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# The Rel subunit of NF-κB-like transcription factors is a positive and negative regulator of macrophage gene expression: distinct roles for Rel in different macrophage populations

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**The role of Rel in the monocyte/macrophage lineage was examined in mice with an inactivated *c-rel* gene. Although the frequency of monocytic cells was normal in Rel$^{-/-}$ mice, we show that Rel serves distinct roles in regulating gene expression and immune effector function in different mature macrophage populations. Stimulated Rel$^{-/-}$ resident peritoneal macrophages produced higher than normal levels of granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6), but tumour necrosis factor-α (TNF-α) production was not induced. Diminished cytotoxic activity exhibited by resident Rel$^{-/-}$ macrophages was consistent with reduced nitric oxide production resulting from impaired up-regulation of inducible nitric oxide synthase expression. While a similar altered pattern of IL-6 and TNF-α expression was observed in stimulated Rel$^{-/-}$ peritoneal effusion macrophages, cytotoxic activity, nitric oxide, GM-CSF and G-CSF production by these cells was normal. The alternate regulation of certain genes in the two macrophage populations coincided with different patterns of nuclear Rel/NF-κB complexes expressed in normal resident and elicited cells. Collectively, these results establish that Rel is a positive or negative regulator of transcription in macrophages and that Rel has distinct roles in different macrophage populations.**

*Keywords*: c-*rel*/cytokines/immune defects/macrophages/nitric oxide

## Introduction

Cells of the monocyte lineage are an integral component of the immune system, serving to phagocytose foreign material, present processed antigen to T cells and regulate immune responses through the secretion of cytokines (Gordon, 1995). Cytokines synthesized by activated macrophages include interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), IL-6, IL-8, IL-12, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) (Ziegler-Heitbrock, 1989). Other biologically active molecules produced by activated macrophages include non-specific esterases, lysozyme, complement components, plasminogen activator, prostaglandin $E_2$ ($PGE_2$), reactive oxygen intermediates (ROI) and nitric oxide (NO) (Ziegler-Heitbrock, 1989).

Macrophages display phenotypic heterogeneity and regional specialization in different tissues. For example, the functional properties of macrophages resident in the mouse peritoneal cavity (resident macrophages) differ markedly from blood monocyte-derived macrophages recruited to the peritoneal cavity by inflammatory stimuli (elicited macrophages) (Gordon, 1995). Yet, despite detailed knowledge of the unique properties exhibited by various types of macrophages, little is currently understood about the regulation of genetic programmes that contribute to this specialization in the different macrophage populations.

Mounting evidence implicates Rel/NF-κB-like transcription factors in the control of a number of genes that encode macrophage immunomodulatory proteins. These genes include G-CSF, GM-CSF, TNF-α, IL-1, IL-6 and inducible NO synthase (iNOS) (Collart *et al.*, 1990; Liebermann and Baltimore, 1990; Shakhov *et al.*, 1990; Drouet *et al.*, 1991; Ohmori and Hamilton, 1993; Widmer *et al.*, 1993). In vertebrates, Rel/NF-κB factors comprise a group of homodimeric and heterodimeric proteins encoded by a small multigene family that includes *nfkb1*, *nfkb2*, *rela*, *relb* and c-*rel* (Grilli *et al.*, 1993). All Rel-like proteins share a conserved N-terminal domain of ~300 amino acids that encompass sequences required for DNA binding, dimerization and nuclear localization (Grilli *et al.*, 1993). Rel, RelA and RelB all possess transcriptional transactivation domains (Nolan and Baltimore, 1992; Grilli *et al.*, 1993), whereas the 50 and 52 kDa versions of NF-κB1 and NF-κB2 respectively, which lack transcriptional transactivating properties, are derived by proteolysis from the N-terminus of larger precursors (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Schmid *et al.*, 1991; Mercurio *et al.*, 1992). Prior to stimulation, a considerable proportion of the Rel/NF-κB factors reside in the cytoplasm as inactive complexes through association with inhibitory proteins collectively termed IκBs. Diverse stimuli promote the translocation of Rel/NF-κB proteins to the nucleus by a mechanism involving IκB phosphorylation (Didonato *et al.*, 1995), which targets IκBs for proteosome-mediated degradation (Chen *et al.*, 1995; Scherer *et al.*, 1995).

In adult vertebrates, c-*rel* expression is largely confined to haemopoietic cells, where it is thought to be involved in the regulation of a number of physiological processes (Brownell *et al.*, 1987; Moore and Bose, 1989; Grumont and Gerondakis, 1990). In the B cell lineage, Rel levels change in a developmental stage-specific manner (Grumont and Gerondakis, 1990), with the 5- to 10-fold increase in the level of Rel that accompanies the transition

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**Table I.** The number of cells in different $Rel^{-/-}$ macrophage populations is normal

| Organ | Resident monocytes and macrophages ($\times 10^{-6}$) | | Elicited macrophages ($\times 10^{-6}$) | |
|---|---|---|---|---|
| | +/+ | –/– | +/+ | –/– |
| Peritoneal cavity | 6.7 ± 0.6 | 4.1 ± 0.1 | 13.8 ± 1.6 | 13.5 ± 2.0 |
| Bone marrow | 51.0 ± 30.6 | 30.0 ± 22.5 | | |
| Spleen | 0.6 ± 1.4 | 2.0 ± 2.0 | | |

The data for the frequency of resident macrophages in the peritoneal cavity, bone marrow and spleen represent the mean values ± SDs for three mice per group of age 6–8 weeks. The frequency of thioglycollate-elicited peritoneal macrophages is the mean values ± SDs of adherent cells cultured for 2 h that were isolated from three mice per group, each 6–8 weeks of age.

from a pre-B to a B cell linked to qualitative changes in NF-κB subunit usage (Grumont and Gerondakis, 1994; Liou *et al.*, 1994; Miyamoto *et al.*, 1994). In both B and T cells, nuclear levels of Rel are highest during mitogen stimulation (Grumont and Gerondakis, 1990, 1994), a finding consistent with a proposed role for Rel in the regulation of lymphocyte proliferation (Grumont and Gerondakis, 1990). While Rel is also expressed in neutrophils (Blackwell *et al.*, 1994) and monocytes (Ziegler-Heitbrock, 1993), detailed characterization of Rel expression in these lineages remains to be determined. To investigate the role of Rel in haemopoietic cells, gene targeting was used to generate $Rel^{-/-}$ mice (Kontgen *et al.*, 1995). While steady-state haemopoiesis appears to be normal, these mice exhibit B and T cell proliferative defects, impaired humoral immunity and reduced production of IL-2, IL-3 and GM-CSF during T cell activation (Kontgen *et al.*, 1995; Gerondakis *et al.*, 1996). In the study described here, we have examined the immune properties of monocyte/macrophage lineage cells in $Rel^{-/-}$ mice. While the capacity of haemopoietic precursors to undergo differentiation into macrophages appears normal, an examination of $Rel^{-/-}$ resident and elicited peritoneal macrophages revealed that resident, but not elicited, macrophages exhibited defects in cytotoxic killing, NO production and the expression of certain cytokines. Consistent with these findings, the regulation of Rel/NF-κB differs in these two murine macrophage populations.

## Results

### The frequency of cells in different macrophage populations is normal in $Rel^{-/-}$ mice

Previously we had shown that the loss of Rel had no effect on the capacity of haemopoetic precursors in the bone marrow to proliferate and differentiate *in vitro* (Kontgen *et al.*, 1995). The heterogeneity and regional specialization displayed by different macrophage populations prompted an examination of the morphology and frequency of monocyte lineage cells from the bone marrow, spleen and peritoneal cavity of $Rel^{-/-}$ mice. The morphological appearance of $Rel^{-/-}$ monocytes/macrophages appeared indistinguishable from $Rel^{+/+}$ cells (results not shown) and the steady-state frequency of $Rel^{-/-}$ cells in the different macrophage populations was normal (Table I). The emigration of monocytic cells from the blood to the site of pathogen replication during an inflammatory response is an important mechanism in the eradication of microorganisms (Ziegler-Heitbrock, 1989). Despite the fact that the expression of I-CAM, N-CAM and V-CAM, cell adhesion molecules crucial to monocyte extravasation and diapadesis, are under the regulatory control of Rel/NF-κB (Baeuerle and Henkel, 1994), no difference was observed in the frequency of elicited macrophages that accumulated in the peritoneal cavity of normal and $Rel^{-/-}$ mice after thioglycollate injection (Table I). Collectively, these findings indicate that Rel does not appear to be essential for the establishment of different murine macrophage populations.

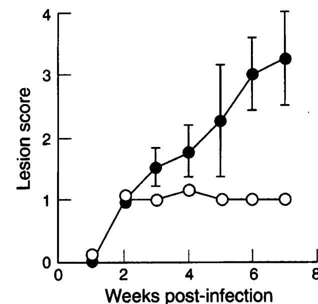


**Fig. 1.** $Rel^{-/-}$ mice are susceptible to infection with *L.major.* Equivalent numbers ($n$ = 4) of litter-matched wild-type and $Rel^{-/-}$ mice were injected intradermally with $10^6$ promastigotes, with disease progression in the wild-type (○) and $Rel^{-/-}$ (●) mice monitored weekly over a 7 week period by examining the size of parasitic lesions. Lesion scores from 1 to 4 represent a scale of increasing disease severity, where 1 represents a small localized swelling and 4 is a large diseminated lesion (Mitchell, 1983). Parasite loads were determined by counting the number of motile promastigotes in sequential 5-fold dilutions of draining lymph node suspensions from infected mice. Parasite numbers are expressed as the geometric mean ($\log_{10}$) and standard error of the last positive reciprocate dilution for each group of mice.

### The loss of Rel results in impaired cell-mediated immunity

To determine if, like $Rel^{-/-}$ B cells (Kontgen *et al.*, 1995), the macrophages from $Rel^{-/-}$ mice exhibit impaired immune function, we examined the capacity of $Rel^{-/-}$ mice to mount an immune response against *Leishmania major*, an intracellular parasite that survives and proliferates within mononuclear phagocytes (Mosser and Rosenthal, 1993). C57BL/6 mice are normally resistant to *Leishmania* infection, with the activation of monocytic cells being an essential part of the host defence mechanism (James and Nacy, 1993). Figure 1 shows that $Rel^{-/-}$ C57BL/6 mice were uniformly susceptible to this parasite, while $Rel^{+/+}$ littermates were resistant, with healing of

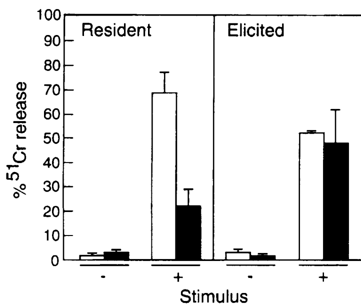


**Fig. 2.** Reduced tumoricidal activity of Rel$^{-/-}$ resident peritoneal macrophages. The cytolytic activity of resident and thioglycollate-elicited peritoneal macrophages from wild-type (open bars) and Rel$^{-/-}$ (solid bars) mice stimulated with LPS (1 μg/ml) and IFN-γ ($10^3$ U/ml) for 16 h was assayed with $^{51}$Cr-labelled P815 mastocytoma cells. The mean and standard deviation for cytotoxic activity were derived from six experiments.

skin lesions in normal animals complete after several weeks. Consistent with the disease patterns and difference in susceptibility, the number of promastigotes in draining lymph nodes (expressed as a geometric mean), a measure of the parasite replicative capacity during infection, was significantly higher in the Rel$^{-/-}$ mice (7 ± 1) compared with the wild-type controls (4 ± 1).

### *Impaired cytotoxic activity displayed by resident but not elicited Rel$^{-/-}$ peritoneal macrophages coincides with the distinct regulation of iNOS expression in these macrophage populations*

The susceptibility of Rel$^{-/-}$ mice to *L.major* prompted an analysis of the immune effector properties of Rel$^{-/-}$ macrophages. While macrophage cytotoxic killing is an important component of the anti-parasitic immune response (James and Nacy, 1993), determining whether the susceptibility was due in part to defects in the Rel$^{-/-}$ macrophages was complicated by the marked phenotypic and functional differences normally exhibited by various macrophage populations (Gordon, 1996). In an attempt to address this issue, the functional and biochemical properties of both activated resident and elicited peritoneal macrophages from normal and Rel$^{-/-}$ mice were compared. Cytotoxic activity was examined after activation with lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) by measuring the capacity of the macrophages to restrict the replication of $^{51}$Cr-labelled P815 tumour cells *in vitro* (Figure 2). Resident peritoneal macrophages from Rel$^{-/-}$ mice were markedly impaired in their ability to kill the tumour cells when compared with those from normal litter mates. Suprisingly, however, elicited macrophages from Rel$^{+/+}$ and Rel$^{-/-}$ mice were indistinguishable.

Numerous studies have linked the production of NO by iNOS as an important component of macrophage microbicidal and tumoricidal activity (Hibbs *et al.*, 1987; Stuehr and Nathan, 1989). The inability of mice lacking the iNOS gene to mount an effective immune response to *L.major* (Wei *et al.*, 1995), combined with the finding that transcription of the iNOS gene appears to be regulated by NF-κB-like factors (Xie *et al.*, 1994), prompted a comparison of iNOS activity in Rel$^{+/+}$ and Rel$^{-/-}$ resident and elicited peritoneal macrophages. $NO_2^-$ levels were measured in the supernatant fluid from cells stimulated

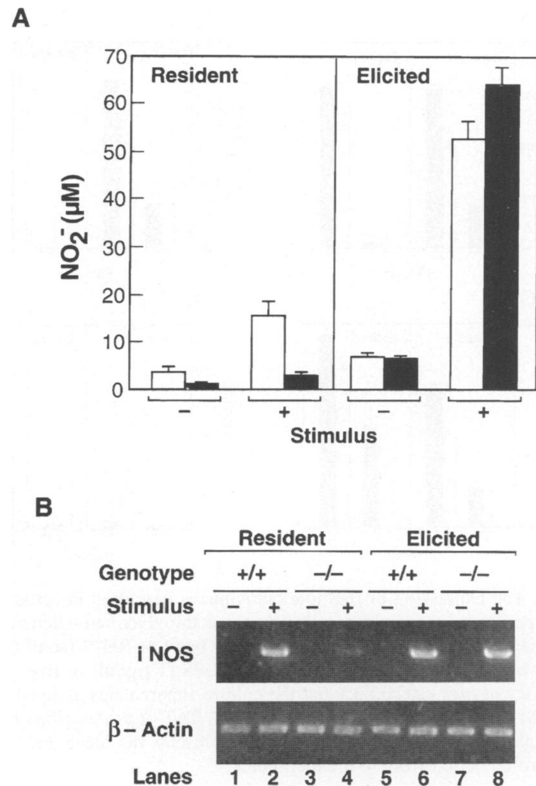


**Fig. 3.** Rel regulates $NO_2^-$ production and iNOS expression in activated resident peritoneal macrophages. (**A**) $NO_2^-$ production. $NO_2^-$ levels in the culture supernatants from resident and thioglycollate-elicited Rel$^{+/+}$ (open bars) and Rel$^{-/-}$ (solid bars) macrophages stimulated for 48 h with LPS (1 μg/ml) and IFN-γ ($10^3$ U/ml) were quantified by the Griess method (Ding *et al.*, 1988). These results represent the mean of six experiments. (**B**) iNOS expression in stimulated macrophages. Total RNA isolated from LPS- and IFN-γ-stimulated Rel$^{+/+}$ and Rel$^{-/-}$ resident and thioglycollate-elicited macrophages stimulated with LPS and IFN-γ for 5 h was subjected to semi-quantitative RT–PCR using iNOS- and β-actin-specific primers. PCR products were fractionated on a 1% agarose gel.

with LPS and IFN-γ for 48 h (Figure 3A). While resident Rel$^{-/-}$ cells produced only 20% of normal $NO_2^-$ levels, $NO_2^-$ production by wild-type and Rel$^{-/-}$ thioglycollate-elicited macrophages was comparable. Consistent with these findings, iNOS mRNA levels were up-regulated markedly in normal but not in Rel$^{-/-}$ stimulated resident macrophages (Figure 3B, lanes 1–4), while similar levels of iNOS mRNA were expressed in elicited macrophages from Rel$^{+/+}$ and Rel$^{-/-}$ mice (Figure 3B, lanes 6 and 8 respectively).

### *The production of certain cytokines is perturbed in the resident but not elicited Rel$^{-/-}$ macrophages*

To determine the role of Rel in the expression of the GM-CSF, G-CSF, IL-6, TNF-α and IL-1β genes, all of which are regulated by NF-κB-like factors (Collart *et al.*, 1990; Shakhov *et al.*, 1990; Drouet *et al.*, 1991; Ohmori and Hamilton, 1993; Widmer *et al.*, 1993), the production of these cytokines was compared in non-stimulated and activated normal or Rel$^{-/-}$ resident and elicited peritoneal macrophages (Figure 4). While basal levels of GM-CSF (Figure 4A) and G-CSF (Figure 4B) were normal in both

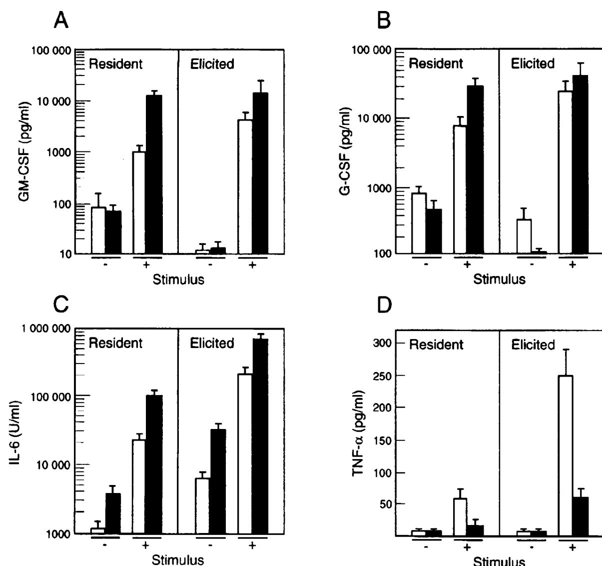


**Fig. 4.** The expression of multiple cytokines is perturbed in activated $Rel^{-/-}$ peritoneal macrophages. Resident and thioglycollate-elicited peritoneal macrophages from normal (open bars) or $Rel^{-/-}$ (solid bars) mice were stimulated for 24 h with either LPS (1 μg/ml) or live *L.monocytogenes* ($10^3$ c.f.u.) and the culture supernatants assayed for (**A**) GM-CSF, (**B**) G-CSF, (**C**) IL-6 and (**D**) TNF-α as described in Materials and methods. Each histogram represents the mean and standard deviation from 4–6 experiments.

non-stimulated $Rel^{-/-}$ macrophage populations, upon LPS treatment, GM-CSF and G-CSF production by resident $Rel^{-/-}$ macrophages was ~10- and 5-fold greater respectively than that produced by normal LPS-stimulated resident macrophages. This finding contrasted with the production of GM-CSF and G-CSF by activated elicited macrophages, where cytokine levels secreted by $Rel^{+/+}$ and $Rel^{-/-}$ cells were not significantly different (Figure 4A and B). Unlike GM-CSF and G-CSF, the levels of IL-6 secreted by unstimulated $Rel^{-/-}$ resident and elicited macrophages were elevated ~3-fold compared with unstimulated $Rel^{+/+}$ cells (Figure 4C). IL-6 production by both $Rel^{-/-}$ macrophage populations stimulated with LPS (Figure 4C) was consistently 3- to 5-fold higher than that of $Rel^{+/+}$ cells.

To monitor TNF-α production by $Rel^{+/+}$ and $Rel^{-/-}$ macrophages, live *Listeria monocytogenes*, a potent activator of TNF-α, was used in preference to LPS, which is a poor inducer of TNF-α in resident peritoneal macrophages (Zhan and Cheers, 1995). While basal levels of TNF-α were unchanged in both unstimulated $Rel^{-/-}$ macrophage populations, TNF-α was not induced in either the stimulated $Rel^{-/-}$ resident or elicited macrophages (Figure 4D). No difference in IL-1β production by wild-type and $Rel^{-/-}$ macrophages was observed in either population (data not shown). Collectively, these results establish that Rel can function as a positive or negative regulator of cytokine production, with the activated $Rel^{-/-}$ resident and elicited peritoneal macrophages exhibiting different cytokine secretory patterns.

### *Different NF-κB complexes are expressed in resident and elicited peritoneal macrophages*

The finding that Rel was important for the regulation of iNOS, G-CSF and GM-CSF in resident, but not elicited, peritoneal macrophages, prompted an analysis of the NF-κB-like complexes in the two macrophage populations. A probe corresponding to one of two putative NF-κB binding sites in the iNOS promoter (κB1: 5′-GGGATTTTCC-3′) was used in electrophoretic mobility shift assays (EMSAs) to analyse Rel/NF-κB complexes in resting and stimulated resident and thioglycollate-elicited peritoneal macrophages. Similar results were obtained using different κB sites as probes (results not shown). Two major nuclear complexes were detected in non-stimulated wild-type resident (Figure 5A, lane 1; here denoted Cα and Cβ) and elicited (Figure 5B, lane 1; here denoted Ca and Cb) macrophages. While there was only a modest up-regulation of both complexes in LPS-stimulated $Rel^{+/+}$ resident cells (Figure 5A, lane 2), Ca, but not the Cb complex, was induced by LPS in the normal elicited population (Figure 5B, lane 2). The regulation of these complexes also differed markedly in the two $Rel^{-/-}$ macrophage populations. While Cα and Cβ levels in non-activated $Rel^{-/-}$ resident cells (Figure 4A, lane 5) were considerably lower than those in unstimulated $Rel^{+/+}$ cells (lane 1), both complexes were induced to normal levels upon LPS stimulation (Figure 5A, lane 6). In unstimulated $Rel^{-/-}$ elicited cells, the pattern of κB1 binding complexes appeared unchanged but, upon LPS treatment, a complex of equivalent mobility to Cb but not Ca was up-regulated (Figure 5B, compare lanes 5 and 6). The κB1 binding specificity of all complexes in both normal (Figure 5A and B, lanes 3 and 4 respectively) and $Rel^{-/-}$ (Figure 5A and B, lanes 7 and 8) macrophage populations was demonstrated by competition with excess κB1 and mutant κB1 probes.

The composition of the κB1 binding complexes in both macrophage populations from wild-type and $Rel^{-/-}$ mice was examined by supershift analysis using antibodies specific for the different Rel/NF-κB subunits (Figure 5C and D). In resting and LPS-stimulated wild-type resident peritoneal macrophages (Figure 5C, lanes 1–8), Cα is comprised of NF-κB1 (lanes 2 and 6), Rel (lanes 3 and 7) and RelA (lanes 4 and 8), while Cβ was only supershifted with NF-κB1-specific antibodies (lanes 2 and 6). In non-stimulated (lanes 9–12) and LPS-activated (lanes 13–16) $Rel^{-/-}$ resident macrophages, supershifts indicate that the Cα complex was comprised mainly of NF-κB1 and RelA, while the Cβ complex was NF-κB1 homodimers (lanes 10 and 14). In elicited macrophages (Figure 5D), NF-κB1 (lanes 2 and 6) and RelA (lanes 4 and 8) are major components of Cα in both non-stimulated and LPS-treated $Rel^{+/+}$ cells. While Rel is only a minor component of the Cα complex in resting wild-type elicited macrophages (Figure 5D, lane 3), it is up-regulated upon LPS stimulation (lane 7). In both non-stimulated and LPS-treated $Rel^{-/-}$ elicited cells, the low abundance Cα complex is composed of NF-κB1 (lanes 10 and 14) and RelA (lanes 12 and 16). Surprisingly, the RelA-specific antibodies (Grumont and Gerondakis, 1994; Kontgen *et al.*, 1995), appear to alter the mobility of Cb in elicited (Figure 5D, lanes 4, 8, 12 and 16) and, to a lesser extent, Cβ in resident (Figure 5C, lanes 4, 8, 12 and 16) macrophages. The molecular basis of this anomaly, which was still observed when several peptide-specific sera raised against unrelated epitopes of murine RelA were used (unpublished results), is currently under investigation. RelB and NF-κB2

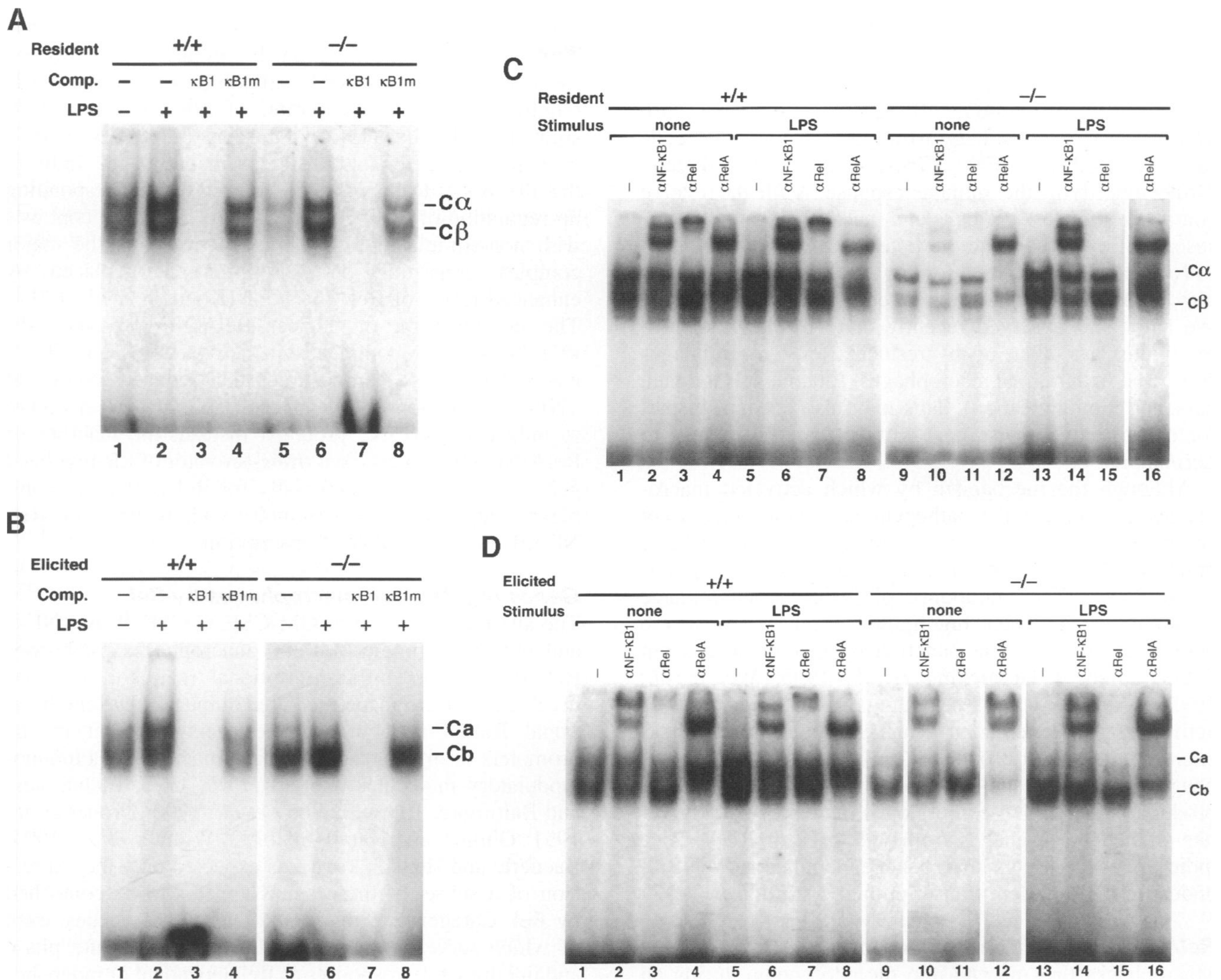


**Fig. 5.** Analysis of Rel/NF-κB complexes in resting and elicited peritoneal macrophages. Nuclear extracts (1–2 μg) isolated from non-stimulated and LPS-activated (4 h) resident and thioglycollate-elicited peritoneal macrophages were incubated with 1 μg of poly d(I–C) and $^{32}$P-labelled κB1 probe. The resulting Rel/NF-κB complexes were resolved on 5% non-denaturing polyacrylamide gels and exposed to autoradiography for 6 h at –70°C. Nuclear κB1 binding complexes in (**A**) resident and (**B**) elicited macrophages. Nuclear extracts from non-stimulated (lanes 1 and 5) and LPS-activated (lanes 2–4 and 6–8) Rel$^{+/+}$ (lanes 1–4) and Rel$^{-/-}$ (lanes 5–8) macrophages were incubated in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of a 50-fold molar excess of unlabelled κB1 (lanes 3 and 7) or κB1m (lanes 4 and 8) probe. Cα and Cβ correspond to the upper and lower complexes from resident macrophages; Ca and Cb are the fast and slow mobility complexes in elicited macrophages. The subunit composition of Rel/NF-κB complexes in (**C**) resident and (**D**) elicited macrophages. Nuclear extracts from non-stimulated (lanes 1–4 and 9–12) or LPS-activated (lanes 5–8 and 13–16) Rel$^{+/+}$ (lanes 1–8) and Rel$^{-/-}$(lanes 9–16) macrophages were incubated for 20 min with pre-immune (lanes 1, 5, 9 and 13) or NF-κB1- (lanes 2, 6, 10 and 14), Rel- (lanes 3, 7, 11 and 15) and RelA- (lanes 4, 8, 12 and 16) specific sera prior to adding κB1 probe.

were not detected in any of the κB1 binding complexes (unpublished results).

## Discussion

In this study, mice lacking the c-*rel* gene were used as a model to determine the role of Rel in macrophage immunomodulatory responses. While Rel was not essential for monocyte/macrophage maturation from precursor cells, resident and elicited Rel$^{-/-}$ peritoneal macrophages exhibited differences in immune function and cytokine gene expression. Collectively, these findings establish Rel both as a positive and negative regulator of gene expression that serves distinct transcriptional regulatory roles in different macrophage populations.

### *Cell-mediated immunity in Rel$^{-/-}$ mice*

Rel previously has been shown to be important in humoral immunity (Kontgen *et al.*, 1995). The susceptibility of Rel$^{-/-}$ mice to *L.major*, combined with the markedly reduced cytotoxic killing capability of Rel$^{-/-}$ resident peritoneal macrophages, indicates that Rel is also a genetic regulator of cell-mediated immune responses. In resistant mouse strains, resolution of leishmanial infections is critically dependent on the induction of T helper cell activity of the inflammatory Th1 type and the conversion of macrophages harbouring parasites to a microbicidal state (Howard, 1986; Liew, 1989). Secretion of IFN-γ by antigen-specific Th1 cells synergizes with TNF-α produced by macrophages to convert cells harbouring parasites to a microbicidal state (Locksley and Louis, 1992)

in which iNOS produces reactive nitrogen radicals toxic to the parasite (Green *et al.*, 1990; Liew and O'Donnell, 1993). Once infected or activated by *Leishmania* antigens, macrophages also produce IL-12 which, together with TNF-α, acts synergistically to trigger natural killer cells to make additional IFN-γ (Tripp *et al.*, 1993), thereby further amplifying the immune response. While the relative contribution of $Rel^{-/-}$ T cells and macrophages to *L.major* susceptibility remains to be determined, the attenuation of NO and TNF-α production by $Rel^{-/-}$ macrophages may play an important role in the defective immune response. We have also observed recently that 10-fold more promastigotes are taken up by resident $Rel^{-/-}$ macrophages compared with normal macrophages (Emanuela Handman, personal communication). This may also account in part for the difference in susceptibility of the $Rel^{-/-}$ mice to *Leishmania*.

Although the mechanism by which activated macrophages kill intracellular pathogens or tumour cells is not completely understood, oxygen- and nitrogen-derived free radicals appear to be crucial in this process (Hibbs *et al.*, 1987). The importance of iNOS in macrophage cytotoxicity has been highlighted recently by severe defects exhibited in this function by macrophages from $iNOS^{-/-}$ mice (MacMicking *et al.*, 1995; Wei *et al.*, 1995). Consistent with these findings, diminished cytotoxic activity is associated with a marked reduction in iNOS expression and NO production by resident $Rel^{-/-}$ macrophages. However, the absence of a difference in NO production and iNOS expression in activated thioglycollate-elicited macrophages from wild-type or $Rel^{-/-}$ mice indicates that, in this macrophage population, iNOS is under Rel-independent transcriptional control.

### Rel/NF-κB regulation in macrophages

Macrophages that migrate into the peritoneum during an inflammatory response are functionally and phenotypically distinct from the terminally differentiated resident macrophage population (Gordon, 1995). The finding that Rel/NF-κB is differentially regulated in these two types of macrophages may indicate that changes in the expression of these transcription factors is an important mechanism in the establishment of the specific genetic programmes that accompany the functional specialization of various macrophage populations. Furthermore, these differences in Rel/NF-κB expression in the resident and elicited macrophages, together with the change in Rel/NF-κB subunit usage and regulation that accompanies B cell differentiation (Grumont and Gerondakis, 1994; Liou *et al.*, 1994; Miyamoto *et al.*, 1994), suggest that changes in Rel/NF-κB could be a common theme in haemopoietic cell differentiation.

The loss of Rel has different consequences for the expression of NF-κB1 homodimers and NF-κB-like complexes in the two peritoneal macrophage populations. Nuclear NF-κB1 homodimer and NF-κB complexes, although markedly diminished in non-activated $Rel^{-/-}$ resident macrophages, are up-regulated to normal levels during LPS stimulation. In contrast, while expression of NF-κB1 homodimers appears normal in non-stimulated $Rel^{-/-}$ elicited cells, NF-κB is not induced upon activation. This indicates that in resident macrophages, Rel appears to regulate basal NF-κB1 homodimer and NF-κB expression, but is not essential for expression of these complexes during macrophage activation. In elicited cells, however, Rel does not alter NF-κB1 homodimer regulation, but is required for the up-regulation of NF-κB during LPS stimulation. Reduced levels of the constitutive Cα complex in non-stimulated $Rel^{-/-}$ resident macrophages indicate that the loss of Rel is not accompanied by a corresponding up-regulation of other Rel-related proteins. This contrasts with non-stimulated $Rel^{-/-}$ B cells, in which the major complex consisting of Rel/NF-κB1 is replaced by enhanced levels of RelA/NF-κB1 (Kontgen *et al.*, 1995). The up-regulation of nuclear RelA/NF-κB1 and NF-κB1 homodimer complexes in activated resident $Rel^{-/-}$ macrophages also demonstrates that reduced iNOS and TNF-α expression does not result simply from a failure to induce NF-κB-like proteins. Instead, the inability of RelA/NF-κB1, an efficient *trans*-activator of κB-regulated genes, to substitute functionally for Rel-containing complexes emphasizes the specificity with which each Rel/NF-κB protein regulates transcription.

### Genes regulated in macrophages by Rel

The altered expression of GM-CSF, G-CSF, IL-6, TNF-α and iNOS in stimulated $Rel^{-/-}$ macrophages establishes Rel as an important transcriptional regulator of genes involved in macrophage-mediated immunity. Despite functional Rel/NF-κB binding sites being present in the promoters of many genes encoding macrophage immunomodulatory molecules (Collart *et al.*, 1990; Liebermann and Baltimore, 1990; Shakhov *et al.*, 1990; Drouet *et al.*, 1991; Ohmori and Hamilton, 1993; Widmer *et al.*, 1993; Baeuerle and Henkel, 1994), as expected only the expression of a subset of these genes appears to be controlled by Rel. Categorizing the regulation of these genes, each of which serves a distinct role in immune, acute phase and inflammatory responses as Rel dependent or independent, may provide important insights into the coordinated expression of certain genes during particular immune responses mounted by different types of macrophages.

Results presented here show that like the *Drosophila* homologue, Dorsal (Anderson, 1987; Levine, 1988), mammalian Rel possess both positive and negative transcriptional regulatory activities. In peritoneal macrophages, Rel can function as an activator of the TNF-α and iNOS genes, but is a repressor of GM-CSF, G-CSF and IL-6 expression. While promoters for the murine GM-CSF (Ghosh *et al.*, 1993), IL-6 (Nakayama *et al.*, 1992) and iNOS (Xie *et al.*, 1994) genes have functional κB elements that bind complexes containing Rel, it remains to be determined whether the Rel-dependent regulation of G-CSF and TNF-α in macrophages is due directly to Rel binding to the κB sites in the G-CSF and TNF-α promoters. In the case of induced TNF-α transcription in monocytes, a role for NF-κB-like factors is supported by the antioxidant pyrrolidine dithiocarbamate (PDTC) inhibiting TNF-α expression (Ziegler-Heitbrock *et al.*, 1993).

The role of Rel in the regulation of particular genes varies in different cell types. For example, induced TNF-α expression is Rel dependent in peritoneal macrophages, but Rel independent in T cells (Gerondakis *et al.*, 1996). For the GM-CSF gene, Rel functions as an activator in T cells (Gerondakis *et al.*, 1996), it represses GM-CSF expression in resident peritoneal macrophages, but is not

required for the regulation of this gene in elicited peritoneal macrophages. While a role for Rel in the control of a particular gene in one cell type but not another can be explained, in some circumstances, by distinct stimuli utilizing different transcription factors, the modulation of Rel activity through interaction with other transcription factors is also likely to be an important mechanism in determining if Rel functions as a transcriptional activator or repressor. These dual activities could result from dimerization with different Rel-related proteins or interaction of Rel with unrelated transcription factors. This latter model is consistent with the ability of Rel/NF-κB proteins to interact with a number of transcription factors including ATF-2 (Kaszubska *et al.*, 1993), SP1 (Perkins *et al.*, 1993) and C/EBP (Stein and Baldwin, 1993). The ability of different high mobility group (HMG) proteins to convert Rel/NF-κB factors to activators or repressors of transcription is one such example. Binding of HMG I (Y) at sites adjacent to the κB element in the IFN-β gene augments both NF-κB binding and transcriptional activity (Thanos and Maniatis, 1992), while the DSP1 HMG protein binds to a negative regulatory element flanking the IFN-β κB site and converts NF-κB from a transcriptional activator to a repressor (Lehming *et al.*, 1994). The identification of genes, the expression of which is induced or repressed by Rel, will offer important clues in the elucidation of the mechanisms governing the transcription regulatory properties of Rel/NF-κB factors.

## Materials and methods

### Macrophage isolation and culture

The generation of the c-*rel*$^{-/-}$ mice has been described elsewhere (Kontgen *et al.*, 1995). Resident peritoneal macrophages were harvested from litter-matched 6–10 week old Rel$^{+/+}$ and Rel$^{-/-}$ mice by first rinsing the peritoneal cavity with 6 ml of Dulbecco's modified Eagle's medium (DMEM). Elicited cells were also obtained by flushing the peritoneal cavity of mice 4 days after intraperitoneal injection with 2 ml of 3% thioglycollate solution. After multiple washes, peritoneal exudate cells were resuspended in DMEM supplemented with 0.216 mg/ml L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS, Gibco) and macrophages enriched to >95% purity by adherence to plastic dishes for 2 h. The viability of cells as determined by trypan blue exclusion was >90%. For all cytokine and NO assays, $1\times10^6$ peritoneal macrophages seeded in 10 mm wells containing 2 ml of media were either left untreated or stimulated for 24–48 h with live *L.monocytogenes*, LPS (1 μg/ml) or LPS (1 μg/ml) plus IFN-γ ($10^3$ U/ml). For the isolation of RNA and nuclear extracts, peritoneal macrophages seeded at a density of $1.5\times10^7$cells/30 mm dish were either untreated or stimulated with LPS (1 μg/ml) plus IFN-γ ($10^3$ U/ml) for 5 h.

### Cytokine assays

The levels of GM-CSF, G-CSF, IL-6, TNF-α and IL-1β in the macrophage culture supernatants were determined by bioassay essentially as described (Kelso, 1990). All cytokine assays were standardized by including a titration of the appropriate purified recombinant cytokine of known activity. The cell lines used were Nob-1 and CTLL for IL-1 (Gearing *et al.*, 1987), WEHI-164 for TNF-α (Espevik and Nissen-Meyer, 1986), FDC-P1 for GM-CSF (Metcalf *et al.*, 1994), Ba/F3GR for G-CSF (Metcalf *et al.*, 1994) and 7TD1 for IL-6 (Kelso, 1990).

### Macrophage production of NO

Adherent macrophages were cultured for 48 h in the presence or absence of LPS (0.1–1 μg/ml) and IFN-γ ($10^3$ U/ml). Synthesis and release of NO by macrophages was determined by assaying culture supernatants for nitrite ($NO_2^-$) content (Ding *et al.*, 1988). Briefly, 100 μl of supernatant was reacted for 10 min at room temperature with an equal volume of Griess reagent [0.5% sulfanilamide and 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid]. The optical density at 570 nm was then determined. The $NO_2^-$ content was quantified by comparison with a standard curve generated with $NaNO_2$ in the range of 0–100 μM.

### Semi-quantitative PCR

Peritoneal macrophages were homogenized in 3 M $LiCl_2$/6 M urea and total RNA precipitated for 12–15 h at 4°C. The homogenate was centrifuged and the RNA was resuspended in TE/0.1% SDS, then purified by phenol–chloroform extraction and ethanol precipitation. Equivalent amounts of total RNA were used for cDNA synthesis in a reaction mix containing 50 mM KCl, 4.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3, 0.01% gelatin, 0.2 μg of random hexamer primers (Boehringer Mannheim), 1 mM dNTPs, 8 U of RNAsin (Promega) and 2 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega), which was incubated at 42°C for 60 min. For the semi-quantitative PCR, cDNA was added to a cocktail comprising 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris–HCl pH 8.3, 0.01% (w/v) gelatin, 0.5 mM dNTPs, 1 U of *Taq* polymerase (Ampli Taq: Perkin-Elmer-Cetus) and 1 μM of each oligonucleotide, adjusted to a final volume of 30 μl and overlaid with paraffin oil. After an initial 5 min denaturation at 94°C, the cDNA was amplified for 25 cycles with each cycle programmed for denaturation at 94°C for 45 s, annealing at 55°C for 60 s, followed by elongation at 72°C for 90 s. Samples were then fractionated on 1.5% agarose gels. The sequence of the oligonucleotides used for the amplification of murine iNOS and β-actin cDNAs were: iNOS mRNA, 5′ oligo GACATGGCTTGCCCCTGGAAGTTTCTC (nucleotides –3 to 24, according to the sequences of Lyons *et al.*, 1992), 3′ oligo TCCAAGTCTTGCTTGGGGTCCATGATG (nucleotides 316–342, Lyons *et al.*, 1992); and β-actin, 5′ oligo CTGAAGTACCCATTGAACATGGC (nucleotides 278–303, according to the sequence of Tokunaga *et al.*, 1986 ), 3′ oligo CAGAGCAGTAATCTCCTTCTGCAT (nucleotides 1016–1040, Tokunaga *et al.*, 1986). The iNOS and β-actin PCR products were 345 and 762 nucleotides in length respectively.

### Cytotoxicity assay

Macrophage-mediated killing was measured using a 16 h $^{51}Cr$ release assay (Pace and Russell, 1981). Target cells (P815 mastocytoma), free of mycoplasma contamination, were pre-labelled for 1.5 h at 37°C with $^{51}Cr$ (100 μCi as sodium chromate/$10^6$ cells), washed four times by centrifugation and allowed to equilibrate for at least 1 h at 37°C in DMEM containing 10% FCS. To assay for cytolytic activity, macrophage monolayers were first pre-incubated for 4 h with 100 μl of a given agonist. Then, $2\times10^5$ freshly washed $^{51}Cr$-labelled P815 cells in a 100 μl volume were seeded into each well. Sixteen hours later, the uppermost 100 μl of supernatant was removed and assayed for radioactivity. Results were expressed as the percentage specific $^{51}Cr$ release, calculated using the following formula: % specific release = experimental release – spontaneous release/total releasable counts – spontaneous release ×100. Spontaneous release was determined from monolayers incubated with $^{51}Cr$-labelled P815 and medium only. Total releasable counts were determined by freezing and thawing of $^{51}Cr$-labelled P815 in hypotonic medium.

### Nuclear extracts

Nuclear extracts were prepared from macrophages essentially as described (Schrieber *et al.*, 1989). Briefly, $10^7$ cells were washed in MTPBS, resuspended in 500 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 4 mg/ml leupeptin], then pelleted. Cells were resuspended gently in 400 μl of buffer A and allowed to swell on ice for 15 min. After the addition of 25 μl of NP-40, samples were vortexed for 10 s and centrifuged at 12 000 *g* for 30 s. The resultant supernatant containing the cytoplasmic fraction was frozen at –70°C. Fifty microlitres of buffer C [420 mM NaCl, 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, 4 mg/ml leupeptin] was added to the pellet and placed on ice with the tube agitated periodically for 20 min. Extracts were centrifuged at 10 000 *g* and the supernatant containing the nuclear fraction frozen at –70°C.

### EMSA

The iNOS κB1 (5′-GATCCTGCTAGGGGGATTTTCCCTCTCTCG-3′) and iNOS κB2 (5′-GATCCCCAACTGGGGACTCTCCCTTTGGGG-3′) probes were prepared by end labelling with [$^{32}P$]ATP as described (Grumont and Gerondakis, 1994). EMSA reactions contained 5000 c.p.m. of $^{32}P$-labelled probe, 0.1–1 μg of poly d(I–C), 1.5–3 μg of

protein extract, 10 μg of bovine serum albumin and reaction buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% NP-40] in a total volume of 15 μl. For competition analysis, a 50-fold excess of unlabelled iNOS κB1 and iNOS κB2 DNA was added to the reaction 15 min prior to the addition of the radiolabelled probe. For supershift analysis, antibodies that specifically recognize NF-κB1, NF-κB2, RelA, RelB and Rel were incubated for 30 min before addition of the radiolabelled probe. All EMSA reactions were then incubated for 20 min at room temperature, 2 μl of Ficoll dye added, and the reactions fractionated on 5% non-denaturing acrylamide gels.

### *Leishmania major* inoculation

Groups of four C57BL $Rel^{+/+}$ and $Rel^{-/-}$ mice were infected intradermally at the base of the tail with $1\times10^6$ stationary-phase (day 7 culture) *L.major* promastigotes derived from a cloned line (Handman *et al.*, 1983). Lesion development was monitored weekly and the size of the lesion given a score of 1–4, where 1 represents a small localized swelling and 4 is a large diseminating lesion (Mitchell, 1983). The number of parasites surviving in the draining lymph nodes of infected mice was determined by limited dilution analysis (Morris *et al.*, 1993).

### Statistical analyses

The data were subjected to Student's *t* test. Results are expressed as mean ± standard deviation (SD); $P < 0.05$ was considered significant.

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