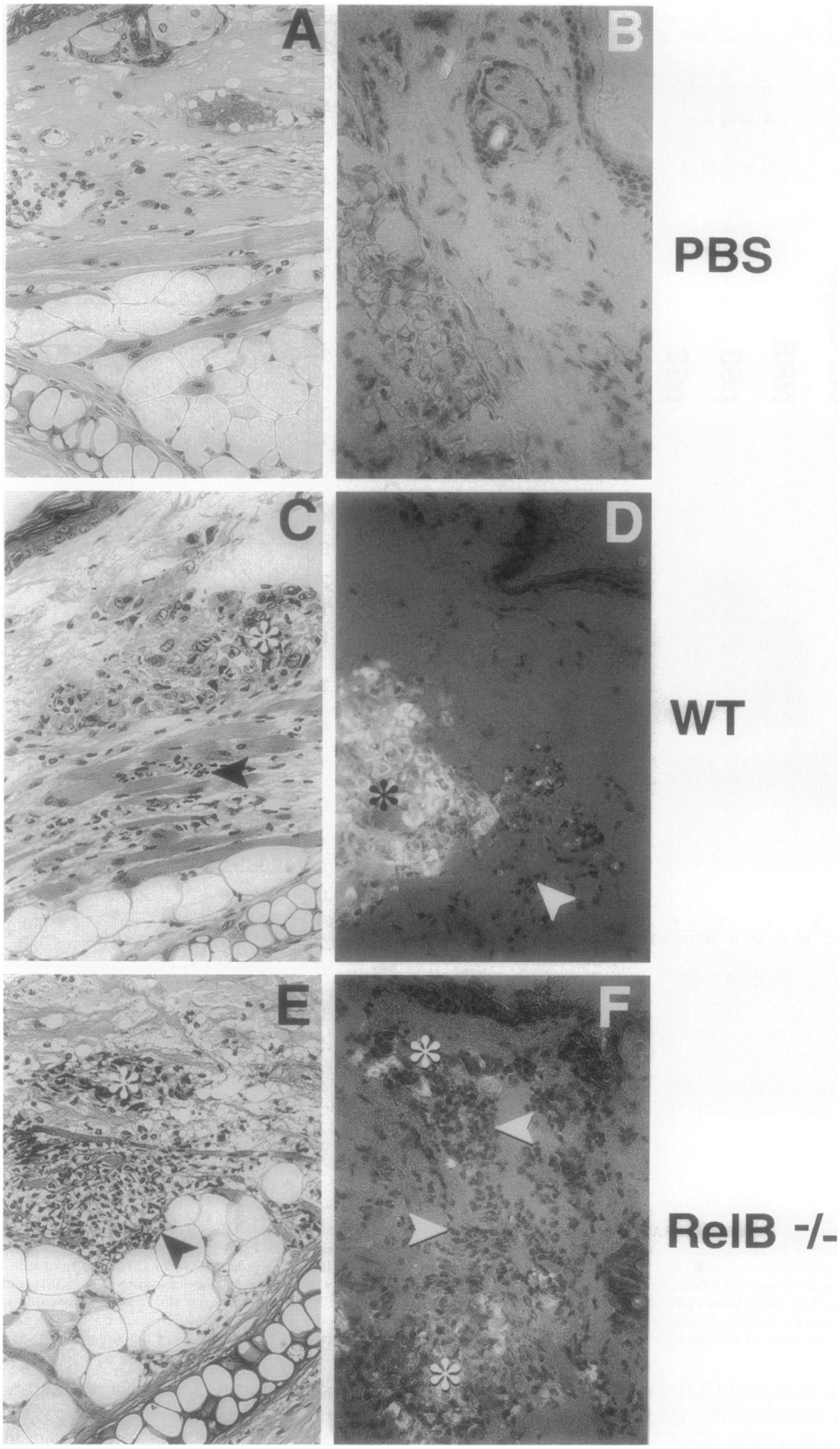

A
B
PBS
C
D
WT
E
F
RelB -/-

Table 1. Comparison of Cell Concentrations Obtained from Peritoneal Lavage Fluid

Experiment	PBS (mean × 10^{-6} cells/ml)	Wild type + LPS (mean × 10^{-6} cells/ml)	SEM	$RelB^{-/-}$ + LPS (mean × 10^{-6} cells/ml)	SEM	P value*
1	0.41 (n=2)	0.48 (n=2)	NA	0.81 (n=2)	NA	NA
2	0.71 (n=2)	0.79 (n=5)	0.06	3.00 (n=3)	1.50	0.047
3	0.04 (n=2)	0.84 (n=4)	0.10	1.86 (n=5)	0.40	0.032

Results are expressed as mean cell concentrations obtained from lavage fluid 6 hours after injection. NA, not available.
*Values obtained from one-tailed unpaired Student's *t*-test.

gether, these data indicate that RelB functions to suppress activation-induced chemokine expression in fibroblasts.

RelB Is Translocated to the Nucleus upon Stimulation of Fibroblasts but Does Not Bind Directly to κB Sites

The interactions between NF-κB/Rel family members result in regulation of gene expression at κB sites, so the effect of fibroblast stimulation on nuclear NF-κB activity was examined in EMSAs. Up-regulation of κB binding activity has been previously documented in wild-type fibroblasts after serum stimulation[15] and was likewise observed after LPS stimulation (Figure 3A). Importantly, this up-regulation is relatively insignificant when compared with that observed in RelB-deficient fibroblasts (Figure 3A). Not only did mutant fibroblasts display a higher basal NF-κB activity than wild-type fibroblasts but the binding activity was also strongly increased after LPS stimulation (Figure 3A). The kinetics of binding were similar to those observed for chemokine mRNA expression in these cells. Supershift assays revealed that the NF-κB subunits p50, p65/RelA, and c-rel are responsible for κB binding in both wild-type and mutant fibroblast nuclear extracts (Figure 3B). Curiously, complexes containing RelB were not detected in these assays. These data suggest that, although RelB has only a modest direct contribution to constitutive NF-κB activity, it has a major regulatory influence serving to dampen NF-κB activity after LPS stimulation in fibroblasts. This modulatory effect may involve a novel mechanism, as it does not appear to involve direct binding of RelB-containing complexes to κB sites.

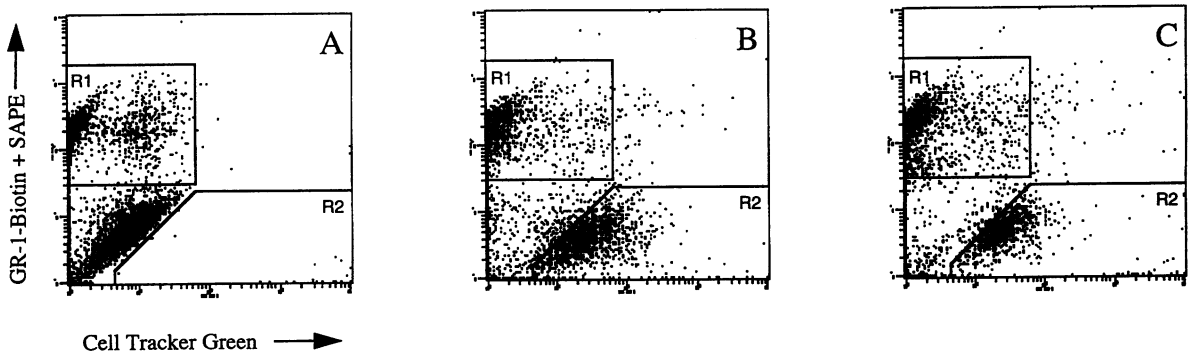
If RelB expression is induced but does not participate in the observed increase in NF-κB activity, are there alterations in the protein function? To address this, the cellular localization of RelB protein was investigated by immunoprecipitation together with immunoblotting (Figure 4A). Despite the fact that RelB did not appear to bind κB sites directly as indicated above, it was present in the cytoplasm of fibroblasts cultured without LPS and translocated into the nucleus upon stimulation. Additional analyses of control and mutant fibroblasts indicated that both cytoplasmic and nuclear forms of RelB are associated with p50 (Figure 4B).

The kinetics of p50, p65, and IκBα mRNA levels were also determined for control and mutant fibroblasts after LPS stimulation using RNAse protection assays (Figure 5, A and B). Stimulation of wild-type fibroblasts resulted in rapid, but short-lived increases in all three NF-κB-related mRNA levels (Figure 5, A and B). As observed with chemokine expression, $RelB^{-/-}$ fibroblasts showed higher basal expression of p50, p65, and IκBα mRNA with a more exaggerated and prolonged expression after stimulation when compared with control fibroblasts. Peak mRNA levels were reached by 2 to 4 hours, with a return to baseline levels not occurring until 24 to 48 hours. The increased expression of p50 and p65 in $RelB^{-/-}$ fibroblasts was expected given the observed increase in κB binding activity seen in these cells. In addition, IκBα, known to inhibit NF-κB activity, is regulated by NF-κB, and so its expression is also increased.[30,31] However, the increased IκBα expression does not appear to affect the overall p65/p50 NF-κB activity. This may be a result of increased IκBα degradation as has been described for embryonic fibroblasts after TNF-α stimulation (data not shown).[20]

RelB Regulation of Chemokine Expression Is Cell Type Specific

The apparent suppressive effect of RelB on chemokine induction in fibroblasts contradicts earlier studies showing strong transcriptional activation activity when RelB is expressed in a variety of cell types.[15,32–35] To determine whether RelB influences chemokine expression in other cell types in a manner similar to fibroblasts, RNAse protection assays were performed using LPS-stimulated peritoneal macrophages. Control and mutant macrophage chemokine mRNA levels did not show the striking differences in the basal level and expression kinetics that were discovered in fibroblasts (compare Figure 6, A with B). In this case, $RelB^{-/-}$ peritoneal macrophages produced slightly lower levels of RANTES, MIP-1β, MIP-1α, IP-10, and JE/MCP-1 chemokine mRNA than wild-type macrophages after LPS stimulation (Figure 6B).

Figure 7. LPS-stimulated fibroblasts mediate interstitial inflammation. **A**, **C**, and **E**: H&E-stained paraffin-embedded sections of SCID mouse ears 6 hours after injection with PBS (**A**), LPS-stimulated wild-type fibroblasts (**C**), or LPS-stimulated RelB-deficient fibroblasts (**E**) show the presence of infiltrating granulocytes. **B**, **D**, and **F**: SCID mouse ear cryostat sections counterstained with hematoxylin and viewed using double exposure (bright-field and fluorescence microscopy) demonstrate the presence of injected wild-type (**D**) or RelB-deficient (**F**) fibroblasts as fluorescent cells (**asterisk**). The infiltrating cells are also visible and have a typical granulocytic morphology (**arrow**). As expected, no fluorescent cells were observed in ears injected with only PBS, although occasional small areas of infiltrating granulocytes were sometimes observed. Magnification, ×400.



Region	% Total
R1	44.06
R2	0.52

Region	% Total
R1	39.92
R2	22.50

R1:R2 Ratio = 1.77

Region	% Total
R1	54.84
R2	14.90

R1:R2 Ratio = 3.68

Figure 8. RelB $^{-/-}$ fibroblasts mediate significantly greater infiltration than wild-type fibroblasts. Representative two-color dot plots of cells were obtained from SCID mice by peritoneal lavage 6 hours after injection with PBS (**A**), LPS-stimulated wild-type fibroblasts (**B**), or LPS-stimulated RelB $^{-/-}$ fibroblasts (**C**). Cells were stained with biotinylated GR-1 and streptavidin phycoerythrin (SA-PE) before flow cytometric analysis. The injected fibroblasts were evident due to their green fluorescence imparted by the cell tracker probe. The percentage of granulocytes (R1), injected fibroblasts (R2), and granulocyte:fibroblast ratios are as indicated for each individual sample.

As in fibroblasts, the stimulated macrophage cell line RAW 265.7 contained κB binding activity attributable to p50, p65, and c-rel proteins. RAW 265.7 cells also contained RelB-mediated κB binding (Figure 6C). In addition, RNAse protection assays using an IκBα-specific probe with control and mutant peritoneal macrophage extracts showed expression kinetics similar to those observed with mutant fibroblasts (compare Figures 6D and 5B). Previous studies have shown that RelB κB binding function is differentially regulated in lymphoid (B220$^+$ splenocytes and S194 plasmacytoma and EL-4 thymoma cells) *versus* nonlymphoid (fibroblasts and transfected COS) cells.[16] Our data show that, even among nonlymphoid cells (macrophages and fibroblasts) RelB κB binding activity is differentially regulated. Thus, RelB may function as a transcriptional activator in stimulated macrophages as has been described in lymphoid cells.[16]

Simulated RelB-Deficient Fibroblasts Can Mediate Inflammation in Vivo

As depicted above, in the absence of RelB expression, fibroblasts were unable to control adequately NF-κB activity resulting in an excessive production of chemokines. To test the physiological relevance of this observation, fibroblasts were stimulated with LPS, labeled with a fluorescent dye, and injected subcutaneously into ears of SCID mice. Frozen and paraffin-embedded sections were examined 6 hours after injection for the presence of inflammatory infiltrates (Figure 7). The fibroblasts could be easily seen by fluorescence microscopy in frozen sections counterstained with hematoxylin. As expected, RelB-deficient fibroblasts caused a localized accumulation of large numbers of inflammatory cells within 6 hours of injection. The injection of stimulated wild-type fibroblasts also produced local inflammation but at a lower magnitude. This minimal inflammation induced by wild-type fibroblasts could have been due to 1) residual LPS present in the cell preparations, although extensive washing was employed, 2) minor levels of chemokines produced by these cells, although injection times were delayed for 16 to 20 hours to minimize this effect, and/or 3) the injection process itself, as was evident to a lesser degree in ears injected with PBS alone.

To quantitate the inflammatory response mediated by injected fibroblasts, cells were injected intraperitoneally followed by analysis of cells recovered by peritoneal lavage. Fibroblasts were prepared as described previously, injected intraperitoneally, and recovered in the peritoneal lavage fluid of SCID mice 6 hours after injection. As shown in Table 1, lavage fluid cell concentrations were significantly higher after injection of RelB$^{-/-}$ fibroblasts than wild-type fibroblasts. Cytospin preparations of cells recovered from mice injected with fibroblasts showed typical granulocyte morphology (data not shown). Lavage samples were stained with the granulocyte-specific monoclonal antibody GR-1 and subjected to two-color flow cytometry (Figure 8). The percentage of GR-1$^+$ and injected fibroblasts (labeled before injection) obtained by flow cytometry were used to generate a ratio for each sample. The mean ratios and standard errors were calculated for each of three experiments (Table 2). As expected, statistical analysis revealed that a significantly higher ratio of granulocytes per injected fibroblast was obtained from mice injected with stimulated RelB mutant fibroblasts as compared with stimulated wild-type fibroblasts.

Discussion

The present study focused on the response of fibroblasts after LPS stimulation as it related to RelB expression. Although up-regulation of RelB expression occurred

Table 2. Comparison of Granulocyte: Fibroblast Ratios in Peritoneal Lavage Fluid

Experiment	Wild type + LPS	SEM	RelB$^{-/-}$ + LPS	SEM	P value*
1	0.95 (n=2)	NA	2.79 (n=2)	NA	NA
2	2.18 (n=5)	0.22	6.26 (n=3)	1.82	≤0.025
3	1.46 (n=4)	0.38	3.26 (n=5)	0.36	≤0.025

Results are expressed as means obtained from the ratio of granulocytes (GR-1$^+$) to fibroblasts recovered from lavage fluid 6 hours after injection. NA, not available.
*Values obtained from one-tailed Wilcoxon rank sum tests.

upon stimulation of wild-type fibroblasts, only brief up-regulation of the additional Rel family proteins p50 and p65 was observed. No change in the overall NF-κB activity was detected, perhaps due to the transient and parallel up-regulation of the inhibitor IκBα. The expression of only four of seven chemokines tested were increased after stimulation. This up-regulation was maintained for only a brief duration in the case of two of the four chemokines, MIP-2 and KC/CINC. The wild-type fibroblast mRNA levels of the remaining two chemokines, JE/MCP-1 and KC/CINC, were consistently lower than observed in RelB-deficient fibroblasts when compared by densitometry (data not shown). In contrast to these observations, RelB mutant fibroblasts exhibited a dysregulated increase in expression of p50, p65, IκBα, and chemokine mRNA. Nuclear extracts showed increased κB binding activity attributable to p50, p65, and c-rel. Aberrant chemokine expression was corrected by the transfection of a single gene, RelB. Therefore, RelB may be an essential regulator required for suppression of NF-κB function and modulation of chemokine expression in activated fibroblasts. This was demonstrated further by *in vivo* analyses comparing the recruitment potential of wild-type and RelB-deficient LPS-stimulated fibroblasts. Stimulated RelB$^{-/-}$ fibroblasts elicited a significantly greater granulocytic infiltrate than control fibroblasts, suggesting a central role for RelB in the resolution of acute inflammation.

Other studies have proposed that RelB-mediated transactivation occurs in lymphoid cells but not in nonlymphoid cells.[16,36] Here we extend this observation to include responses of both LPS-stimulated fibroblasts and macrophages. Supershift assays demonstrated that a macrophage cell line, RAW 265.7, contains RelB-mediated κB binding activity. Although RelB played a minor role in limiting the extent of chemokine expression, the expression was still increased after LPS stimulation of peritoneal macrophages independent of the cells' ability to express RelB. In contrast, fibroblast responses to LPS did not include RelB-mediated κB binding as detectable by EMSA. The emerging role of RelB appears to be cell specific with transactivating functions manifested in cells of hematopoietic origin (eg, macrophages and dendritic cells) but not necessarily in nonhematopoietic cells such as fibroblasts. The interesting exception is thymic medullary epithelium,[12] where RelB expression is associated with a dendritic cell phenotype (dendritic morphology, high levels of major histocompatibility complex class II and B7, and antigen presentation).

How might RelB be exerting its suppressive function in fibroblasts? Immunoprecipitation and immunoblot analysis revealed that RelB is associated with p50 in both the cytoplasm of nonstimulated cells and the nucleus of stimulated cells. RelB may compete with p65 and c-rel to sequester p50 in an inactive form after fibroblast stimulation. Post-translational modifications of RelB, as have been suggested by others, could account for the differential transactivation attributed to RelB derived from differing cell populations.[16] Other alternatives are equally viable, such as cell-specific limited access to cofactors necessary for p50/RelB transactivation or the use of alternately spliced forms of RelB.

How could the inflammatory process be initiated in RelB-deficient mice? As the lack of a thymic medulla in RelB mutant mice results in autoreactive peripheral T cells, one possible mechanism involves cytokine production by the constitutively activated circulating T cells.[27,28] Cytokines produced by the T cells, such as TNF-α, stimulate fibroblasts.[16,37,38] The fibroblasts then produce chemokines in a dysregulated manner due to the lack of RelB. This leads to accumulation of granulocytic/monocytic infiltrates. In the case of TNF-α, our data would suggest a preferential attraction of granulocytes due to the favored up-regulation of MCP-2 and KC/CINC, both powerful granulocytic chemoattractants. Without RelB regulation, RelB$^{-/-}$ mice are unable to curtail the inflammatory process, leaving them stranded in a progressing inflammatory state. The significant involvement of nonhematopoietic cells in this inflammatory process is further supported by bone marrow chimera studies using wild-type donor cells and RelB$^{-/-}$ recipients.[21,27] The inflammatory syndrome of the RelB$^{-/-}$ recipient mice is not resolved in these chimeras, suggesting that bone-marrow-derived cells are not solely responsible for the inflammation. Also, reconstitution of wild-type recipients with RelB$^{-/-}$ bone marrow did not induce the inflammatory syndrome.

RelB and the Regulation of Acute Inflammation

Although chemokines are readily induced from hemopoietic cells such as macrophages, initial tissue infection most likely triggers responses from resident tissue cells such as fibroblasts and vascular endothelium. It is the activation of these cells and induction of adhesion molecules on vascular endothelium that subsequently leads to the recruitment of inflammatory cells such as macrophages, granulocytes, and lymphocytes. Thus, the contribution of resident nonhemopoietic cells to the progression of tissue inflammation cannot be underestimated.

As described here, activation of fibroblasts by danger signals such as LPS results in a transient production of

chemokines. The relatively brief expression of chemokines is consistent with the nature of acute inflammation, which persists in the range of hours to days. Recruitment of blood-borne inflammatory cells helps sustain the local inflammatory response, as these cells are also triggered to produce chemokines. The chemokines themselves trigger activation of cells, which may feed a cycle of increasing cytokine production and activation. How is this cycle regulated normally in the tissue? Presumably, the recruited inflammatory cells are critical in the destruction of the pathogen producing the danger signal, but resident cells should not remain in a persistently activated state. In the case of the recruited blood-borne inflammatory cells, their very brief lifetime (approximately 12 hours for granulocytes) removes part of the problem, but resident tissue stromal cells such as fibroblasts and vascular endothelium are not short-lived and so must develop another mechanism for returning to a resting state. Our studies suggest that, for fibroblasts, induction of RelB may serve this purpose by modulating chemokine expression, perhaps in part by regulating overall NF-κB activity.

These results contrast with the demonstrated transactivator function of RelB in other cell types, including macrophages. Moreover, previous studies have indicated a role for RelB in the maturation of dendritic cells, which are antigen-presenting cells responsible for the initiation of immune responses *in vivo*. Differential RelB activity is further supported by studies of lck-RelB transgenic mice where thymocyte expression of transgenic RelB was associated with constitutive κB binding activity; as thymocytes do not normally express RelB, no functional consequences were observed.[39] In contrast, IκBα and transforming growth factor-β knock-out mice exhibit extensive granulopoiesis and skin lesions similar to $RelB^{-/-}$ mice, suggesting a possible link between RelB, IκBα, and transforming growth factor-β function.[20,40,41] Together, these disparate inducible functions of RelB build a compelling picture of the regulation of inflammation. In nonlymphoid cells, RelB induction helps with the resolution of acute inflammation, whereas in dendritic cell precursors, it leads to maturation and the initiation of cellular immune responses. Thus, the same molecule induced in different cell types under similar conditions appears critical in driving the transition from innate to adaptive immunity *in vivo*. Indeed, the multi-organ inflammatory syndrome and absence of secondary lymphoid tissue in RelB-deficient mice are exactly what might be expected in animals unable to make the transition; that is, the mice are chronically in a state of acute inflammation.

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References

1. Detmers PA, Lo SK, Olsen-Egbert E, Walz A, Baggiolini M, Cohn ZA: Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J Exp Med 1990, 178:1493–1500
2. Vaddi K, Newton RC: Regulation of monocyte integrin expression by β-family chemokines. J Immunol 1994, 153:4721–4732
3. Rot A: Neutrophil attractant/activation protein-1 (interleukin-8) induces in vitro neutrophil migration by haptotactic mechanism. Eur J Immunol 1993, 23:303–306
4. Geiser T, Dewald B, Ehrengruber MR, Clark-Lewis I, Baggiolini M: The interleukin-8-related chemotactic cytokines GROα, GROβ, and GROγ activate human neutrophil and basophil leukocytes. J Biol Chem 1993, 268:15419–15424
5. Walz A, Meloni F, Clark-Lewis I, von Tscharner V, Baggiolini M: $[Ca^{+2}]_i$ changes and respiratory burst in human neutrophils and monocytes induced by NAP-1/interleukin-8, NAP-2, and gro/MGSA. J Leukocyte Biol 1991, 50:279–286
6. Sylvester I, Suffredini AF, Boujoukos AJ, Martich GD, Danner RL, Yoshimura T, Leonard EJ: Neutrophil attractant protein-1 and monocyte chemoattractant protein-1 in human serum: effects of intravenous lipopolysaccharide on free attractants, specific IgG autoantibodies and immune complexes. J Immunol 1993, 151:3292–3298
7. Chaudhuri A, Zbrzenzna V, Polyakova J, Pogo A, Hesselgesser J, Horuk R: Expression of the Duffy antigen in K652 cells: evidence that it is the human erythrocyte chemokine receptor. J Biol Chem 1994, 269:7835–7838
8. Furie MB, Randolph GJ: Chemokines and tissue injury. Am J Pathol 1995, 146:1287–1301
9. Gilmore TD, Koedood M, Piffat KA, White DW: Rel/NF-κB/IκB proteins and cancer. Oncogene 1997 (in press)
10. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature 1995, 376:176–170
11. Baeuerle PA, Baltimore D: NF-κB: ten years after. Cell 1996, 87:13–20
12. Burkly L, Hession C, Ogata L, Reilly C, Marconi LA, Olson D, Tizard R, Cate R, Lo D: Expression of relB is required for the development of thymic medulla and dendritic cells. Nature 1995, 373:531–536
13. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck RP, Lira SA, Bravo R: Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell 1995, 80:331–340
14. Baldwin AS Jr: The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996, 14:649–681
15. Ryseck RP, Bull P, Takamiya M, Bours V, Siebenlist U, Dobrzanski P, Bravo R: RelB, a new Rel family transcription activator that. can interact with p50-NF-kappa B. Mol Cell Biol 1992, 12:674–684
16. Lernbecher T, Kistler B, Wirth T: Two distinct mechanisms contribute to the constitutive activation of RelB in lymphoid cells. EMBO J 1994, 13:4046–4069
17. Grilli M, Chiu JS, Lenardo MJ: NF-kappa B and Rel: participants in a multiform transcriptional regulatory system. Int Rev Cytol 1993, 143: 1–62
18. Baeuerle PA, Henkel T: Function and activation of NK-kappa B in the immune system. Annu Rev Immunol 1994, 12:141–179
19. Widmer U, Manogue KR, Cerami A, Sherry B: Genomic cloning and promoter analysis of macrophage inflammatory protein (MIP)-2, MIP-1 α, and MIP-1 β, members of the chemokine superfamily of proinflammatory cytokines. J Immunol 1993, 150:4996–5012
20. Beg AA, Sha W, Bronson R, Baltimore D: Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα deficient mice. Genes Dev 1995, 9:2736–2746
21. Lo D, Quill H, Burkly L, Scott B, Palmiter RD, Brinster RL: A recessive defect in lymphocyte or granulocyte function caused by an integrated transgene. Am J Pathol 1992, 141:1237–1246
22. Feng L, Xia Y, Kreisberg JI, Wilson CB: Interleukin-1α stimulates KC synthesis in rat mesangial cells: glucocorticoids inhibit KC induction by IL-1. Am J Physiol 1994, 266: F713–F722
23. Conrad RE: Induction and collection of peritoneal exudate macrophages. Manual of Macrophage Methodology. Edited by HB Herscowits, HT Holden, JA Bellanti, A Ghaffar. New York, Marcel Dekker, 1981, pp 1–11
24. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
25. Xia Y, Feng L, Yoshimura T, Wilson CB: LPS-induced MCP-1, IL1β,

and TNF-α mRNA expression in isolated erythrocyte-perfused rat kidney. Am J Physiol (Renal) 1993, 266: F774–F780
26. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983, 11:1475–1489
27. DeKoning J, DiMolfetto L, Reilly C, Wei Q, Havran W, Lo D: T cell development in relB deficient mice: positive selection and acquisition of function do not require thymic medulla. J Immunol 1997, 158:2558–2566
28. Laufer TM, DeKoning J, Markowitz JS, Lo D, Glimcher LH: Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. Nature 1996, 383:81–85
29. Schall TJ, Bacon KB: Chemokines, leukocyte trafficking, and inflammation. Curr Opin Immunol 1994, 6:865–873
30. Ito CY, Kazantsev AG, Baldwin AS Jr: Three NF-KB sites in the IKB-α promoter are required for induction of gene expression by TNFα. Nucleic Acids Res 1994, 22:3787–92
31. Sun SC, Ganchi PA, Ballard DW, Greene WC: NF-kappa B controls expression of inhibitor I kappa Bα: evidence for an inducible autoregulatory pathway. Science 1993, 259:1912–1915
32. Dobrzanski P, Ryseck R, Bravo R: Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipper-like motif. Mol Cell Biol 1993, 13:1572–1582
33. Bours V, Azarenko V, Dejardin E, Siebenlist U: Human RelB (I-Rel) functions as a κB site-dependent transactivating member of the family of Rel-related proteins. Oncogene 1994, 9:1699–1702
34. Lernbecher T, Miller U, Wirth T: Distinct NF-kappa B/Rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature 1993, 365:767–770
35. Dobrzanski P, Ryseck RP, Bravo R: Specific inhibition of RelB/p52 transcriptional activity by the C-terminal domain of p100. Oncogene 1995, 10:1003–1007
36. Cheng Q, Cant CA, Moll T, Hofer-Qarbinek R, Wagner E, Birnstiel ML, Bach RH, de Martin R: NF-kappa B subunit-specific regulation of the I kappa B α promoter. J Biol Chem 1994, 269:13551–13557
37. Bombara MP, Webb DL, Conrad P, Marlor CW, Sarr T, Ranges GE, Aune TM, Greve JM, Blue ML: Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. J Leukocyte Biol 1993, 54:399–406
38. Mauviel A, Teitamo S, Remitz A, Lapiere JC, Ceska M, Baggiolini M, Walz A, Evans CH, Uitto J: Leukoregulin, a T cell-derived cytokine, induces IL-8 gene expression and secretion in human skin fibroblasts: demonstration and secretion in human skin fibroblasts: demonstration of enhanced NF-kappa B binding and NF-kappa B-driven activity. J Immunol 1992, 149:2969–2976
39. Weih F, Lira SA, Bravo R: Overexpression of RelB in transgenic mice does not affect I kappa B α levels: differential regulation of RelA and RelB by the inhibitor protein. Oncogene 1996, 12:445–449
40. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S: Transforming growth factor β1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci USA 1993, 90:770–774
41. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T: Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature 1992, 359:693–699