Technology and Development Team for BioSignal Program

Subteam for BioSignal Integration

Subteam Leader

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Goal

Life always receives stimulation from the outside and maintain itself in response to stimulation through a complex network of signal transduction. The analysis of this mechanism is a means to elucidate the bioresponse mechanism and characterize bioresource materials. The main goal of our subteam is to develop new techniques for elucidating the signal transduction mechanism underlying bioresponse through the analysis of gene and protein expressions using a microchip array technique and a database in each step including genes, cells, tissues and whole bodies. Another goal is to elucidate the effect of abnormal cytokine networks and the mechanisms of diseases including cancers on life by analyzing the signal transduction pathways of cytokines. Still another goal is to develop mouse models of human diseases by reconstructing abnormal networks in vivo on the basis of bioinformation stored in BRC for a more detailed study and development of new therapeutic techniques for such diseases. We focus on the characterization of NF-κB and the tumor necrosis factor (TNF) in the analysis of signal transduction mechanisms during life.

Activities

1. Analysis of the essential roles of NF-κB in tumorigenesis:
2. Analysis of the essential roles of NF-κB in apoptosis pathway
3. Analysis of the essential roles of NF-κB in innate immunity
4. Analysis of the essential roles of NF-κB in development

Members

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Specific Aims

Transcription factor NF-κB

The transcription factor NF-κB was identified as the regulatory factor for lymphocyte development. Over these past years, NF-κB has been demonstrated to be a key regulator for the signal transduction of stimuli from the outside into the nucleus. NF-κB regulates genes related to development, differentiation, proliferation and tumorigenesis as well as to the immune system (Fig. 1). The NF-κB family consists of five members, each carrying the Rel homology domain (Fig. 2). In the cytoplasm, NF-κB is present as a heterodimer complex. After activation, NF-κB translocates to the nucleus and transcriptionally regulates many genes.

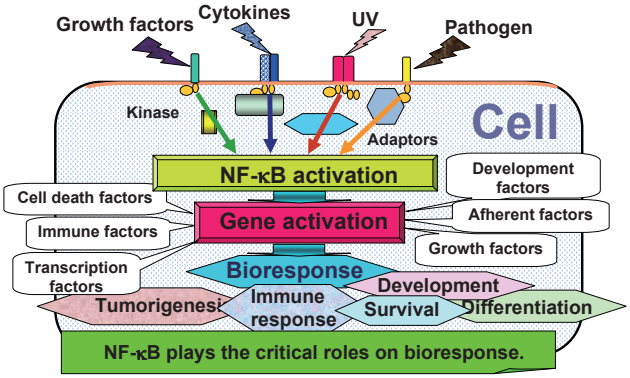


Figure 1. NF-κB and bioresponse
NF-κB regulates the genes related with development, differentiation, proliferation and tumorigenesis as well as immune system. In cytoplasm, NF-κB is present as hetero dimmer complex. After activation, NF-κB translocates to nucleus and transcriptionally regulates a lot of genes.

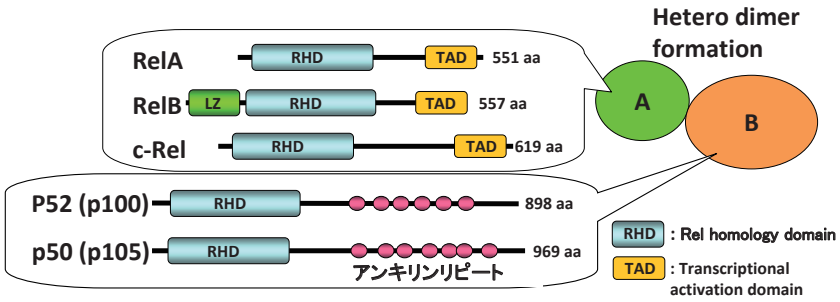


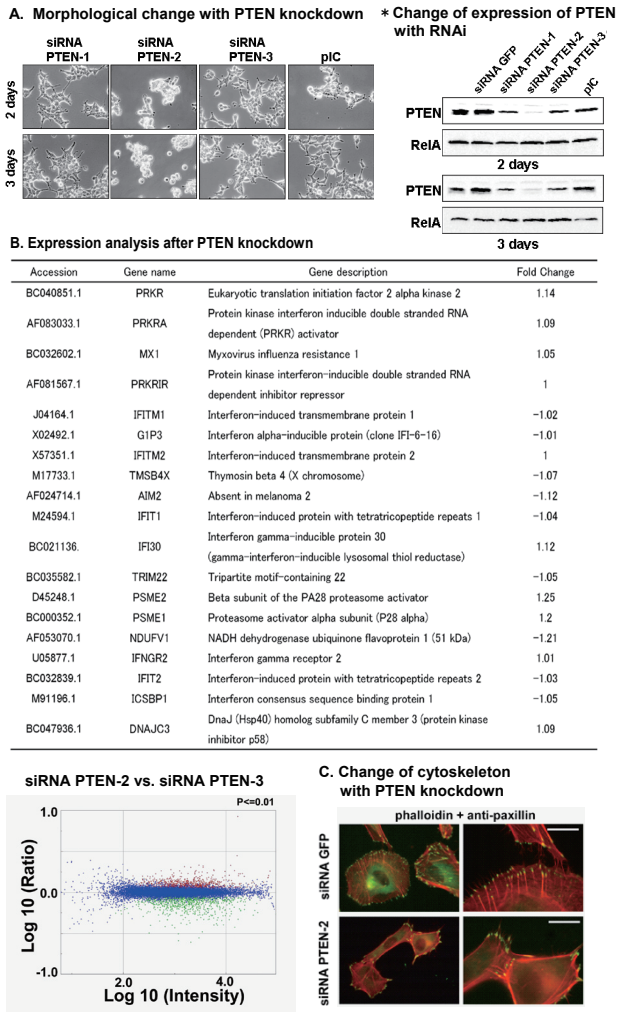
Figure 2. NF-κB family
NF-κB family consists of five members carrying Rel homology domain.

1. Analysis of the essential roles of NF-κB in tumorigenesis

There are many factors related to the steps in tumorigenesis. We have focused on the characterization of NF-κB among factors for the analysis of the mechanism of tumorigenesis.

a. Analysis of the function of PTEN using RNA interference method

PTEN is a tumor suppressor gene closely related to tumorigenesis. However, It is very difficult to characterize its function, because PTEN knockout mice are lethal. We generated cells with an extremely decreased PTEN activity by the RNA interference method for PTEN function analysis. Those cells lost their adhesion activity. We demonstrated that such a phenotype is due to the dysregulated cytoskeleton with decreased amounts of FAK and actin fibers. We also demonstrated that the phenotype is not caused by nonspecific effects of RNA interference on PTEN (Figure-3).



Accession	Gene name	Gene description	Fold Change
BC040851.1	PRKR	Eukaryotic translation initiation factor 2 alpha kinase 2	1.14
AF083033.1	PRKRA	Protein kinase interferon inducible double stranded RNA dependent (PRKR) activator	1.09
BC032602.1	MX1	Myxovirus influenza resistance 1	1.05
AF081567.1	PRKRIR	Protein kinase interferon-inducible double stranded RNA dependent inhibitor repressor	1
J04164.1	IFITM1	Interferon-induced transmembrane protein 1	-1.02
X02492.1	G1P3	Interferon alpha-inducible protein (clone IFI-6-16)	-1.01
X57351.1	IFITM2	Interferon-induced transmembrane protein 2	1
M17733.1	TMSB4X	Thymosin beta 4 (X chromosome)	-1.07
AF024714.1	AIM2	Absent in melanoma 2	-1.12
M24594.1	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	-1.04
BC021136.	IFI30	Interferon gamma-inducible protein 30 (gamma-interferon-inducible lysosomal thiol reductase)	1.12
BC035582.1	TRIM22	Tripartite motif-containing 22	-1.05
D45248.1	PSME2	Beta subunit of the PA28 proteasome activator	1.25
BC000352.1	PSME1	Proteasome activator alpha subunit (P28 alpha)	1.2
AF053070.1	NDUFV1	NADH dehydrogenase ubiquinone flavoprotein 1 (51 kDa)	-1.21
U05877.1	IFNGR2	Interferon gamma receptor 2	1.01
BC032839.1	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	-1.03
M91196.1	ICSBP1	Interferon consensus sequence binding protein 1	-1.05
BC047936.1	DNAJC3	DnaJ (Hsp40) homolog subfamily C member 3 (protein kinase inhibitor p58)	1.09

Figure 3. Analysis of PTEN function with RNA interference method
Cells treated with RNA interference of PTEN lost lost the adhesion activity due to dysregulated cytoskeleton with decrease of FAK and actin fibers.

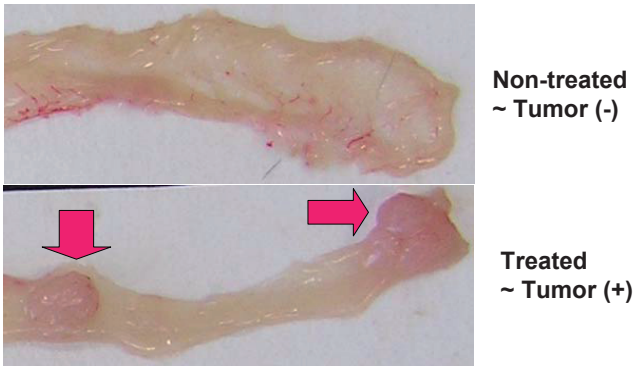


Figure 4. Colon tumors are induced with AOM and DSS
All of C57BL/6 mice developed colon tumors 10 weeks after, as far as we did the same way; on Day-1 Azaoxymethane (AOM) i.p.and on Day-8~14 Dextran sulfate (DSS) p.o.

b. Analysis of the function of NF-κB in tumorigenesis

We are trying to elucidate the NF-κB function in colon tumorigenesis. Mice develop colon tumors with the administration of Azaoxymethane (AOM) and dextran sulfate (DSS). The incidence of colon tumorigenesis induced in this manner has been reported to be almost 100%. In our experiment performed using the same method, all C57BL/6 mice developed colon tumors 10 weeks after the administration: Day 1 AOM i.p., Days 8~14 DSS p.o. (Fig. 4) Then we treated TNF-deficient mice and c-Rel-deficient mice in the same manner. We demonstrated that the incidence of tumors decreases in TNF-deficient mice and that there is no significant difference between wild-type and c-Rel-deficient mice. We now are trying to induce colon tumors in mice lacking other members of the NF-κB family.

2. Analysis of the essential roles of NF-κB in apoptosis pathway

a. Analysis of apoptosis pathway by TNF

Previously, we generated NF-κB/RelA knockout mice and found them to be embryonically lethal owing to TNF cytotoxicity. Such a phenotype demonstrated that NF-κB/RelA has an anti-apoptotic activity against TNF cytotoxicity. We then tried to elucidate anti-apoptotic factors that are transcriptionally activated by RelA. By analysis using fibroblast cells derived from RelA-deficient embryos, it was found that the caspase-dependent pathway is activated, whereas the mitochondrion-dependent pathway is not activated by TNF. For the identification of anti-apoptotic genes directly mediated by RelA, we conducted a high-throughput analysis of gene expression profiling with chip

arrays using RelA-deficient and normal cells. Among several genes, Bcl-2 family genes, which are typical antiapoptotic genes, were not detected; however, IAP (an inhibitor of apoptosis protein family genes were detected. RelA-deficient cells were not rescued from TNF cytotoxicity by the introduction of those IAP genes. Meanwhile, we detected a decrease in the expression level of c-FLIP, an inhibitor of caspase-8, in RelA-deficient cells by Western blotting. The introduction of c-FLIP rescued RelA-deficient cells from TNF cytotoxicity. Therefore, it was speculated that RelA regulates c-FLIP expression indirectly. We are now analyzing how RelA regulates c-FLIP expression. We are also analyzing macrophages lacking RelA. RelA-deficient macrophages are sensitive to TNF, although such cells have a c-Rel protein, another NF-κB family member. This suggests that only RelA plays a critical role in anti-apoptosis against TNF cytotoxicity in specific tissues.

b. Lipopolysaccharide (LPS)-induced apoptosis and NF-κB

RelA-deficient macrophages are made to undergo apoptosis by LPS, although normal macrophages are activated by LPS. We found that the caspase-dependent pathway and mitochondrion-dependent pathway are both activated in the apoptosis of RelA-deficient macrophages by LPS. We confirmed that the apoptosis of RelA-deficient macrophages by LPS is not due to TNF-induced LPS, but directly due to LPS, while demonstrating that TNF/RelA-deficient macrophages are also made apoptotic by LPS. We also demonstrated that there might be a mechanism of the localization of activated caspases immediately downstream of the receptor for LPS, e.g., TLR4, which might be similar to that in the case of TNF receptor 1. No types of c-Rel-deficient cell are made apoptotic by either TNF or LPS. This demonstrates that only RelA plays a critical role in anti-apoptosis by TNF/LPS among the NF-κB family members (Figure-5).

3. Analysis of the essential roles of NF-κB in innate immunity

NF-κB has been hypothesized to play the critical roles in innate immunity, although no unequivocal evidence has been demonstrated thus far. We obtained some evidence supporting such a hypothesis.

a. NF-κB function in host defense against infection

Mice lacking the RelA, a subunit of the NF-κB family, are embryonically lethal at ED14.5. To elucidate the role of RelA in infectious diseases, we transferred an RelA-deficient fetal liver at ED13.5 into lethally irradiated C57B6 CD45.1^{+} host mice to prepare mice having RelA-deficient lymphoid

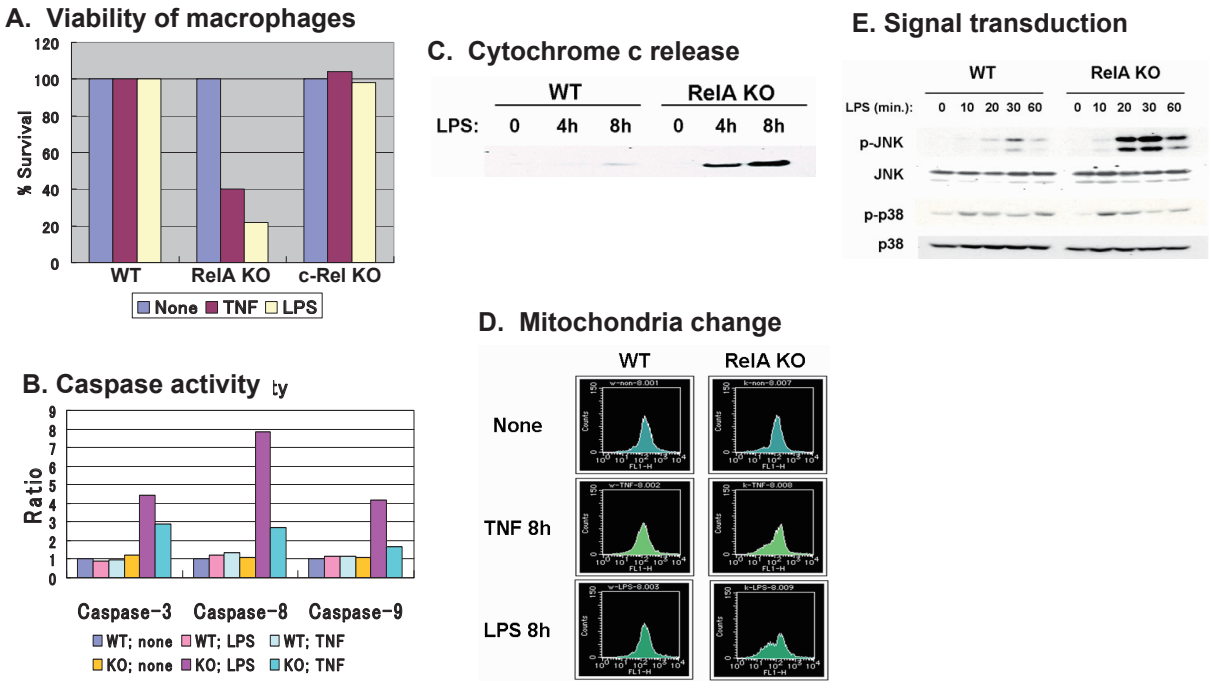


Figure 5. RelA regulates macrophage apoptosis

RelA-deficient macrophages are apoptotic by LPS, although normal macrophages are activated by LPS. Any types of c-Rel-deficient cells are not apoptotic neither by TNF nor LPS. RelA plays a critical role on anti-apoptosis by TNF/LPS among NF-κB family members.

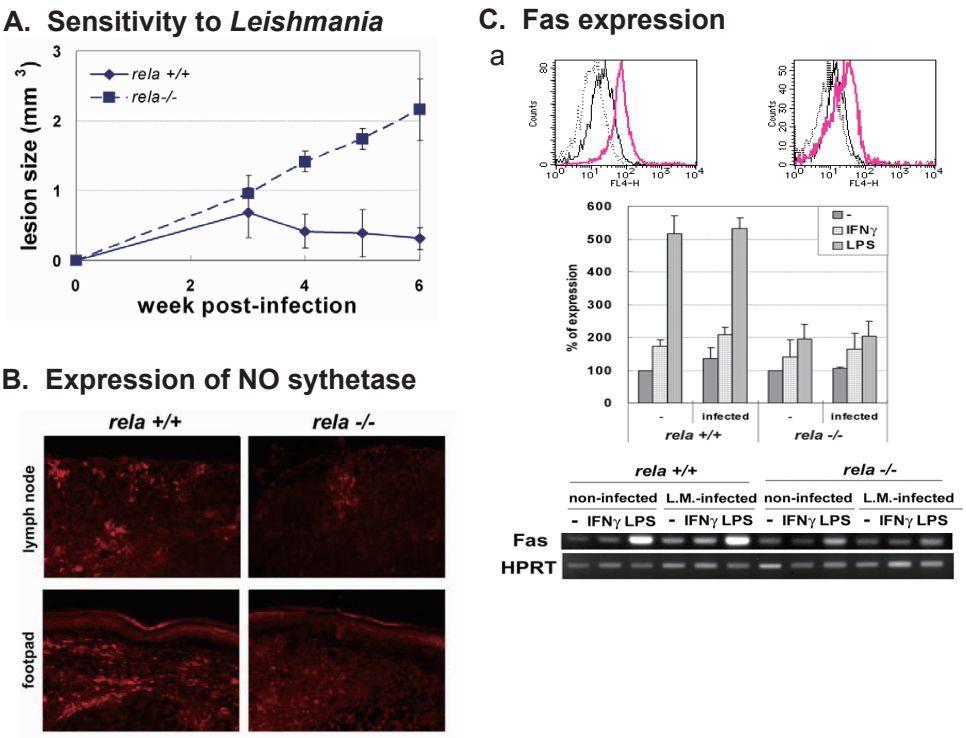


Figure 6. Leishmania infection for NF-κB deficient mice
RelA deficient macrophages had less killing activity to intracellular parasites. Compared with wild type macrophages, RelA deficient macrophages produced less amount of NO in response to suboptimal dose of LPS and IFNγ stimulation. Fas expression after IFNγ or LPS stimulation was impaired in RelA deficient macrophages.

cells. The chimeric mice with RelA-deficient fetal liver were found to be highly sensitive to *Leishmania major* infection, exhibiting progressive lesions and succumbing within 11 weeks after infection. Despite the severity of the disease, *Leishmania* antigen-reactive Th1 cells normally developed in these chimeric mice. In contrast, RelA-deficient macrophages had a lower killing activity to against intracellular parasites. Compared with wild-type macrophages, RelA-deficient macrophages produced a smaller amount of NO in response to a suboptimal dose of LPS or IFNγ stimulation. Fas expression after IFNγ or LPS stimulation was impaired in RelA-deficient macrophages, suggesting that the defect in the elimination of infected cells mediated by the Fas/FasL pathway resulted in nonhealing lesions in the chimeric mice with RelA-deficient fetal liver. Thus, RelA, a member of the NF-κB family, is a prerequisite for macrophage function to eradicate intracellular parasites (Fig. 6).

b. Analysis of NF-κB deficient mice as severely immunodeficient mice

We generated TNF-/-RelA-/ and TNF-/-c-Rel-/-RelA+/- mice for further analysis. Both types of mice died several weeks after birth owing to infection (Fig. 7). An analysis of the cause of death revealed that the mice died of infection by *Pasteurella pneumotica* and *Staphylococcus aureus*, both of which are weakly pathogenic. This demonstrated that both types of mice were much more severely immunodeficient mice than the SCID or nude mice. Further analysis revealed that the TNF-/-RelA-/- mice had no activity for producing nitric oxide, which kills bacteria, and that the TNF-/-c-Rel-/-RelA+/- mice had defect in the production of reactive oxygen and nitric oxide, which kill bacteria. However, no TNF+/+c-Rel-/-RelA+/- mice were susceptible to bacteria. These suggest that NF-κB plays critical roles in the mechanism of host defense against infection, synergistically with TNF.

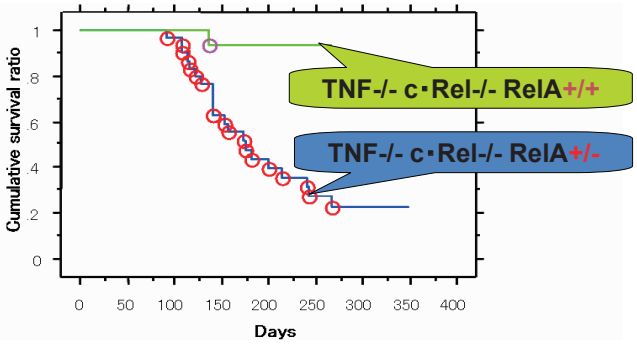


Figure 7. Viability of TNF/c-Rel/RelA deficient mice
TNF-/-c-Rel-/-RelA+/- mice f died several weeks after birth due to infection. Analysis of causal death revealed that those mice died due to *Pasteurella pneumotica* and *Staphylococcus aureus* both of which had quite weak pathogenic.

c. NF-κB function in cytokine production

Interleukin-23 (IL-23) is a heterodimeric cytokine composed of a unique p19 subunit and a common p40 subunit shared with IL-12. In the pathogenesis of experimental autoimmune diseases, IL-12 is important for the differentiation of interferon-γ-producing T-helper type 1 (Th1) cells. In contrast, IL-23 promotes inflammatory response by inducing the expansion of pathogenic $CD4^+$ cells producing IL-17. The expression of the p40 subunit in macrophages is well-known to depend on NF-κB; however, the regulation of p19 gene expression is little examined. We thus examined the expression level of the p19 subunit in NF-κB deficient macrophages. p19 expression level was markedly reduced in c-Rel-deficient macrophages and reduced at half level in RelA-deficient macrophages. To verify whether p19 gene expression is dependent on NF-κB, we examined the involvement of three putative κB sites at the positions of -642 to -632, -513 to -503, and -105 to -96 of the p19 promoter region by a promoter activity assay using luciferase-based reporter genes. In contrast to the report by Carmody et al., a mutation of -105 to -96, but not of -642 to -632 or -513 to -503, abolished the induction of p19 expression (Fig. 8). We demonstrated by EMSA that the sequence from -105 to -96 but not from -513 to -503 are bound to NF-κB complexes in macrophages activated by lipopolysaccharides (LPSs). Although the sequence from -642 to -632 also bound to NF-κB complexes, the association was weaker than that with the sequence from -105 to -96. Chromatin immunoprecipitation also revealed *in vivo*-specific associations of RelA, c-Rel, and p50 with the sequence from -105 to -96 of the p19 promoter. Our results demonstrate that p19 expression is dependent on NF-κB molecules, c-Rel and RelA, and that the sequence from -105 to -96 acts as a functional κB site *in vivo*.

b. NF-κB function in inflammatory diseases

NF-κB is activated immediately after bacterial infections and promotes serial inflammation steps. in the primary steps among multiple inflammatory steps, NF-κB plays a critical role, inducing proinflammatory cytokines, IL6, and TNF among others. Therefore, NF-κB is thought to be a novel therapeutic target for inflammatory bowel diseases (IBDs). However, how NF-κB acts on IBD remains to be elucidated. We analyzed here the contribution of NF-κB to dextran sulphate sodium (DSS)-induced acute colitis using NF-κB- deficient mice. We induced colitis in such mice by administering 2.5% DSS for seven days, and evaluating colitis in terms of symptomatic parameters, such as weight loss, survival, and severity of diarrhea, daily. The c-Rel-deficient and wild-type mice lost weight with the administration of DSS, whereas the TNF-deficient mice did not lose weight as well as all types of mice drinking water. Moreover, colon length was significantly reduced in the c-Rel-deficient and wild-type mice after drinking DSS. However, the colon length of the TNF-deficient mice remained almost the same as that of the mice drinking water. The disease symptoms of the c-Rel knockout mice were much severer than those of the wild-type mice after drinking DSS. 37.5% of the c-Rel deficient mice died within seven days, whereas all the wild-type mice, as well as the TNF-deficient mice, survived even after drinking DSS for seven days. Taken together, the results indicate that c-Rel deficiency aggravates DSS-induced acute colitis and that the absence of TNF reduces the severity of DSS-induced colitis. These data suggest that c-Rel plays a protective role in DSS-induced acute colitis (Fig. 9).

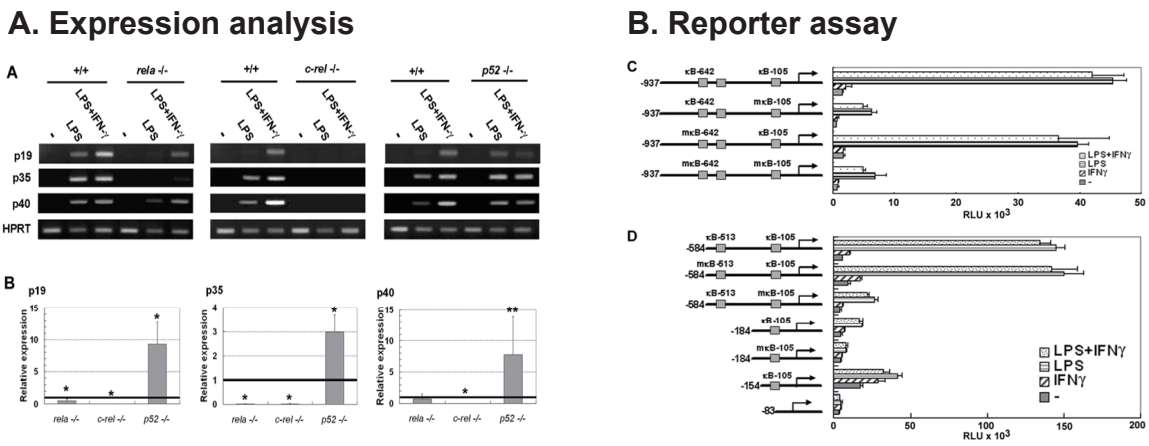


Figure 8. Regulation of cytokine genes with NF-κB

p19 expression is dependent on NF-κB molecules, c-Rel and RelA, and the sequence from -105 to-96 acts as a functional κB site *in vivo*.

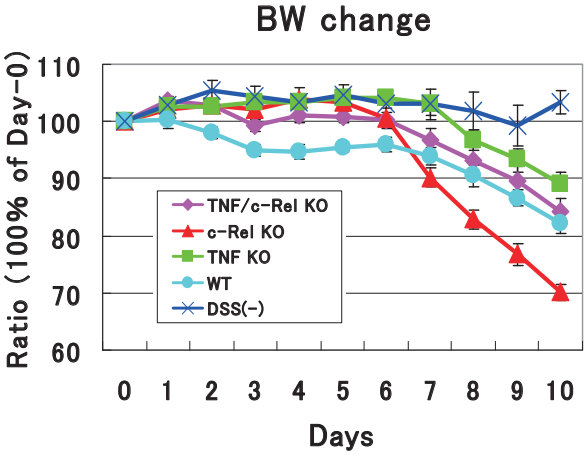


Figure 9. Induction of colitis with DSS
Colitis was induced in mice with administration of 2.5%DSS for seven days, and daily evaluated colitis in terms of symptomatic parameters, such as weight loss, survival, severity of diarrhea. c-Rel deficiency aggravates DSS-induced acute colitis and absence of TNF reduces severity of DSS-colitis.

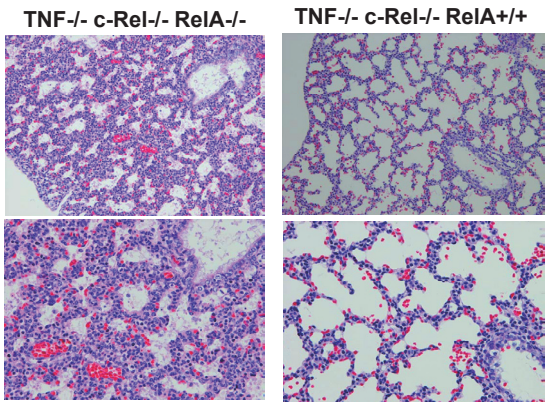


Figure 10. Hhistological examination of newborn lungs
TNF-/-c-Rel-/-RelA-/- mice were born, while TNF+/+c-Rel-/-RelA-/- mice died at 13.5 d.p.c. However those mice died several hours after birth. Pathological findings demonstrated that lungs of newborn mice were not dilatated .

4. Analysis of the essential roles of NF-κB in development

a. NF-κB function in development processes

We have been analyzing how NF-κB functions in development. Thus far, there has been no evidence that RelA or c-Rel has any potential for development, although the dorsal, a Drosophila homolog of NF-κB, plays a critical role in determining the dorsal-ventral axis in embryonic stages. However, here, we show that NF-κB plays a critical role in organogenesis. We generated TNF-/-c-Rel-/-RelA-/- mice for the rescue of c-Rel-/-RelA-/- mice from TNF cytotoxicity. The TNF-/-c-Rel-/-RelA-/- mice were born alive, whereas the TNF+/+c-Rel-/-RelA-/- mice died at 13.5 d.p.c. However, the mice that were born alive died several hours after birth. Pathological findings demonstrated that lungs of the newborn mice were not dilatated (Fig. 10). The cause of this was the lack of pulmonary surfactants in the lung epithelial cells of these mice. This suggests that NF-κB plays a critical role in lung organogenesis in the neonatal stage.

b. Essential roles of NF-κB in hematopoietic development:

We tried to analyze the essential functions of c-Rel and RelA using mice lacking c-Rel and RelA. These mice as well as RelA-deficient mice were embryonically lethal. For determining the functions of RelA and c-Rel with focus on hematopoiesis, we conducted bone marrow reconstitution with the transplantation of fetal livers derived from c-Rel/RelA double-deficient embryos into lethally irradiated Ly5.1 mice. Bone marrows were reconstituted from fetal livers of c-Rel/RelA double-deficient embryos. FACS analysis revealed significant features (Fig. 11): (i) few lymphocytes (both T and B cells), (ii) granulocyte hyperproliferation, and (iii) the presence of macrophages. The spleens derived from the c-Rel/RelA double-deficient fetal livers were ten times larger than those derived from the littermate livers (c-Rel-/- RelA+/- and c-Rel-/- RelA+/+). Moreover, almost all cells of those large spleens consisted of granulocytes. These findings suggest that maturation arrest occurs specifically in lymphocytes. We also conducted an expression analysis of hematopoiesis-related genes by an RT-PCR technique. The expressions of IL-7R, Pax-5 and EBF (Early B-cell factor) were lost in bone marrow cells lacking c-Rel/RelA. Taken together, the results indicate that RelA and c-Rel play critical roles in lymphopoiesis through gene regulation for lymphocyte development.

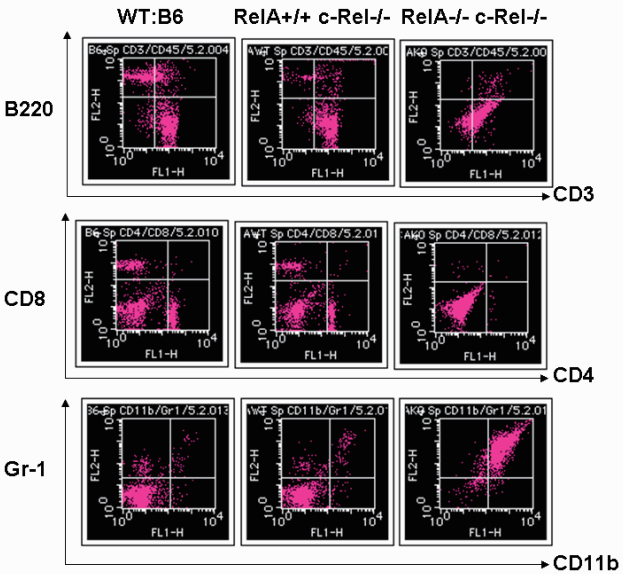


Figure 11. FACS analysis of mice lacking RelA and c-Rel RelA/c-Rel
FACS analysis, for characterization of function of RelA and c-Rel focused on hematopoiesis, revealed the significant features: (i) few lymphocytes (both of T and B cells), (ii) granulocyte hyperproliferation, and (iii) the presence of macrophages.

Publications

【Original Papers】(*Peer reviewed journals)

1. Mise-Omata S., Obata Y., Iwase S., Mise N., Doi T. : "Transient strong reduction of PTEN expression by specific RNAi induces loss of adhesion of the cells." Biochem. Biophy. Res. Comm. 328: 1034-1042 (2005).*

2. Suzuki S., Singhirunnusorn P., Nakano H., Doi T., Saiki I., Sakurai H.: "Identification of TNF-alpha-responsive NF-kappaB p65-binding element in the distal promoter of the mouse serine protease inhibitor SerpinE2." FEBS Lett., 580(13):3257-62 (2006).*

3. Akashi-Takamura S., Doi H., Takahashi K., Tanimura N., Saitoh S., Adachi Y., Doi T., Shimozato T., Miyake K: "Agonistic Antibody to Toll-like receptor 4/MD-2 Protects Mice from Acute Lethal Hepatitis Induced by Tumor Necrosis Factor." J. Immnnol., 176:4244-4251 (2006).*

4. Mise-Omata S., Kuroda E., Niikura J., Yamashita U., Obata Y., Doi T. S.: "A Proximal κB Site in the IL-23 p19 Promoter Is Responsible for RelA- and c-Rel-Dependent Transcription." J. Immunol., 179:6596-6603 (2007).*

5. Kuroda E., Noguchi J., Doi T., Uematsu S., Akira S., Yamashita U.: "IL-3 is an important differentiation factor for the development of prostaglandin E2-producing macrophages between C57BL/6 and BALB/c mice." European J. Immunol. 37:2185-2195 (2007).*

6. Piao J.-H., Yoshida H., Yeh W.-C., Doi T., Xue X., Yagita H., Okumura K., Nakano H.: "TRAF2-dependent canonical pathway is critical for the development of peyer's patches." J. Immunol.. 178(4):2272-7 (2007).*

Oral Presentations

【International Conferences】

1. Doi T., Mise S., Hiraiwa N., Ike F., Yoshiki A., Kasai K., Obata Y.: "Transcription factor NF-κB is essential at neonatal stage." 20th IUBMB, Kyoto (2006).

2. Mise S., Kuroda E., Obata Y., Doi T.: "NF-kB RelA subunit is required for NOS2 and Fas expression but not TH1 induction to acquire the resistance to Leishmania Major." Gene Expression and Signaling in the Immune System, Cold Spring Harbor, NY, USA (2008).

【Domestic Conferences】 Total 13