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NFκB2/p100 Is a Key Factor for Endotoxin Tolerance in Human Monocytes: A Demonstration Using Primary Human Monocytes from Patients with Sepsis

Carolina Cubillos-Zapata,*†,† Laura Esteban-Burgos,*† Irene Fernández-Ruiz,*† Vanesa Gómez-Piña,*† Carlos del Fresno,† María Siliceo,*† Patricia Prieto-Chinchina,‡ Rebeca Pérez de Diego,†,‖ Lisardo Boscá,†,‖ Manuel Fresno,* Francisco Arnalich,*† and Eduardo López-Collazo,*†

Endotoxin tolerance (ET) is a state of reduced responsiveness to endotoxin stimulation after a primary bacterial insult. This phenomenon has been described in several pathologies, including sepsis, in which an endotoxin challenge results in reduced cytokine production. In this study, we show that the NFκB2/p100 pathway might be crucial for the development of the ET refractory state (4, 13). Along these lines, previous findings suggested a potential role for NFκB2/p100 in the ET of human monocytes further indicated that NFκB2/p100 expression is a crucial factor in the progression of ET. The monocytes derived from patients with sepsis had high levels of NFκB2/p100, and a downregulation of NFκB2/p100 in these septic monocytes reversed their ET status.

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NFκB2 IS A KEY FACTOR FOR ENDOTOXIN TOLERANCE IN MONOCYTES

μmol/L), HIV/AIDS, hepatitis B or C, pregnancy, and age >80 y. All procedures were in accordance with the Helsinki Declaration of 2000, and informed consent was obtained from all participants. This study was approved by the La Paz Hospital Ethics Committee.

Reagents

The following Abs were used: anti-CD14 aliphophycocyanin (Immunostep), anti-CD16b, anti-CD1a, and anti-CD89 (Serotec), and anti-p50, anti-p100/ p52, and anti-actin (Cell Signaling). The medium used for the cell culture was DMEM from Invitrogen. The LPS from Salmonella abortus was a gift of C. Galanos (Max Planck Institut für Immunobiologie, Freiburg, Germany).

Isolation and culture of human monocytes

Mononuclear cells from peripheral blood were isolated from the buffy coats. The monocytes were obtained by Percoll-Plus gradient (GE Healthcare Bio-Sciences), as previously described (8). The purity of the monocyte cultures was tested by CD14 labeling and flow cytometry analysis (average 89% of CD14-positive cells). Other cell surface markers were also tested (CD89, 90%; CD1a, 5.2%; CD16b, 5.1%; see Supplemental Fig. 1). The same protocol was used to obtain the monocytes from all of the patients. All of the reagents used for the cell culture were endotoxin free, as assayed with the Limulus amebocyte lysate test (Cambrex).

The workflow to establish the ET model was as follows: The monocytes were treated with 10 ng/ml LPS during the time of tolerization (8 h). After LPS treatment, the cells were washed three times with PBS and kept in complete medium for various times during the recovery phase. Then cells were restimulated with 10 ng/ml LPS for time periods ranging from 1 to 24 h. The control cells were not treated with LPS during the tolerization and/or the restimulation phase.

RNA isolation

The cells were washed once with PBS, and the RNA was isolated using the High Pure RNA Isolation Kit (Roche Diagnostics). The cDNA was obtained by reverse transcription of 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

mRNA quantitation

Gene expression levels were analyzed by real-time quantitative PCR using the LightCycler system (Roche Diagnostics) and cDNA obtained, as described above. Real-time quantitative PCRs were performed using the Quantimix Easy SYG kit from Biotools and specific primers. Results were normalized to the expression of the β-actin (actin), and the cDNA copy number of each gene of interest was determined using a 7-point standard curve, as described previously (4, 5, 7, 20, 21).

The products were amplified using primers for TNF-α, 5′-GCC TCT CCT TCC TTC TCA TGG TAC T3′ (forward) and 5′-CGT GGA AAA GTC GAG ATG GTC GAT GTC G3′ (reverse); IL-1β, 5′-GGA TAT GGA GCA GCA ACA AGT GG-3′ (forward) and 5′-ATT CAC TAT CAG TGG GAC AAT TCT G3′ (reverse); I-κBα, 5′-GAT CTC AGT GCA GAG GCT G-3′ (forward) and 5′-ATT CTT GGG TTG TGG AGT GAG TGT TCA-3′ (reverse); chemokine ligand 18 (CCL18), 5′-CCG TCT GTC TCT GCT TGG GCT-3′ (forward) and 5′-GTC CTA GTG CCC TCT ATT-3′ (reverse); and p100, 5′-TAC CGA CAG CAG ACC TCA C-3′ (forward) and 5′-CTT CAG CAC CCT ATC T-3′ (reverse). All primers were synthesized, desalted, and purified by Bionsa Biotech.

Flow cytometry analysis

For the surface marker staining, the cells were labeled with the following mAbs: anti-CD14 aliphophycocyanin (Immunostep), and anti-CD1a FITC, anti-CD16b FITC, and anti-CD89 FITC (Serotec). Matched isotype Abs were used as negative controls. The cells were incubated in the dark for 30 min at 4°C. The data were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences). The data were analyzed with FlowJo software (Tristar).

Cytometric bead array

The cytokine levels in the culture supernatants from the human samples were determined using the cytometric bead array (CBA) Flex Set (BD Biosciences) following the manufacturer’s protocol. Supernatants from the murine cultures were evaluated using the CBA Mouse Inflammation kit (BD Biosciences). The data collected were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences).

Western blot

The monocyte cultures were harvested and washed with ice-cold PBS containing 2 mM PMSF and 2 mM CaCl2 (pH 7.4). They then were lysed by sonication (Micronase Heat System) in a solubilization buffer containing protease inhibitors (200 μg/ml soybean trypsin inhibitor, 1 mg/ml benzamidine, 1 mg/ml aminocaproic acid, and 2 mM PMSF) and phosphatase inhibitors (20 mM Na3P2O7 and 100 mM NaF).

Proteins were measured by aliquots of cell lysates using the Bio-Rad protein assay. Briefly, proteins were resolved in 8% SDS-PAGE. Gels were then blotted onto nitrocellulose and electrotransferred. Blots showing lanes with equal amounts of proteins were incubated with 5% nonfat milk in TBS (pH 7.4) for 30 min at room temperature. Blots were then incubated overnight at 4°C with Abs diluted in 5% nonfat milk in TBS. Abs used for Western blots were rabbit anti-p100, rabbit anti-p52, mouse anti-p56, and rabbit anti-actin (all from Santa Cruz Biotechnology). Blots were then rinsed repeatedly in TBS and incubated for 1 h at room temperature with alkaline phosphate-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary Abs diluted in 5% nonfat milk in TBS. After rinsing with TBS, blots were incubated with the alkaline phosphate substrate (5-bromo-4-chloro-3-indolyl phosphate/NBT tablets, Sigma-Aldrich).

Immunoprecipitation

The cell pellets were suspended in radiiimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% NaDodSO4) supplemented with protease inhibitors at a concentration of 10 μl/ml and phosphatase inhibitors at a concentration of 40 μl/ml. The cell extracts were centrifuged at 14,000 × g for 30 min. The supernatant was collected and precleared with incubation of protein A agarose beads (Roche Applied Science) for 1 h at 4°C. Protein A agarose beads were collected by centrifugation at 14,000 × g for 10 min at 4°C. The supernatant was recovered and incubated with 1 μg primary Ab for 2 h at 4°C. A volume of 20 μl protein A agarose was added to each 1 ml lysate and incubated overnight at 4°C. The agarose beads bound to the Ab–protein complex were collected by centrifugation at 14,000 × g for 10 min at 4°C. The supernatant was discarded, and the beads were washed with PBS and centrifuged three times at 14,000 × g for 10 min at 4°C. Finally, the beads were resuspended in 40 μl 2× sample buffer and electrophoresed using polyacrylamide gels.

Small interfering RNA

Life Technologies designed and synthesized the NFκB2 (p100) and the control small interfering RNAs (siRNAs). The monocytes were transfected with siRNAs using the Amaxa Nucleofector system (Amaxa Biosystems). Briefly, 1 × 106 monocytes, mixed with 25 μM siRNA in 100 μl transfection buffer, were transferred to an electroporation cuvette and nucleofected, according to the manufacturer’s instructions. The cells were then immediately transferred into a six-well culture plate (Costar) containing 2 ml prewarmed RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen). The nucleofected cells were cultured at 37°C with 5% CO2 for 1 h before the assays.

Bone marrow–derived macrophages from p100 knockout mice

The p100−/− mice were a gift of M. Fresno (Centro de Biología Molecular Severo Ochoa, Madrid, Spain). The bone marrow–derived macrophages were prepared, as previously described (22). Briefly, the tibia and femur were flushed with DMEM to obtain bone marrow. The bone marrow cells were cultured in 10 ml at an initial density of 4 × 107 cells/ml in a 100-mm petri dish (BD Biosciences) at 37°C with 10% CO2 for 5 d. On the third day, 5 ml medium was added to each culture. The medium used for the bone marrow–derived macrophages was DMEM supplemented with 20% FBS, glutamine (Invitrogen), and 90% L929 supernatant containing macrophage-stimulating factor. After 5 d, the cells were harvested with cold Dulbecco’s PBS (Invitrogen), and then were washed and resuspended at a density of 2 × 106 cells/ml in DMEM supplemented with 10% FBS. The cells were allowed to rest for a minimum of 4 h at 37°C in 10% CO2 prior to further handling.

Statistical analysis

The number of experiments analyzed is indicated in each figure legend. The data were collected from a minimum of three experiments and are expressed as mean ± SD. The statistical significance was calculated using a one-way ANOVA with the Student Newman–Keuls post hoc test, except when indicated. The correlations were assessed using Spearman’s rank–order correlation for nonnormally distributed data. The statistical significance was set at p < 0.05, and all statistical analyses were conducted using Prism 5.0 software (GraphPad).

Results

NFκB2/p100 is overexpressed during ET in human monocytes

Several previous studies have demonstrated that patients with bacteremia subsequently develop ET. ET does not allow the patient’s
The innate immune system to respond to new pathogens as it did prior to infection (2). This situation can be modeled by two consecutive LPS treatments that are separated in time. Data from a study that used this model indicated that 8 h of LPS exposure (tolerance induction) was enough to induce a refractory state in human monocytes. After entering a refractory state, the monocytes were unable to produce an inflammatory response against new endotoxin stimulation. This refractory state was not permanent, because after 5–6 d the cells reverted to a proinflammatory phenotype in response to endotoxin stimulation (8).

We cultured human monocytes following the experimental design shown in Fig. 1A to study the putative role of NFκB2/p100 (hereafter p100) in the control and development of ET (8). As expected, the production of TNF-α was downregulated in tolerant cells (Fig. 1B). Other cytokines such as IL-1β, CCL2, and CCL18 were analyzed (Supplemental Fig. 1A). We also investigated the monocyte purity of our human cultures (Supplemental Fig. 1B). The transcript levels of p100 were significantly increased after 3 h of LPS treatment. However, the

![FIGURE 1. ET model.](image)

![FIGURE 2.](image)
induction of p100 was faster in the tolerant cultures (Fig. 1C). These data were also confirmed at the protein level (Fig. 1D). The Western blot analysis of the cytosolic fraction showed high levels of p100 in the tolerant cultures. No p100 was detected in the nucleus (data not shown). The immunoprecipitation of p100 using Abs for p65 and p50 revealed that p100 interacts with members of the canonical NF-κB pathway during tolerance (Supplemental Fig. 2). These findings are in agreement with previously published data (17).

The accumulation of p100 correlates with a refractory state of human monocytes

Given that our findings suggested that p100 might play a role in ET in human monocytes, we decided to study the long-term p100 expression after LPS stimulation and its correlation with the development of a refractory state.

After 8 h of LPS challenge (tolerance induction), the cells were washed and kept in complete medium for 1–9 d. The Western blot analysis showed that p100 accumulation remained high from day

FIGURE 3. Correlation between p100 accumulation and TNF-α expression after LPS challenge. (A) Schematic representation of the ET model used for this study. (B and C) Cultures of human monocytes were pretreated (tol) with 10 ng/ml LPS for 8 h, washed twice with PBS, cultured in medium for the indicated time (1–9 d), and restimulated with 10 ng/ml LPS for 24 h (B) or 1 h (C). (B) TNF-α protein levels were analyzed using CBA (n = 5). *p < 0.05, ***p < 0.0005 compared with the nontolerant culture, respectively. (C) Expression of TNF-α mRNA was analyzed by real-time quantitative PCR; the ratio [TNF-α]/[Actin] is given (n = 3). **p < 0.005, ***p < 0.0005 compared with the nontolerant culture, respectively. (D) The correlation between TNF-α mRNA levels and densitometry of p100 band (arbitrary units [AU]) (see Fig. 2) was calculated.

FIGURE 4. Specific p100 downregulation. The siRp100- and siRcontrol-transfected cultures, along with nontransfected cultures, were subjected to the ET model described in Fig. 1A. (A) Western blot analysis of p100 and actin in the cytosolic fraction (the final LPS challenge was 3 h); a standard blot is shown (n = 3). (B) Soluble TNF-α protein levels were evaluated in cultures by CBA (final LPS challenge was at the indicated times) (n = 3). *p < 0.05 (tol + LPS, siRp100) compared with (tol + LPS, no-siRNA). *p < 0.05 (tol + LPS, siRp100) compared with (tol + LPS, siRcontrol).
FIGURE 5. The p100 expression commands the cytokine profile. The siRp100- and siRcontrol-transfected cultures, along with nontransfected cultures, were subjected to the ET model (the final LPS challenge was 1 h). Then mRNA levels of TNF-α (A), IL-1β (B), CCL2 (C), and CCL18 (D) were evaluated by real-time quantitative PCR. The fold induction compared with the LPS condition is shown (n = 3). *p < 0.05, **p < 0.005 compared with (LPS, no-siRNA).

1 to day 3. However, p100 showed reduced expression from day 6 onward. It was nearly undetectable on days 7 and 9 after the endotoxin challenge (Fig. 2). Considering these data, we modified our in vitro ET model and prolonged the gap between the first LPS stimulation (tolerance induction) and the second by several days (see scheme in Fig. 3A). Using this model, we detected a marked reduction of the tolerant state after day 6, as shown by the increased production of TNF-α (Fig. 3B, 3C). We also observed a correlation between ET and p100 expression. Note that, after the LPS challenge, TNF-α production was low when p100 was high and vice versa (Fig. 3D).

Specific p100 downregulation reverts the ET status

To study the impact of p100 on ET status, we knocked p100 down using siRNA. The human monocytes were transfected with a siRNA for p100 (siRp100) or siRNA control (siRcontrol) as a negative control. In contrast to untransfected cells (no siRNA) and siRcontrol-transfected cells, there was no p100 induction in the tolerant cultures transfected with siRp100 and stimulated with LPS for 3 h (Fig. 4A). Of particular note, TNF-α production was significantly restored in siRp100-trasfected monocytes after a second LPS challenge (Fig. 4B). The downregulation of p100 restored TNF-α and IL-1β expression. However, CCL2 and CCL18 levels were reduced in the siRp100 cultures (Fig. 5). In this human ET model, siRcontrol transfection reduced cytokine production in a nonsignificant manner, as a consequence of cell viability reduction (data not shown).

Bone marrow–derived macrophages from p100 knockout mice (p100−/−) were also unable to reproduce ET (Supplemental Fig. 3). Our findings demonstrate that the downregulation of p100 reverses the ET phenotype in monocytes.

Patients with sepsis locked into an ET state upregulate p100, and the downregulation of p100 in primary septic monocytes restores the inflammatory response

As we previously reported, a clinically relevant example of ET was observed in patients with sepsis (23, 24). Monocytes from these patients exhibit ET (2, 4, 23, 25, 26) and fail to produce proinflammatory cytokines after an ex vivo LPS challenge (1, 2, 4, 25).

To explore the pathophysiological implications of our findings, we studied the p100 expression in a cohort of 17 patients with sepsis (68 ± 10.6 y of age; see clinical details in Table I). An ex vivo LPS assay confirmed that all the patient-derived monocytes were locked into a refractory state (Fig. 6A). Monocytes from the same patients were taken for baseline measurements 3 mo after recovery from sepsis. As expected, the p100 levels were significantly higher during sepsis in comparison with baseline p100 levels (Fig. 6B). Moreover, when the monocyte cultures from patients with sepsis were transfected with siRp100 and stimulated with LPS, TNF-α and IL-1β expression returned to baseline levels (Fig. 6C, 6D). In contrast, the mRNA levels of CCL2 and CCL18 were considerably downregulated (Fig. 6E, 6F). Protein levels of TNF-α, IL-1β, and CCL2 corroborated the mRNA data (Fig. 7).

Discussion

Although the underlying mechanisms implicated in ET have been extensively studied, including reprogrammed epigenetic, microRNAs, and several molecules (1, 7, 27, 28), a complete picture of this process is still lacking. In-depth studies of ET have analyzed the participation of a number of factors and have established the role of several negative regulators, such as IRAK-M, ST2, suppressor of cytokine signaling 1, short version of MyD88 and SHIP, by guest on May 30, 2017 http://www.jimmunol.org/ Downloaded from

Table I. Clinical parameters (n = 17)

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<tr>
<th>Data</th>
<th>Age</th>
<th>Cardiac frequency</th>
<th>Respiratory frequency</th>
<th>Temperature</th>
<th>Creatinine</th>
<th>Hematocrit</th>
<th>Leukocytes</th>
<th>Neutrophils</th>
<th>Glucose</th>
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<td></td>
<td>68 ± 10.6 y</td>
<td>106.2 ± 3.9</td>
<td>24.1 ± 1.9</td>
<td>38.4 ± 0.4°C</td>
<td>2.4 ± 0.3 mg/dl</td>
<td>36.1 ± 3.7</td>
<td>18,896.4 ± 3,674.9 cells/mm3</td>
<td>15,910 ± 3,674.9 cells/mm3</td>
<td>134.7 ± 47.7 mg/dl</td>
<td>4.8 ± 0.6 mEq/L</td>
<td>138.5 ± 2.7 mEq/L</td>
<td>17.6 ± 1.2 mmol/L</td>
<td>13.9 ± 0.86</td>
<td>Gram-negative bacteria</td>
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<td>This study was approved by the La Paz Hospital Ethics Committee.</td>
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<td>aGlasgow Coma Scale (Glasgow-CS) is a neurologic scale that aims to give a reliable and objective way to evaluate the neurologic damage in patients. Glasgow-CS is part of the APACHE II score. The normal value of Glasgow-CS is 15 (a healthy-normal parameter matched with age).</td>
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as well as the dysregulation of TLR4 and TREM-1 (4, 12, 29, 30), roles that have been observed occasionally in different models (1, 9). Among them, the pseudokinase IRAK-M is one of the genes that is consistently induced into ET regardless of the model used (13, 31). Apparently, the mechanism by which IRAK-M regulates the LPS response is related to the inhibition of one of the earliest steps of the canonical NF-κB pathway: the formation and split of the TLR4/MyD88/IRAK complex (32, 33).

However, our data confirm a new important player in the control of ET. Previous studies from our group and others had suggested the involvement of p100 in this phenomenon (8, 14). In addition to the high expression of p100 during endotoxin tolerance, our findings demonstrated that p100 knockdown restored tolerant cells to an inflammatory status, including the following: tolerant human monocytes from an in vitro model, bone marrow–derived macrophages from p100−/− mice, and monocytes from septic patients after an endotoxin challenge.

Given that ET takes place in sepsis, we used a cohort of 17 patients with Gram-negative bacteremia whose innate immune system was locked in a refractory state. The monocytes from these patients displayed an increase in p100 expression during sepsis versus baseline, suggesting a potential use for p100 as a biomarker for refractory states. In addition, knocking down p100 with siRNAs in these cells avoids the ET phenotype. After siRp100 transfection, the tolerant monocytes and the monocytes from septic patients re-established their TNF-α and IL-1β levels after LPS challenge.

Interestingly, Basak et al. (17) previously reported the ability of p100 to form inhibitory complexes with members of the canonical NF-κB pathway. In this regard, p100 could be considered as a fourth IκB protein, sequestering latent NF-κB dimers (17). In line with their data, our findings indicated an impairment of p65/p50 translocation into the nucleus by p100, leading to a down-regulation of these inflammatory cytokines. In addition, IRAK-M interferes with the canonical NF-κB pathway; our findings suggest

FIGURE 6. Patients with sepsis. (A and B) Monocytes were isolated from the same patients with sepsis after they had been admitted to the hospital (sepsis) and 3 mo after they had recovered from sepsis (baseline). (A) The monocytes were cultured in the presence of 10 ng/ml LPS for 1 h, and TNF-α mRNA levels were analyzed by real-time quantitative PCR. Control monocytes did not receive LPS (nonstimulated). Fold induction compared with nonstimulated cultures is shown for the baseline and sepsis conditions (n = 17); ***p < 0.0005 compared with LPS (basal). The p values were calculated using the Mann–Whitney U test. (B) p100 transcript levels were evaluated in the monocytes with sepsis and at baseline (n = 17). Fold induction is represented as sepsis over baseline; ***p < 0.0005 compared with basal. The p values were calculated by paired t test. (C–F) Cultures from randomly selected patients with sepsis were transfected with siRp100 and siRcontrol. An untransfected control was also taken. All conditions were exposed to 10 ng/ml LPS for 1 h. Then TNF-α (C), IL-1β (D), CCL2 (E), and CCL18 (F) transcript levels were evaluated by real-time quantitative PCR. The fold induction of siRp100-transfected cells or siRcontrol-transfected cells over nontransfected cells is shown (n = 7). *p < 0.05, **p < 0.005, ***p < 0.0005 compared with (LPS, no-siRNA).
that p100 inhibited the same signal progression. However, the evidence indicated that IRAK-M could block the formation and split of TLR/MyD88/IRAK complex, which was crucial in terms of inflammatory progression (33), whereas p100 directly interfered with NF-κB translocation into the nucleus. These data highlight the relevance of the rigorous regulation at the various levels of the NF-κB canonical pathway. Future studies should focus on elucidating the coexistence and overlap of these two control points of the ET.

Interestingly, we found high levels of CCL2 and CCL18 in both tolerant and septic monocytes. Although CCL18 is induced by Th2-type cytokines such as IL-4, IL-10, and IL-13 (34), CCL2 has been implicated in the recruitment of monocytes/macrophages to the inflammatory site (35, 36) and in bacterial clearance in a murine model of sepsis (37). Our findings concur with published data demonstrating that CCL2 was increased in plasma from septic patients (38) and that CCL2 and CCL18 were found to be significantly downregulated. Therefore, we hypothesized that the production of these two chemokines would be p100 dependent in this context.

In conclusion, the data presented in this study indicate that p100 accumulation plays a key role in the development of a refractory state in human monocytes. During bacteremia, there is a fine line between a refractory state and an inflammatory state. The balance of these states is crucial for patient survival. The significance of our data might be useful for the design of a clinical trial on the control of bacteremia. Antimicrobial therapy requires early diagnosis, and avoiding delays is crucial to avoiding mortality. Therefore, data presented in this study should be considered when planning future clinical trials on bacteremia and other infectious diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


