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Induction of RelB Participates in Endotoxin Tolerance

Barbara K. Yoza, Jean Y.-Q. Hu, Sue L. Courset, Lolita M. Forrest, and Charles E. McCall

Using a THP-1 human promonocyte model of endotoxin tolerance that simulates the sepsis leukocyte phenotype, we previously showed that tolerant cells remain responsive to LPS endotoxin with degradation of IκB in the cytosol and nuclear translocation and accumulation of p50 and p65 NF-κB transcription factors. Despite this, endotoxin-inducible NF-κB-dependent innate immunity genes, like IL-1β, remained transcriptionally unresponsive in the tolerant phenotype, similar to the endotoxin tolerance observed in sepsis patients. In this study, we examined this paradox and found that RelB, another member of the NF-κB family, is induced during the establishment of tolerance. RelB expression correlated with IL-1β repression, and sepsis patients showed increased RelB when compared with normal controls. Transient expression of RelB inhibited IL-1β in endotoxin-responsive cells. In the inverse experiment, small inhibitory RNAs decreased RelB expression in tolerant cells and restored endotoxin induction of IL-1β. When we examined tolerant cell extracts, we found transcriptionally inactive NF-κB p65/RelB heterodimers. Taken together, our findings demonstrate that RelB can repress proinflammatory gene expression, and suggest that RelB expression in sepsis patient blood leukocytes may play a role in the endotoxin-tolerant phenotype. The Journal of Immunology, 2006, 177:4080–4085.

Human sepsis is associated with an immunosuppressed phenotype that involves both innate and adaptive immunity and that is associated with a poor clinical outcome (1). NF-κB plays an essential, albeit controversial role in sepsis (2–6). Although the expression of many NF-κB-dependent genes, like IL-1β and TNF-α, are repressed in leukocytes from patients with septic shock (1), several studies have demonstrated elevated levels of p65 in sepsis nuclei (6, 7). Indeed, elevated levels of p65, the transcription-activating subunit of NF-κB, correlate well with poor outcomes in these patients (2, 3). Animal and tissue culture models also show increased nuclear p50 levels, and formation of p50 homodimers is promoted by some investigators as a mechanism for transcription repression during endotoxin tolerance (8–10). Other studies (11) find no such connection.

Several lines of evidence also revealed that down-regulation of NF-κB signaling pathways, for example IL-1R-associated kinase (IRAK)3 (12–16) and IκB kinase, may significantly impact LPS response in the tolerant phenotype (17, 18). These dysfunctions, characteristic of LPS tolerance, may have developed as a mechanism to fine-tune the balance between a pro- and anti-inflammatory response that would ensue from the initial encounter with an infectious agent (19, 20). Many studies have found that expression of some anti-inflammatory genes is sustained during sepsis and LPS tolerance, and the physiological consequence of this immunosuppressed state continues to be explored (21–26).

We reported that cytosolic NF-κB activation occurs in the human LPS-tolerant phenotype, as evidenced by LPS-mediated IκB degradation (27), nuclear translocation of NF-κB p50 and p65, and formation of DNA-binding competent NF-κB complexes, as assessed by EMSAs (28). Two notable defects in LPS-mediated response were observed in tolerance when compared with normal cells. In examining LPS-dependent TLR4 signaling pathways, we found that IRAK1 was inactivated, degraded, and absent in tolerant cells (29–31). In examining LPS repression of the IL-1β gene, chromatin immunoprecipitation (ChIP) studies revealed that the chromatin structure at or near the IL-1β TATA box was altered and in a transcriptionally inactive state (32, 33). To further understand this latter phenomenon, we also found that p50 did not bind to the NF-κB site within the IL-1β promoter, although inducible and present in tolerant nuclei (32). This finding was in contrast to our findings that p65 NF-κB complexes could bind in EMSA (28) and highlights the differences in vitro (EMSA) and in vivo (ChIP) DNA-binding assays.

In this study, we sought an explanation to the increased levels of p65 in the nucleus of tolerant cells and the inability of p50 to access the IL-1β promoter in vivo and activate transcription. We tested the hypothesis that RelB may be induced as a negative feedback mechanism for repressing NF-κB transcription during LPS tolerance. We find increased expression of RelB that is coincident with the establishment of LPS tolerance. Overexpression of RelB significantly reduces LPS-induced IL-1β expression in naive cells. We also show that decreased expression of RelB by RelB small interfering RNA (siRNA) can restore endotoxin induction of IL-1β expression in tolerant cells. Finally, we find that blood leukocytes from sepsis patients expressed RelB, whereas normal healthy controls did not. Taken together, our findings suggest that RelB expression, as a negative feedback, may provide a necessary and sufficient molecular mechanism involved in the repression of NF-κB-dependent innate immunity genes observed in LPS tolerance.

Abbreviations used in this paper: IRAK, IL-1R-associated kinase; ChIP, chromatin immunoprecipitation; DC, dendritic cell; PMN, polymorphonuclear blood leukocyte; siRNA, small interfering RNA.

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Abbreviations used in this paper: IRAK, IL-1R-associated kinase; ChIP, chromatin immunoprecipitation; DC, dendritic cell; PMN, polymorphonuclear blood leukocyte; siRNA, small interfering RNA.
Materials and Methods

Cell culture and stimulation

THP-1 cells obtained from the American Type Culture Collection were maintained in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10 U/ml penicillin G, 10 μg/ml streptomycin, 2 mM L-glutamine, and 10% FCS (low LPS FCS; Sigma-Aldrich) at 37°C and 5% CO2 in a humidified incubator. LPS-tolerant THP-1 cells were made and are characterized in detail, as described previously (27). Briefly, THP-1 cells were rendered LPS tolerant by preculture for 16 h with LPS (1.0 μg/ml Gram-negative LPS (Escherichia coli 0111:B4; Sigma-Aldrich)). Normal (no preculture) and tolerant THP-1 cells were washed with FCS-free RPMI 1640 medium, then resuspended in FCS-supplemented RPMI 1640 medium at 1 × 106 cells/ml and stimulated with LPS (1.0 μg/ml) for the times as indicated in the figure legends. Nonspecific and RelB siRNAs (Santa Cruz Biotechnology) were transfected using an AMAXA THP-1 transfection kit, according to the manufacturer’s protocol. pcDNA3-HA (Santa Cruz Biotechnology) were transfected using an AMAXA THP-1 transfection kit, according to the manufacturer’s protocol.

PCR using TaqMan methodology and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Real-time PCR were performed in 25 μl of a mixture containing 1 μl of cDNA (1 μg), 300 nM IL-1β primers, 100 nM FAM/TAMRA TaqMan probe, and 12.5 μl of TaqMan Universal PCR Master Mix, containing AmpliTaq Gold DNA polymerase, reaction buffer, and dNTP mix (Applied Biosystems). Results from at least three independent experiments were quantitated by continuous measurement during 40 cycles. All data were normalized to GAPDH quantitated in parallel samples (Applied Biosystems).

Peripheral blood leukocyte, polymorphonuclear cell blood leukocyte (PMN), and PBMC isolation

The selection of patients was based on a modified criteria of Bone (35), as previously described (36). Using these criteria, 95% of patients reproducibly have reduced IL-1β production in response to LPS (23, 36). Patients enrolled in this study were positive for Gram-positive and/or Gram-negative sepsis. The patient without sepsis was a trauma patient with pulmonary contusion. Patients with known HIV infection, hematological malignancies affecting leukocyte counts, current cytotoxic chemotherapy, and high-dose glucocorticoid treatment were excluded. The Internal Review Board endorsed the study for Clinical Research associated with the General Clinical Research Center of the Medical Center. A venous or arterial heparinized blood sample (up to 30 ml) was drawn from patients within 1–4 days after the onset of sepsis or from normal healthy controls and immediately processed. PBL were isolated by Isolymph sedimentation (Gallard-Schlesinger), followed by centrifugation for 5 min at 200 × g at room temperature. The remaining erythrocytes were lysed in LPS-free H2O for 20 s before isotonicity was restored with 3.6% NaCl. Isolated PBL were washed and counted in PBS. PBL whole cell lysates were immediately prepared in SDS-Tris sample solubilization/loading buffer (reducing) and stored at −20°C until further use. PMN and PBMC were isolated, as previously described (23, 27). The proportions of PMN in PBL vary in sepsis (85–95%) when compared with normal (60–80%). In two patients, one without sepsis and one with sepsis, PBL were further separated into PMN and PBMC by a second centrifugation over 3 ml of Isolymph for 30 min at 400 × g. The PMN pellet and PBMC interface were washed and counted in PBS, and lysates were immediately prepared in SDS-Tris sample solubilization/loading buffer (reducing) and stored at −20°C until further use in Western blot analysis, as described below.

Immunoprecipitation and Western blot analysis

Whole cell and nuclear extracts (28) were prepared, using the protocols described previously. Whole cell and nuclear extract proteins (50 μg) were separated by SDS-PAGE and transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad). RelB, B cell lymphoma protein 2 (Bcl-2), β-actin, IκB (α), aggresome-conjugated p50 and p65 NF-κB Abs, and appropriate HRP-conjugated secondary Abs (Santa Cruz Biotechnology) were used to immunoprecipitate and/or visualize proteins using Western blot protocols, as specified by the manufacturer. Blots were stripped and reprobed with control Abs (Bcl-2, β-actin), as indicated. Protein levels on the blots were quantitated using ImageQuant software (Amersham Biosciences).

Real-time PCR analysis

Total RNA was isolated from 1 × 106 cells/condition using RNA STAT-60 (Tel-Test), according to the manufacturer’s instructions. cDNA was reverse transcribed in a final volume of 20 μl containing 1 μg of total RNA, 10× PCR buffer, 200 μM deoxy nucleotides, 1 μM oligo(dT), 2 mM MgCl2, and 2.5 U of murine leukemia virus reverse transcriptase (Applied Biosystems) for 1 h at 42°C. IL-1β mRNA was quantitated by real-time PCR using TaqMan methodology and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Real-time PCR were performed in 25 μl of a mixture containing 1 μl of cDNA (1 μg), 300 nM IL-1β primers, 100 nM FAM/TAMRA TaqMan probe, and 12.5 μl of TaqMan Universal PCR Master Mix, containing AmpliTaq Gold DNA polymerase, reaction buffer, and dNTP mix (Applied Biosystems). Results from at least three independent experiments were quantitated by continuous measurement during 40 cycles. All data were normalized to GAPDH quantitated in parallel samples (Applied Biosystems).

Statistical analysis

Statistical analysis of data is presented as the average of at least three independent experiments ± SEM. Analyses were performed after consultation with a statistician using Student’s t test to determine significant changes (p ≤ 0.05) using Microsoft Excel 2002 statistical analysis software, as indicated.

Results

RelB is expressed in LPS-tolerant cells

We first determined the time course of RelB expression in the THP-1 human promonocyte model of LPS tolerance. We found that LPS-tolerant cells express increased levels of RelB, as shown in Fig. 1A. We next examined nuclear extracts for RelB expression and found low levels of RelB in normal cells that is increased in tolerant cell nuclei. Thus, the appearance and localization of RelB are consistent with a potential role in LPS tolerance. We also compared sepsis PBL with normal PBL (Fig. 1B) and also found increased RelB in sepsis patients’ PBL, further supporting our hypothesis that RelB may mediate LPS tolerance. To further associate RelB expression with LPS tolerance, PMN and PBMC were isolated from two patients, without (Fig. 1C, P4) and with sepsis (Fig. 1C, P5). Consistent with our previous results, RelB was found only in the patient with sepsis.

Expression of RelB in normal cells mimics tolerance and inhibits IL-1β expression

We and others had observed that tolerant cells do not respond to LPS with an immediate and robust induction of proinflammatory cytokines.
genes. Paradoxically, we found that LPS-tolerant cells still showed LPS-stimulated \(\text{I\kappa B}\) degradation and p50/p65 nuclear translocation, despite the continued repression of IL-1\(\beta\) expression. Our recent ChIP studies provided a partial explanation for this discrepancy, in which we found that p65 was unable to bind the IL-1\(\beta\) promoter in tolerant cells (32). Given the correlation between the appearance of RelB and transcription inhibition, we next tested the hypothesis that RelB mediated decreased expression of IL-1\(\beta\).

RelB-transfected THP-1 cells were transfected with a human RelB expression vector (Bren and colleagues (34)) or empty vector (control). As shown in Fig. 2A, cells transfected with RelB showed increased levels of RelB protein compared with cells transfected with the empty vector. Levels of transfected RelB were comparable to RelB protein levels in tolerant cells (Fig. 2B). RelB-transfected or control cells were stimulated with LPS and IL-1\(\beta\) mRNA measured by real-time PCR at 1 and 3 h after stimulation. In these experiments (Fig. 2C), we found that RelB expression decreased IL-1\(\beta\) mRNA levels after LPS stimulation. Control cells (empty vector) showed the rapid and transient expression of IL-1\(\beta\) mRNA that is typical of the immune response to LPS. In contrast, RelB-expressing cells showed a significant decrease in IL-1\(\beta\) expression that is characteristic of LPS tolerance. Thus, this experiment supports the hypothesis that RelB can repress LPS-induced expression of IL-1\(\beta\) and is consistent with a role in the LPS-tolerant phenotype. We further examined whether expression of NF-\(\kappa B\) p65 might overcome the LPS tolerance induced by RelB. As shown in Fig. 2D, p65 was able to overcome RelB-mediated suppression.

**Gene silencing of RelB by siRNA can reverse LPS tolerance**

The introduction of siRNA as a molecular tool has developed into a powerful technique for selective gene silencing (reviewed in Ref. 37). We used RelB-specific siRNA to test whether the knockdown of RelB protein levels in tolerant cells could restore LPS responsiveness and increase IL-1\(\beta\) mRNA. As shown in Fig. 3. intro-duction of RelB siRNA significantly reduced RelB protein levels in tolerant cells. This decrease persisted even with LPS stimulation. In contrast, control siRNA still showed the high levels of RelB associated with LPS tolerance.

Given the demonstration of significantly reduced RelB levels by RelB-specific siRNA, we further asked whether this gene silencing could restore LPS response and induce IL-1\(\beta\) expression, i.e., reverse the LPS-tolerant phenotype. Fig. 4A shows the decreased LPS response to induction of IL-1\(\beta\) that is a salient feature of LPS-tolerant cells (compare control \(\pm\) LPS with tolerant \(\pm\) LPS). We found that introduction of control siRNA still showed this repressed LPS response and decreased IL-1\(\beta\) expression in tolerant cells (Fig. 4B). In contrast, RelB siRNA was able to reverse the repression and near normal levels of IL-1\(\beta\) mRNA were induced by LPS in RelB knockdown tolerant cells (Fig. 4C). These data strongly support a role for RelB in mediating LPS tolerance.

**RelB inhibition of LPS-stimulated immune response**

Studies in relb\(^{-/-}\) mice implicate an important role for RelB in limiting multiorgan inflammation and showed that RelB is a key transcriptional regulator in suppressing proinflammatory cytokine expression.

**FIGURE 2.** RelB expressed in normal cells mimics tolerance and inhibits IL-1\(\beta\) expression. A, A RelB expression vector (HA-RelB) was transfected into THP-1 cells, as described in Materials and Methods. The empty vector was used as a control. RelB levels in expression vector- and empty vector-transfected cells were assessed by immunoblot, as shown on the right. RelB levels in tolerant THP-1 cells are shown on the left for comparison. B, RelB protein levels in transfected cells were quantitated on Western blot, as described in Materials and Methods. Increased RelB levels in transfected cells are the average of two experiments \(\pm\) SEM; RelB levels in normal and tolerant cells (nontransfected) are shown for comparison. C, RelB (\(\square\)) and empty vector (\(\square\)-transfected cells were stimulated with LPS and total RNA isolated at various times after stimulation. IL-1\(\beta\) and GAPDH mRNA were measured by real-time PCR, as described in Materials and Methods. Relative IL-1\(\beta\) mRNA levels are normalized to GAPDH mRNA and are shown as the average (\(\pm\) SEM) for each of three independent experiments. D, RelB (\(\square\)), empty vector (\(\square\))-, or RelB + NF-\(\kappa B\) p65 (\(\square\))-transfected cells were stimulated with LPS and total RNA isolated at various times after stimulation. IL-1\(\beta\) and GAPDH mRNA were measured by real-time PCR, as described in Materials and Methods. Relative IL-1\(\beta\) mRNA levels are normalized to GAPDH mRNA in each sample. Data shown are representative of two independent experiments, with mRNA measured in duplicate for each experiment.
expression (38). In particular, RelB was implicated in delimiting the LPS response in relb−/− fibroblasts and, in the absence of RelB, IL-1β was overexpressed (39). This study revealed that relb−/− mice have lowered levels of IκB and, consequently, augmented NF-κB activation and enhanced expression of a number of proinflammatory cytokines and chemokines. As we had previously implicated IκB as a labile transcription inhibitor in LPS tolerance (27), we first tested whether RelB knockdown (i.e., RelB siRNA-transfected) cells, like relb−/− cells, had lowered levels of IκB, and whether this decrease could provide a mechanism for enhanced IL-1β and the reversal of tolerance in knockdown cells. However, as shown in Fig. 3A, we did not observe a decrease in IκB levels in RelB knockdown cells.

Alternatively, RelB has also been shown to have differential transcription activity dependent on interaction with variable cofactors. A number of NF-κB (34, 40) and non-NF-κB proteins (41, 42) have been implicated in this role. In particular, several studies suggest that p65/RelB heterodimers are unable to bind DNA and, as such, form transcriptionally inactive complexes (43, 44). As shown in Fig. 5, we examined immunoprecipitates from tolerant THP-1 cells for formation of p65 and RelB complexes. In these experiments, we found RelB associated with p65, suggesting that repressed transcription of IL-1β observed in tolerant cells is due, at least in part, to inactive NF-κB.

**Discussion**

**RelB as a mediator of immunosuppression**

In this study, we show a novel role for RelB in the transcription-repressed phenotype of LPS-tolerant cells. We found that RelB expression is induced as part of a primary NF-κB response to inflammation, which includes IκB degradation, NF-κB nuclear translocation, and transcription of proinflammatory genes as immediate early responses. Increased levels of RelB were observed after this initial wave of proinflammatory response and are consistent with the observation that induction of RelB itself is NF-κB-dependent. Additionally, RelB has been shown to have differential transcription activity dependent on interaction with variable cofactors, such as p65/RelB heterodimers, which are unable to bind DNA and form transcriptionally inactive complexes. As shown in Fig. 5, our experiments revealed RelB associated with p65, suggesting that RelB's role in transcription repression is due, at least in part, to its association with non-NF-κB proteins.
Regulation of RelB

The canonical NF-κB pathway is largely dependent on IκB kinase β to promote phosphorylation and degradation of IκB and translocation of NF-κB to the nucleus (48). More recently, an alternative pathway to NF-κB activation has been proposed, in which IκB kinase α and NF-κB-inducing kinase play a more essential role and in which nuclear localization of RelB regulates the expression of a subset of chemokines involved in spleen development (49). In addition to nuclear localization, RelB is regulated by phosphorylation (50) and dimerization (49, 51). The roles that these pathways may play in the establishment of the tolerant phenotype remain to be elucidated.

The LPS-induced RelB, acting as a negative feedback loop to curtail an overwhelming and potentially devastating inflammatory response, can be implicated in the sustained LPS-tolerant phenotype that is observed during the course of sepsis (3–8). Consistent with this model, RelB was constitutively elevated in the sepsis patient leukocytes that we examined. More detailed temporal studies of sepsis patients will be required to assess whether RelB expression correlates with the onset of the immunosuppressed phenotype and whether RelB is lost when the disease state is resolved and immunoresponsiveness is restored. Sepsis is a complicated and not very well-understood syndrome that will require further investigations to examine the participation of RelB in this immune response.

RelB as a mediator of innate and adaptive immunity

Although mechanisms of NF-κB activation are relatively well understood, the specificity and diversity of transcription control imparted by the activity of this family of proteins continue to be discovered. Given the central role these proteins play in inflammatory processes, we and others (for a review, see Ref. 52) have focused on NF-κB proteins in immune response and disease. In particular, we have studied the immunosuppressed state associated with LPS tolerance that is related to clinical immunosuppressed state observed in sepsis. Our finding of a role for RelB in immunosuppression is consistent with the experimental findings in reBl-deficient mice that showed increased expression of proinflammatory cytokines and an increased susceptibility to opportunistic infection (53). RelB-deficient mice also show a generalized multorgan inflammation (54, 55). More recently, it has been demonstrated that RelB plays a unique role in the transition from immediate early innate immune responses to that of more prolonged adaptive immune responses. In particular, RelB is thought to be a critical regulator of dendritic cell (DC) maturation that assures a functional transition from innate to adaptive immunity (56). RelB-deficient mice show a dramatic decrease in thymic and splenic DCs and are impaired in Ag-presenting function and cellular immunity (57). In this study, our results extend this regulatory role and suggest that RelB is essential to an immune response program. Within innate immunity, RelB may down-regulate acute inflammation; as an activator, RelB facilitates the maturation of DCs that are necessary in Ag presentation, T cell activation, and acquisition of adaptive immunity (58).

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Disclosures

The authors have no financial conflict of interest.

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