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Evidence for Monocyte Reprogramming in a Long-Term Postsepsis Study

Raquel Bragante Gritte, PhD[1]

Talita Souza-Siqueira, PhD[1]

Eliane Borges da Silva, PhD[1]

Laiane Cristina
   dos Santos de Oliveira, PhD[1]

Rodrigo Cerqueira Borges, PhD[2]

Heloísa H de Oliveira Alves, PhD[1]

Laureane Nunes Masi, PhD[1]

Gilson Masahiro Murata, PhD[3]

Renata Gorjão, PhD[1]

Adriana Cristina Levada-Pires, PhD[1]

Antônio Carlos Nogueira, PhD[2]

Tânia Cristina Pithon-Curi, PhD[1]

Ricardo Bentes de Azevedo, PhD[4]

Francisco Garcia Soriano, MD, PhD[5]

Rui Curi, PhD[1,6]

Marcel Cerqueira Cesar Machado, MD, PhD[5]

OBJECTIVES: This study sought to identify monocyte alterations from septic patients after hospital discharge by evaluating gene expression of inflammatory mediators and monocyte polarization markers. It was hypothesized that sepsis reprograms the inflammatory state of monocytes, causing effects that persist after hospital discharge and influencing patient outcomes.

DESIGN: The gene expression patterns of inflammatory receptors, M1 and M2 macrophage polarization markers, NLRP3 inflammasome components, and pro- and anti-inflammatory cytokines in monocytes were assessed.

PATIENTS: Thirty-four patients from the University of São Paulo Hospital, during the acute sepsis phase (phase A), immediately after ICU discharge (phase B), and 3 months (phase C), 6 months (phase D), 1 year (phase E), and 3 years (phase F) after discharge, were included. Patients that died during phases A and B were grouped separately, and the remaining patients were collectively termed the survivor group.

MEASUREMENTS AND MAIN RESULTS: The gene expression of toll-like receptor (*TLR*)*2* and *TLR4* (inflammatory receptors), *NLRP3, NFκB1*, adaptor molecule apoptosis-associated speck-like protein containing a CARD, *caspase 1, caspase 11*, and *caspase 12* (NLRP3 inflammasome components), *interleukin-1α, interleukin-1β, interleukin-18,* and high-mobility group box 1 protein (proinflammatory cytokines), *interleukin-10* (anti-inflammatory cytokine), C-X-C motif chemokine ligand 10, C-X-C motif chemokine ligand 11, and *interleukin-12p35* (M1 inflammatory polarization markers), and C-C motif chemokine ligand 14, C-C motif chemokine ligand 22, transforming growth factor-beta (*TGF-β*), *SR-B1*, and peroxisome proliferator-activated receptor γ (M2 anti-inflammatory polarization and tissue repair markers) was upregulated in monocytes from phase A until phase E compared with the control group.

CONCLUSIONS: Sepsis reprograms the inflammatory state of monocytes, probably contributing to postsepsis syndrome development and mortality.

KEY WORDS: inflammasome; M1 macrophage; M2 macrophage; postsepsis syndrome

S epsis is caused by an intense unregulated immune response to infection that damages human tissues and organs (1). Progression to septic shock, characterized by persistent hypotension requiring vasopressors to maintain a mean blood pressure above 65 mm Hg and elevated plasma lactate concentrations (>18 mg/dL) after adequate blood volume replacement, often causes death (1, 2). Septic shock patients in ICUs present the characteristic inflammatory response followed by organ function loss (3). Annually, there are approximately 49 million sepsis cases and 11 million sepsis-related deaths worldwide (4).

Sepsis survivors often present sequelae collectively called postsepsis syndrome (PSS) (5) associated with a prolonged cognitive and functional decline, chronic renal and respiratory dysfunction, fatigue, depression, and

reduced physical function, resulting in poor quality of life (6–11). In general, septic shock patients typically exhibit more sequelae post-ICU, persisting for 6 months or longer (12), than those with sepsis (13). The causes of the sequelae in postsepsis patients remain under investigation.

It has been reported that infections commonly occur in sepsis survivor patients (5, 14). For example, a recent study investigated 1,731 postsepsis patients and reported higher incidences of abdominal cavity (20.6%) and urinary tract (19.2%) infections, and lung pneumonia (11.3%). Furthermore, 47% of postsepsis patients were diagnosed with an infection less than 1-year post-ICU, a value reduced to 38.7% after 1–5 years, 7.5% after 6–10 years, and 6% after greater than 10 years (6).

Concerning sepsis-related mortality, a study reported that 66% and 70% of septic shock patients die within 1 and 2 years following hospital discharge, respectively (7). In the same study, all septic shock patients died less than 5 years post-ICU, whereas 18.3% of the septic patients survived 8 years. Jagodic and Podbregar (15) observed 30% and 70% mortality rates in postsepsis patients after 1 and 2 years, respectively. Another study reported that 41.3% of patients die within 90 days, and 81.9% within 5 years post-ICU (16). Furthermore, a study with 1,083 participants found that 27.5% of the patients died 90-day postsepsis, and 44.2% died after 1 year (11). However, the specific underlying factors contributing to these statistics remain unknown.

It has been proposed that immature circulating monocytes migrate to sites of tissue inflammation (17), releasing pro- and anti-inflammatory cytokines and antigenic phagocytosis-produced particles (18) and inducing systemic inflammation (19). Indeed, depending on microenvironment factors, monocyte-derived macrophages can differentiate into proinflammatory (M1) or anti-inflammatory and tissue repair (M2) subtypes (17, 20, 21).

Toll-like receptor (TLR) agonists (e.g., interferon-γ) can transform macrophages into the proinflammatory M1 subtype. In contrast, interleukin (IL)-4, IL-13, IL-10, and transforming growth factor-beta (TGF-β) cytokines induce M2 polarization (17, 22). Concomitant to macrophage polarization, there are marked changes in cell metabolism, known as meta-inflammation (23–25). Studies have shown that M1 macrophages generate high adenosine triphosphate (ATP) levels in the cytosol through aerobic glycolysis, whereas the Krebs cycle and mitochondrial oxidative phosphorylation are primarily responsible for M2 macrophage ATP production (26). Previously, a marked increase in the proportion of proinflammatory M1 macrophages during the acute sepsis phase was reported (27). Interestingly, the sepsis-induced macrophage inflammatory state persists after hospital discharge (28), possibly contributing to PSS development (29–33) and involving monocyte reprogramming (18, 34).

Previous studies reported that sepsis-induced epigenetic changes in leukocyte progenitor cells from bone marrow persist in peripheral macrophages, impairing their function even after sepsis resolution (35–38). Monocytes collected during the acute sepsis phase and after 7 days of hospitalization exhibited increased phagocytosis capacity and reactive oxygen species and nitric oxide production in vitro (32). However, IL-6 and tumor necrosis factor-α (TNF-α) production was attenuated in monocytes from the septic patients compared with healthy volunteers, as observed in endotoxin-stimulated monocytes in vitro (29, 30).

In addition to epigenetic mechanisms, immune cell reprogramming involves marked alterations in cell metabolism (33), switching from a proinflammatory to an immunosuppressed state in septic patients (31). Septic patients' monocytes display multiple energy metabolism defects that have been correlated with immunometabolism paralysis characterized by reduced cytokine production capacity. Consequently, reduced leukocyte function in the late sepsis phase results in immunoparalysis, increasing the patient's susceptibility to secondary and opportunistic infections (39).

Most of the above studies followed patients for up to 1 year after hospital discharge. Herein, we hypothesized that sepsis reprograms the inflammatory state of monocytes, causing effects that persist for an extended period after hospital discharge and influence patient outcomes. To address the proposed hypothesis, the gene expression levels of inflammatory receptors, macrophage polarization markers, NLRP3 inflammasome components, and pro- and anti-inflammatory cytokines in monocytes collected during the acute sepsis phase and up to three years post-ICU discharge were monitored to investigate sepsis-induced monocyte reprogramming.

MATERIALS AND METHODS

Patient Selection

Sixty-two clinically diagnosed sepsis patients admitted to the University Hospital at the University of São Paulo (HU-USP), São Paulo, Brazil, were enrolled in the study. Blood was collected by day 4 of ICU admission (phase A), immediately after ICU discharge (phase B), and 3-month (phase C), 6-month (phase D), 1-year (phase E), and 3 year (phase F) post-ICU.

The study included patients diagnosed with sepsis or septic shock according to the diagnostic criteria of Sepsis 3 (1). We excluded patients diagnosed with HIV, chronic hepatitis, cancer, and autoimmune and inflammatory diseases from the study and volunteers who did not participate in all blood collections.

Fourteen participants failed to meet the inclusion criteria, 10 were not located (three attempts), and four withdrew. Of the remaining 34 patients, 11 died during hospitalization (phase A death group), six died after ICU discharge (phase B death group), 17 survived 1 year (phase A–E survivor groups), and eight survived 3 years post-ICU (phase F survivor group). Control individuals who never had sepsis and were not regularly taking anti-inflammatory medication were compared with septic patients. The control group's age and sex distributions were similar to those of the septic patient groups.

The Ethics Committee of HU-USP (Process 1513/01/29/2016) approved this study. All participants or relatives provided informed written consent before enrolling in the study. The characteristics of the selected patients are in **Table 1**, and **Tables S1 and S2** (http://links.lww.com/CCX/B32). Overall the study.

Blood Collection and Monocyte Preparation

Approximately 20 mL of blood from the antecubital vein were collected into BD vacutainer tubes containing 1-mg/mL EDTA (Becton Dickinson, San Diego, CA). Samples were diluted 1:1 in phosphate-buffered saline, layered onto Histopaque-1077 (Sigma-Aldrich, St. Louis MO), and centrifuged (400 × g, 30 min) at room temperature. Peripheral blood mononuclear cells (mixture of monocytes and lymphocytes) were isolated from the interphase and cultured in RPMI-1640 culture medium containing 10% fetal bovine serum (Sigma-Aldrich) in a 5% CO_2 incubator at 37°C

TABLE 1.

Characteristics of Patients (*n* = 34), São Paulo, 2016−2017

Sex	Female	13 (38.2%)
	Male	21 (61.8%)
Age	Average (SD)	59.7 (13.5)
	Median (min−max)	60 (34-87)
Comorbidities	Absent, *n* (%)	Present, *n* (%)
Arterial hypertension	14 (41.2)	20 (58.8)
Diabetes mellitus	21 (61.8)	13 (38.2)
Acute renal insufficiency	25 (73.5)	9 (26.5)
Heart disease	26 (76.5)	8 (23.5)
Chronic renal insufficiency	32 (94.1)	2 (5.9)
Chronic obstructive pulmonary disease	33 (97.1)	1 (2.9)
Cirrhosis	33 (97.1)	1 (2.9)
Congestive heart failure	33 (97.1)	1 (2.9)
Hyperthyroidism	33 (97.1)	1 (2.9)
Dementia	33 (97.1)	1 (2.9)
Chronic osteomyelitis	33 (97.1)	1 (2.9)
Liver disease	33 (97.1)	1 (2.9)
Sequential Organ Failure Assessment, *n* (%)	2–6	5 (14.7)
	7–9	9 (26.5)
	10–12	11 (32.4)
	13–14	5 (14.7)
	15	0
	16–19	4 (11.8)
Length of stay in the ICU (d)	Average (SD)	13.1 (13.9)
	Median (min−max)	9.5 (2–75)
Sepsis focus		
Abdominal		9 (26.5)
Leptospirosis		1 (2.9)
Osteomyelitis		1 (2.9)
Pancreatitis		1 (2.9)
Soft parts		1 (2.9)
Lungs		10 (29.4)
Kidney		6 (17.6)
Skin		1 (2.9)
Another		4 (11.8)
Status vital	Survivors	17 (47.1)
	Deaths	17 (52.9)

min = minimum value, max = maximum value.
Length of stay in the ICU (d): 2 (not informed).

for 1 hour. After incubation, monocytes adhered to the culture plate were resuspended in Trizol (Invitrogen, Carlsbad, CA) and stored at –80°C. The HU-USP Clinical Analysis Laboratory performed the biochemical and hematological plasma measurements.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from monocytes using the RNAqueous Micro Kit following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) by measuring the absorbance at 260 and 280 nm. The extracted RNA (1 μg) was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

qPCR

For the qPCR assays, 12.5-μL Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), and 40-ng, 1-μM forward and reverse primers (Exxtend, Paulínia, SP, Brazil) were combined. Then, 6.9-μL nuclease-free water was added to the wells of a 96-well plate. The reactions were performed on a QuantStudio 3 (Thermo Fisher Scientific) following the SYBR Green protocol. Melt curves were analyzed to determine the qPCR product specificity and monitor primer-dimer formation. Results were normalized to the reference gene *Syntaxin 5A*. Primer sequences are provided in **Table S3** (http://links.lww.com/CCX/B32).

Statistical Analysis

The central tendency and dispersion measures of the quantitative variables (mean, median, SD, minimum value, maximum value, first quartile, and third quartile) were calculated. The normality of the data was tested by the Kolmogorov-Smirnov test under the null hypothesis that the data follow a normal distribution. Comparison between independent variables was performed using the nonparametric Mann-Whitney *U* test. The generalized estimating equations method was used to analyze the data over time, considering vital status and time as factors. Paired comparisons were performed using the Bonferroni method, and the significance level adopted was 5%. Statistical analyses were performed using the SPSS for Windows Version 25 software (IBM, Armonk, NY).

RESULTS

Clinical Assessment

This study included 34 patients (17 survivors and 17 deaths) with sepsis and septic shock and 17 control individuals without any history of the disease. Patients were evaluated from phase A (disease onset) to phase F (3 years after ICU discharge) of the disease. Most patients were male (61.8%), and the mean age was 59.7 years (minimum of 34 and maximum of 87 yr) (Table 1).

The most frequent comorbidity was arterial hypertension (58.8%), followed by diabetes mellitus (38.2%), renal failure (26.5%), and heart disease (23.5%) (Table 1).

Sequential Organ Failure Assessment scores ranged from 2 to 19, with 32.4% of patients scoring 10 to 12. The mean length of stay in the ICU was approximately 13 days, ranging from 2 to 75 days. Of the total number of enrolled patients, 52.9% died during the study.

As shown in Table 1, the incidences of Gram-positive and Gram-negative bacteria in septic patients were similar. The most frequent infectious foci included the lungs (29.4%), abdominal cavity (26.5%), kidney (17.6%), skin (2.9%), soft tissue (2.9%), osteomyelitis (2.9%), pancreatitis (2.9%), leptospirosis (2.9%), and other (11.8%).

The plasma biochemical, hematological, and inflammatory data are presented in **Table S4** (http://links.lww.com/CCX/B32).

Gene Expression of M1 and M2 Macrophage Polarization Markers

The C-X-C motif chemokine ligand 10 (*CXCL10*), C-X-C motif chemokine ligand 11 (*CXCL11*), *IL-12p35*, and *IL-1β* genes were used as M1 markers and the C-C motif chemokine ligand 14 (*CCL14*), C-C motif chemokine ligand 22 (*CCL22*), *TGF-β*, scavenger receptor class B type 1 (*SR-B1*), and peroxisome proliferator-activated receptor γ (*PPARγ*) genes were used as M2 macrophage polarization markers, following Jaguin et al (20).

The expression of *CXCL10* was downregulated ($p < 0.05$) in survivor phase D (**Fig. 1A**; and **Table S5**, http://links.lww.com/CCX/B32). *CXCL11* expression was downregulated ($p < 0.05$) in phase F (**Fig. 1B**; and Table S5, http://links.lww.com/CCX/B32), *IL-12p35*
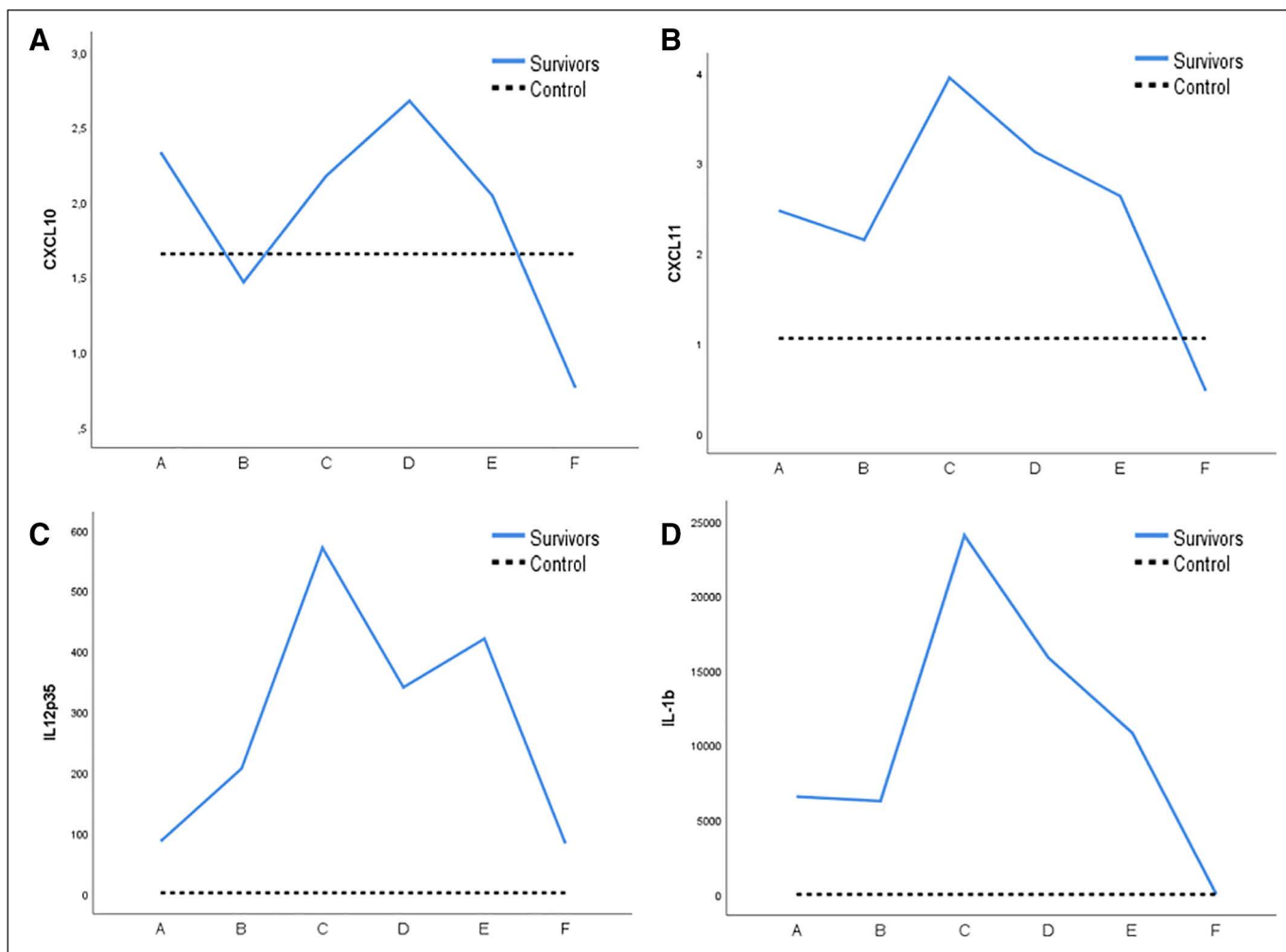
Figure 1. Messenger RNA expression of M1 macrophage markers. **A**, C-X-C motif chemokine ligand 10 (*CXCL10*). **B**, C-X-C motif chemokine ligand 11 (*CXCL 11*). **C**, Interleukin 12 p35 (*IL-12p35*). **D**, Interleukin 1-beta (*IL-1β*). Description of the phases: A—acute period; B—immediately after ICU discharge; C—3-mo post-ICU; D—6-mo post-ICU; E—1-yr post-ICU; and F—3-yr post-ICU. Results are presented as the median.

was upregulated ($p < 0.05$) in all survivor phases (A–F) (**Fig. 1C**; and Table S5, http://links.lww.com/CCX/B32), and *IL-1β* expression was upregulated ($p < 0.05$) in phases A–E (**Fig. 1D**; and Table S5, http://links.lww.com/CCX/B32) compared with the control group.

Comparisons among the study survivors showed that the expression of *CXCL10* during phase F was attenuated ($p < 0.05$) compared with phases A, B, and D ($p < 0.05$) and that phase D expression was reduced ($p < 0.05$) compared with phases B, C, and E (**Table S7**, http://links.lww.com/CCX/B32). Similar to *CXCL10*, peer comparisons revealed that phase F *CXCL11* expression levels were decreased ($p < 0.05$) compared with phases A, B, and D (Table S7, http://links.lww.com/CCX/B32). Furthermore, phase F *IL-12p35* expression levels were significantly less ($p < 0.05$) than that in phase E (Table S7, http://links.lww.

com/CCX/B32). There was no difference in *IL-1β* expression between the death and the survivor groups ($p > 0.05$).

Concerning M2 macrophage marker gene expression, the expression of *PPARγ* was upregulated ($p < 0.05$) during survivor phases A–E (**Fig. 2A**; and Table S5, http://links.lww.com/CCX/B32), *TGF-β* was upregulated ($p < 0.05$) in phases A–F (**Fig. 2B**; and Table S5, http://links.lww.com/CCX/B32), *SR-B1* was upregulated ($p < 0.05$) in phases A–F (**Fig. 2C**; and Table S5, http://links.lww.com/CCX/B32), and *CCL14* was upregulated ($p < 0.05$) in phases A, C, D, and E (**Fig. 2D**; and Table S5, http://links.lww.com/CCX/B32). Interestingly, *CCL22* expression was upregulated ($p < 0.05$) in phases C and E but was downregulated ($p < 0.05$) in phase F (**Fig. 2E**; and Table S5, http://links.lww.com/CCX/B32) compared with the control group.
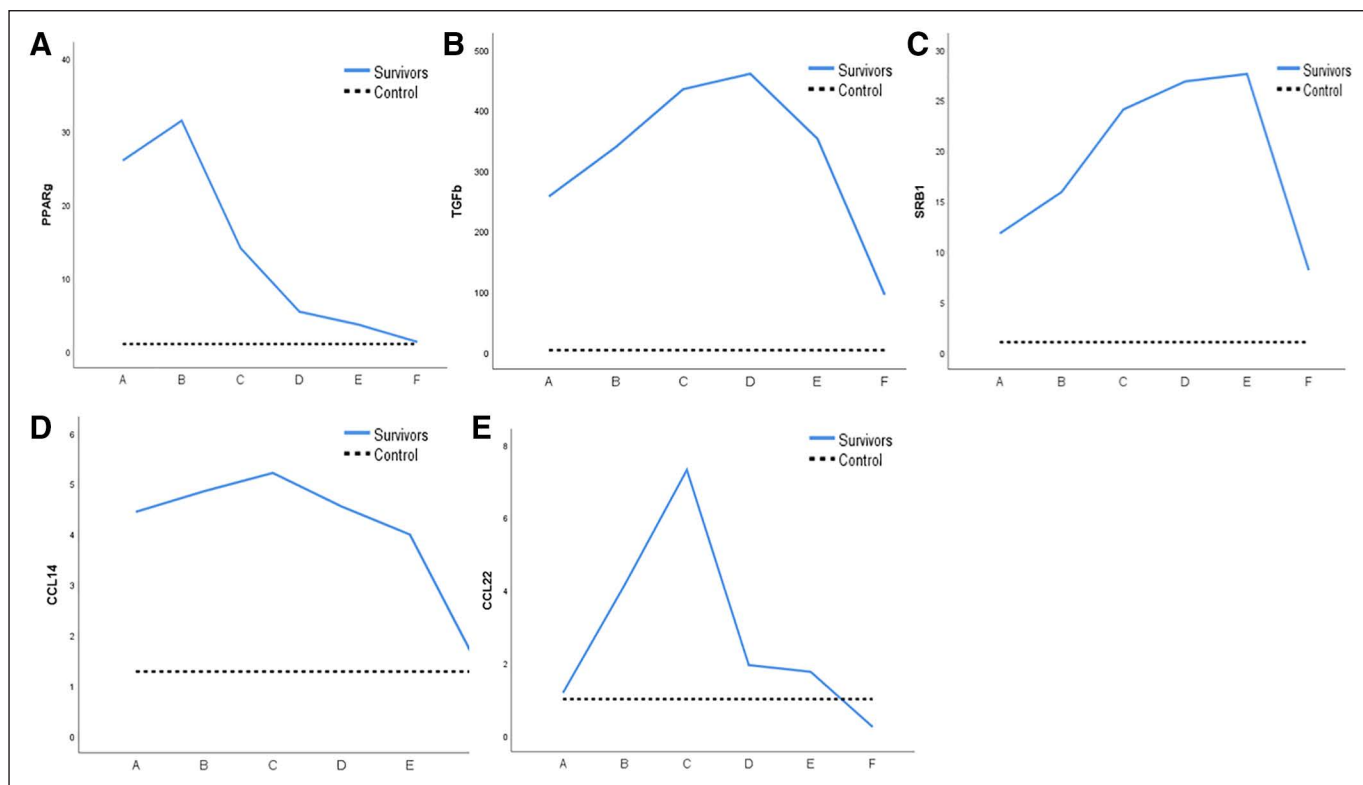
Figure 2. Messenger RNA expression of M2 macrophage markers. **A**, Peroxisome proliferator-activated receptor γ (PPARγ). **B**, Transforming growth factor β (TGF-β). **C**, scavenger receptor class B type 1 (SR-B1). **D**, C−C motif chemokine ligand 14 (CCL14). **E**, C−C motif chemokine ligand 22 (CCL22). Description of the phases: A—acute period; B—immediately after ICU discharge; C—3-mo post-ICU; D—6-mo post-ICU; E—1-yr post-ICU; and F—3-yr post-ICU. Results are presented as the median.

Comparisons among the study survivors demonstrated that *PPARγ* expression was higher in phase F than in phases A and E ($p < 0.05$) (Table S7, http://links.lww.com/CCX/B32), *TGF-β* expression was higher in phase F ($p < 0.05$) compared with phase E (Table S7, http://links.lww.com/CCX/B32), *SR-B1* expression levels were greater ($p < 0.05$) than those in phase E (Table S7, http://links.lww.com/CCX/B32), *CCL14* expression in phase F was less ($p < 0.05$) than that in phase E (Table S7, http://links.lww.com/CCX/B32), and *CCL22:* expression was downregulated in phase E ($p < 0.05$) compared with phase A, and downregulated in phase F expression ($p < 0.05$) compared with phases A and E (Table S7, http://links.lww.com/CCX/B32).

Analysis over time according to mortality identified differences between the survivor and death groups ($p < 0.05$) for the gene expression of M1 polarization markers *CXCL10* and *IL-12p35* (**Table S6**, http://links.lww.com/CCX/B32) and M2 polarization markers *TGF-β* and *SR-B1* (Table S6, http://links.lww.com/CCX/B32). The expression of *CXCL10* was upregulated ($p < 0.05$) in the survivor group, whereas the expression levels of *IL-12p35, TGF-β* and *SR-B1* were downregulated ($p < 0.05$) in the death group (Table S6, http://links.lww.com/CCX/B32).

The expression of M1 and M2 marker genes is upregulated for up to 1 year after hospital discharge. Three years after hospital discharge (phase F), the expression levels of all polarization markers returned to control group levels. These findings suggest that sepsis survivors undergo monocyte reprogramming, which favors the M1 type and lasts up to a year.

Gene Expression of Inflammasome Components

The expression of *NLRP3* was upregulated ($p < 0.05$) in phases A, B, D, E, and F compared with the control group (**Fig. 3A**; and Table S5, http://links.lww.com/CCX/B32), but there were no significant differences (p greater than 0.05) among survivor group patients over time (Table S7, http://links.lww.com/CCX/B32). Additionally, *NFκB1* (**Fig. 3B**; and Table S5, http://links.lww.com/CCX/B32) and Adaptor molecule
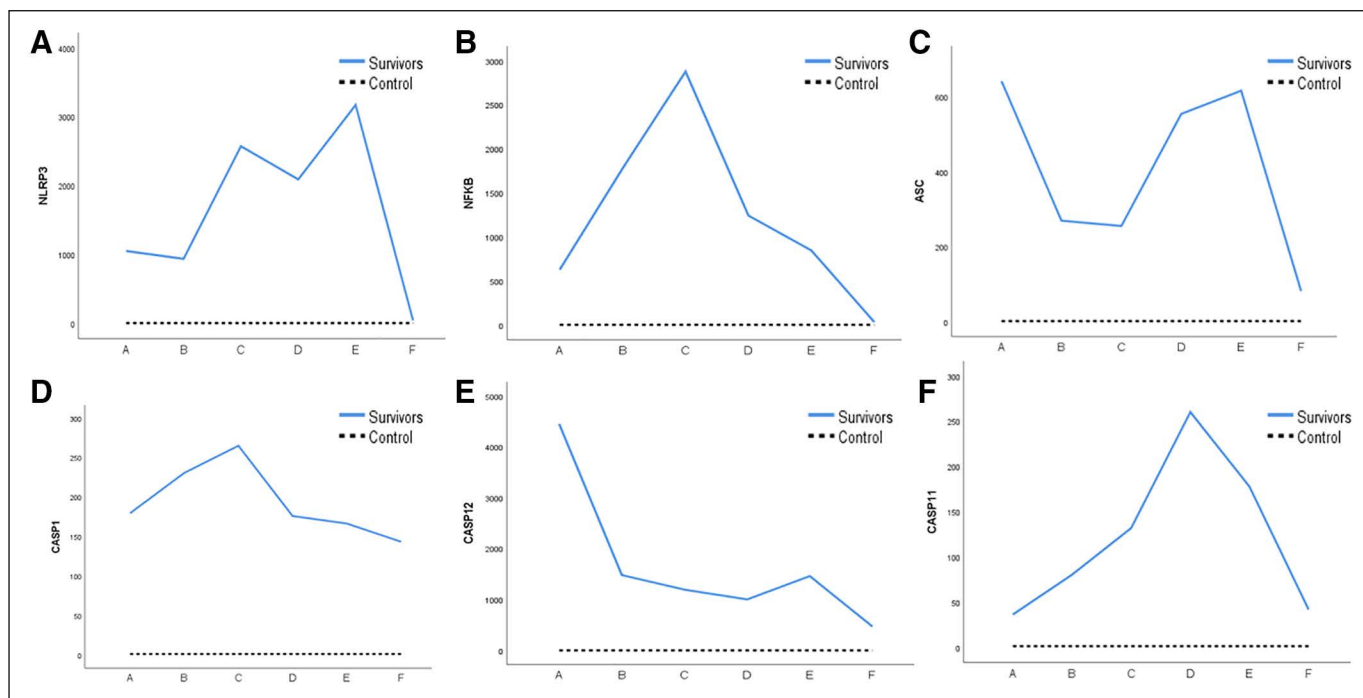
Figure 3. Messenger RNA expression of inflammasome components. **A**, NOD-, LRR-, and pyrin domain-containing protein 3 (*NLRP3*). **B**, Nuclear factor kappa B (*NFκB1*). **C**, Adaptor molecule apoptosis-associated speck-like protein containing a CARD (*ASC*). **D**, Caspase 1 (*CASP1*). **E**, Caspase 12 (*CASP12*). **F**, Caspase 11 (*CASP11*). Description of the phases: A—acute period; B—immediately after ICU discharge; C—3-mo post-ICU; D—6-mo post-ICU; E—1-yr post-ICU; and F—3-yr post-ICU. Results are presented as the median.

apoptosis-associated speck-like protein containing a CARD (*ASC*) (**Fig. 3C**; and Table S5, http://links.lww.com/CCX/B32) expression levels were augmented in survivor phases A–F ($p < 0.05$) compared with the control group. It is worth mentioning that nuclear factor kappa B (*NF-κB*) *p105* subunit expression in phase C of the survivor group was upregulated ($p < 0.05$) compared with phase F (Table S7, http://links.lww.com/CCX/B32), but there was no difference between the death and survivor groups (Table S6, http://links.lww.com/CCX/B32).

The expression of caspase 1 (*CASP1*) and caspase 12 (*CASP12*) was upregulated ($p < 0.05$) in all survivor phases (A–F) compared with the control (**Fig. 3**, *D* and *E*; and Table S5, http://links.lww.com/CCX/B32). Additionally, caspase 11 (*CASP11*) expression ($p < 0.05$) was increased in phases B, D, and F compared with the control group (**Fig. 3F**; and Table S5, http://links.lww.com/CCX/B32). A comparison between the survivor and death groups revealed significant alterations ($p < 0.05$) in *CASP1* and *CASP12* expressions (Table S6, http://links.lww.com/CCX/B32). For example, the expression of *CASP1* was increased ($p < 0.05$) in the death group, whereas the expression of *CASP12* was increased (p

< 0.05) in the survivor group (Table S6, http://links.lww.com/CCX/B32).

Gene Expression of Pro- and Anti-Inflammatory Cytokines

In addition to upregulated *IL-1β* expression, *IL-1α* gene expression was found to be increased ($p < 0.05$) in survivor group phases A–E compared with the control group (Table S5, http://links.lww.com/CCX/B32) and downregulated ($p < 0.05$) in monocytes from the death groups compared with the survivor group (**Fig. 4A**; and Table S6, http://links.lww.com/CCX/B32). *IL-18* and high-mobility group box 1 protein (*HMGB1*) (late proinflammatory cytokine) gene expression was upregulated in monocytes from all survivor group phases ($p < 0.05$) compared with the control group. The former remained elevated ($p < 0.001$) in phase F (**Fig. 4B**; and Table S5, http://links.lww.com/CCX/B32), whereas the expression of the latter cytokine was reduced ($p < 0.05$) in the death group compared with the survivor group (**Fig. 4C**; and Table S6, http://links.lww.com/CCX/B32). Finally, the expression of the anti-inflammatory cytokine *IL-10* was elevated ($p < 0.05$) in survivor
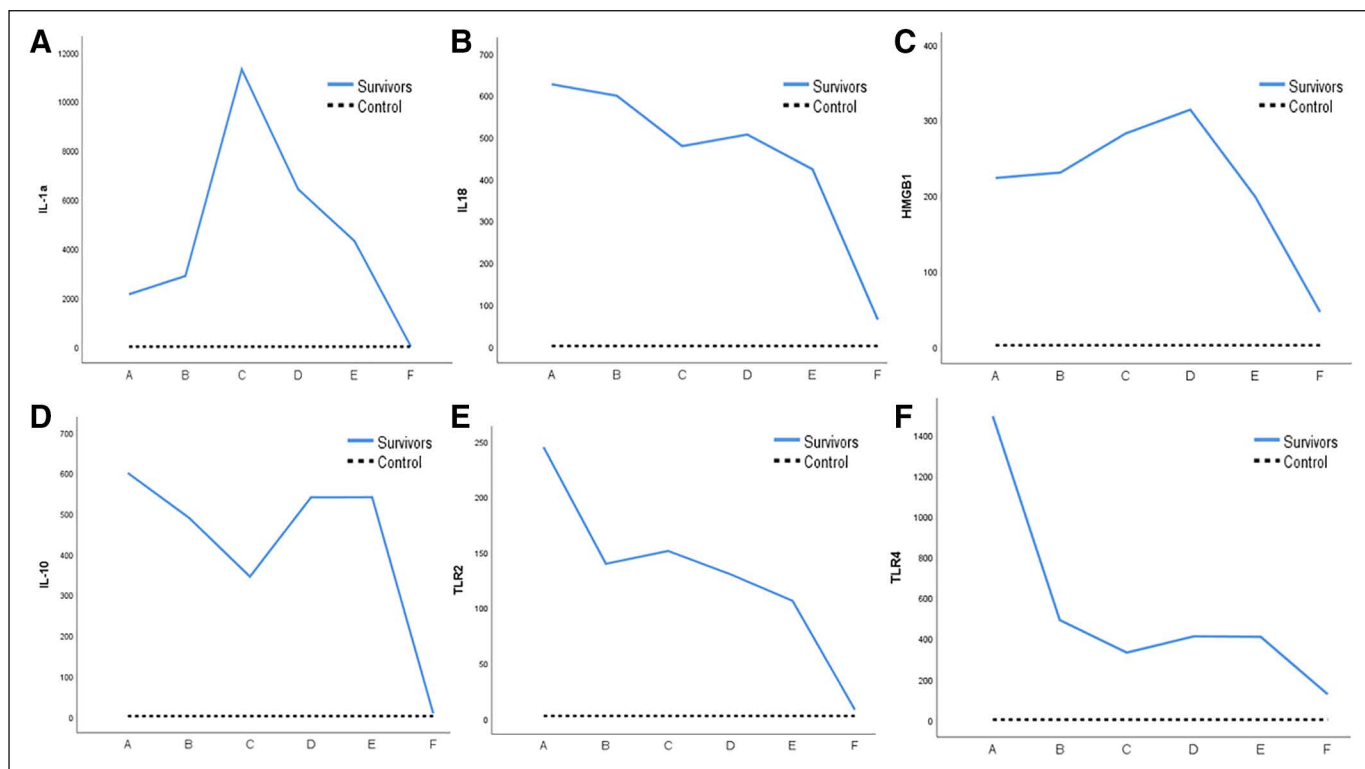
Figure 4. Messenger RNA expression of inflammatory receptors and pro- and anti-inflammatory cytokines. **A**, Interleukin 1 alpha (*IL-1α*). **B**, Interleukin-18 (*IL-18*). **C**, High-mobility group box 1 protein (*HMGB1*). **D**, Interleukin-10 (*IL-10*). **E**, Toll-like receptor 2 (*TLR2*). **F**, Toll-like receptor 4 (*TLR4*). Description of the phases: A—acute period; B—immediately after ICU discharge; C—3-mo post-ICU; D—6-mo post-ICU; E—1-yr post-ICU; and F—3-yr post-ICU. Results are presented as the median.

phases A–F (**Fig. 4D**; and Table S5, http://links.lww.com/CCX/B32).

Gene Expression of Inflammatory Receptors

Compared with controls, *TLR2* gene expression was upregulated ($p < 0.05$) in phases A–E (**Fig. 4E**; and Table S5, http://links.lww.com/CCX/B32). *TLR2* gene expression in phases B, C, D, E, and F was downregulated ($p < 0.05$) compared with phase A (**Fig. 4F**; and Table S7, http://links.lww.com/CCX/B32). Similarly, *TLR4* gene expression was increased ($p < 0.05$) in phases A–F compared with the control group (Fig. 4F; and Table S5, http://links.lww.com/CCX/B32), with significantly lower ($p < 0.05$) expression levels in phase F than in phase A (Table S7, http://links.lww.com/CCX/B32).

DISCUSSION

The present study showed that M1 and M2 marker messenger RNA expression levels were higher in monocytes from septic patients than in controls. Additionally, the expression of M1 markers, *CXCL10*, *CXCL11*, and *IL-1β*, was upregulated during phase

A and remained above control levels until phase E, whereas *IL-12p35* remained elevated in phase F. M2 polarization markers *PPARγ, CCL14*, and *CCL22* remained above control levels for up to 1 year. *TGF-β* and *SRB1* continued to be upregulated for up to 3 years post-ICU. The observed upregulation of *TLR2, TLR4, NF-κB, NLRP3, ASC, CASP1, CASP11, CASP12, IL-1α, HMGB1, IL-1β, IL-18,* and *IL-10* during phase A is consistent with previous studies (40–45). Interestingly, the gene expression of *NLRP3, NFκB, TLR2, IL-1α, IL-1β,* and *IL-10* remained upregulated 1-year post-ICU, and *CASP1, CASP11, CASP12, ASC, TLR4, IL-18,* and *HMGB1* expression was augmented 3-year post-ICU. These results are summarized in **Figure 5**.

In healthy subjects, monocytes are nonpolarized circulating cells (20). These cells express chemokine and adhesion receptors that mediate their migration from the blood to tissue during inflammatory processes. When there is an insult, these cells are recruited and undergo maturation into macrophages (17). Herein, monocytes from healthy individuals exhibited low messenger RNA expression levels for M1 and M2 polarization markers, NLRP3 inflammasome components,
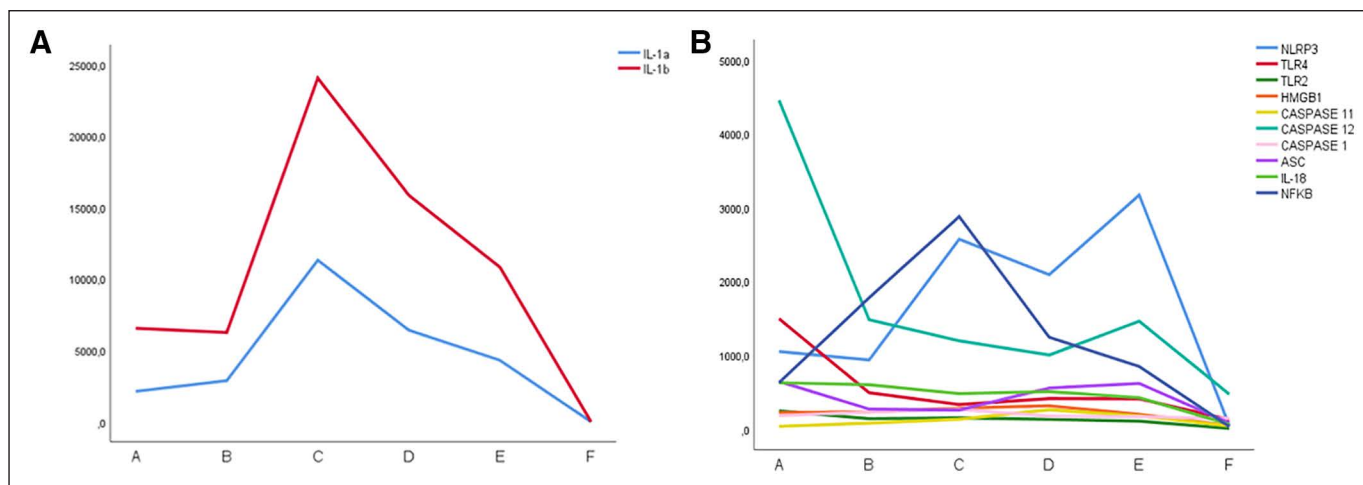
Figure 5. Messenger RNA expression of NLRP3 inflammasome compounds. **A**, Interleukin 1 alpha (*IL-1α*) and interleukin 1 beta (*IL-1β*). **B**, *NLRP3*, Toll-like receptor 4 (*TLR4*), Toll-like receptor 2 (*TLR2*), High-mobility group box 1 protein (*HMGB1*), Caspase 11 (*CASP11*), Caspase 12 (*CASP12*), Caspase 1 (*CASP1*), adaptor molecule apoptosis-associated speck-like protein containing a CARD (*ASC*), Interleukin-18 (*IL-18)*, and nuclear factor kappa B (*NFκB*). Description of the phases: A—acute period; B—immediately after ICU discharge; C—3-mo post-ICU; D—6-mo post-ICU; E—1-yr post-ICU; and F—3-yr post-ICU. Results are presented as the median.

and pro- and anti-inflammatory cytokines, opposite of what was observed in patients with and after sepsis. We found that monocytes from the control group have basal and constant M1 and M2 polarization marker messenger RNA expression. Indeed, monocytes from the survivor group displayed increased M1 or M2 polarization marker gene expression, with some changes lasting for up to 1 year after discharge and mostly returning to control levels after 3 years.

Sepsis causes marked changes in macrophage functions and increases M1 cell proportion, particularly in the acute phase. Several groups reported that macrophages undergo cell metabolism reprogramming during sepsis (29–33). Notably, our results indicate that sepsis-induced macrophage reprogramming remains for up to 1 year and is elevated for up to 3-year post-ICU in some cases.

As mentioned above, the expression of *IL-1α, HMGB1*, and *CASP-12* was higher ($p < 0.05$) in the survivor group than in the death group (Table S6, http://links.lww.com/CCX/B32). *CASP1* is linked to the assembly of the inflammasome complex through the classical pathway, cleaving and releasing the active forms of the cytokines IL-1β and IL-18. On the other hand, *CASP12* inhibits the inflammasome assembly. Interestingly, monocytes of the death group express more *CASP1* and less *CASP12* than the survivor group. This result indicates an association between NLRP3 inflammasome downregulation via *CASP12* and survival. The expression of proinflammatory cytokines

from the nonclassical NLRP3 inflammasome pathway corroborates this proposal. It should also be pointed out that survivors' monocytes expressed more *HMGB1* and *IL-1α* than patients who died. Thus, it is plausible that the downregulation of the classical pathway and the nonclassical NLRP3 inflammasome might play a role in sepsis survival.

As shown in Figure 5, monocytes from septic patients remained activated for up to 1 year and slightly activated for 3-year post-ICU, contributing to persistent systemic inflammation. The expression of *IL-1α* and *IL-1β* was markedly enhanced during and after sepsis (Fig. 5*A*), with values greater than 5,000 times higher than the control group 1-year post-ICU. However, the values were not different from the control after 3 years. Previous reports demonstrated that some stimuli cause long-term cellular reprogramming (35–37), supporting our evidence of such an event in the monocytes of septic patients. Several parameters did not return to control levels after 3 years (Fig. 5).

Concerning TLRs, these receptors play an important role in the pathogenesis of sepsis (40). Our data show that TLR2 and TLR4 messenger RNA expression in the survivor group during phase A increased compared with the control group. It already has reported that TLR signaling pathways were upregulated during sepsis and correlated with disease severity (41). Although *TLR2* and *TLR4* gene expression was upregulated in monocytes of septic patients compared with the control group, mortality was only associated with

TLR2 in blood monocytes and correlated with reduced *IL-10* and *TNF-α* expressions.

The initial inflammatory response of monocytes/macrophages is associated with the release of TNF-α, IL-1β and other early inflammatory mediators of shock and tissue injury. In sepsis, HMGB1 is released by activating innate immune cells in the late phase of the disease (42, 43). Another study demonstrated that plasma HMGB1 concentration correlates with disease severity mortality levels in ICU patients with severe pneumonia (44). Plasma HMGB1 concentrations have also been correlated with the trauma severity and are significantly higher in patients with multiple organ failure and those who die (43, 45, 46). Furthermore, plasma HMGB1 was higher in individuals who died during the first year after sepsis when compared with survivors (28).

The small number of septic patients that survived 3-year post-ICU ($n = 8$) is one limitation of the study. However, the results are highly relevant to sepsis research, especially postsepsis diagnosis and follow-up. Most previous studies focused only on the acute sepsis phase, used animal models/immortalized cell lines, or did not follow up with the patients. It remains unclear whether cell reprogramming occurs in the bone marrow and/or plasma or if epigenetic and metabolic mechanisms are involved, but these studies are currently underway.

In conclusion, we demonstrated that sepsis induces monocyte reprogramming. Some gene expression of macrophage polarization markers, NLRP3 inflammasome components, and proinflammatory cytokines remained elevated for 3 years after ICU discharge, thus representing potential postsepsis survival indicators. It is plausible that these reprogrammed monocytes with a sepsis-induced memory (Msepsis monocytes) could contribute to PSS onset and development, influencing post-ICU outcomes.

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1   Interdisciplinary Post-Graduate Program in Health Sciences, Cruzeiro do Sul University, São Paulo, SP, Brazil.

2   University Hospital, University of São Paulo, São Paulo, Brazil.

3   Internal Medicine Department, School of Medicine, University of São Paulo, São Paulo, Brazil.

4   Department of Genetics and Morphology, Institute of Biological Sciences, Brasília University, Brasília, Brazil.

5   Department of Emergency Medicine, University of São Paulo, São Paulo, Brazil.

6   Bioindustrial Center, Butantan Institute, São Paulo, Brazil.

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