

Negative Regulation of Toll-Like Receptor Signaling by NF- κ B p50 Ubiquitination Blockade

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Toll-like receptors (TLRs) trigger the production of inflammatory cytokines and shape adaptive and innate immunity to pathogens. We report the identification of B cell leukemia (Bcl)-3 as an essential negative regulator of TLR signaling. By blocking ubiquitination of p50, a member of the nuclear factor (NF)- κ B family, Bcl-3 stabilizes a p50 complex that inhibits gene transcription. As a consequence, Bcl-3-deficient mice and cells were found to be hypersensitive to TLR activation and unable to control responses to lipopolysaccharides. Thus, p50 ubiquitination blockade by Bcl-3 limits the strength of TLR responses and maintains innate immune homeostasis. These findings indicate that the p50 ubiquitination pathway can be selectively targeted to control deleterious inflammatory diseases.

Toll-like receptor (TLR) activation is essential for the development of innate immunity to pathogens (1, 2). However, repeated or prolonged activation of TLRs can render them insensitive or hyporesponsive to

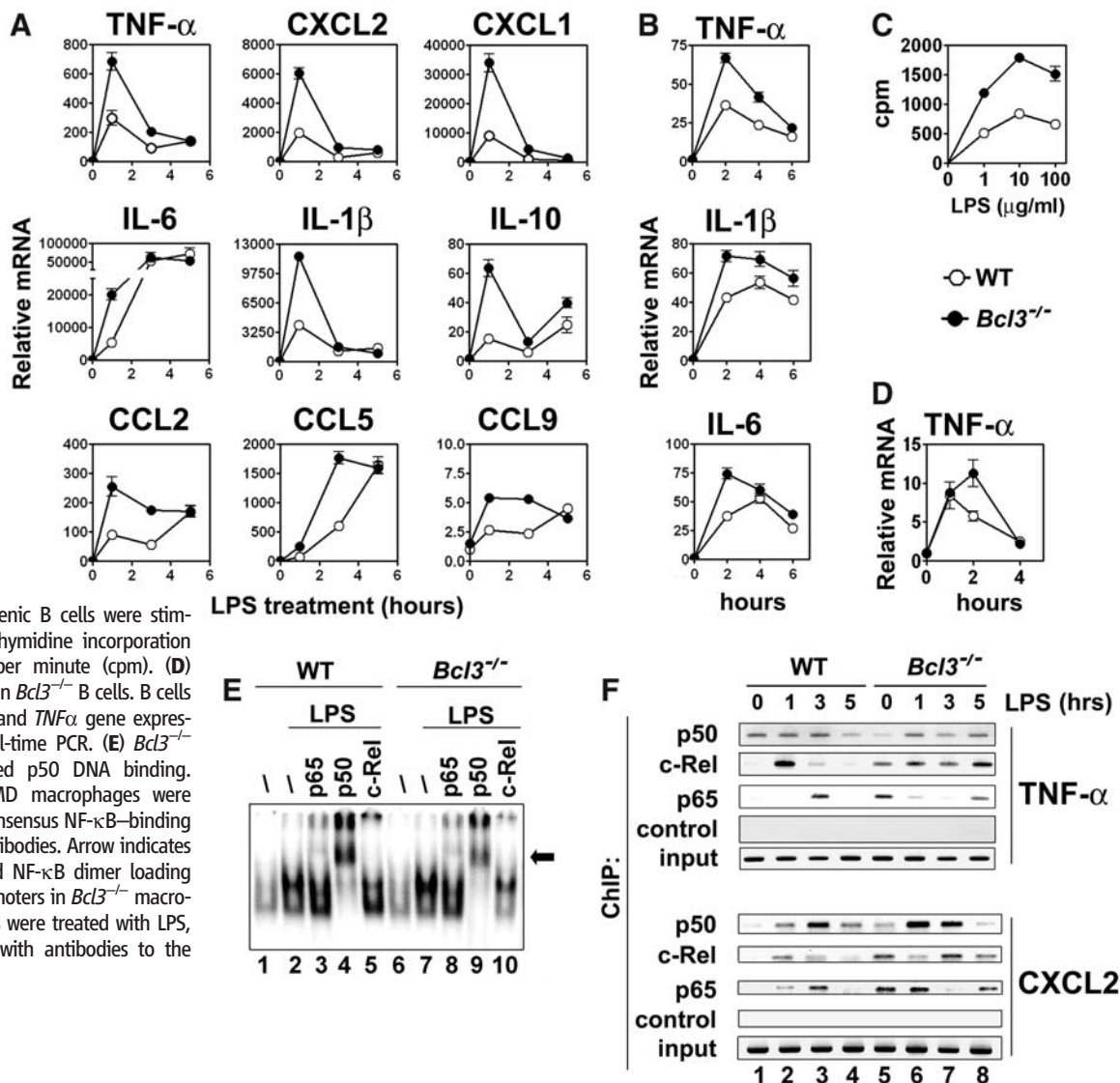
subsequent ligand stimulation. This phenomenon is referred to as TLR tolerance (3). The molecular mechanisms of TLR tolerance are not well understood, although several negative regulators of TLR signaling have been implicated

(4–10). Bcl-3 is a nuclear member of the inhibitor of NF- κ B (I κ B) family, which interacts exclusively with the transcriptionally inactive homodimers of p50 and p52, two members of the NF- κ B family (11–13). Bcl3 deficiency in mice disrupts the microarchitecture of lymphoid organs but does not affect the development of lymphoid or myeloid cells (14, 15). To explore the roles of Bcl-3 in immunity and tolerance, we examined the phenotype and function of Bcl3-deficient cells. We found that, although Bcl3 deficiency did not affect cell surface marker expression or phagocytotic function (fig. S1), Bcl3^{-/-} macrophages, dendritic cells, and B cells produced significantly more cytokines than wild-type (WT) cells upon stimulation with several TLR ligands (Fig. 1 and figs. S2 and S3).

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Fig. 1. Bcl-3 inhibits cytokine gene expression and controls NF- κ B dimer exchange at gene promoters. **(A)** Bcl3^{-/-} macrophages are hyperresponsive to LPS. Bone marrow-derived (BMD) macrophages were stimulated with LPS, and cytokine mRNA was quantified by real-time polymerase chain reaction (PCR) (25). Error bars indicate \pm SEM. **(B)** Bcl3^{-/-} dendritic cells are hyperresponsive to LPS. BMD dendritic cells were stimulated with LPS, and gene expression was measured by real-time PCR. **(C)** Enhanced proliferation of Bcl3^{-/-} B cells to LPS. Splenic B cells were stimulated with LPS, and ³H-thymidine incorporation was measured as count per minute (cpm). **(D)** Enhanced gene expression in Bcl3^{-/-} B cells. B cells were stimulated with LPS, and TNF α gene expression was measured by real-time PCR. **(E)** Bcl3^{-/-} macrophages have reduced p50 DNA binding. Nuclear extracts from BMD macrophages were tested by EMSA with the consensus NF- κ B-binding sequence and indicated antibodies. Arrow indicates p50 complexes. **(F)** Altered NF- κ B dimer loading and exchange at gene promoters in Bcl3^{-/-} macrophages. BMD macrophages were treated with LPS, and ChIP was performed with antibodies to the indicated factors.



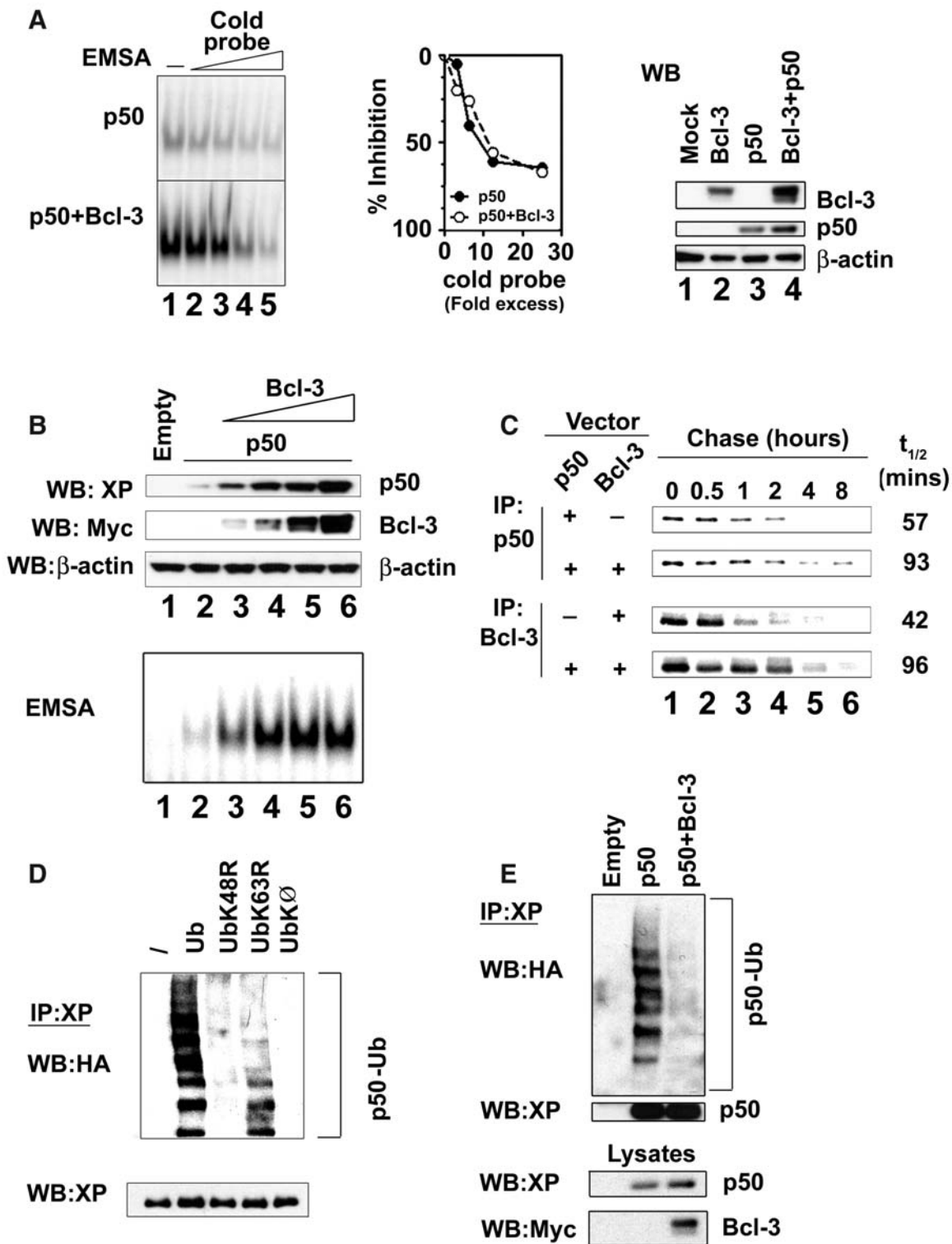
Additionally, *Bcl3*^{-/-} macrophages were also hypersensitive to interleukin (IL)-1 β and tumor necrosis factor (TNF)- α stimulation (fig. S3).

The hyperresponsiveness of *Bcl3*^{-/-} cells was not due to increased receptor-proximal signaling (fig. S4A) but could be blocked by a NF- κ B

inhibitor (fig. S3F). In response to lipopolysaccharides (LPS), *Bcl3*^{-/-} cells displayed a marked reduction in nuclear p50 DNA binding relative to WT controls (Fig. 1E), although nuclear translocations of p50, p65, and c-Rel were normal (fig. S4B). Chromatin immunoprecipitation

(ChIP) analysis revealed a similar reduction in p50 binding to promoters of *TNF α* and *CXCL2* (*chemokine C-X-C motif ligand 2*) genes in *Bcl3*^{-/-} cells (Fig. 1F). In unstimulated WT macrophages, *TNF α* and *CXCL2* promoters contained only the p50 subunit of NF- κ B, indicating the

Fig. 2. Bcl-3 inhibits p50 ubiquitination and degradation. **(A)** Bcl-3 increases p50 homodimer binding to DNA without altering its affinity. Human embryonic kidney (HEK) 293T cells were transfected with p50 and Bcl-3 expression plasmids. EMSA was performed with increasing amounts of unlabeled NF- κ B consensus oligonucleotides (cold probe) (left), and p50 homodimer DNA binding was measured by densitometry (middle). Relative protein levels were determined by immunoblotting of whole-cell extracts (right). WB, Western blot. **(B)** Increased p50 homodimer DNA binding is associated with increased amounts of p50 protein. HEK 293T cells were transfected with XP-p50 and increasing amounts of myc-Bcl-3 plasmids. p50 and Bcl-3 levels were measured in whole-cell lysates by immunoblotting (top), and p50 homodimer DNA binding was measured by EMSA (bottom). Empty, empty vector alone. **(C)** Bcl-3 increases the half-life of p50. HEK 293T cells were cotransfected with XP-p50 and empty vector or myc-Bcl-3, and the half-life ($t_{1/2}$) of proteins was determined (25). IP, immunoprecipitation. **(D)** p50 undergoes Lys⁴⁸ (K48) polyubiquitination. HEK 293T cells were transfected with XP-p50 and expression vectors encoding either WT, Lys⁴⁸ \rightarrow Arg⁴⁸ (K48R) mutant, Lys⁶³ \rightarrow Arg⁶³ (K63R) mutant, or lysine-less (K \emptyset) HA-tagged ubiquitin (Ub). Lysates were immunoprecipitated with antibody against XP and immunoblotted with antibody against HA. **(E)** Bcl-3 inhibits p50 ubiquitination. HEK 293T cells were transfected with XP-p50 and HA-ubiquitin with or without myc-Bcl-3. Ubiquitination was determined as in (D).



presence of p50 homodimer binding. After stimulation with LPS, p50 was transiently replaced by c-Rel and p65 dimers (Fig. 1F). Five hours after stimulation, both *TNF α* and *CXCL2* promoters reverted to the state of p50 occupancy (Fig. 1F). In contrast, unstimulated *Bcl3*^{-/-} macrophages lacked p50 homodimers on the *TNF α* and *CXCL2* promoters, which were instead occupied by p65, c-Rel, and p50 dimers (Fig. 1F). The order of p65 and c-Rel dimer exchange on both *TNF α* and *CXCL2* promoters was severely disrupted in LPS-stimulated *Bcl3*^{-/-} cells (Fig. 1F), indicating an essential role for Bcl-3 and p50 homodimers in regulating NF- κ B DNA binding and transcriptional output of target genes.

Although Bcl-3 has previously been reported to enhance p50 homodimer DNA binding (16, 17), the established picomolar dissociation constant of p50 homodimers suggests that any further increase in their affinity is unlikely to be significant (18, 19). In support of this, we observed no measurable differences in the binding affinity of

p50 homodimers in the presence or absence of overexpressed Bcl-3 despite a clear increase in p50 DNA binding in cells overexpressing Bcl-3 (Fig. 2A). Overexpressing Bcl-3 increased p50 protein levels in a dose-dependent manner, leading to increased p50 DNA binding, as shown in an electrophoretic mobility shift assay (EMSA) (Fig. 2B). Pulse chase analysis demonstrated that the half-life of p50 protein (57 min) was almost doubled in cells overexpressing Bcl-3 (93 min) (Fig. 2C). These results indicate that Bcl-3 increases p50 DNA binding by extending the half-life of p50 rather than by enhancing its affinity to DNA.

To determine the potential roles of Lys⁴⁸-mediated ubiquitination pathway in the turnover of p50 homodimers, we examined the polyubiquitination of both overexpressed and endogenous p50. Overexpressed p50 underwent constitutive Lys⁴⁸-mediated polyubiquitination, which was dramatically inhibited by Bcl-3 (Fig. 2E). Significant ubiquitination of endogenous p50 was detected only after LPS stimulation, which

was markedly increased in *Bcl3*^{-/-} macrophages (Fig. 3A). The increased ubiquitination of p50 in *Bcl3*^{-/-} cells led to a fourfold reduction in its half-life (63 min versus 240 min in WT cells) (Fig. 3B). Importantly, *Bcl3* deficiency did not affect the half-life of p65, c-Rel, or the p50 precursor, p105. Mutation of Tyr⁵⁷ and Gly⁶⁰ in the DNA binding domain of p50 (20) gave rise to a DNA binding-defective mutant, p50^{Y57A,G60D}, that was unable to bind to DNA (Fig. 3C) but retained the ability to interact with p65 and Bcl-3 (fig. S5). Ubiquitination of p50^{Y57A,G60D} was dramatically reduced compared with that of WT p50 (Fig. 3D). This was associated with a significant increase in p50^{Y57A,G60D} half-life (Fig. 3E). Taken together, these results establish that p50 homodimer binding to DNA triggers its polyubiquitination and degradation, which are effectively blocked by Bcl-3.

We next examined whether Bcl-3 played a role in TLR tolerance, because p50 homodimers have previously been implicated in this process (21). WT and *Bcl3*^{-/-} macrophages were treated with LPS for 24 hours (to induce tolerance), rested, and restimulated with LPS. Pretreatment of WT cells with LPS induced tolerance, characterized by reduced cytokine gene expression upon restimulation (Fig. 4A). By contrast, LPS pretreatment of *Bcl3*^{-/-} macrophages not only failed to repress cytokine gene expression but significantly increased *IL6* and *CCL2* expression upon restimulation (Fig. 4A). *Bcl3* deficiency did not significantly alter the expression of other negative regulators of TLRs or receptor-proximal signals in tolerized cells (fig. S6). However, like IRAK-M (IL-1 receptor-associated kinase-M), SOCS-1 (suppressor of cytokine signaling 1), and A20, Bcl-3 was significantly up-regulated in tolerized macrophages (fig. S6). As expected, Bcl-3 knockdown by RNA interference diminished LPS tolerance (fig. S7), whereas Bcl-3 overexpression significantly inhibited *TNF α* promoter activity (fig. S8). Re-stimulation of tolerized WT macrophages with LPS led to p50 homodimer binding to the *TNF α* promoter, whereas in *Bcl3*^{-/-} macrophages restimulation led to p65 dimer binding (Fig. 4B). Thus, Bcl-3 mediates LPS tolerance by stabilizing the p50 homodimer on the *TNF α* promoter and by preventing the binding of transcriptionally active p65 dimer.

To determine the roles of Bcl-3 in vivo, we studied TLR tolerance in bone marrow chimeric mice that did or did not express Bcl-3 in their hematopoietic cells. Mice were first tolerized with low doses of LPS and challenged with increasing doses of LPS. LPS pretreatment protected all mice that received WT bone marrow from septic shock. By contrast, the vast majority of mice that received *Bcl3*^{-/-} bone marrow died of the disease a few days after LPS challenge (Fig. 4C).

Taken together, these results establish that Bcl-3 promotes p50 homodimer occupancy of target gene promoters by inhibiting the ubiquitination and subsequent degradation of DNA-bound p50 homodimers. We propose that this state of Bcl-3-p50 homodimer-mediated pro-

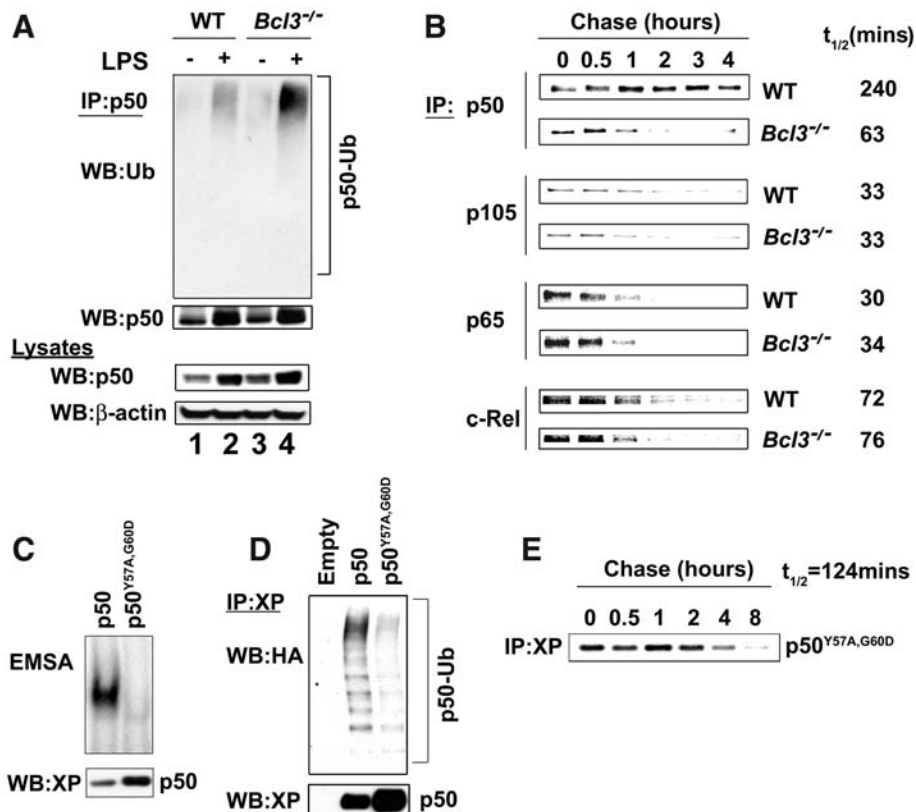


Fig. 3. *Bcl3*^{-/-} macrophages have increased p50 ubiquitination and degradation. (A) Increased ubiquitination of p50 in *Bcl3*^{-/-} macrophages. BMD macrophages were treated with (+) or without (-) LPS for 16 hours. Equal amounts of protein were immunoprecipitated with antibody against p50 and immunoblotted with antibody against ubiquitin. (B) Reduced p50 half-life in *Bcl3*^{-/-} macrophages. BMD macrophages were treated with LPS, pulse-labeled with ³⁵S-methionine cysteine, and tested as in Fig. 2C. (C) Generation of a p50 mutant that does not bind to DNA. HEK 293T cells were transfected with XP-p50 or an XP-p50 mutant containing Lys⁵⁷→Ala⁵⁷ (Y57A) and Gly⁶⁰→Asp⁶⁰ (G60D) substitutions in the DNA binding domain. EMSA was performed with the consensus NF- κ B binding sequence. (D) The p50 mutant is resistant to ubiquitination. Cells were transfected with HA-ubiquitin plus XP-p50, XP-p50^{Y57A,G60D}, or empty vector. Ubiquitination was determined as in Fig. 2D. (E) The p50 mutant has a significantly increased half-life. HEK 293T cells were co-transfected with XP-p50 or XP-p50^{Y57A,G60D}, and protein half-life was determined (25).

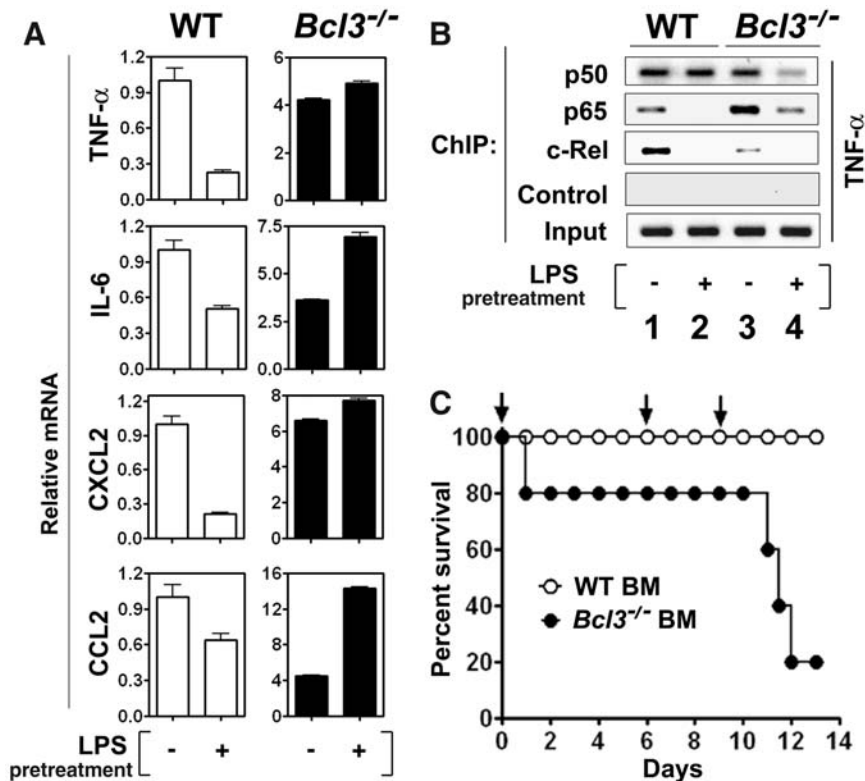


Fig. 4. *Bcl3* deficiency in mice and macrophages abolishes LPS tolerance. (A) Lack of LPS tolerance in *Bcl3*^{-/-} macrophages. BMD macrophages were pre-treated with (+) or without (-) LPS for 24 hours. After 1 hour of resting, cells were restimulated with LPS for an additional hour. mRNA levels were determined by real-time PCR. (B) Reduced p50 homodimer binding to *TNFα* promoter in *Bcl3*^{-/-} macrophages under tolerizing conditions. BMD macrophages were treated as in (A). ChIP was performed with antibodies to p50, p65, and c-Rel. (C) *Bcl3* deficiency in hematopoietic cells renders mice hypersensitive to septic shock. WT mice were lethally irradiated and reconstituted with either WT or *Bcl3*^{-/-} bone marrow (BM) cells (*n* from 3 to 5) (25). Eight weeks later, chimeric mice were tolerized with two consecutive injections of low dose LPS (5 mg/kg on day -5 and 10 mg/kg on day -3) and then challenged with three high doses of LPS (15 mg/kg on day 0, 30 mg/kg on day 6, and 90 mg/kg on day 9 as indicated by arrows). Data shown are survival curves of the two groups. The difference between the two groups is statistically significant (*P* < 0.01). Error bars indicate ± SEM.

motor hyporesponsiveness is the molecular basis of TLR tolerance. Neither Bcl-3 nor p50 alone is sufficient to maintain the tolerant state of gene promoters (Fig. 4) (22). In the absence of Bcl-3-p50 complex, the loading of NF-κB subunits on target promoters and the subsequent dimer exchange, critical for appropriate gene expression (23, 24), are disrupted, leading to aberrant expression of inflammatory cytokines. Thus, TLR tolerance and suppression are dependent on the coordinated action of both the inhibitor p50 and its stabilizer, Bcl-3 (fig. S9 and SOM text). These findings provide important insights into the molecular mechanisms of TLR signaling and suggest that deleterious inflammatory responses can be effectively controlled by targeting the NF-κB p50 ubiquitination pathway.

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25. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material
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 Materials and Methods
 SOM Text
 Figs. S1 to S9
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Immune-like Phagocyte Activity in the Social Amoeba

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Social amoebae feed on bacteria in the soil but aggregate when starved to form a migrating slug. We describe a previously unknown cell type in the social amoeba, which appears to provide detoxification and immune-like functions and which we term sentinel (S) cells. S cells were observed to engulf bacteria and sequester toxins while circulating within the slug, eventually being sloughed off. A Toll/interleukin-1 receptor (TIR) domain protein, TirA, was also required for some S cell functions and for vegetative amoebae to feed on live bacteria. This apparent innate immune function in social amoebae, and the use of TirA for bacterial feeding, suggest an ancient cellular foraging mechanism that may have been adapted to defense functions well before the diversification of the animals.

Phagocytes that engulf bacteria, first described by Metchnikoff in 1883, form part of the innate immune system of animals in the defense against pathogens (1–4). Both plants and animals also use innate signaling

pathways as a means of sensing microbial pathogens; mainly through Toll-like receptors (TLRs) in animals and resistance (R) proteins in plants (5, 6). Both TLRs and R proteins bind to bacterial elicitors through leucine-rich repeats (LRRs) and

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