Mitochondrial DNA

An Endogenous Trigger for Immune Paralysis

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ABSTRACT

Background: Critically ill patients are at high risk to suffer from sepsis, even in the absence of an initial infectious source, but the molecular mechanisms for their increased sepsis susceptibility, including a suppressed immune system, remain unclear. Although microbes and pathogen-associated molecular pattern are accepted inducers of sepsis and septic immunosuppression, the role of endogenous Toll-like receptor (TLR) ligands, such as mitochondrial DNA (mtDNA), in altering the immune response is unknown.

Methods: Mitochondrial DNA serum concentrations of the mitochondrial genes D-Loop and adenosine triphosphatase 6 were determined (quantitative polymerase chain reaction) in 165 septic patients and 50 healthy volunteers. Furthermore, cytotoxic T-cell activity was analyzed in wild-type and TLR9 knockout mice, with/without previous mtDNA administration, followed by injection of an ovalbumin-expressing adenoviral vector.

Results: Mitochondrial DNA serum concentrations were increased in septic patients (adenosine triphosphatase 6, 123-fold; D-Loop, 76-fold, $P < 0.0001$) compared with volunteers. Furthermore, a single mtDNA injection caused profound, TLR9-dependent immunosuppression of adaptive T-cell cytotoxicity in wild-type but not in TLR9 knockout mice and evoked various immunosuppressive mechanisms including the destruction of the splenic microstructure, deletion of cross-presenting dendritic cells, and up-regulation of programmed cell death ligand 1 and indoleamine 2,3-dioxygenase. Several of these findings in mice were mirrored in septic patients, and mtDNA concentrations were associated with an increased 30-day mortality.

Conclusions: The findings of this study imply that mtDNA, an endogenous danger associated molecular pattern, is a hitherto unknown inducer of septic immunoparalysis and one possible link between initial inflammation and subsequent immunosuppression in critically ill patients. **(Anesthesiology 2016; 124:923-33)**

TISSUE injury and destruction, *e.g.*, after cerebral infarction and burns, are associated with systemic immune suppression and early bacteremia.[1,2] Although a causal relationship between severe tissue trauma and an altered immune response is intuitively plausible, mechanistic evidence for such a link has not been achieved, possibly due to the complexity and heterogeneity of disease states, especially in critically ill patients. However, animal studies at least allow to draw causal conclusions, mimicking parts of the situation in critically ill humans.[3] To this end, mice suffering from experimentally evoked stroke develop sepsis within a few hours, and various immunosuppressive phenotypes, such as depletion of T cells and natural killer cells, have also been found.[1]

Furthermore, translational research led to the well-accepted concept that the complex sepsis syndrome is evoked by both microbes and pathogen-associated molecular patterns (PAMPs) and believed to result in a (dysregulated)

What We Already Know about This Topic

- Critically ill patients are at high risk to suffer from sepsis, even in the absence of an initial infectious source, but the molecular mechanisms for their increased sepsis susceptibility, including a suppressed immune system, remain unclear. Namely, the role of endogenous Toll-like receptor ligands, such as mitochondrial DNA, in altering the immune response is unknown.

What This Article Tells Us That Is New

- Mitochondrial DNA (mtDNA) serum concentrations of the mitochondrial genes D-Loop and adenosine triphosphatase 6 were determined (quantitative polymerase chain reaction) in 165 septic patients and 50 healthy volunteers. Furthermore, cytotoxic T-cell activity was analyzed in wild-type and TLR9 knockout mice, with/without previous mtDNA administration, followed by injection of an ovalbumin-expressing adenoviral vector.
- It was found that mtDNA concentration is increased in sepsis and, besides inflammatory effects, has powerful immunosuppressive effects and can evoke immunosuppression on its own.

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two-phased immune response.[3,4] This is, an initial phase of hyperinflammation (systemic inflammatory response syndrome [SIRS]), often associated with septic shock and multiorgan failure, followed by a phase of "immune paralysis," contributing to late morbidity and death by the incapability to develop adaptive immunity against secondary opportunistic infections or viral reactivation.[5–8] Investigations and treatments for decades mainly focused on the initial SIRS phase, but most clinical trials, targeted to dampen the hyperinflammatory initial phase, failed to decrease mortality.[9–11] Accordingly, current research increasingly focuses on the second immunosuppressive phase.[7,12,13]

Initial findings from us and others suggested that the defect in mounting adaptive immune responses was induced by bacteremia.[3,4,12,14,15] Mechanistically, immune paralysis was associated with a variety of phenotypes, such as deletion of immune cells (*e.g.*, dendritic cells [DCs]), overproduction of suppressive mediators (indoleamine 2,3-dioxygenase [IDO], transforming growth factor-β), or up-regulation of counterregulatory cells (regulatory T cells) or receptors (programmed cell death ligand 1 [PD-L1]), or cytotoxic T-lymphocyte–associated protein 4.[1,12–16] In addition, not only intact bacteria but also PAMPs such as the toll like receptor (TLR)-4 ligand lipopolysaccharide or unmethylated, bacterial DNA sequences, so-called CpG-DNA, which are known to bind to TLR9, can evoke septic shock and may lead to immune paralysis.[17–20]

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However, all these findings do not explain why intensive care unit (ICU) patients frequently show signs of SIRS and immunosuppression without identification of infectious agents, despite better and more sensitive diagnostic techniques and tools.[3,12,21] This may be explained either by bacteria being confined to the site of the infection and only PAMPs appearing in the patient's blood or by endogenous mediators altering the immune response irrespective of infectious agents themselves. In this regard, mitochondrial DNA (mtDNA) was recently identified as an endogenous immunostimulatory trigger. mtDNA has a structure similar to bacterial CpG-DNA and stimulates the immune response in a TLR9-dependent manner. However, if cells are exposed to mtDNA for a longer time, this may evoke endotoxin tolerance.[22,23] mtDNA serum concentrations are increased in trauma patients, and mtDNA strongly stimulated neutrophils.[22] Furthermore, increased mtDNA serum concentrations were recently shown to be a predictor for mortality in medical ICU patients, *i.e.*, with sepsis or acute respiratory distress syndrome, however, without providing a molecular explanation.[24] Accordingly, we (1) performed a confirmatory study to test that mtDNA serum concentrations are increased in septic patients without trauma and correlate with mortality and (2) tested the hypothesis that mtDNA evokes immune system alterations associated with immunosuppression.

We now show that a single injection of mtDNA markedly suppresses the adaptive immune response in wild-type mice in a TLR9-dependent fashion. In addition, we identified a variety of immunosuppressive mechanisms common to both mice and critically ill humans with sepsis, suggesting that mtDNA may be one of the links between inflammation and immunoparalysis.

Material and Methods

Experimental Design

For this translational study, we conducted both a prospective observational trial including 165 septic patients and 50 healthy volunteers and performed *in vivo* experiments using wild-type and TLR9 knockout mice ($TLR9^{-/-}$).

Human Studies. Healthy volunteers were recruited by bulletins posted at the University Duisburg-Essen and enrolled after obtaining written informed consent. The characteristics of volunteers were documented, and blood (24 ml) was drawn from a peripheral vein. Adult patients with sepsis treated in the ICU of Essen University Hospital, Essen, Germany, between June 2011 and June 2014 were eligible for study enrollment. Patients with sepsis were considered eligible and were enrolled on the day of diagnosing sepsis when they fulfilled the sepsis criteria as defined by Bone *et al.*[25] Of 496 patients admitted to an ICU with severe sepsis, 400 could be screened for study inclusion. Of these patients, 165 were finally included. Two hundred thirty-five patients had to be excluded as they or their guardians refused study participation, because they did not meet the inclusion criteria (more than 18 yr, mechanical ventilation at the time of study inclusion, no preexisting immunologic

disorder, *i.e.*, HIV infection), had mental dysfunction, or whenever blood sampling and/or immediate preparation and storage was not available within 24 h after first diagnosing sepsis.[14] Blood was taken for the isolation of mtDNA, cytokine concentration measurements, blood tests, and microbiology cultures at the time of study inclusion. In addition, Simplified Acute Physiology Score II and blood culture results were documented, and the patients were followed up for the length of their hospitalization and 30-day mortality.[14]

Power considerations for two independent groups revealed that a sample size of 165 sepsis patients and 50 healthy volunteers has a comparison-wise power of 80% to detect expected (medium to larger) mean differences of 0.45 in units of a SD of a standardized normal distribution (two-sided significance level α of 5%—planning for Student's *t* test).

Cell Culture. The human monocytic cell line THP-1 was obtained from the German Collection for Microorganisms and Cells (DSMZ, Germany). Cells were cultured as described previously.[14] THP-1 cells were kept in medium only (control) or were incubated with 1 μg/ml mtDNA for 6 h. At the end of the experiments, total RNA was extracted for the determination of specific mRNA by quantitative polymerase chain reaction (qPCR; real-time polymerase chain reaction [PCR]) for hypoxia-inducible factor-1α, TNF-α, interleukin-1β, and GAPDH as described.[14] Experiments were performed using duplicates for each condition and repeated at least three times.

Mice Studies. Wild-type mice were obtained from Janvier Labs (Le Genest Saint Isle Saint Berthevin Cedex, France) and backcrossed to C57BL/6 at least 10 times. $TLR9^{-/-}$ were obtained from Professor Hermann Wagner (Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, München, Germany) and bred as described.[12,26] All animal experiments were performed using littermates as controls and repeated twice with animal numbers of four mice per group. Mice of both sexes were used to rule out gender-specific effects.

Study Approval. The human studies were reviewed and approved by the local ethics committee (06-3078, University of Duisburg-Essen, Essen, Germany) and registered by the German clinical trial database (no. DRKS00006012, Deutsches Register für klinische Studien). Experiments in mice were approved by Bezirksregierung Köln of the German state of North Rhine-Westphalia (9.93.2.10.35.07.268; 9.93.2.10.35.07.269, Köln, Germany), and all experiments were performed in accordance with the local animal welfare guidelines.

mtDNA

Isolation of mtDNA from Serum of Septic Patients and Healthy Volunteers. Mitochondrial DNA was isolated from the serum of septic patients and healthy volunteers using the peqGOLD Blood DNA Mini Kit (peqlab, Germany) following the manufacturer's instructions. mtDNA serum concentration was assessed by qPCR (real-time PCR) for the mitochondrial genes adenosine triphosphatase (ATPase) 6 (165 septic patients; 50 healthy volunteers) and D-Loop (154 septic patients; 41 healthy volunteers). mtDNA serum concentrations were determined using a primer from the conserved, noncoding region of the mitochondrial genome, the so-called control region (D-Loop primer), as well as ATPase 6 as a primer from a coding region, because this primer yielded the best results even with samples containing very low mtDNA concentrations. The primers for qualitative and quantitative PCR were designed using the National Center for Biotechnology Information Primer-BLAST (USA) or Primer Express 3.0 software (Applied Biosystems, Germany) and were obtained from Eurogentec (Belgium).[10,14] Real-time PCR was performed for the mitochondrial genes ATPase 6 (5′ primer: TCC CCA TAC TAG TTA TTA TCG AAA CCA; 3′ primer: GCC TGC AGT AAT GTT AGC GGT TA) and D-Loop 1 (5′ primer: TGC ACG CGA TAG CAT TGC; 3′ primer: AGG CAG GAA TCA AAG ACA GAT ACT G) using a Step One Plus Real-Time PCR system (Applied Biosystems) as described,[14] and values below the standard curve range were set to 10^{-5} fg/ml. No homology between mtDNA primers and bacterial or fungal DNA was found using National Center for Biotechnology Information Primer-BLAST. To further ensure that ATPase 6 and D-Loop primers are specific for mtDNA and do not amplify bacterial DNA sequences, we performed real-time PCR using DNA isolated from various bacteria and fungi commonly observed in patients with sepsis, *i.e.*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Candida glabrata*, and *Candida albicans*. Bacterial and fungal DNA were isolated using the Qiagen DNeasy blood and tissue kit (Qiagen, Germany) with the DNA extraction protocol for bacterial cultures. Specific amplification products could not be seen in any sample (data not shown).

Preparation of mtDNA from Cells. Mitochondria were isolated from HepG2 cells using institutional protocols based on the method of Frezza *et al.*[27] Afterward, mtDNA was extracted using the DNeasy blood & tissue kit (Qiagen) following the manufacturers protocol. In addition, mitochondria from HepG2 cells were isolated using the Mitochondria Isolation Kit (Pierce, USA) according to the manufacturer's instructions. mtDNA was consecutively isolated by phenol/chloroform extraction. Concentration of mtDNA was measured by spectrophotometry, and mtDNA concentration was diluted to 0.1 or 1 μg/μl and immediately stored at −80°C.

Determination of Endotoxin Contamination. Endotoxin contamination of the mtDNA preparations were tested by the Limulus Amebocyte Lysate Assay (Lonza Cologne GmbH, Germany). Only mtDNA preparations with an endotoxin concentration less than 0.01 EU/ml (approximately 0.001 ng/ml) were used in the experiments.

Interleukin-2RA and Kynurenine Serum Concentration Measurements from Human Serum

Interleukin-2R α-chain concentration in human serum was measured using the Procarta Cytokine Kit (Affymetrix

Inc., USA) following the manufacturer's protocol.[14] Kynurenine concentration in human serum was determined using high-performance liquid chromatography, as described previously.[12]

In Vivo *Cytotoxicity Assay in Mice*

In vivo cytotoxicity assays after mtDNA injection were done as described previously,[12] and saline and murine eukaryotic DNA (eukDNA) served as a negative and unmethylated CpG as a positive control, respectively. Briefly, mice were injected with 100 μg CpG (TIB MolBiol, Germany) mtDNA or eukDNA and 1 day later infected with 1×10^6 colony-forming units Adeno-ovalbumin.[12] After 5 days, carboxyfluorescein-*N*-hydroxysuccinimide ester (CFSE)-labeled target cells were prepared and injected into the tail vein of the respective mice.[12] After 4 h, the survival of target cells in the spleen was analyzed by flow cytometry. Specific lysis was calculated with the following formula: % specific cytotoxicity = 100 − (100 × [CFSEhi/CFSElo] primed/[CFSEhi/CFSElo] control).[12]

Splenocyte Isolation and Measurements

Single-cell suspensions of splenocytes and lymph nodes were generated by digesting the tissues DNAse and collagenase at 37°C for 20 min and passing it through a metal sieve and a sterile 50-μm nylon mesh afterward.

Flow Cytometry. Murine cells were stained with fluorochrome-conjugated antibodies against CD8a (Becton, Dickinson and Company, USA; clone 53–6.7), anti-CD11b (eBioscience Inc., USA), anti-CD11c, CD169, Clec9a, F4/80, and PD-L1 (all Bio Legend, USA). Unspecific binding was blocked by Fc receptors with 1.5 mg/ml human IgG (Privigen, Germany). Dead cells were excluded using Hoechst 33342 dye (Invitrogen, Germany).

Isolation of CD11c-positive Dendritic Cells. Dendritic cells were isolated from cell suspensions of murine splenocytes using magnetic cell separation (Miltenyi, Germany) according to the manufacturer's protocol. Briefly, single-cell suspensions were incubated with anti-CD11c antibody-labeled nanoparticles (Miltenyi). The cells were then washed with MACS buffer (Miltenyi) and separated from the nonlabeled cells on LS separation columns according to the manufacturer's protocol.

T-cell Activation Assay for *In Vitro* Studies. T cells were isolated from OTI mice as described.[12,28] Purified OTI T cells (1×10^5) were cultured for 2 days with 1×10^5 splenic DCs from wild-type or TLR9$^{-/-}$ mice. Then soluble ovalbumin (Sigma-Aldrich, USA) was added for 5 h. Afterward, DCs were digested for 20 min by incubation with collagenase and DNase (both from Sigma-Aldrich). To further purify T cells, a CD11c$^+$ isolation kit (Miltenyi) was applied. In addition, to serve as positive control, DCs were pulsed for 20 min with the ovalbumin peptide SIINFEKL (Anaspec Inc., USA). Coculture supernatants were analyzed for interleukin-2 and interferon-γ concentrations after 18 to 20 h of incubation.[12,28,29]

Immunofluorescence Staining of Tissue Sections. Cryosections, 5 μm in thickness, from the shock-frozen spleens were prepared. Sections were fixed with iced acetone and blocked for 1 h with 1% (wt/vol) bovine serum albumin in phosphate-buffered saline. Indicated antigens were stained with the antibodies against mouse antigens (CLEC9a, MAdCAM1, CD11c, PD-L1, CD169) and human antigens (CD11c, CD11b, HLA Class II DRB1, CLEC9a, MAdCAM1, CD3, CD19, CD274). Images were captured with an Olympus IX71 microscope (Olympus, Zeiss AG, Germany).

Statistics

Standard descriptive statistics were used to summarize the data (continuous variables: quartiles or mean ± SD)/count data: absolute and relative frequencies). For the comparison of quantitative continuous variables between patients with sepsis and healthy volunteers, we either applied nonparametric Wilcoxon Mann–Whitney *U* tests or parametric Student's *t* tests depending on the graphical inspection of a potential violation of the normality assumption. In case of large variability differences between the groups, Welch *t* test was used. To test for differences in frequency distributions, Fisher exact test was applied. Spearman correlations are reported for the association between serum concentrations of ATPase 6, D-Loop, kynurenine, and interleukin-2RA. Analyses of patients' materials are displayed as dot plots with median and interquartile range to delineate the heterogeneity of critically ill patients. Results of mice studies and cell culture experiments are depicted as means ± SD.

One-way ANOVA with *post hoc* Student's *t* test ($\alpha_{corr} = 0.05/n$) was used to compare wild-type and TLR9$^{-/-}$ mice following different incubation conditions tested. All reported *P* values are two sided, and we applied, unless otherwise stated, a two-sided significance level α of 5%. All statistical analyses were done using GraphPad Prism 5.0f (USA), R version 3.1.0 or IBM SPSS Statistics V.21 (SPSS Inc., Chicago, USA).

Results

mtDNA Serum Concentrations in Critically Ill Patients with Sepsis and Healthy Volunteers

In a first step, we speculated that mtDNA serum concentrations are increased in critically ill patients with sepsis and associated with worse outcomes. The characteristics of 165 septic patients and 50 healthy volunteers are displayed in table 1. Indeed, we detected increased mtDNA serum concentrations (D-Loop, 76-fold increase; ATPase 6, 123-fold increase; all $P < 0.0001$) in septic patients compared with healthy volunteers (fig. 1, A and B), with D-Loop and ATPase 6 serum concentrations showing a strong correlation ($r = 0.906$; $P < 0.0001$, table 2). Of interest, mtDNA serum concentrations on the day of first diagnosing sepsis[25] were further increased in patients who died within 30 days when compared with survivors (D-Loop, 1.6 fg/μl ± 3.6 *vs.*

Table 1. Demographic and Clinical Characteristics of Patients with Sepsis and Healthy Volunteers

Characteristics	Septic Patients (n = 165)	Healthy Individuals (n = 50)	*P* Value (Two Sided)
Demographic variables*			
Median age (IQR), yr	57 (47–70)	40 (27–56)	< 0.001†
Females/males, N (%)	60/95 (39/61)	30/20 (60/40)	0.009‡
Mean height (SD), m	1.73 (0.11)	1.74 (0.85)	0.564§
Mean body weight (SD), kg	83 (22)	72 (12)	< 0.001‖
Mean body mass index (SD), kg/m^2	27 (6)	24 (3)	< 0.001‖
Median arterial pressure (IQR), mm Hg	80 (70–87)	92 (90–100)	< 0.001†
Median heart rate (IQR), per minute	95 (80–108)	69 (64–75)	< 0.001†
Clinical characteristics#			
Infectious disease variables			
Median pro-calcitonin concentration (IQR), µg/l	4 (1–17)	0 (0)	< 0.001†
Median C-reactive protein concentration (IQR), g/l	17 (9–25)	0 (0)	< 0.001†
Median leukocyte concentration (IQR), 10^9/l	14 (9–19)	6 (5–7)	< 0.001†
Severity of sepsis			
Median SAPS II (IQR)	32 (23–42)		
Median hospital stay (IQR), days	26 (13–40)		
30-d survival (%)**	64		
Primary diagnoses; N (row%)			
Gastrointestinal cancer	19 (14)		
Gastrointestinal disease	34 (24)		
Lung disease	41 (29)		
Cardiovascular disease	6 (4)		
Urogenital disease	6 (4)		
Hematological disease	8 (6)		
Cancer, other	10 (7)		
Intraabdominal pathology and other	17 (12)		
Blood cultures; N (row%)			
Gram-positive isolates only	39 (24)		
Gram-negative isolates only	12 (7)		
Fungal isolates only	4 (2)		
Mixed isolates	45 (27)		
Negative blood cultures	64 (39)		

*Total N of missing values for each variable (in order of the variables): 10, 10, 64, 56, 65, 171, and 41. †Based on Wilcoxon Mann–Whitney *U* test. ‡Based on Fisher exact test. §Based on Student's *t* test. ‖Based on Welch *t* test. #Total N of missing values for each variable (in order of the variables): 94, 47, 30, 70, 28, not applicable, 24, and 1. **Derived from the Kaplan–Meier estimator at day 30.

IQR = interquartile range; SAPS II = Simplified Acute Physiology Score II.

0.4 fg/µl ± 1.2; P = 0.003; ATPase 6, 1.3 fg/µl ± 3.4 *vs.* 0.55 fg/µl ± 2.3; P = 0.005). Importantly, as our primers were selective for mtDNA, cross-reactivity with DNA from various common bacterial and fungal isolates was excluded (data not shown).

Stimulatory Effect of mtDNA on Human Monocytes

It is well established that sepsis is induced by systemic bacteremia or PAMPs, and even DAMPs like mtDNA have been shown recently to stimulate isolated neutrophils or monocytes.[22,23] To confirm the stimulatory effect of mtDNA, we analyzed the human monocytic cell line THP-1 and could show that 1 µg/ml mtDNA acts in a proinflammatory way, as shown by an increased mRNA expression of proinflammatory genes (data not shown). Specifically, mtDNA increases TNF-α (11.3-fold ± 7.6; P = 0.001), interleukin-1β (10-fold ± 8.0; P = 0.005), and hypoxia-inducible factor-1α (3.6-fold ± 3.5; P = 0.03) mRNA expression.

To this end, we can confirm the immune stimulatory effect of mtDNA on cell populations of the innate immune response. As a next step, we injected mtDNA in wild-type and TLR9$^{-/-}$ mice to assess its potential immunomodulatory effects *in vivo*.

Cytotoxic T-cell Activity in Wild-type and TLR9$^{-/-}$ Mice after mtDNA Injection

To investigate functional effects of mtDNA on adaptive immune responses, we employed an adenoviral mouse model, because adenoviral vectors are well-known inducers of adaptive T- and B-cell responses (fig. 2). This model has been used previously in mice with bacteremia (*E. coli*) to reveal that lipopolysaccharide can suppress adaptive immunity.[12] To investigate a potential suppressive effect of mtDNA, we injected 100 µg of the TLR9-ligands mtDNA or CpG-DNA, respectively, and murine genomic DNA into wild-type and TLR9$^{-/-}$ mice before applying an adenoviral

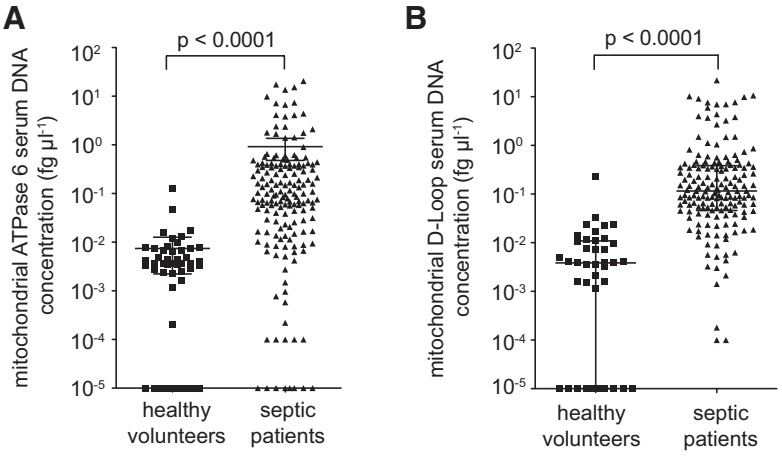

Fig. 1. Increased serum concentrations of mitochondrial DNA in septic patients. Quantitative polymerase chain reaction (real-time polymerase chain reaction) analysis of mitochondrial adenosine triphosphatase (ATPase) 6 (*A*) and D-Loop (*B*) serum concentrations in patients with sepsis and healthy volunteers.

Table 2. Characteristics of Septic and Nonseptic Patients Undergoing Splenectomy

Characteristics	Septic Patients (n = 5)	Trauma Patients (n = 5)	*P* Value (Two Sided)
General variables			
Median age (IQR), yr	61 (26–71)	61 (18–77)	0.7*
Females/males, N (%)	1/4 (20/80)	1/4 (20/80)	1.0†
Indication for splenectomy, N (row%)			
Splenic bleeding	2	2	
Intraoperative ligation of splenic artery	3	1	
Splenic congestion due to portal hypertension	0	2	

*Based on Student's *t* test. †Based on Fisher exact test.
IQR = interquartile range.

vector expressing ovalbumin and measured the antiadenoviral immune response. In wild-type mice, a single mtDNA or CpG-DNA injection evoked a profound loss of splenic cytotoxic T-cell activity (control: 80 ± 12%; mtDNA: 1 ± 0.8%; $P = 0.001$; CpG: 0.3 ± 1%; $P = 0.01$; fig. 2A). Importantly, injection of murine genomic DNA (eukDNA: 86 ± 4%) did not influence the cytotoxic T-cell activity (P = not significant). In TLR9$^{-/-}$ mice, in contrast, mtDNA and CpG injection did not alter cytotoxic T-cell activity, demonstrating that the ovalbumin-specific immune reaction following mtDNA or CpG injection requires the TLR9 receptor (control: 70 ± 9%; mtDNA: 84 ± 4%; CpG: 73 ± 9%; all $P >$ 0.05). Thus, mtDNA suppresses the cytotoxic T-cell activity in wild-type mice but not in TLR9$^{-/-}$ mice.

Suppression of Cytotoxic T-cell Activation by Splenic Dendritic Cells after mtDNA Injection in Wild-type and TLR9$^{-/-}$ Mice

Several potential mechanisms associated with suppression of adaptive immunity have been postulated.[5,12] Among the most prominent are those preventing T-cell activation by DCs specialized to cross-present antigen.[3,5,9,12] Lacking CD8 T-cell activation after mtDNA could represent a DC-intrinsic defect or the result of cell deletion. Thus, we analyzed interferon-γ and interleukin-2 secretion of ovalbumin-specific OTI T cells incubated with splenic CD11c$^+$ DCs isolated from mtDNA or CpG injected wild-type and TLR9$^{-/-}$ mice. OTI T cells secreted much less interferon-γ and interleukin-2 when stimulated with DCs from wild-type but not TLR9$^{-/-}$ mice (fig. 2B).

As the observed decrease in interferon-γ and interleukin-2 secretion might be due to the deletion of DCs, we also determined the number of splenic CD11c$^+$ DCs in relation to all splenocytes. Although the overall CD11$^+$ DC count was increased in wild-type mice after mtDNA and CpG injection compared with controls, the total CD11c$^+$ DC count was not altered in TLR9$^{-/-}$ mice (fig. 2C). Furthermore, in wild-type mice but not in TLR9$^{-/-}$ mice, we observed a depletion of a subpopulation of DCs known to be crucial for antigen cross-presentation, the so-called CD8$^+$ CD11c$^+$ DCs (fig. 2C).[28]

Interestingly, although the overall CD11$^+$ DC population was increased, we observed a depletion of a subpopulation of DCs known to be crucial for antigen cross-presentation, the so-called CD8$^+$ CD11c$^+$ DCs.[28] Only in wild-type mice but not in TLR9$^{-/-}$ mice, CD8$^+$ CD11c$^+$ DCs were highly diminished after mtDNA and CpG injections (fig. 2C).

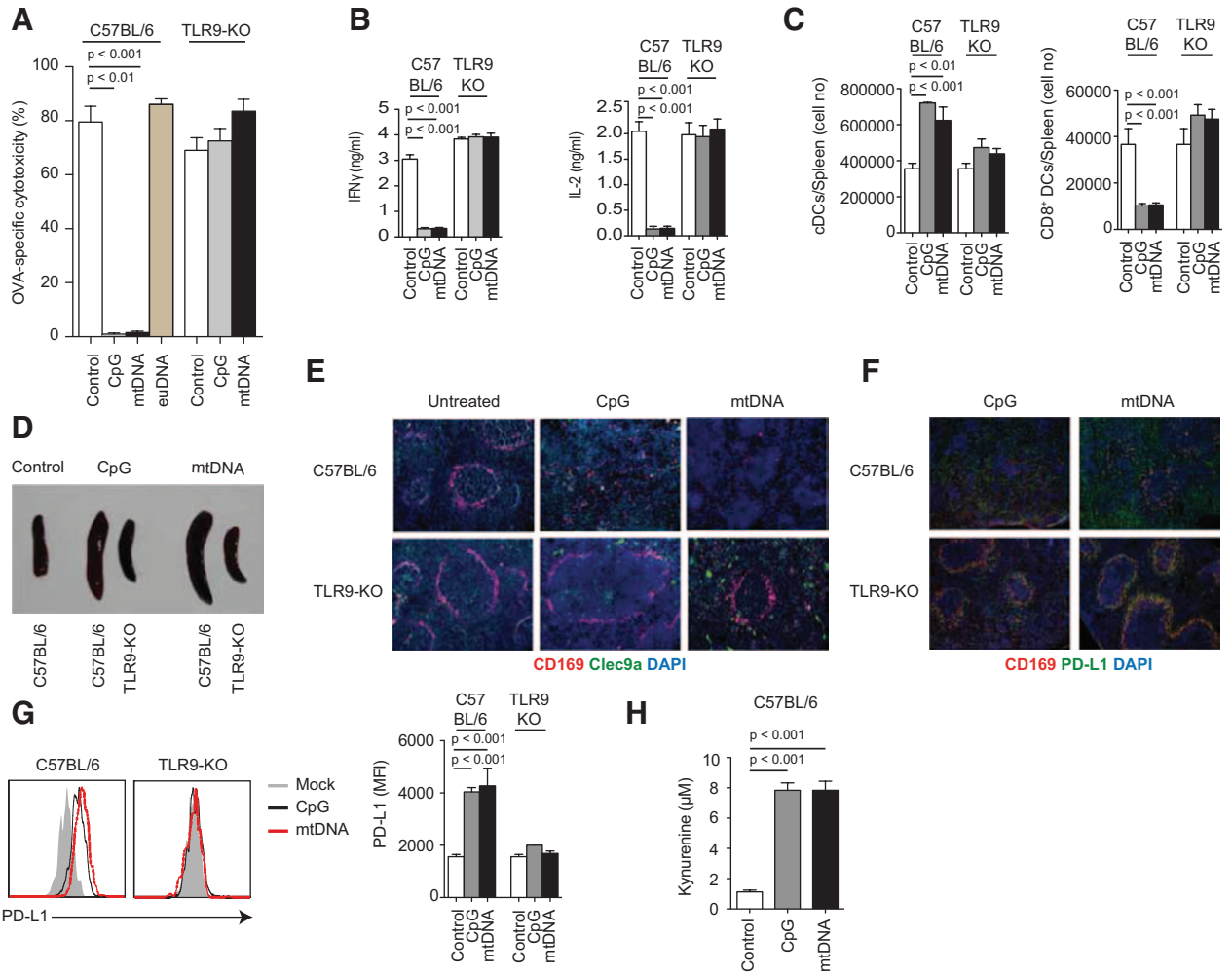

Fig. 2. Mitochondrial DNA (mtDNA) suppresses adaptive immune responses *via* various mechanisms. Ovalbumin (OVA)-specific cytotoxicity (*A*) in B6 wild-type and TLR9-knockout (KO) mice after immunization with adeno-OVA. Mice were either untreated (*white bars*) or treated with CpG oligonucleotides (*gray bar*), mtDNA (*black bar*), or murine eukaryotic DNA (euDNA; *brown bar*); (*B*) interferon γ (IFN-γ; *left graph*) and interleukin (IL)-2 concentrations (*right graph*) measured in supernatants of cocultures of T cells and dendritic cells (DCs). (*C*) Total number of splenic conventional DCs (*left* graph) and splenic CD8+ Clec9a+ DCs (*right* graph). (*D*) macroscopic images, spleen cryosections from mice either untreated or treated with CpG/mtDNA, stained for 4',6-diamidino-2-phenylindole (DAPI; *blue*), CD169 (*red*), and (*E*) Clec9a, or (*F*) programmed cell death ligand 1 (PD-L1; *green*) expression (*G*) on splenic macrophages (flow cytometry). Left histogram showing PD-L1 expression from untreated (Mock, *gray*) CpG- (*black line*) or mtDNA-treated mice (*red line*) and *right* graph showing median fluorescence intensity (MFI) of PD-L1. (*H*) Kynurenine serum concentration in untreated (*white bar*) or treated mice (CpG: *gray bar*; mtDNA: *black bar*).

To this end, DCs isolated from mtDNA or CpG-DNA–treated wild-type mice but not from TLR9$^{-/-}$ mice were unable to induce T-cell differentiation, as demonstrated by the strong decrease of interleukin-2 and interferon-γ concentrations in the supernatant of cocultures. This implies that mtDNA can cause deletion of CD8+ CD11c+ DCs, as described in mice and critically ill patients, even in the absence of bacteria or PAMPs.[5,12]

Spleen Histology after mtDNA Injection

From previous studies, we know that for the generation of protective CD8+ T-cell responses, the spleen and therein an intact marginal zone is crucially required.[4,7,12,28] Because septic patients often have splenic alterations, we also focused on the spleen.[5] To this end, after a single mtDNA injection, we not only observed a pronounced splenomegaly (fig. 2D) but also detected a severe, TLR9-dependent disruption of its marginal zone, demonstrated by the depletion of splenic CD169+ marginal zone macrophages (fig. 2, E and F). These findings alone demonstrate that a single mtDNA injection induces alterations associated with immunosuppressive mechanisms. In addition, we found that expression of the immunosuppressive molecule PD-L1 on splenic macrophages (1,562 median fluorescence intensity [MFI] ± 153) of wild-type mice was strongly increased after mtDNA (4,272 MFI ± 1,169) and CpG-DNA (4,045 MFI ± 285) treatments, respectively (fig. 2, F and G). In contrast, this effect was absent in TLR9$^{-/-}$ mice (all $P > 0.05$).

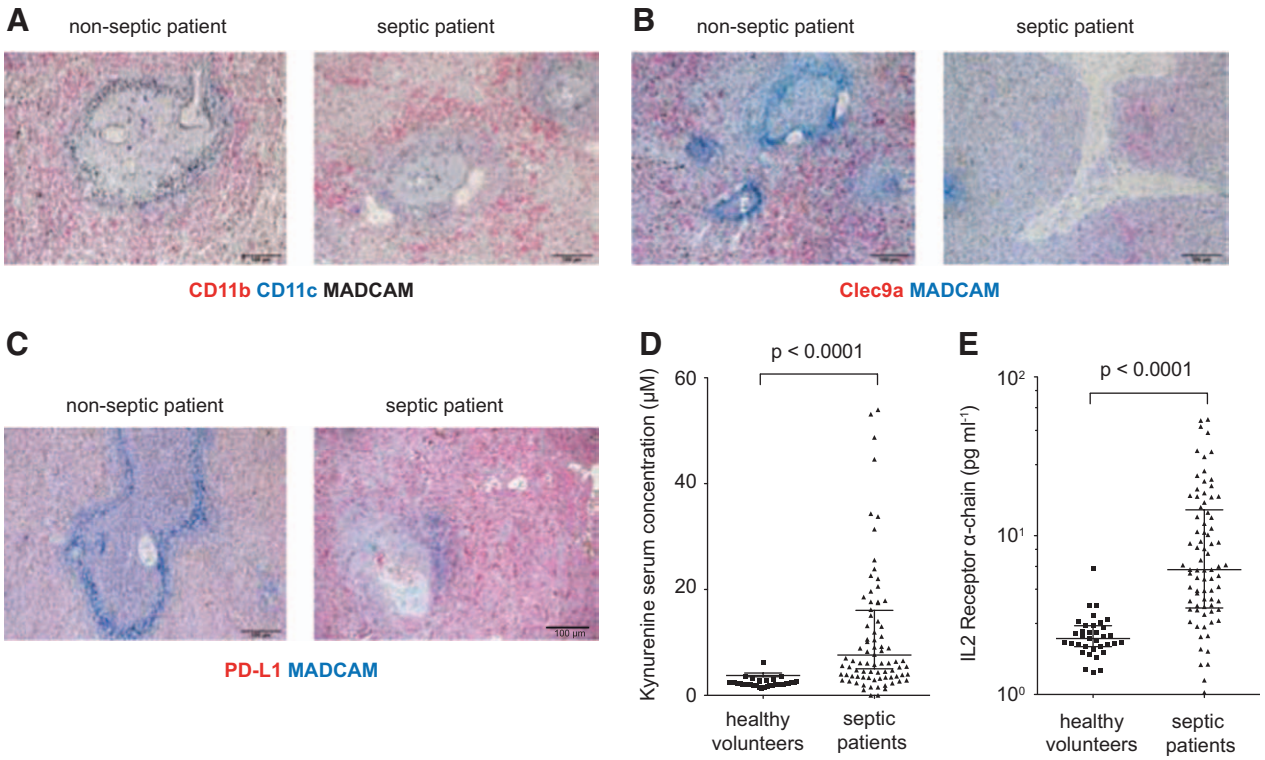

Fig. 3. Immunosuppressive mechanisms found in mice reflect findings in patients with sepsis. Spleen tissue sections from nonseptic (*left* picture) or septic patients (*right* picture) undergoing splenectomy stained for (*A*) CD11b (*red*), CD11c (*blue*), and mucosal addressin cell adhesion molecule (MADCAM; *black*), (*B*) Clec9a (*red*) and MADCAM (*blue*), or (*C*) PD-L1 (*red*) and MADCAM (*blue*). Kynurenine (*D*) and soluble interleukin-2R α-chain (*E*) serum concentrations of healthy individuals and of patients with sepsis.

To relate our results obtained in mice to humans with sepsis, we also analyzed spleens from septic and nonseptic patients undergoing splenectomy (fig. 3). Patients' characteristics are depicted in table 2. We observed strikingly similar structural alterations in the spleens obtained from septic patients to those in mice, with an almost complete loss of mucosal addressin cell adhesion molecule expression in their marginal zone when compared with the spleens from nonseptic individuals (fig. 3A). In a next step, we analyzed splenic cross-presenting DCs' in humans with and without sepsis. Please note, that in mice, cross-presenting $CD8^+$ $CD11c^+$ DCs co-express CD8 and Clec9a, whereas in humans, these cross-presenting DCs express Clec9a only. We could now show a high count of $Clec9a^+$ cells in the spleens from nonseptic patients, whereas almost none $Clec9a^+$ cells were seen in the spleens from septic patients (fig. 3B). These findings correspond to the depletion of $Clec9a^+$ DCs in the spleens of mtDNA-treated mice (fig. 2E). Furthermore, similar to wild-type mice with a single mtDNA injection, we found PD-L1 to be highly induced in the spleens from septic patients (fig. 3C).

Kynurenine Serum Concentrations

Besides mechanisms affecting splenic structure and immune cell survival, other mechanisms such as the up-regulation of counterregulatory receptors (PD-L1) and the induction of suppressive acting enzymes such as IDO may play a role in immune paralysis, as IDO catalyzes the degradation of tryptophan to kynurenine, which causes T-cell suppression by two mechanisms. Although deprivation of tryptophan indirectly blocks T-cell proliferation, kynurenine directly induces T-cell apoptosis.[20] In our patient cohort, kynurenine serum concentrations were increased in both wild-type mice injected with mtDNA or CpG (control: 1.1 ± 0.2 µM *vs.* mtDNA: 7.8 ± 1 µM or CpG: 7.8 ± 1.2 µM; all $P = 0.001$; fig. 2H) and in septic patients compared with healthy volunteers (11.3 ± 13 µM *vs.* 2.8 ± 2.6 µM; $P = 0.0001$; fig. 3D). Furthermore, in humans with sepsis, soluble interleukin-2R α-chain serum concentrations, a phenotype strongly associated with IDO activity, was highly increased too (754 ± 1,500 pg/ml *vs.* 25 ± 50 pg/ml; $P = 0.0001$; fig. 3E).

When focusing on patients with the symptoms and signs of sepsis without confirmed bloodstream infection, despite multiple cultures and also the use of highly sensitive diagnostic tools, *i.e.*, Septifast® (Roche Molecular Systems, USA), kynurenine (18.2 ± 18.4 µM) and interleukin-2R α-chain (2,205 ± 2,624 pg/ml) serum concentrations, which are known to be associated with immunosuppression, were even more increased when compared with septic patients with confirmed bloodstream infection (kynurenine: 7.7 ± 6.3 µM; $P = 0.0001$; interleukin-2R α-chain: 602 pg/ml ± 1037; $P < 0.0001$), supporting our hypothesis that endogenous TLR ligands can induce immunosuppression even in the

absence of bacterial infections. This is further confirmed by our finding that increased mtDNA serum concentrations correlated with both kynurenine (r = 0.59; $P < 0.0001$) and interleukin-2R α-chain (r = 0.37; $P < 0.0001$) serum concentrations. Taken together, these results demonstrate that mtDNA concentration is increased in sepsis and, besides inflammatory effects, has powerful immunosuppressive effects and can evoke immunosuppression on its own.

Discussion

According to recent definitions, sepsis is considered a (dysregulated) immune response of the host to injury and/or infectious stimuli in the presence of a known (or strongly suspected) infection.[4] In this respect, improved therapeutic, *i.e.*, antimicrobial strategies indeed helped many septic patients to survive the first stage of sepsis, the "cytokine storm" of the initial hyperinflammatory crisis.[11] However, despite its resolution, patients still die from opportunistic or nosocomial secondary infections.[8] Interestingly, even patients without confirmed bloodstream infection, despite the use of new and sensitive diagnostic tools, such as multiplex PCR (Septifast), are at risk of developing "immunoparalysis."[21,30] A solution to this conundrum might be that endogenous triggers of an immune response like mtDNA, besides microbes, could play a so far unrecognized role.[1,2]

We show that the endogenous TLR-ligand mtDNA is a strong suppressor of the adaptive immune response in wild-type but not in $TLR9^{-/-}$ mice. This is in accordance with our previous finding that unmethylated, bacterial CpG-DNA, which as a structural similarity to endogenous mtDNA, suppresses the adaptive immune response as well.[20] Addressing these results in humans, we further show that mtDNA serum concentrations are increased in septic patients and that high mtDNA serum concentrations on the first day of diagnosing sepsis are associated with increased 30-day mortality. This confirms the finding from Nakahira *et al.* that mtDNA serum concentrations are increased in medical ICU patients and are associated with the patients' mortality.[24] Even more important, we identified a variety of suppressive mechanisms common to both mice and critically ill humans. Thus, our findings show that mtDNA is an endogenous DAMP previously unrecognized to evoke immunoparalysis in humans.

Recently, mtDNA was identified as an endogenous TLR9 ligand considered capable of triggering a proinflammatory response, with mtDNA concentrations found to be increased in the sera of patients after severe trauma.[22] Because of its bacterial-like, unmethylated DNA structure, we hypothesize that mtDNA is one of the possible links from inflammation to immunosuppression in both critically ill humans and mice. Indeed, we were able to show that the concentration of mtDNA, an endogenous TLR9 ligand, was increased in the serum of critically ill patients with sepsis and that even a single mtDNA injection in otherwise healthy wild-type mice suppressed cytotoxic T-cell activity and induced a variety of immunosuppressive mechanisms such as deletion of $CD8^+$ DCs, disruption of the splenic marginal zone, up-regulation of PD-L1, and induction of IDO.

Of particular novelty, endogenous mtDNA, a DAMP so far only described to act stimulatory, was able to induce various mechanisms inhibiting antigen-specific T-cell immunity.[22] Furthermore, most of our observations in mice seem to be of translational value, as they were found in septic patients as well.

First, a suppressive role of PD-L1 has been reported in diseases such as chronic viral infection,[31] tumors,[32] or sepsis.[33] In all these conditions, interaction of PD on T cells and of PD-L1 on antigen-presenting cells has been shown to prevent T-cell function, as exhausted T cells were no longer able to clear viral infections or reject tumors.[34] Although already applied successfully in various mouse models (viral infections and tumors), the successful treatment of tumor patients with antibodies against PD-L1 has recently demonstrated the importance of this molecule.[35]

Second, cross-presentation of antigen by specialized $CD8^+$ $Clec9a^+$ DCs is an essential mechanism for activation of CD8 T cells against viral infections and tumors, and $CD8^+$ cells were diminished in wild-type mice after even a single mtDNA injection.[12] Third, IDO is an enzyme initially identified to be crucial during pregnancy to prevent rejection of the fetus.[36] Furthermore, IDO is also operative outside the placenta, such as in tumors and bacterial and parasitic infections.[37] In all settings, T cells were functionally impeded by degradation of the essential amino acid tryptophan and by the direct suppressing action of kynurenine.[20,38] Of note, kynurenine serum concentrations were increased in both mtDNA-injected wild-type mice and septic patients. Furthermore, we have previously shown that $TLR9^{-/-}$ mice fail to increase IDO activity after TLR9 stimulation by unmethylated, bacterial CpG-DNA.[20] Therefore, in this study, we did not perform IDO measurements after injection of the TLR9 ligand mtDNA.

Interestingly, all these suppressive mechanisms play an important role in protecting the body against (auto-) immune damage.[32] Counter regulation of an overwhelming immune activation is presumably essential to prevent immune system–related collateral damage. If sepsis cannot be controlled, the concentration of (aseptic) DAMPs like mtDNA, which are released from the necrotic cells and not from bacteria, increases, and thus, the immune system is counterregulated, eventually switching from immune activation to long-lasting immunoparalysis.[33,39] Recent approaches to interfere with these counter regulatory suppressive mechanisms are highly promising strategies to mitigate late immunoparalysis of sepsis.[19,35]

Due to the diversity of the sepsis syndrome, it seems unreasonable that there will be one "magic bullet" for treatment, and a successful therapy most likely will have to be custom tailored, based on a clear definition of the immunologic stage of each individual septic patient.[3,7,40] This will require biomarkers for assessing the immune status.[41] In this respect, Volk and coworkers[42] had demonstrated that patients showing a "paralyzed" monocytic phenotype ("lipopolysaccharide tolerance"), as determined by low levels of major

histocompatibility complex class II molecules on the cell surface and a lack of TNF production after challenge with lipopolysaccharide, had a poor prognosis. Possibly, the measurement of mtDNA concentrations could be helpful in this respect. Thus, our finding that mtDNA is increased in septic patients even without confirmed bacteremia and associated with increased mortality is in accordance with a recent report describing mtDNA as highly significant and independent prognostic marker for the survival of critically ill patients.[24] Accordingly, our findings along with those of others refocuses the current perspective to mtDNA or other DAMPs as inducers of immunosuppression in critically ill patients.[12,24,28,43]

All results taken together, mtDNA alters various pathways associated with immune suppression in mice. Furthermore, evidence for immune suppression by mtDNA in mice can be translated to humans, as increased serum concentrations of mtDNA, kynurenine, and soluble interleukin-2R α-chain and many splenic phenotypes were not only observed in mice but also in patients with sepsis. Mechanistically, our data now strongly suggest that these observations are related and explained by previously unrecognized immunosuppressive effects of mtDNA potentially representing one missing link between initial inflammation and immunoparalysis.

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Competing Interests

The authors declare no competing interests.

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