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SHORT COMMUNICATION

Outlining key inflammation-associated parameters during early phase of an experimental gram-negative sepsis model in rhesus macaques (*Macaca mulatta*)

Jose J. Rosado-Franco[1] | Marcos J. Ramos-Benitez[1] | Laura M. Parodi[2] | Derick Rosario[3] | Nicole Compo[4] | Luis D. Giavedoni[2] | Ana M. Espino[1]

[1]Department of Microbiology and Medical Zoology, Medical Sciences Campus, University of Puerto Rico, San Juan, Puerto Rico

[2]Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio, TX, USA

[3]Chemistry Department, Rio Piedras Campus, University of Puerto Rico, San Juan, Puerto Rico

[4]Medical Sciences Campus, Caribbean Primate Research Center University of Puerto Rico, Toa Baja, Puerto Rico

Correspondence
Ana M. Espino, Department of Microbiology and Medical Zoology, University of Puerto Rico-Medical Sciences Campus, PO BOX 365067, San Juan, PR 00936-5067.
Email: ana.espino1@upr.edu

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Abstract

The aim of this study was to identify inflammation-associated markers during the early phase of sepsis in rhesus macaque. Four rhesus macaques were given an intravenous dose of 10^{10} CFU/kg of *E. coli*. Blood samples were collected before, or 30 minutes, 2, 4, 6 and 8 hours after *E. coli* infusion. Physiological parameters, bacteremia, endotoxemia, C-reactive protein (CRP), procalcitonin (PCT), and plasma cytokines/chemokines were determined for each animal. Bacteremia was present in all animals from 30 minutes to 3 hours after *E. coli* infusion whereas endotoxin was detected during the full-time course. CRP and PCT levels remained at detectable levels during the whole experimental window suggesting an ongoing inflammatory process. Signature cytokines and chemokines such as TNF-α, MIP-1α, and MIP-1β peaked about 2 hours after *E. coli* infusion and decreased thereafter. Plasma IL-6, IL-12p40, IFN-γ, and IL-1Ra, as well as I-TAC, MIG, IP-10 and MCP-1, remained at detectable levels after 4 hours of *E. coli* infusion. This nonhuman primate model could be useful for the assessment of new therapeutics aiming to suppress key inflammatory markers throughout sepsis early phases.

KEYWORDS
chemokines, cytokines, *Macaca mulatta*, sepsis

1 | INTRODUCTION

Sepsis is a major healthcare problem that affects more than 30 million people worldwide resulting in 8 million deaths annually.[1] In the United States, sepsis is the second leading cause of death among patients in intensive care units and the 10th leading cause of death overall.[2] In sepsis caused by Gram-negative bacteria, endotoxin (LPS) activates the immune system through TLR4 and induces activation of monocytes and macrophages to release excessive and dysregulated discharge of potent inflammatory cytokines and other

Jose J. Rosado-Franco and Marcos J. Ramos-Benitez contributed equally to this work.

inflammatory regulators, which may lead to vasodilation, increased capillary permeability and hypotension leading to multiple organ failure and death.[3] Although the development of new and more effective antibiotics over the past decade has improved the prognosis of sepsis, neutralization of inflammation is crucial to prevent the most severe consequences of this condition.[4]

The use of anti-inflammatory cytokines as a novel therapeutic concept for the treatment of endotoxemia has been developed. However, although the knowledge about variations in inflammation-associated markers during endotoxemia has been well-addressed, the alterations and dynamics of inflammatory cytokines/chemokines during early phase are still not clear and are worth elucidating, as they may provide insights for new therapeutic targets, potentially inhibiting progression into septic shock. Treatments at early phase of endotoxemia are much more effective.

The use of nonhuman primates (NHPs) is considered a favorable choice for basic and preclinical models of sepsis primarily due to their phylogenetic proximity to humans. The primary goal of this study was to develop a rhesus macaque model of Gram-negative sepsis in order to investigate changes in the levels of inflammatory cytokines/chemokines as well as of other inflammatory markers during the early phase (within 8 hours) of endotoxemia in rhesus macaques.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (US), 2011) and was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico-Medical Sciences Campus (Protocol No. 7870116).

2.2 | Animals

Four adult male (6-7 years old) rhesus macaques (*Macaca mulatta*), weighing 6.0-11 kg, were facilitated by the Caribbean Primate Research Center. Prior to inclusion in the experiment, the monkeys received a physical examination and were tested for hematological, serological, and microbiological abnormalities. Only the animals confirmed healthy by a veterinarian were included.

2.3 | Bacterial culture

The *Escherichia coli* strain 086a: K61 serotype used for sepsis induction was purchased from the American Type Culture Collection ATCC (ATCC 33985). Prior to the experiment a fresh *E. coli* culture was set up in Luria Broth (LB) agar and cultured overnight at 37°C. The following day a single colony was cultured in 300 mL of LB broth for 16-18 hours, a final concentration of 1 x 10^{10} CFU/mL was harvested and washed three times to remove free endotoxin.

2.4 | Animal preparation and establishment of baseline physiological parameters

The day before the procedure, monkeys were sedated with ketamine hydrochloride (10 mg/kg) (AST Pharma) intramuscularly and transported to a holding room for overnight fasting. The day of the procedure monkeys were sedated with ketamine as described above, induced under anesthesia with inhaled isoflurane gas (1%-5%) via mask, then intubated and maintained on isoflurane for the remainder of the procedure. The cephalic vein was cannulated and used for intravenous (IV) infusion of live *E. coli* and isotonic saline (0.9%NaCl) containing 2.5% Dextrose at a rate of 3.3 mL/kg/h. Animals were connected to a BIONET monitor for one hour prior to sepsis induction. The levels of body temperature (BT), heart rate (HR), respiratory rate (RR), and mean arterial blood pressure (MAP), were recorded for establishment of baseline values. Veterinary staff continuously monitored animals throughout the procedure.

2.5 | *E. coli* infusion and blood collection

Individual body weights were used to calculate the *E. coli* dose (10^{10} CFU/kg), which was previously established as lethal for this species.[5] Sterile isotonic saline was used to adjust to a final volume of 50 mL and given as an IV constant rate infusion (CRI) (0.42 mL/min for 2 hours). Eight hours following the bacterial infusion, animals were euthanized in accordance with *the AVMA Guidelines for the Euthanasia of Animals (2013).* Gross necropsy was completed immediately following euthanasia. Following *E. coli* infusion, 5 mL of blood were collected from the femoral vein using a 20-gauge Vacutainer needle into heparinized tubes at 0, 30 minutes, 2, 4, 6 and 8 hours. Blood samples were centrifuged at 450 rcf x 10 minutes and the plasma was collected. Prior to centrifugation, an aliquot of 100 μL from each blood sample was allotted for determining bacteremia levels.

2.6 | Bacteremia and endotoxemia assessment

To assess bacteremia, the allotted blood samples were diluted with equal volume of sterile PBS, spread onto LB agar plates, and cultured overnight at 37°C. After incubation, colonies were counted, and the total CFU/mL was calculated and adjusted based on the dilution factor. Endotoxin levels were assessed directly from plasma samples using the Chromogenic Limulus Amoebocyte Lysate QCL-1000 Assay (Lonza, Walkersville, MD) following the manufacturer's instructions.

2.7 | C-reactive protein levels (CRP) and procalcitonin assay (PCT)

Plasma samples were tested for the presence of CRP and PCT. Quantification of CRP levels was performed at a local clinical laboratory (Martin Clinical Lab, Bayamon, PR) using an Architect c8000 Clinical Chemical Analyzer (Abbot, Illinois, US). Procalcitonin levels

were determined as per the manufacturer's instructions using a Human Procalcitonin ELISA kit (Abcam, UK, ab100630).

2.8 | Cytokine and chemokine profiling

Plasma cytokine and chemokine levels were determined using Luminex technology employing established protocols for old world primates.[6] The assay included evaluation of interleukin-6 (IL-6), gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12p40 (IL-12p40), interleukin-1 receptor antagonist (IL-1Ra), macrophage inflammatory protein-1α/β (MIP-1α & MIP-1β), interferon gamma induced protein-10 (IP-10), monokine induced by gamma interferon (MIG), monocyte chemoattractant protein-1 (MCP-1), and interferon-inducible T-cell alpha chemoattractant (I-TAC).

2.9 | Statistical analysis

All determinations of cytokines/chemokines, PCT and CRP were performed in duplicate and the results are expressed as mean value (pg/mL or mg/dL) for each determination. Significance was determined using a student's *t* test for single comparison or analysis of variance test for multiple comparisons, with a $P < .05$ established as significant (GraphPad Prism 6.0 software).

3 | RESULTS AND DISCUSSION

To better understand the complexity of pathogenesis of sepsis in humans it is necessary to establish experimental animal models with physiological functions and anatomical features comparable to humans. Nonhuman primates (NHP) display identical cardiopulmonary anatomy and physiology, as well as host response to infection.[7] In particular, rhesus macaques have shown to be similar to the responses of humans to Gram-negative sepsis.[8] Moreover, the outbred nature of monkeys is more representative of the human population as a whole. Thus, rhesus macaque constitutes a realistic model to study the acute inflammatory process of sepsis after live bacteria infusion.

Compared to humans, NHPs are notably less sensitive to the lethal effects of LPS, as has been documented in rodents.[9] Boluses of 10-20 mg/kg of LPS are usually required to develop endotoxic shock in NHPs.[10] This dose is > 1 000 000 times greater than the typical dose of LPS (2-4 ng/kg), which has been used in human volunteers to induce symptoms and release detectable levels of TNF-α into the circulation.[11] However, the sepsis model based on the bolus injection with LPS commonly induces an immediate hypo-dynamic cardiovascular state as well as an overwhelming innate immunity response, failing to reproduce the hemodynamic changes observed in human sepsis.[9,12] In our study sepsis induction was achieved by introducing live *E. coli* by CRI to mimic human bacterial exposure.

At baseline the BT, HR and RR ranged between 36.1-37.7°C (median 37°C), 104-124 beat/min (median 121 beats/min), and 16-31 breaths/min (median 21 breaths/min), respectively. The MAP ranged between 46-65 mm Hg (median 50.5 mm Hg). Low values for MAP during baseline are associated with oscillometric monitoring of blood pressure (BP). Recent literature showed that oscillometric BP measurements underestimate MAP values in rhesus macaque when compared to direct BP measurement.[13,14] We were unable to include a mock-infected group. However, considering the high immune response heterogeneity of this model, using preinfusion physiological parameters values as each individual controls, appropriately achieve the goal of this work, and accurately measured the dynamics of targeted immune parameters during disease progression.

Two hours after bacterial infusion completion animals BS81 and CB22P showed minor episodes of bradycardia and hypotension. At the conclusion of the procedure, the HR and BP parameters were similar or higher than baseline (Table 1) and the animals were euthanized without clinical signs of septic shock. In contrast, HR for animals 702 and MA107 dropped to 94 and 72 beats/min, respectively and the RR towards the end was 10 breaths/min for 702 and 2 breaths/min for MA107. Both animals died approximately 15 minutes before the study scheduled 8 hours end point. However, since the animals remained continuously monitored, we were able to collect the corresponding blood samples. To simplify the data presentation, terminal samples collected from animals 702 and MA107 were graphed as an 8 hours time point. Although there were clinical manifestations suggesting septic shock in these two animals, postmortem examination showed no gross abnormalities, with the exception of mild peripheral lymphadenopathy, which was also observed in the BS81 and CB22P animals.

TABLE 1 Main vital signs monitored in rhesus macaques during 8 h of experimental sepsis induced by intravenous infusion with live *E. coli*

Animal ID	Physiological parameter	Baseline Value	Value at 8 h of *E. coli* infusion
BS81	Body temperature (°C)	36.1	34.1
	Heart rate (bpm)	104	128
	Mean arterial pressure	65	61
	Respiratory rate (rpm)	19	24
CB22P	Body temperature (°C)	36.3	37
	Heart rate (bpm)	124	172
	Mean arterial pressure	46	71
	Respiratory rate (rpm)	16	24
702	Body temperature (°C)	37.7	37.9
	Heart rate (bpm)	118	94
	Mean arterial pressure	52	24
	Respiratory rate (rpm)	23	10
MA107	Body temperature (°C)	37.7	36.8
	Heart rate (bpm)	123	72
	Mean arterial pressure	49	23
	Respiratory rate (rpm)	31	2

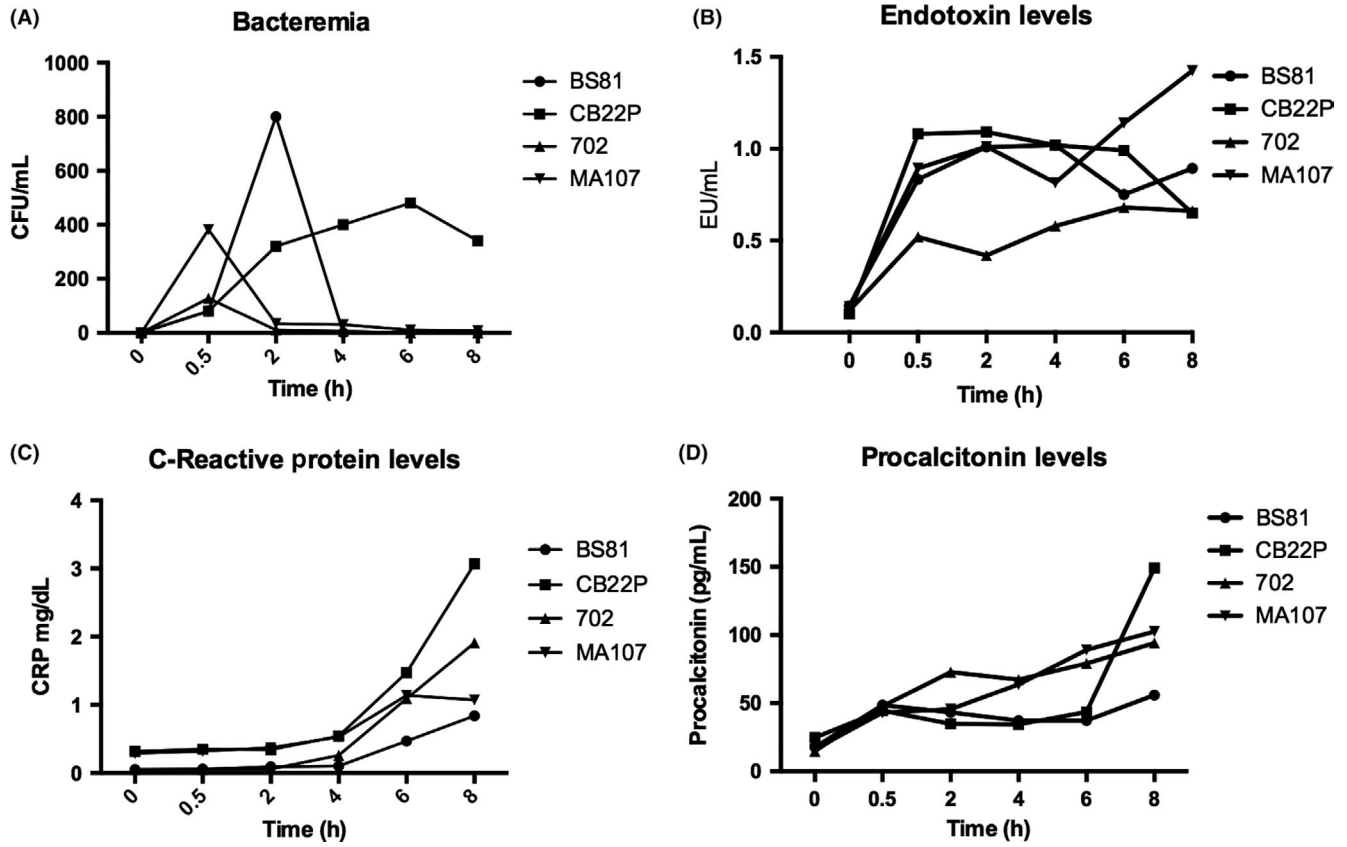

FIGURE 1 Evaluation of bacterial infection, inflammation, and sepsis markers during acute sepsis induction. A, Whole blood samples diluted 1:1 with sterile PBS were spread on LB-agar and incubated overnight. Total Colony forming units (CFU) per milliliter was calculated and adjusted by diluting factor. B, Endotoxin levels were assessed directly from plasma samples using the Chromogenic Limulus Amoebocyte Lysate Assay and the results are expressed as Endotoxin Units (EU per milliliter). (C) Plasma C-reactive protein (CRP) and (D) Procalcitonin were quantified by using an Architect c8000 Clinical Chemical Analyzer and a Human Procalcitonin ELISA kit, respectively

Bacteremia was detectable in three animals (BS81, 702 and MA107) from 30 minutes to 2 hours after *E. coli* infusion, but not thereafter. In contrast, bacteremia of CB22P was steadily increasing and remained high through the end of the procedure (Figure 1A). Undetectable bacteremia in three animals suggests that the bacteria failed to colonize or replicate inside the animals and was rapidly lysed by the complement system, as it has been previously reported for high bacterial inoculum to induced sepsis.[15] In that regard, and because all animals received the same bacterial dose adjusted to individual body weight, the detection of bacteremia in CB22P through all time points may suggest the inability of the complement system to efficiently lyse all bacteria. This may be a consequence of the immunological variability among outbred individuals. Importantly, the levels of endotoxemia increased notably following 30 minutes of *E. coli* infusion and remained at high levels during the course of the study, with a tendency to increase as the number of viable bacteria decreased (Figure 1B). This finding is consistent with disruption of the bacterial membrane and subsequent release of LPS into the bloodstream, which could cause the development of an acute inflammatory response in all animals. The detection of high levels of CRP and PCT in the plasma of all animals (Figure 1C,D) supports this assumption. C-reactive protein, produced by the liver, is classified as an acute phase reactant, meaning that its levels should rise in response to ongoing inflammation.[16] Procalcitonin serves as a biomarker of high specificity for bacterial infections and is a good predictor of bacterial sepsis in humans.[17] The finding that all animals showed elevating levels of CRP and PCT (Figure 1D) despite that three of four animals had no detectable bacteremia, reinforces the validity of these two parameters as functional predictors of an ongoing inflammatory process due to bacterial infection in rhesus macaques.

We also evaluated the levels of several plasma cytokines and chemokines in our experiment. Tumor necrosis factor-α, a component of the acute phase of systemic inflammation, is primarily produced by monocytes and macrophages,[18] but can also be produced by neutrophils and natural killer cells (NK). Following *E. coli* infusion, high levels of TNF-α were detected in all four monkeys at 30 minutes (354.75 ± 207.03 pg/mL), peaked at 2 hours (39 399 ± 1045 pg/mL), (Figure 2A) and gradually decreased thereafter; however, mean concentration of TNF-α in the four animals remained significantly higher than baseline at the final time point (3203.5 ± 4710 pg/mL; $P < .0001$). These results are in agreement with previously established endotoxic shock models in mice and rabbits in which TNF-α levels were reported to increase 30 minutes after LPS injection and reach a peak about 60-120 minutes after LPS injection.[19,20]

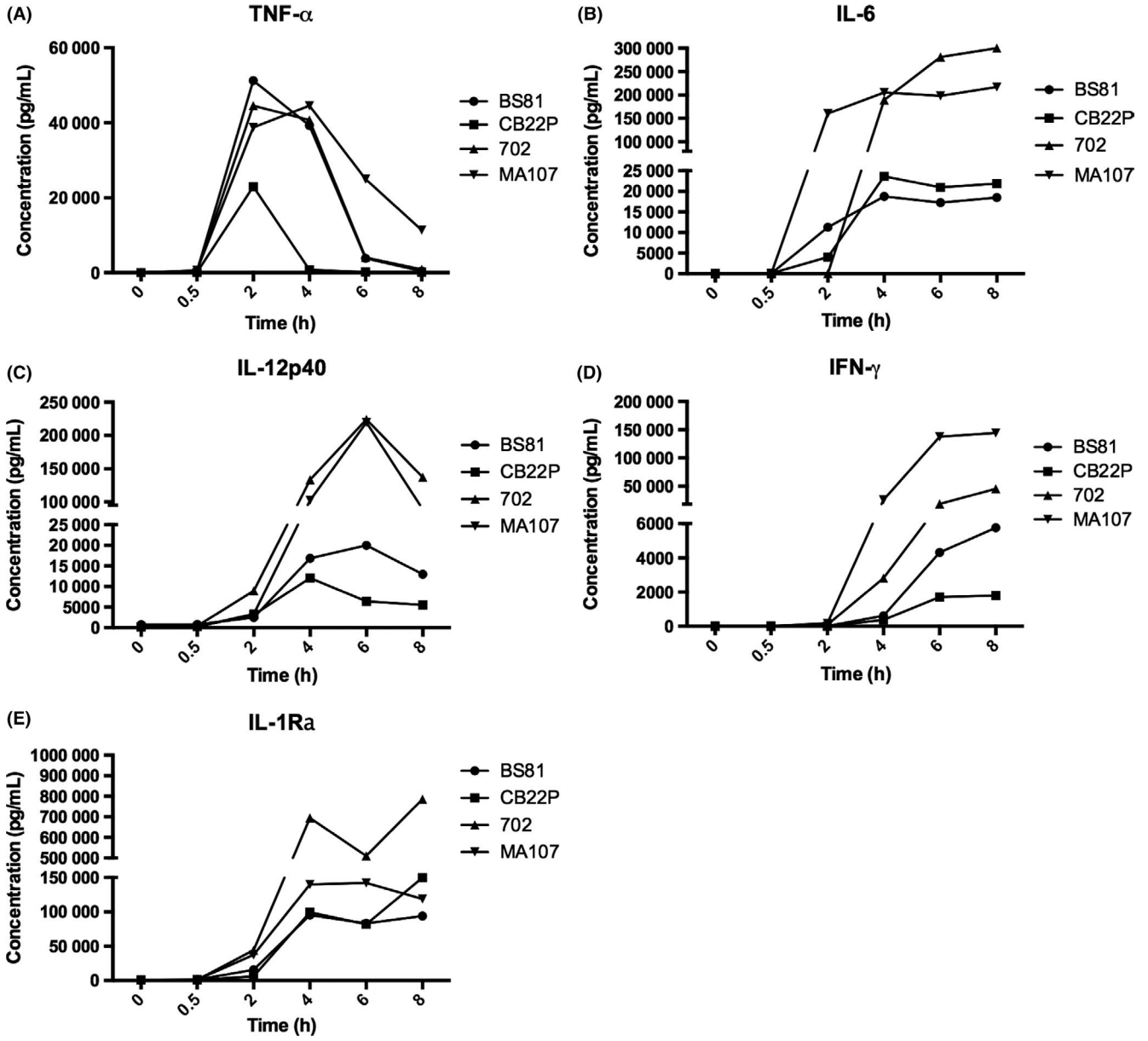

FIGURE 2 Plasma cytokines of rhesus macaques during the early phase of an experimentally induced sepsis model by intravenous infusion of live *E. coli*. (A) Levels of TNF-α, (B) IL-6, (C) IL-12p40, (D) IFN-γ and (E) IL-1Ra in plasma samples were quantified using Luminex technology and expressed in pg/mL. Each determination was done in duplicate. Each graph represents the dynamic of a determined cytokine during the experimental window of 8 h after *E. coli* infusion for each animal

Interleukin-6 is produced by a variety of cells in response to LPS, TNF-α, and IL-1.[21] In this study, IL-6 was detectable in plasma after 30 minutes of *E. coli* infusion and increased gradually throughout the experiment with mean concentrations of 139 388.35 ± 122 749.1 pg/mL at 8 hours of experimentation (Figure 2B). Interestingly, although the four animals showed a similar IL-6 dynamics, we noticed that rhesus macaques 702 and MA107, which died shortly before completion of the study, had 120-fold increase in IL-6 when compared to rhesus macaques BS81 and CB22P (animals with no clinical symptom of septic shock). These results are in agreement with studies that showed elevated levels of IL-6 in human sepsis in correlation with the occurrence of multiple organ failure and septic shock and mortality.[22] Another cytokine with a similar dynamic to IL-6 was IL-12p40 (Figure 2C). Interleukin-12p40 was detectable in all animals at 4 hours of *E. coli* infusion as compared to baseline (66 196.5 ± 52 874 pg/mL; $P < .0001$), and peaked at 6 hours (117 546.5 ± 104 479.9 pg/mL). Collectively the rhesus macaques that died (702 and MA107) had 30-fold more IL-12p40 than rhesus macaques BS81 and CB22P. The high levels of plasma IL-12p40 are consistent with our previous findings in a mouse model of septic shock.[23] Interleukin-12p40 is a subunit essential for biological activity of IL-12p70 and has shown to be a subunit of another composite cytokine designated IL-23.[24] Both, IL-12 and IL-23, induce the production of IFN-γ from both activated or memory $CD4^+$ T-lymphocytes and have been found increased in patients with sepsis,[25,26] suggesting a role in sepsis. Consistent with these observations, we also found high concentrations of IFN-γ in all

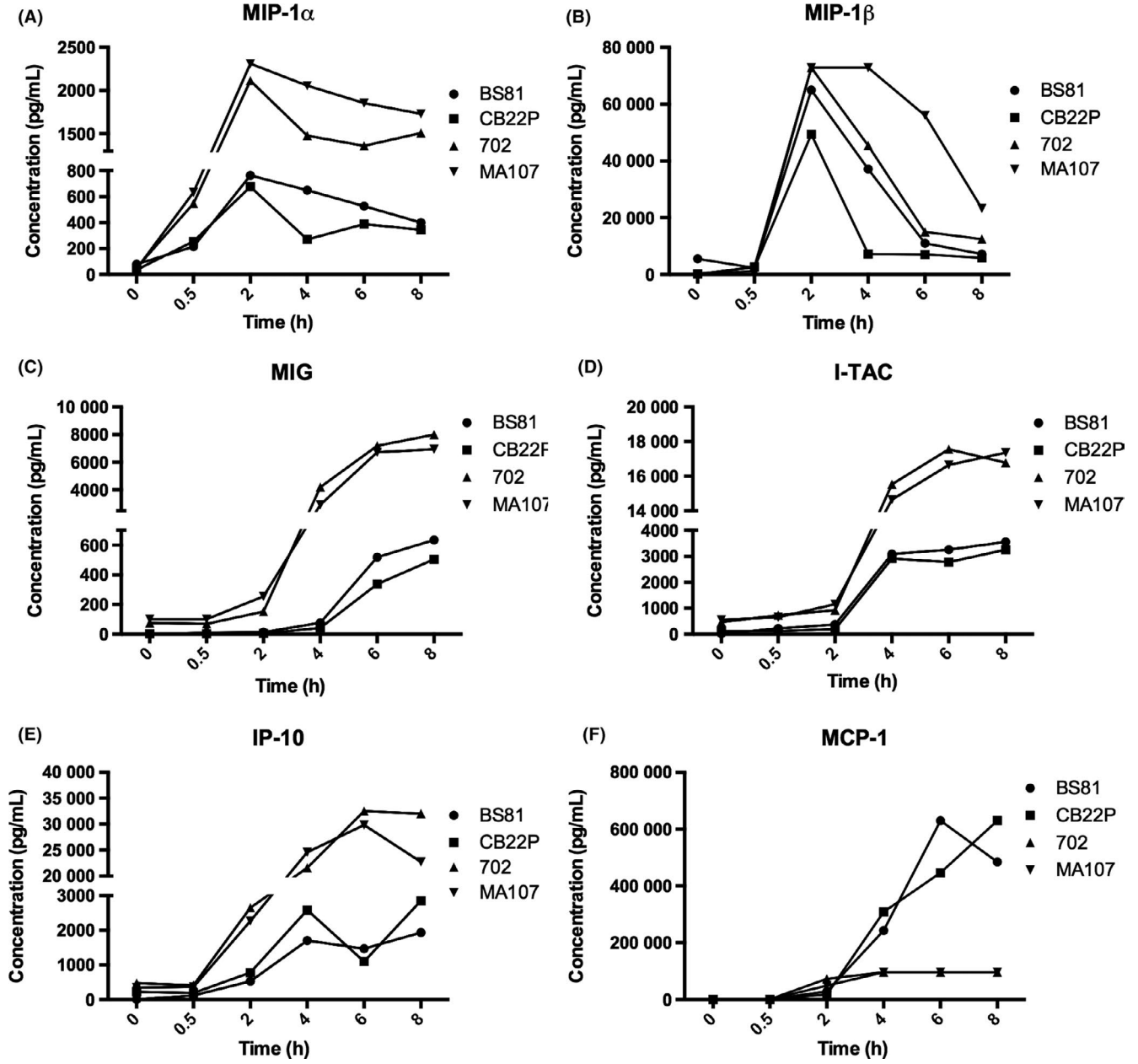

FIGURE 3 Plasma chemokines of rhesus macaques during the early phase of an experimentally induced sepsis model by intravenous infusion of live *E. coli*. (A) Levels of MIP-1α, (B) MIP-1β, (C) MIG, (D) I-TAC, (E) IP-10 and (F) MCP-1 were quantified in plasma samples using Luminex technology and expressed in pg/mL. Each determination was done in duplicate. Each graph represents the dynamic of a determined cytokine during the experimental window of 8 h after *E. coli* infusion for each rhesus macaque

animals over 4 hours of *E. coli* infusion with mean concentrations of 49 366.17 ± 57 489.6 pg/mL, which were steadily increasing during the course of the study. Several studies indicate that IFN-γ promotes the production of proinflammatory responses during septic shock.[27] Moreover, our observation that rhesus macaques 702 and MA107 showed a 25-fold increase in IFN-γ when compared to BS81 and CB22P (Figure 2D) is also consistent with previous reports that demonstrated enhanced and persistent levels in patients who died of sepsis.[26]

Interestingly, IL-1Ra, a major anti-inflammatory cytokine[28] that modulates the action of IL-1,[29] was also found at detectable levels in all animals from 2 hours after *E. coli* infusion (25 820.75 ± 15 430 pg/mL) and increased gradually throughout the experiment reaching mean concentrations of 287 211.00 ± 288 683 pg/mL at the end of the study (Figure 2E). It has been reported that IL-1Ra along with TNF-α, amplies the inflammatory cascades by activating macrophages to secrete other proinflammatory cytokines and inflammatory mediators, leading to sepsis-mediated organ dysfunction.[30] High concentrations of IL-1Ra have been also found in septic patients and have also been correlated with disease severity in humans.[31]

Chemokines are a family of chemoattractant cytokines that play a central role in recruiting immune effector cells to a site of

infection to target and destroy invading pathogens. Although there are some reports about the change of plasma chemokines, such as MIP-1β, MIP-1α, IP-10, I-TAC and MIG, during endotoxic shock in humans and experimental models of sepsis,[32,33] to our knowledge there are no reports evaluating these chemokines in a rhesus macaque model. MIP-1α and MIP-1β have chemotactic activity for inflammatory and immune effector cells, and play a role in stimulating the production of IL-1 and TNF-α.[34] In our study, the concentration of MIP-1α and MIP-1β peaked at 2 hours after *E. coli* infusion (1465.98 ± 864.77 pg/mL and 129 942.20 ± 11 055.478 pg/mL, respectively) (Figure 3A,B), which coincided with the peak of TNF-α and the disappearance of bacteremia from three of four animals, perhaps because the recruitment of macrophages, neutrophils and NK cells synchronized with the complement system. Subsequently, the levels of both chemokines declined. This is consistent with the report of O'Grady et al [35] who reported elevated levels of MIP-1α, MIP-1β that fell in parallel with TNF-α in patients with sepsis. MIG, I-TAC and IP-10, are chemokines that attract CXCR3-bearing cells, such as T cells, NK cells, B cells and Th1 cells. Plasma levels of these chemokines reached their peak at 6 hours after *E. coli* infusion with mean values of 1811.95 ± 2088.06 pg/mL, 9043.7 ± 6983.87 pg/mL and 16 225.3 ± 17 288.46 pg/mL, for MIG, I-TAC and IP-10, respectively (Figure 3C-E). MCP-1 (Figure 3F), a chemokine secreted by macrophages, promotes neutrophil migration towards sites of infection. It is also involved in the production of TNF-α, IL-6, and other inflammatory mediators, such as reactive oxygen species, nitric oxide, and myeloperoxidase. This chemokine was found to be elevated in all animals at 6 hours of *E. coli* infusion (317,237.2 ± 265,751.52 pg/mL). MIG, IP-10, I-TAC and MCP-1, also known as CXCL9, CXCL10, CXCL11 and CCL2, respectively, have been also found elevated in a large number of inflammatory diseases in close association with high levels of IFN-γ indicative of strong Th1-immune responses.

4 | CONCLUSIONS

To the best of our knowledge, this is the first work describing the dynamic of MIP-1α/β, MIG, I-TAC, IP-10 and MCP-1 chemokines, as well as IFN-γ, IL-12p40, IL-1Ra, CRP and PCT during the first 8 hours of an experimental endotoxemia induced by IV injection of live *E. coli* in a rhesus macaque model. Our work demonstrates that plasma TNF-α, MIP-1β and MIP-1α peaked within 2 hours and declined thereafter whereas plasma CRP and PCT increased and remained high at the end of the experiment. Concentrations of IL-12p40, IFN-γ, IL1Ra, MIG, I-TAC, MCP-1 and IP-10 increased slightly later (~4 hours) and remained elevated during the 8-hour experimental window. Although bacteremia was undetectable in three of four animals after 4 hours following *E. coli* infusion, the combined detection of these biomarkers may help in the selection of new experimental drugs and/or administration timing directed to inhibit the progression of the proinflammatory phase during early endotoxemia to prevent septic shock and organ damage and death.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

AME and MJRB developed the study concept and the study design. NC and her staff were responsible for selecting animals to be included in the experiment, administration of *E. coli* infusion, monitoring and gathering the physiological parameters. JJRF, MJRB and DR prepared the *E. coli* infusion and performed CRP, PCT, endotoxin and bacteremia determinations. LMP and LDG performed the cytokine determinations. JJRF and MJRB performed the data analysis and interpretation under supervision of AME. AME and JJRF drafted the manuscript and the final version. MJRB, NC and LDG provided critical revisions. All authors approved the final version of the manuscript for submission.

ORCID

Jose J. Rosado-Franco https://orcid.org/0000-0002-7394-0680
Ana M. Espino https://orcid.org/0000-0001-8456-2550

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