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ORIGINAL RESEARCH PAPER

FK506 impairs neutrophil migration that results in increased polymicrobial sepsis susceptibility

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Abstract

Objective This study aimed to investigate the effects of FK506 on experimental sepsis immunopathology. It investigated the effect of FK506 on leukocyte recruitment to the site of infection, systemic cytokine production, and organ injury in mice with sepsis.

Methods Using a murine cecal ligation and puncture (CLP) peritonitis model, the experiments were performed with wild-type (WT) mice and mice deficient in the gene *Nfat1* (*Nfat1*$^{-/-}$) in the C57BL/6 background. Animals were treated with 2.0 mg/kg of FK506, subcutaneously, 1 h before the sepsis model, twice a day (12 h/12 h). The number of bacteria colony forming units (CFU) was manually counted. The number of neutrophils in the lungs was estimated by the myeloperoxidase (MPO) assay. The expression of CXCR2 in neutrophils was determined using flow cytometry analysis. The expression of inflammatory cytokines in macrophage was determined using ELISA. The direct effect of FK506 on CXCR2 internalization was evaluated using HEK-293T cells after CXCL2 stimulation by the BRET method.

Results FK506 treatment potentiated the failure of neutrophil migration into the peritoneal cavity, resulting in bacteremia and an exacerbated systemic inflammatory response, which led to higher organ damage and mortality rates. Failed neutrophil migration was associated with elevated CXCL2 chemokine plasma levels and lower expression of the CXCR2 receptor on circulating neutrophils compared with non-treated CLP-induced septic mice. FK506 did not directly affect CXCL2-induced CXCR2 internalization by transfected HEK-293 cells or mice neutrophils, despite increasing CXCL2 release by LPS-treated macrophages. Finally, the CLP-induced response of *Nfat1*$^{-/-}$ mice was similar to those observed in the *Nfat1*$^{+/+}$ genotype, suggesting that the FK506 effect is not dependent on the NFAT1 pathway.

Conclusion Our data indicate that the increased susceptibility to infection of FK506-treated mice is associated with failed neutrophil migration due to the reduced membrane availability of CXCR2 receptors in response to exacerbated levels of circulating CXCL2.

Keywords FK506 · Tacrolimus · Sepsis · CXCR2 · Neutrophil migration · CXCL2

Introduction

Sepsis is defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. Immunocompromised hosts with defects in innate or adaptive immune responses, such as neutropenic and transplant patients, elderly populations, and HIV-positive individuals, are more susceptible to conventional and opportunistic pathogens [2]. It is well known that solid organ and

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hematopoietic cell transplant recipients show higher susceptibility to opportunistic pathogens and are more vulnerable to developing severe infections [3–5]. One of the main factors that contribute to this inherent feature of transplanted patients is the use of immunosuppressive drugs, such as the calcineurin inhibitors cyclosporin A and tacrolimus (FK506), to prevent graft-versus-host disease (GvHD) and organ rejection.

Calcineurin is a serine-threonine phosphatase activated by the calcium (Ca^{2+}) calmodulin complex, and it is known to be an activator of the nuclear factor of activated T cells (NFAT) family members [6]. The immunosuppressive drug FK506 first binds to FK506-binding protein 12 (FKBP12) and complexes with calcineurin, suppressing its phosphatase activity and consequently NFAT activation [7–9]. Although NFAT is a pharmacological target of FK506 in regulating T cell-mediated responses, the FKBP12/calcineurin/NFAT axis affects a wide range of cellular responses [10–15]. Furthermore, in addition to NFAT, calcineurin can act on other intracellular targets, including fork head transcription factors (FOXO), myocyte-specific enhancer factor 2 (MEF2), and transcription factor EB (TFEB), which are important to most cells and systems [6]. Additionally, the FK506-mediated displacement of FKBP12 from the endoplasmic reticulum has been studied as a modifier of intracellular calcium balance, which is associated with FK506 side effects in the cardiovascular system [16]. Nevertheless, the immunosuppressive effects of FK506 on innate immunity, especially in systemic infectious conditions, such as sepsis, are still poorly understood and conflicting.

Neutrophils and macrophages are vital cells of the innate immune system that participate in the local control of infection during the acute events that precede sepsis, preventing bacterial spread into the bloodstream [17]. When the infectious process begins, the recognition of bacterial components by resident tissue cells, mainly macrophages, culminates in the release of the chemotactic mediators that contribute to the recruitment and activation of circulating neutrophils toward the site of infection. Subsequently, pathogens are killed by different neutrophil bactericidal mechanisms, including phagocytosis, proteinase degranulation, reactive oxygen and nitrogen species release, and neutrophil extracellular traps (NETs) [18]. In severe CLP-induced sepsis, our group demonstrated that neutrophil migration is impaired, which results in deficient control of infection, leading to increased bacteremia, worsened multiorgan dysfunction, and death [19–21]. Mechanistically, we demonstrated that the impairment of neutrophil migration toward infectious foci is a consequence of GRK2-dependent CXCR2 receptor internalization, which could be induced by ligands, such as TLR agonists and chemokines [22, 24]. Here, we show that FK506 can affect the innate immune system, exacerbating the release of chemokines by macrophages, which impairs neutrophil migration to the infectious site and consequently controls bacterial growth in polymicrobial peritonitis.

Materials and methods

Animals

The experiments were performed using wild-type (WT) mice and mice deficient in the gene *Nfat1* (*Nfat1*$^{-/-}$) in the C57BL/6 background at 8–10 weeks of age. Animals were housed in temperature-controlled rooms (22–25 °C) and given water and food ad libitum at the animal facility in the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Brazil. We performed all experiments according to the guidelines of the Animal Welfare Committee of the School of Medicine of Ribeirão Preto, University of São Paulo (animal protocol number: 002/2013-1).

FK506 treatment

Animals treated with FK506 (Prograf, Astelllas Pharma) received dosages of 0.7, 2.0 or 6.0 mg/kg, subcutaneously, 1 h before the sepsis model, twice a day (12 h/12 h). In the survival rate experiments, treatment was extended for 3 days. In some survival experiments, 30 mg/kg ertapenem disodium (ERT) was also administered intraperitoneally (i.p.) 6 h after cecal ligation and puncture (CLP) surgery and maintained twice a day (12 h/12 h) for 3 days. Finally, in the biological sample collection experiments, the animals were anesthetized 6 or 24 h after CLP. In both groups, animals received the first administration of FK506 1 h before CLP; in the group in which the collection was performed at 24 h, the animals received a second dose 12 h after CLP.

Cecal ligation and puncture

After mice were exposed to isoflurane anesthesia by a vaporizer (Dräger-Vapor®/2000) (2% for induction and 1% for maintenance), an incision of approximately 1 cm was carefully made in the anterior abdomen to allow cecal exposure. The ileocecal junction was then ligated with cotton thread to prevent the retrograde flow of cecal content. Two punctures with 21 G or 18 G gage needles were made in the cecum of the animals for induction of stimuli. Then, the cecum was repositioned in the abdomen, the incision was sutured, and 1 mL of sterile saline was administered subcutaneously to prevent acute distributive shock. For the survival analysis, the number of CLP survivor mice was recorded every 24 h for 10 days and expressed as a percentage of survival (%).

Blood bacteria count

Immediately after euthanasia, heparinized blood was collected by cardiac puncture and seeded (undiluted and diluted 1:1000) under sterile conditions on Petri plates containing Mueller Hinton agar (Difco Laboratories, Detroit, USA). The culture plates were incubated for 24 h at 37 °C, and then the number of colony forming units (CFU) was manually counted.

Myeloperoxidase assay

The number of neutrophils in the lungs was estimated by the myeloperoxidase (MPO) assay. After euthanasia, the animals were perfused with PBS, and the left pulmonary lobe was collected and immersed in buffer solution (0.1 M NaCl; 0.02 M $NaPO_4$; 0.012 M NaEDTA; pH 4.7). The tissue was macerated using a homogenizer (Polytron®, Polytron PT 3100, USA) at 13,000 rpm, the red blood cells were lysed in 0.2% NaCl solution, and the leukocytes in 0.05 M $NaPO_4$ solution with 0.5% H-TaB. The MPO reaction proceeded by adding TMB substrate and was stopped with 2 N H_2SO_4 solution. The absorbance was measured at 450 nm in a spectrophotometer (Spectra Max-250, Molecular Devices), and the results are expressed as the number of neutrophils/mg of tissue. The absorbance of the samples was associated with the number of neutrophils from a curve made with a known number of peritoneal neutrophils obtained by the carrageenan stimulus.

Flow cytometry analysis

Blood was collected, and the red blood cells were lysed. The remaining leukocytes were resuspended at a final count of 5.0×10^4 cells/100 µL in FACS buffer (1 × PBS, 20 mM glucose and 0.5% BSA). For Fc receptor blocking, the samples were incubated with 10 µL of rabbit serum for 40 min at 4 °C. Then, the cells were incubated with PerCP-conjugated monoclonal anti-Ly6G (1:200; BD Biosciences) and PE-conjugated anti-CXCR2 (1:50; R&D Systems) antibodies for 30 min at 4 °C. Subsequently, the cells were washed with 2 mL of FACS buffer and resuspended in 200 µL of 1% PBS-Formol. Fluorescence was analyzed on a FACSort device (Becton Dickinson, San Jose, CA, USA) using the program Lysis II or WinMDI 2.8.

Cytokine

The dosages of CXCL1, CXCL2, and IL-6 were determined using ELISA kits (R&D System), and the organ injury biochemical markers were measured by commercial kits (Labtest Brazil) according to the manufacturer's instructions. The readings were performed in a spectrophotometer (Spectra Max-250, Molecular Devices, Sunnyvale, CA, USA).

Killing assay

The C57BL/6 J mice were subcutaneously treated with 2.0 mg/kg FK506 or vehicle and, after 1 h, stimulated intraperitoneally with 1 mL of 3% thio-glycolate. The neutrophils were obtained after 6 h by peritoneal lavage with 3 mL of 10% fetal bovine serum in RPMI medium (RPMI-SBF 10%). The cells were plated (5×10^5 cells/well) in 96-well plates. In addition to in vivo treatment, vehicle-treated WT mouse cells also received treatment with FK506 at concentrations of 0.1, 0.3 and 1.0 µM, which started 30 min before the bacterial co-culture. The *Escherichia coli* suspension was opsonized with WT mouse serum (10%) for 30 min at 37 °C. After that, the bacteria were washed and resuspended in RPMI-SBF 10%. The cells were incubated with *E. coli* at a ratio of 1:10 (5×10^5 cells to 5×10^6 bacteria) for 45 min at 37 °C and 5% CO_2. After incubation, the plates were centrifuged (450 g, 4 °C, 10 min), and the supernatant was collected to quantify the bacteria in the extracellular medium. To remove the bacteria in the extracellular medium, the cells were washed once with RPMI-SBF 10% and subsequently incubated with 50 µg/mL gentamicin in RPMI-SBF 10% for 30 min. After that, the plates were centrifuged and washed. Then, the cells were lysed with 200 µL of 0.2% Triton X-100 for 20 min at 4 °C for the quantification of bacteria in the intracellular medium. For the final calculations, wells containing only bacteria in RPMI-SBF 10% were included in the incubation. The percentage of killing was estimated by the difference between the counts of the wells with only bacteria and the counts in the supernatants of neutrophil cultures added to the counts inside the cells. Bacterial measurements were made by culturing on plates containing Mueller Hinton agar (Difco Laboratories, Detroit, USA) and manual counting of CFUs after incubating the plates for 24 h at 37 °C.

Macrophage cultures

Bone marrow-derived macrophages (BMDMs) were obtained using L929 cell-conditioned medium as a source of M-CSF, as previously described [25]. Briefly, bone marrow cells were obtained from 6- to 8-week-old mice by flushing both mouse femurs. After centrifugation, the cells were resuspended in RPMI 1640 supplemented with 20% L929 cell-conditioned medium, 10% FBS (Thermo Fisher, Waltham, MA, USA), L-glutamine (2 mM), penicillin (100 U/mL), and fungizone (2.5 µg/mL). Bone marrow cells were seeded in non-treated Petri dishes (Corning, 430,591) and incubated at 37 °C and 5% CO_2. Four days after seeding, supplemented RPMI 1640 medium was added, and the cells were incubated for an additional three days. At the end

of this period, BMDMs were harvested, and 2×10^5 cells were plated in 96-well flat button culture dishes (Corning, CLS3997) and incubated with RPMI for 24 h for BMDM adherence. BMDMs were treated with FK506 (1 μM), 2APB (50 μM), dantrolene (30 or medium for 30 min. Then, BMDMs were stimulated with LPS (3 ng/mL) for an additional 12 h at 37 °C and 5% CO_2. The supernatants were then collected for CXCL2 quantification.

Neutrophil purification

Bone marrow (BM) mouse neutrophils were isolated by a Percoll (Sigma–Aldrich) density gradient, as previously described [26]. Briefly, two different gradients were prepared in a 15 mL polystyrene tube with 3 mL each (72% and 65% Percoll solutions). After centrifugation at 1200 g for 30 min at 25 °C, the cell layer at the 72% upper interface was collected as the neutrophil fraction. Erythrocytes were removed by lysis (NH_4), and the remaining neutrophil fractions were washed twice in PBS. The pelleted cells were resuspended in 1 mL of RPMI 1640 medium (Sigma Chemical Co., St Louis, USA), and the number of neutrophils was determined by Neubauer chamber counting and purity by Wright-Giemsa staining. A total of 10^5 BM neutrophil cells were plated in 96-well culture dishes and treated with 1.0 μM FK506 or vehicle for 30 min. Afterward, neutrophils were incubated with 500 ng/mL CXCL2/MIP-2 for 1 h. Then, the CXCR2 content on the surface of neutrophils was determined.

Bioluminescence resonance energy transfer (BRET) assay

The plasmid encoding the enhanced bystander BRET-based biosensor rGFP-CAAX was generated as previously reported and provided by Michel Bouvier [27]. To construct the plasmid encoding CXCR2 fused to RLucII (CXCR2-RLucII), the sequence encoding the receptor was amplified from the plasmid pReceiver-M51-CXCR2 (Gene Copoeia, Inc, MD, USA) and inserted into the N-terminus of RLucII in the pcDNA3.1 + GFP10-RLucII plasmid digested with Nhel and BamHI enzymes to remove GFP10. We verified the construct by DNA sequencing. HEK-293T (human embryonic kidney) cells maintained in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin were transiently transfected as previously described [28, 29]. Briefly, cells in suspension were transiently transfected with the prepared plasmids utilizing 25 kDa poly-ethylenimine (PEI) (Polysciences, PA, EUA) at a 3:1 PEI/DNA ratio. Next, cells were distributed in 96-well white plates (Optiplate; PerkinElmer, MA, EUA) (4×10^4 cells per well) and maintained in culture at 37 °C with 5% CO_2. BRET experiments were performed 48 h after cell transfection. To assess CXCR2 internalization, cells transiently expressing CXCR2-RLucII and the plasma membrane marker rGFP-CAAX were washed once with PBS and incubated with Tyrode's buffer for 30 min at 37 °C. Cells were incubated with vehicle or increasing concentrations of FK506 for 30 min. Cells were also incubated with 1.3 μM Prolume Purple (Nanolight Technology, AZ, USA) for 5 min and then stimulated with rmCXCL2/MIP-2 (R&D Systems; Minneapolis, MN, USA) at a fixed concentration of 500 ng/mL. Luminescence values were monitored for 50 min by the Synergy2 (BioTek, VT, USA) microplate reader using the filters 410/40 nm and 515/20 nm to detect the emission of donor (RLucII) and acceptor (rGFP), respectively. To determine the BRET signals, we calculated the ratio of the luminescence value derived from the acceptor to that derived from the donor.

Statistical analysis

Statistical analyses were performed using the Prism® 5 program (GraphPad Software Corporation, USA). For statistical analysis of survival curves, the Mantel–Cox log-rank test was used. The results were analyzed by one-way ANOVA followed by the Newman–Keuls test to determine the significance between the groups. The number (n) of animals per experimental group is described in the figures. The results are expressed as the means ± standard error of the mean (SEM). The differences were considered significantly different at $p < 0.05$.

Results

FK506 treatment results in higher mouse susceptibility to CLP-induced sepsis

It is recognized that the severity of CLP-induced sepsis is directly proportional to the level of bacteremia and inversely proportional to the number of neutrophils that migrate into the peritoneal cavity (primary infection site). To establish our model, we previously standardized CLP induction at 2 degrees of severity and recorded survival over a 10-day study period. CLP with 21-gage (21G-CLP) and 18-gage (18G-CLP) needles result in survival rates of 60% and 0%, respectively. Compared to 18G-CLP, 21G-CLP results in lower bacteremia, assessed 24 h after induction, and higher neutrophil migration into the peritoneal cavity, assessed after 6 h (data not shown). We performed the following in vivo protocols using the moderate 21G-CLP stimulus. Initially, we administered FK506 (0.7, 2.0, and 6.0 mg/kg) subcutaneously (s.c.) 1 h prior to CLP (21G-CLP) surgery, and then twice a day for 3 days. Given that the 2.0 and 6.0 mg/kg doses reduced the survival of CLP mice similarly (0% and 9%, respectively) compared with the vehicle-treated group

(survival of 55%; Fig. 1A), we conducted all the subsequent in vivo experiments using the 2.0 mg/kg dose, and since more than 60% of the mice treated with this dose of FK606 are expected to die between 24 and 48 h after CLP, we proceeded the other analysis at the 6 and 24 h time points. Septic mice treated with FK506 showed elevated bacteremia (Fig. 1B) and plasma organ damage markers, such as lactate dehydrogenase (LDH) (Fig. 1C), urea nitrogen (BUN, Fig. 1D), and creatine kinase-MB (CK-MB, marker of cardiac arrest; Fig. 1E), compared with vehicle-treated mice. Moreover, the FK506-treated group also presented an increase in MPO levels in the lungs (Fig. 1F), a marker used to evaluate the level of tissue infiltrating neutrophils. Finally, we observed that FK506 treatment resulted in higher plasma levels of IL-6 (Fig. 1G) and CXCL2 (Fig. 1H), confirming an exacerbated systemic inflammatory response.

FK506 induces a failure of CLP-induced neutrophil migration to the peritoneal cavity

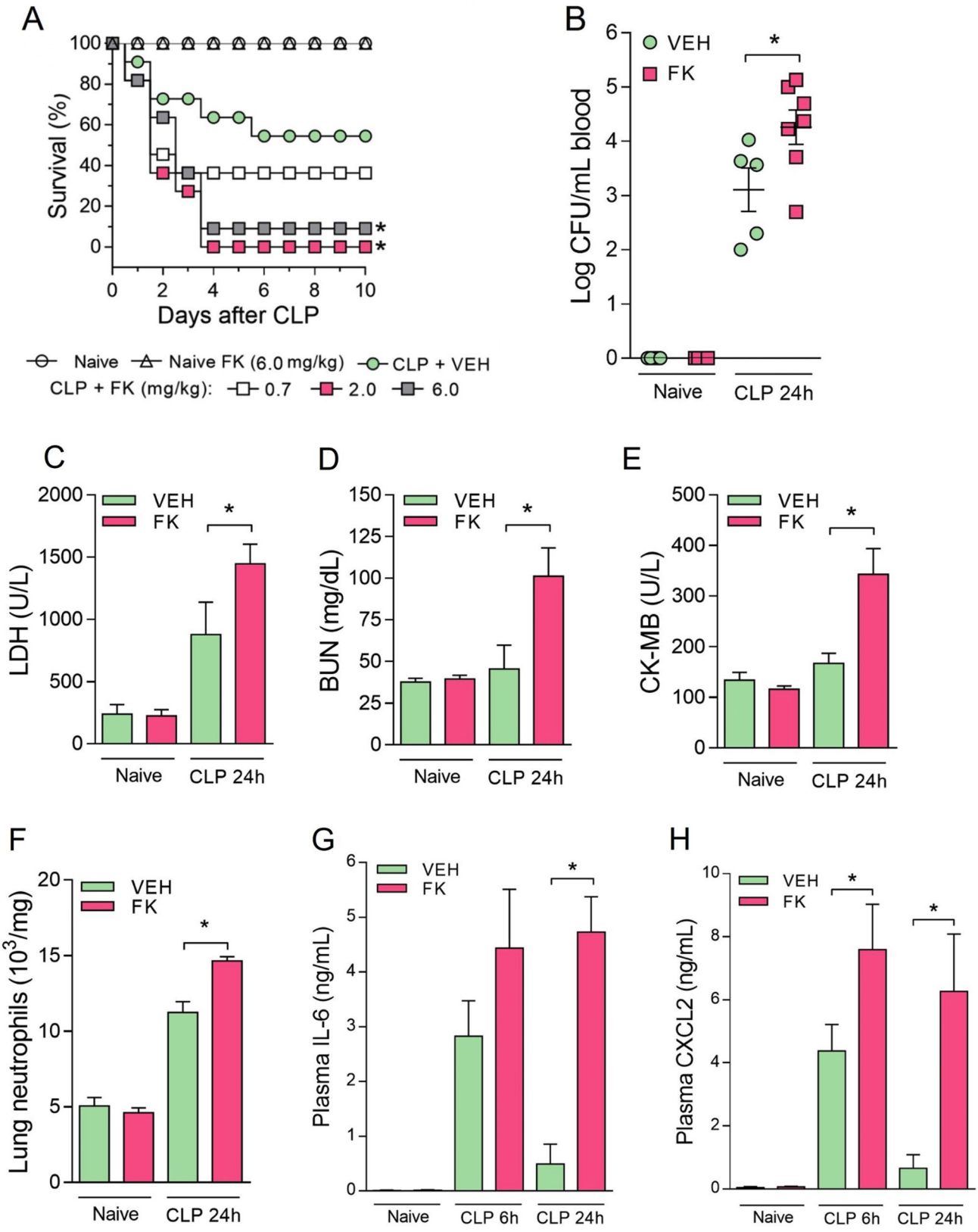
Since FK506 treatment aggravates the septic condition, we investigated the mechanism involved, focusing on neutrophil recruitment, which is considered the first line of defense against invading pathogens. Septic animals treated with FK506 showed a significant reduction in neutrophil migration to the peritoneal cavity (Fig. 2A) and a reduced expression of CXCR2 on the plasma membrane of circulating neutrophils (characterized as $Ly6G^+$ cells) collected 6 and 24 h after sepsis induction (Fig. 2B). The FK506-induced failure in neutrophil migration was not a consequence of a lower release of inflammatory mediators at the infection site, since the IL-6 (Fig. 2C) and CXCL2 (Fig. 2D) levels in the peritoneal lavage fluid were similar to the levels in the vehicle group during the initial 6 h. Moreover, the levels of these cytokines remained elevated in the FK506-treated septic mice even 24 h after the surgery, in contrast with the drastic reduction observed in the vehicle-treated mice (Fig. 2C and D). Next, to investigate whether FK506 has a direct effect on the microbicidal activity of neutrophils, we investigated the killing properties of neutrophils obtained from mice treated with FK506 (2 mg/kg) or vehicle (control group). There was no change in the killing of *E. coli* by neutrophils isolated from mice treated with FK506 (2 mg/kg) or neutrophils treated directly with FK506 (0.1, 0.3 and 1.0 μM) (Fig. 2E). Finally, to confirm that FK506 administration reduces the ability to control bacterial dissemination, we performed a survival experiment using a severe CLP stimulus followed by antibiotic therapy. When CLP animals were treated with ertapenem (ERT), a broad-spectrum antimicrobial agent, we observed an improvement in the survival index. Interestingly, in the antibiotic-treated septic mice, FK506 treatment did not reduce the survival rate, as shown by differences in the mortality index after administration of FK506 (Fig. 2F).

Effect of FK506 treatment on CXCR2 internalization and CXCL2 production

To explore the mechanism by which FK506 affects neutrophil migration to the infectious site in CLP mice, we investigated whether FK506 directly enhances CXCR2 internalization in HEK-293T cells after CXCL2 stimulation. Initially, as expected, CXCL2 incubation induced CXCR2 internalization, as indicated by the decrease in ebBRET signals. Curiously, FK506 at concentrations ranging from 0.01 to 30 μM was not able to induce a significant change in the ΔebBRET values compared to the control (Fig. 3A). Furthermore, when we incubated bone marrow neutrophils with both CXCL2 and FK506, no synergistic effects on the reduction of CXCR2 expression were detected, demonstrating that FK506 does not act downstream of CXCR2 activation (Fig. 3B). Next, to evaluate whether FK506 can affect chemokine release, we quantified the production of CXCL2 in the supernatants of murine LPS-stimulated bone marrow-derived macrophage (BMDM) cultures. When the FK506-treated cells (1 μM; 30 min) were stimulated with LPS (3 ng/mL; 12 h), higher levels of CXCL2 release were detected. Finally, we investigated if IP3 and ryanodine receptors (IP3R and RyR, respectively) are involved in FK506-induced CXCL2 release. It was observed that incubation with 2APB (50 μM), an IP3R inhibitor, but not dantrolene (30 μM), a RyR inhibitor, abrogated the FK506-induced CXCL2 release by macrophages, suggesting that this effect is dependent on IP3 channels (Fig. 3C). Curiously, dantrolene increased the CXCL2 release by LPS, increasing even more if combined with FK506, indicating possible opposing effects by the two Ca^{2+} channels inhibitors.

NFAT1 deficiency did not mimic the FK506 effect on sepsis outcome

Given that FK506 treatment of septic mice promoted a reduction in neutrophil migration, we investigated the participation of the NFAT signaling pathway in sepsis development. For this, wild-type mice (WT; $Nfat1^{+/+}$) and NFAT1-deficient mice ($Nfat1^{-/-}$) were subjected to CLP surgery. NFAT1 deficiency did not result in a worsening of infection after CLP surgery (Fig. 4A). In addition, no differences were observed in the migration of neutrophils to the infectious site 6 and 24 h after sepsis induction (Fig. 4B), as well as no significant differences in the concentration of CXCL2 in the peritoneal exudates (Fig. 4C) or in circulation (Fig. 4D), as observed with FK506 treatment (Figs. 1 and 2). In vitro experiments with neutrophils isolated from $Nfat1^{+/+}$ and $Nfat1^{-/-}$ mice indicated that after CXCL2 stimulation (30 ng/mL), $Nfat1^{-/-}$ neutrophils showed CXCR2 internalization similar to that of neutrophils obtained from WT mice (Fig. 4E). Considering that NFAT1 is described as a


A
Survival (%)
Days after CLP
Naive
Naive FK (6.0 mg/kg)
CLP + VEH
CLP + FK (mg/kg):
0.7
2.0
6.0
B
Log CFU/mL blood
VEH
FK
Naive
CLP 24h
C
LDH (U/L)
D
BUN (mg/dL)
E
CK-MB (U/L)
F
Lung neutrophils (10³/mg)
G
Plasma IL-6 (ng/mL)
CLP 6h
H
Plasma CXCL2 (ng/mL)

◂**Fig. 1** FK506 treatment results in higher mouse susceptibility to CLP-induced sepsis. **A** CLP survival curves of C57BL/6J mice treated with FK506 (FK, 0.7, 2.0 or 6.0 mg/kg, s.c.) or saline 1 h before surgery and then twice a day for 3 days. The non-operated group (naive) was treated with saline or 6.0 mg/kg FK506. The number of survivors expressed as a percentage was recorded every 24 h, and the results were analyzed by the log-rank Mantel–Cox test (*$p < 0{,}05$; $n = 5$–11). **B**–**H** FK506 (2 mg/kg) treatment started 1 h before CLP and then occurred twice a day after surgery until the time of euthanasia (6 and/or 24 h after CLP). Samples were collected for the following assessments: **B** logarithm of colony forming units per milliliter of blood (Log CFU/mL); **C** lactate dehydrogenase (LDH) in plasma (U/L); **D** blood urea nitrogen (BUN) in plasma (mg/dL); **E** creatine kinase-MB (CK-MB) in plasma (U/L); **F** amount of neutrophils per milligram of lung (10^3 cells/mg) determined through the myeloperoxidase assay; **G** plasma IL-6 levels (ng/mL); **H** plasma CXCL2 levels (ng/mL). The parameters were analyzed by one-way ANOVA followed by the Newman–Keuls test (*$p < 0{,}05$; $n = 5$–11)

regulator of CXCL2 expression in microglia [30], we also evaluated the amount of CXCL2 in the supernatants obtained from BMDM cultures of *Nfat1*$^{+/+}$ and *Nfat1*$^{-/-}$ mice in response to LPS stimulation (3 ng/mL, 12 h). As expected, no additional release of CXCL2 was observed (Fig. 4F), suggesting that FK506 does not operate through inhibition of NFAT1 activation.

Discussion

Here we demonstrated that FK506 treatment results in failed neutrophil migration to the primary infectious site, leading to higher mortality in murine polymicrobial sepsis. FK506 alters CXCR2 expression on the surface of circulating neutrophils due to excessive release of CXCL2, one of the CXCR2 ligands. Given that *Nfat1*$^{-/-}$ mice did not present the same phenotype as those septic animals that received FK506 treatment and considering that the IP3R inhibitor 2APB abrogated the in vitro FK506 effects, we suggest that FK506-mediated CXCL2 release is not dependent on NFAT inhibition but due to changes at the calcium trafficking via IP3 channels.

Immunosuppressive therapy with calcineurin inhibitors has been widely associated with greater susceptibility to infections [31, 32]. A previous study demonstrated that lung transplanted subjects showed increased pulmonary aspergillosis infections due to immunosuppression caused by treatment with calcineurin inhibitors and steroids [28]. In the same study, FK506 administration also increased *Aspergillus fumigatus* susceptibility in hydrocortisone-immunocompromised mice, reducing macrophage killing of *Aspergillus fumigatus* with no significant changes in the neutrophil migration to the infection site [28]. In Down syndrome subjects, the expression of regulator of calcineurin 1 (RCAN1), a regulatory protein of calcineurin activity, is increased, and this alteration has been associated with an immunodeficiency condition [33]. Curiously, RCAN1-deficient mice intranasally infected with *Pseudomonas aeruginosa* showed a lower survival index associated with higher NFAT1 nuclear translocation and systemic inflammatory response, the intriguing fact in this study is that the higher mortality and inflammatory response are associated with increased neutrophil migration to the lungs and decreased bacterial burden, locally and systemically [34]. In the present study, CLP-induced septic mice treated with FK506 presented high bacteremia, excessive neutrophil infiltration in the lungs, high plasma levels of CXCL2 and IL-6, which came associated with increased organ damage and mortality. With the systemic inflammatory response, the neutrophils activated in the blood stream fail in migrating to the infection site and, consequently, are not able to contribute in the pathogen proliferation control. On the other hand, there is the accumulation of neutrophils in the organs distant from the infection, as well as the lungs, where they contribute to the organ injury. The increase of neutrophils in the lung tissue of animals with sepsis may be due to increased release of CXC chemokines via alveolar macrophages [35, 36]. Thus, the presence of neutrophils in the locals other than that of primary infection are indicative of systemic inflammatory response, as well as the levels of pro-inflammatory cytokines. In this sense, IL-6 could be considered as an atemporal marker in the diagnosis and prognosis of sepsis [37]. It was proven to be precise in the diagnoses of sepsis, septic shock and the mortality prediction from these conditions, in accordance to the criteria proposed in the current sepsis definition known as Sepsis 3 [38]. The same was observed in the studies with CLP-induced sepsis in mice [39, 40]. The FK-506 treatment is also associated with higher plasma levels of IL-6 in the septic mice, and even if it could be just a severity marker, it was described that both FK506 and ciclosporin A increase the IL-6 release by LPS-stimulated monocytes [41].

Neutrophils are cells of the innate immune system essential for the immediate control of bacterial and fungal infections, preventing the systemic spread of infection [42]. Our research group has shown that failed neutrophil migration in severe sepsis occurs due to reduced expression of the chemotactic receptor CXCR2 on circulating neutrophils [22]. CXCR2 belongs to a large family of G protein-coupled receptors (GPCRs), and its internalization occurs in a GPCR kinase 2 (GRK2)-dependent manner in the presence of high chemokine levels [43]. CXCR2 ligands cause β-arrestin recruitment and CXCR2 internalization in a concentration-dependent manner [44]. The FK506-treated septic mice showed a more pronounced reduction in CXCR2 expression on circulating neutrophils associated with their impaired migration to the infectious site, resulting in increased cytokines in the peritoneal lavage fluid. Consistent with our data, a previous study demonstrated that

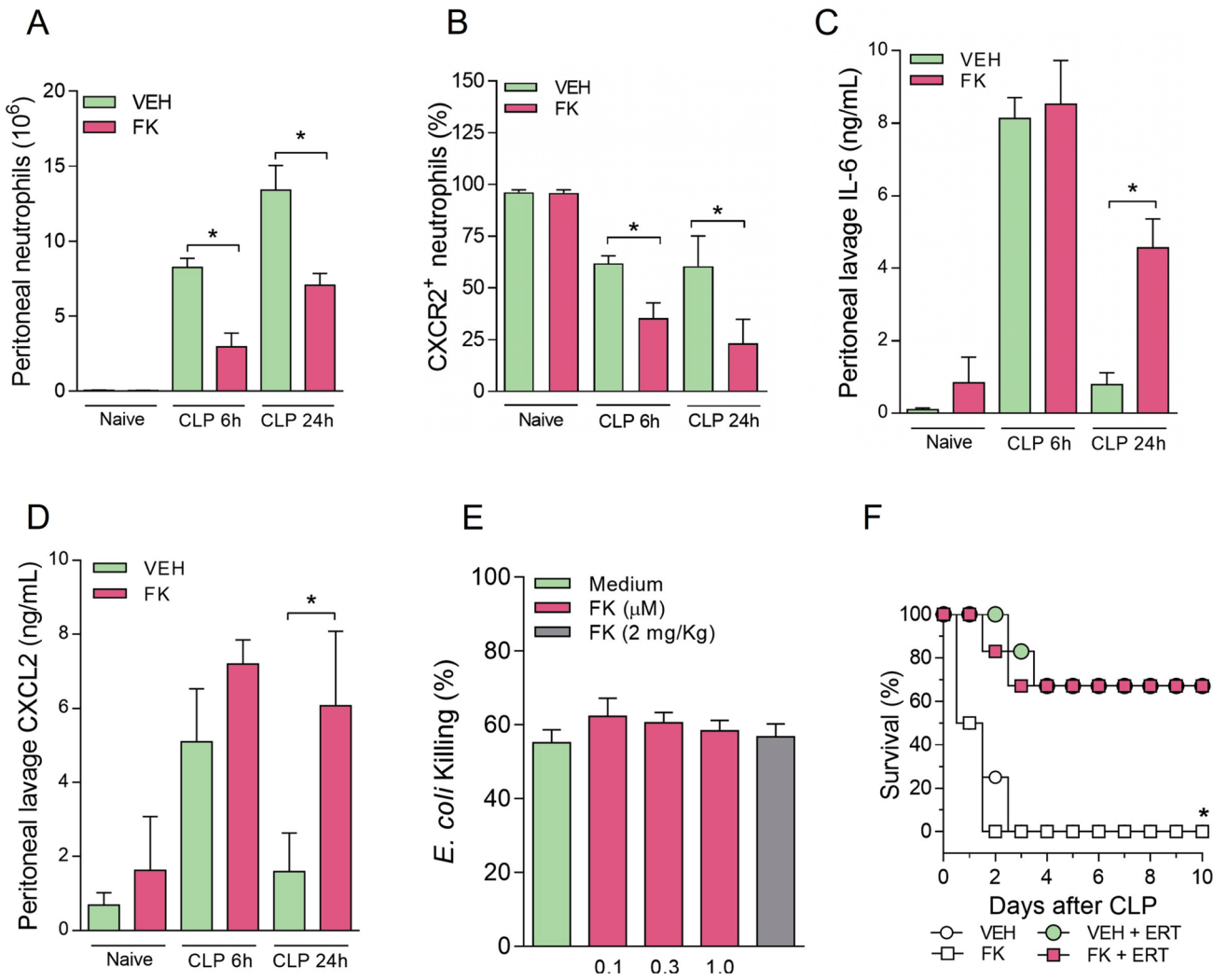


Fig. 2 FK506 induces a failure of CLP-induced neutrophil migration to the peritoneal cavity. FK506 (2 mg/kg) treatment started 1 h before CLP and was maintained twice a day until euthanasia (6 and/or 24 h after CLP), when samples were collected for assessment of (**A**) the absolute number of neutrophils (10^6) per peritoneal cavity; **B** the percentage of circulating CXCR2-positive neutrophils; **C** levels of IL-6 in nanograms per milliliter (ng/mL) of peritoneal lavage fluid; and **D** CXCL2 levels in ng/mL. The cytokine and chemokine levels were quantified by ELISA. **E** In vitro *E. coli* killing by FK506 (FK 0.03, 0.1 and 0.3 μM)-treated neutrophils or neutrophils harvested from FK506 (2 mg/kg)-treated mice. The percentage of killing was determined after 45 min of co-culture between neutrophils and *E. coli* at a 1:10 ratio. **F** Lethal CLP (18GCLP) survival curves of mice that received additional ertapenem therapy (ERT, 30 mg/kg, i.p.) started 1 h before surgery and maintained twice a day for 3 days or until death. In the survival curves, the number of survivors recorded every 24 h was expressed as a percentage and analyzed by the log-rank Mantel–Cox test ($n=6$). The other parameters were analyzed by one-way ANOVA followed by the Newman–Keuls test compared with the vehicle group or as indicated in the figures (*$p<0,05$; $n=5$–11)

FK506 treatment reduced CXCR2 expression on circulating granulocytes, as well as their migration to the infectious site, exacerbating *E. coli* urinary tract infection in female mice [45]. In the present study, we did not find differences in the *E. coli* killing function of neutrophils isolated from FK506-treated mice or incubated in vitro with this drug, suggesting an indirect effect on this cellular property. Herein, antibiotic therapy abolished the FK506 effect in septic mice, confirming the deleterious effect of FK506 lies in the impairment of infection control.

Although our data and those from Emal and collaborators [45] demonstrate that in vivo FK506 administration reduces CXCR2 expression on blood neutrophils under infectious stimuli, our in vitro data showed no significant differences in CXCR2 expression after FK506 incubation in CXCR2-transfected HEK-293 cells stimulated with CXCL2 or in CXCL2-stimulated C57 wild type bone marrow neutrophils, as well as human neutrophils stimulated with IL-8 (data not shown) suggesting that FK506 does not lead directly to CXCR2 desensitization. Considering that the ability

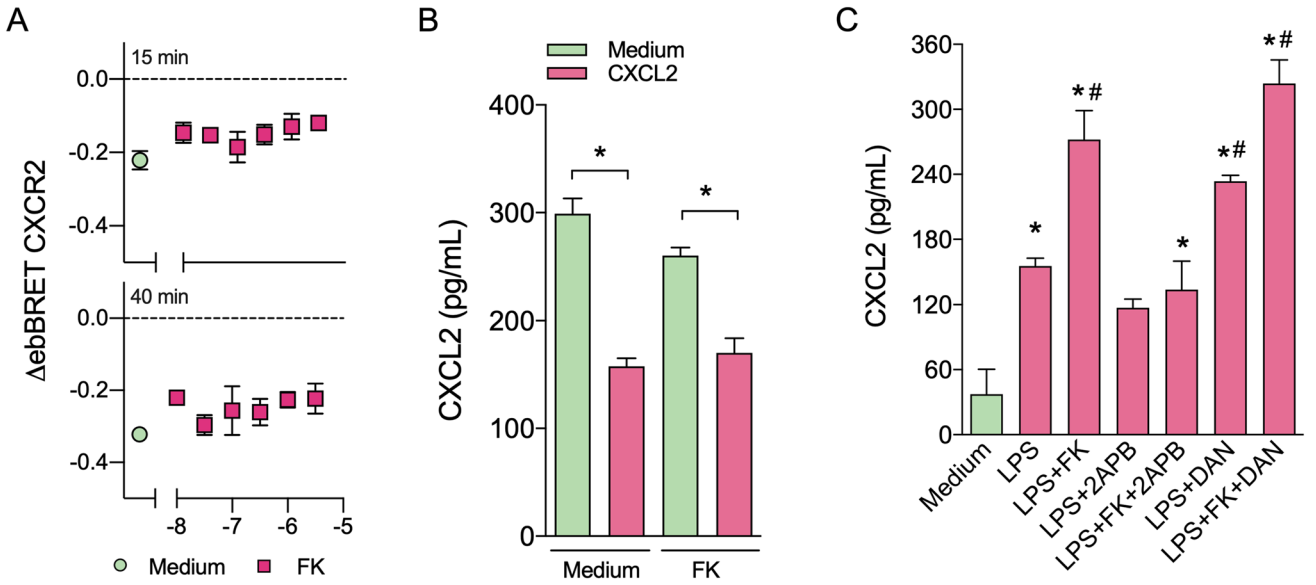


Fig. 3 Effect of FK506 treatment on CXCR2 internalization and CXCL2 production. **A** Concentration-dependent effect of FK506 (log of molar concentration) on internalization of the CXCR2 receptor fused to RLucII (CXCR2-RLucII) in HEK-293T cells. Internalization was measured by an enhanced bystander bioluminescence resonance energy transfer (ebBRET) assay. ΔebBRET represents the difference between the ebBRET values of CXCL2-stimulated cells and the ebBRET values of cells incubated with vehicle (dashed line). The FK506 (Log [FK] -8 to -5.5, same as 1×10^{-8} to 3×10^{-5} molar [M]) treatments were given 30 min before CXCL2 (500 ng/mL) stimulus. ebBRET signals following CXCL2 (500 ng/mL) incubation were monitored for 50 min, and the graphs represent the time points of 15 and 40 min of CXCL2 stimulation. **B** CXCR2 expression on FK506 (1 μM) or medium-treated C57 wild type bone marrow neutrophils stimulated with CXCL2 (30 ng/mL). The results are expressed as the mean fluorescence intensity (MFI). **C** CXCL2 release by macrophages stimulated with LPS (3 ng/mL, 12 h) pretreated (30 min before LPS) with medium, FK506 (FK, 1×10^{-6}, same as 1 μM), 2APB (50 μM), dantrolene (DAN, 30 μM), FK506 + 2APB or FK506 + DAN. The chemokine levels in the culture supernatants were quantified by ELISA and expressed as nanograms per mL (pg/mL). The data were analyzed by one-way ANOVA followed by the Newman–Keuls test (*$p<0.05$ compared to the control (medium) group, #$p<0.05$ compared to the LPS group; $n=3$)

of FK506 to induce neutrophil CXCR2 internalization is restricted to only in vivo models, we hypothesize that this effect may be due to the excessive release of CXCL2 by host cells. Further studies should investigate the effects of FK506 on the expression of other chemokines and their potential to signalize or desensitize CXCR2 and other chemokine receptors in neutrophils, or other cell types in sepsis, as well as other inflammatory conditions.

The ability of FK506 to inhibit calcineurin occurs after its binding to the FKBP proteins. In some cell types, it was described that FKBPs are associated with RyR and IP3R on the endoplasmic reticulum (ER) [16]. The dissociation of FKBP12 from IP3R through the binding with FK506 results in decreased Ca^{2+} release via IP3 channels [46]. However, this same study and other show that the combination of FK506 with calcineurin, on the other hand, results in the opening of the same channel, and its consequent ER Ca^{2+} release into the cytoplasm of smooth muscle cells [46] and endothelial cells [47]. Thus, FK506 has the potential to both stimulate and inhibit the ER Ca^{2+} release through IP3 channels. The RyR, in turn, has its opening probability increased with the displacement of FKBPs, thus increasing the ER Ca^{2+} release to the cytosol in arterial smooth muscle cells [48], colonic myocytes [49] and portal vein myocytes [50, 51], in a calcineurin-independent way. These studies were focused on the cardiovascular system and there is still no evidence that supports similar pathways in macrophages. Interestingly, our results showed opposing effects for 2APB and dantrolene, IP3 and RyR inhibitors, respectively. In summary, LPS causes CXCL2 release by BMDMs, which is higher in the presence of FK506. 2APB did not induce changes in the LPS response, but inhibited the FK506 effect, suggesting that the FK506 effect is dependent on IP3 channel, probably by increasing the ER Ca^{2+} release. However, different conclusions are possible in the RyR perspective, increased LPS-induced CXCL2 release was observed with the dantrolene treatment, which is even more prominent in the presence of FK506. By acting on RyR, we expected that FK506 would increase the ER Ca^{2+} release, but the synergism observed with one RyR blocker indicates RyR independency. These results can postulate that inhibiting ER Ca^{2+} release in LPS-stimulated macrophages could be a signal to intensify the CXCL2 release.

NFAT nuclear translocation is also an event tightly associated to CXCR2 internalization, but the repertoire of gene expression that NFAT regulates after CXCR2 stimulation

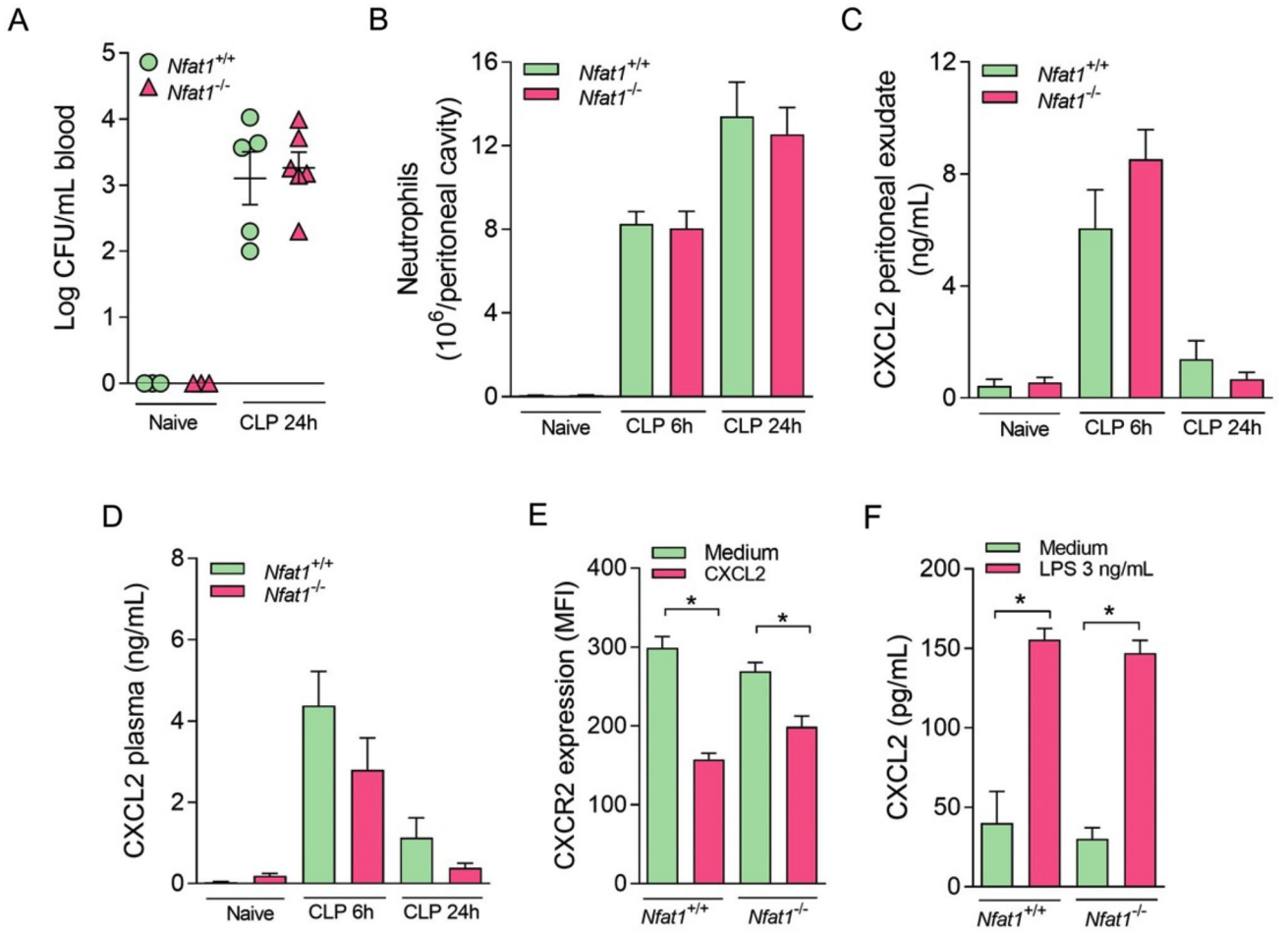


Fig. 4 NFATI deficiency did not mimic the FK506 effect on sepsis outcome. **A**–**D** *Nfat1*$^{+/+}$ and *Nfat1*$^{-/-}$ mice were submitted to sham surgery or CLP surgery and samples were collected 6 or 24 h after for assessment of (**A**) logarithm of colony forming units per milliliter of blood (Log CFU/mL); (**B**) number (10^6) of neutrophils in the peritoneal lavage fluid; and (**C**) CXCL2 quantification in peritoneal lavage fluid or in (**D**) plasma. **E** CXCR2 expression of C57 wild type bone marrow neutrophils stimulated in vitro with CXCL2 (30 ng/mL). CXCR2 expression was expressed as the mean fluorescence intensity (MFI). **F** CXCL2 release by BMDMs harvested from *Nfat1*$^{+/+}$ and *Nfat1*$^{-/-}$ mice and stimulated (12 h) with LPS (3 ng/mL). The chemokine levels were expressed as nanograms per milliliter (ng/mL) or picograms per milliliter (pg/mL) as indicated in the panels. The data were analyzed by one-way ANOVA followed by the Newman–Keuls test (*$p < 0.05$ compared to the control group or as indicated in the figure; $n = 3$–6 for in vivo experiments; $n = 3$ for in vitro experiments)

and whether this transcription factor plays a role in CXCR2 internalization are not yet understood [52]. In our present study, *Nfat1*$^{-/-}$ septic mice showed no change in peritoneal neutrophil migration or bacteremia, indicating that NFAT1 inhibition by FK506 may not be associated with the biological effects observed. In septic models, NFAT transcriptional activity is increased in different organs, and treatment with A-285222, a calcineurin-independent NFAT inhibitor, results in a reduction in lung neutrophil sequestration and chemokine production [53, 54]. Similarly, in experimental pancreatitis, this drug also reduced neutrophil migration and pancreatic CXCL2 production [55]. In fact, it was demonstrated that calcineurin-NFAT1 signaling is required for the CXCL2 expression in microglia [30]. Corroborating these findings, in our NFAT1-deficient mice subjected to CLP, we see a tendency of lower CXCL2 release in both peritoneal exudate and plasma. We do believe that FK506-induced NFAT1 inhibition could be happening but the FK506 effects on the other targets, as IP3R, could be more prominent in defining the final balance. It is important as well considering the inhibition of other calcineurin substrates. It is difficult to compare data from experiments with cyclosporin A and FK506 because, in addition to different drug binding sites on different immunophilins, the responses triggered are the result of the activation of different pathways. Consistent with the A-285222 findings, cyclosporin A treatment also reduced the release of CXCL1 and CXCL2 and consequently resulted in reduced neutrophil migration in acute

pyelonephritis [56]. On the other hand, a transcriptomic and bioinformatics analysis study identified the mechanisms by which FK506 induces nephrotoxicity, revealing that FK506 treatment is associated with higher expression of CXCL1, CXCL2, CXCL3 and CXCR2 [56].We reinforce that future studies are essential to clarify the phenomenon observed in this study and, as conclusion, the FK506 treatment is associated with higher mortality of CLP-induced septic mice due to a failure in the neutrophils migration to the infection site explained by the reduced plasma membrane expression of CXCR2 in response to exacerbated levels of circulating CXCL2.

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Author contributions VFB, LSG, AK, and FQC designed the study. VFB, LSG, AK, FVSC, VVSM, DAD, FCR, AHS, GCMC, CMSS, and MHFL performed the mouse experiments. VFB, LSG, AK, and FQC wrote the manuscript. VFB, LSG, AK, FVSC, VVSM, AHS, GCMC, CMSS, MHFL, JPBV, TMC, CMCN, JCFAF, ASP, and FQC reviewed the manuscript.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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