THE IMMUNOLOGY OF SEPSIS – UNDERSTANDING HOST SUSCEPTIBILITY, PATHOGENESIS OF DISEASE, AND AVENUES FOR FUTURE TREATMENT

EDITED BY: Luregn J. Schlapbach, Johannes Trück and Thierry Roger
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THE IMMUNOLOGY OF SEPSIS – UNDERSTANDING HOST SUSCEPTIBILITY, PATHOGENESIS OF DISEASE, AND AVENUES FOR FUTURE TREATMENT

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EDITORIAL
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Editorial: The Immunology of Sepsis—Understanding Host Susceptibility, Pathogenesis of Disease, and Avenues for Future Treatment

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Editorial on the Research Topic

The Immunology of Sepsis—Understanding Host Susceptibility, Pathogenesis of Disease, and Avenues for Future Treatment

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Most recent estimations suggest that sepsis affects about 49 million people and accounts for around 11 million deaths each year worldwide (2), making it one of the leading causes of preventable death in all age groups. Sepsis is a health priority according to the World Health organization (3), which provided recommendations to improve the prevention, diagnosis and management of sepsis. Although sepsis mortality has decreased over the past two decades (4, 5), the incidence of sepsis continues to rise due to a higher number of patients with complex conditions such as immunosuppression as well as an increase in more elderly people. A basic requirement for effective sepsis management is timely and appropriate antimicrobial therapy (6, 7). With the increase of infections caused by multidrug resistant bacteria, the advances made toward rapid multidrug testing to enable personalized antimicrobial therapies is promising (Sun et al.). However, a major obstacle to improving sepsis outcomes is the lack of knowledge on the intricate interplay between host defense, infection, and pathogen virulence, as well as timing and nature of interventions. Over the past decade, our understanding of sepsis has evolved from the earlier concept of sepsis as a result of an excessive inflammatory response to the current notion that sepsis outcomes are more likely related to the dynamic balance between pro-inflammatory and counteracting anti-inflammatory mechanisms. To this end, animal models and a number of human observational studies have paved the way toward precision trials on immunotherapy for sepsis (Zijlstra et al.).

The aim of the Frontiers in Immunology Research Topic "The Immunology of Sepsis–Understanding Host Susceptibility, Pathogenesis of Disease, and Avenues for Future Treatment" was to collect state-of-the art articles and reviews on the role of the host immune system affecting susceptibility, presentation, and outcome of sepsis. We hereby provide an overview of this Frontiers in Immunology topic which includes 11 original articles, 13 review articles, 1 commentary, and 1 case report.

SUSCEPTIBILITY TO SEPSIS

Sepsis susceptibility may result from maturational, genetic, or acquired alterations of the immune system. The incidence of sepsis is highest in newborns (8), and an increasing body of studies characterize distinct patterns of immune responses in this age group compared to children and adults. Schneider et al. for example, demonstrate that neonatal macrophages express substantially less tumor necrosis factor compared to adult cells, regulated through the transcription factor interferon regulatory factor 5 (IRF5).

Considering how few patients develop sepsis in comparison to the exposed population, there is biological plausibility and epidemiologic evidence for underlying genetic mechanisms affecting susceptibility to sepsis (9, 10). These may affect both very rare variants associated with extreme phenotypes (11) and common variants that may be of relevance at the populational level (12). Deficiency in mannose-binding lectin (MBL) emerged two decades ago as a promising candidate to investigate this hypothesis (13). Although polymorphisms affecting MBL serum levels are common and the role of complement in host defenses against bacterial infections is well known, previous reports on the relevance of MBL deficiency in susceptibility to infection remained conflicting (14). Using a well-phenotyped prospective intensive care unit cohort, Levy et al., genotyped 420 pediatric patients with influenza-associated organ dysfunction for variants in the *MBL2* gene expected to result in low serum MBL levels. No clear relationship was observed between genetic variants and overall outcomes in the cohort, neither in within-cohort, nor in trio or control analyses. Interestingly, and similar to a previous study, low-MBL producing variants were more common in a subset of fatal cases with methicillin-resistant *Staphylococcus aureus* (MRSA), but the relevance of this finding remains to be confirmed in larger studies.

HOST-PATHOGEN INTERACTION

Children are prone to severe infections caused by encapsulated bacteria such as *Streptococcus pneumoniae*, *Streptococcus agalactiae* (Group B streptococcus, GBS), *Neisseria meningitis* and *Haemophilus influenzae*. The polysaccharide capsule is a key bacterial virulence factor hindering opsonophagocytosis and complement-mediated bacteriolysis. However, it also represents an effective target for vaccine development. In his review, Sadarangani describes the mechanisms of vaccine-induced protection and illustrates successful examples of pneumococcal and meningococcal capsular conjugate vaccines, while challenges remain in the development of effective vaccines against non-type b *H. influenzae* and GBS that also carry polysaccharide capsules. The article by Kadhim et al., demonstrates a novel potential drug candidate to block staphylococcus enterotoxin-induced organ damage. Finally, Deng et al. investigate mechanisms of complement evasion in streptococcal strains.

Very timely considering the current coronavirus disease 2019 (COVID-19) pandemic, and giving credit to an often overlooked problem, Lin et al., provide a thorough overview of the epidemiology and pathogenesis of viral sepsis with a focus on herpes simplex, influenza, and dengue viruses, as well as entero- and parechoviruses. Recognition of a viral origin of sepsis could allow targeted treatment and reduce the unnecessary use of antibiotics.

UNDERSTANDING THE DYSREGULATED HOST RESPONSE TO INFECTION

While the concept of a "dysregulated host response to infection leading to organ dysfunction" in sepsis is broadly accepted, our understanding of the underlying mechanisms remains very limited. The contributions to this Frontiers topic shed light on a number of pathways that are likely involved.

Previous studies have shown that a subgroup of septic children and adults reveal similar patterns to patients with hemophagocytic lymphohistocytosis (HLH), or macrophage activation syndrome (MAS) such as cytokine storm, hyperferritinemia, and multi-organ dysfunction. This entity has been termed macrophage activation-like syndrome (MALS). Karakike et al. provide an overview of the available evidence on this topic, while the results of the first randomized clinical trials (RCTs) on MALS are eagerly awaited. Zarjou et al. assessed the inhibitory role of myeloid ferritin heavy chain and ferritin light chain in a mouse model on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation, illustrating a potential immunomodulatory role of ferritin light chain.

More recently, insight into pro- and anti-inflammatory responses to the exposure of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) suggest a dynamic and heterogenous process (15). A fascinating research area in this regard relates to epigenetic processes affecting gene expression during sepsis. Cross et al. provide an overview on the literature on this topic. They summarize the rapidly growing number of mainly laboratory studies indicating that epigenetic mechanisms are thoroughly perturbed during sepsis, and are related to endothelial dysfunction and immunosuppression. These observations may open up new treatment options, such as drug interventions with histone deacetylase inhibitors for which pre-clinical animal studies suggest a potential benefit. It will be interesting to see whether candidate drugs progress to clinical studies on immune modulation in sepsis in the coming decade. Another possible drug approach that could support a more balanced immune response is the blocking of the Src family of tyrosine kinases with dasatinib, a drug for the treatment of chronic myeloid leukemia and acute lymphoblastic leukemia. Indeed, Gonçalves-de-Albuquerque et al. report promising results of using dasatinib in a mouse model of polymicrobial sepsis.

Several other proteins and cells represent both encouraging biomarkers and therapeutic targets for future strategies to combat sepsis. For example, Schrijver et al. report that myeloid-derived suppressor cells (MDSCs) are a heterogenous group of immature cells that expand in a number of conditions including sepsis. MDSCs suppress immune responses of different cell types in the early and late phases of sepsis, and pre-clinical studies show distinct patterns of expansion of MDSC subpopulations

Observational studies have already reported an association between high proportions of blood MDSCs and poor outcome in sepsis patients. In a mouse model, Bomans et al. have elucidated mechanisms involved in the functional reprogramming of naïve bone marrow monocytes after sepsis, which lead to a "memory" of the innate immune system.

Other articles submitted to this Research Topic illustrate a number of recent discoveries of cells and pathways that contribute to the host response to sepsis. Yin et al., demonstrate that phosphatase regenerating liver 2 (PRL2) regulates the generation of reactive oxygen species in macrophages and thereby contributes to bactericidal activity. Sjaastad et al., assessed the response to polymicrobial sepsis using a mouse model and observed features consistent with chronic immunoparalysis, measured by reduced antigen-specific T cell-dependent B cell responses.

Although the intestinal system has not been commonly considered in organ dysfunction scores (1), it represents an extraordinary body surface area containing a high density of lymphatic tissues and immune cells. The regulation between the host immune system and intestinal pathogens as well as bacterial translocation play a key role in the dynamic processes during and after shock. Increasing our understanding of these events may point to ways for secondary interventions, as summarized in the review by Haussner et al., on the role of the intestinal mucosa during sepsis. Expanding from this work, the importance of intestinal microbiota in sepsis is explored in the excellent review by Haak et al. shedding light on the therapeutic potential of microbiome-modifying strategies for prevention and treatment of sepsis and sepsis-related late mortality.

BIOMARKERS, MONITORING THE IMMUNE SYSTEM, AND THE FUTURE OF IMMUNOMODULATION IN SEPSIS

The immune status of patients with sepsis is subject to rapid progression, and hyperinflammation and immunoparalysis may change dynamically. The failures to characterize the predominant immunological phenotype of sepsis patients coupled with considerable disease and patient heterogeneity represent major obstacles to develop effective immunomodulation-based trials. Peters van Ton et al. discuss the critical need for accurate monitoring of associated immune dysregulation to select patients more likely to benefit from targeted immunomodulatory interventions, and to facilitate enrolment in clinical studies toward personalized medicine.

The initial response to infection includes activation of innate immunity through soluble and cell-bound pattern recognition receptors. Porte et al. for example, review the available evidence on the role of the long pentraxin 3 (PTX3) in sepsis, a molecule with high homology with C-reactive protein. PTX3 binds to various microorganisms and has protective effects against sepsis in animal models. In human studies, high blood levels of PTX3 are associated with sepsis severity, suggesting a potential use as a biomarker. Furthermore, Tipoe et al. performed a systematic review and meta-analysis of plasminogen activator inhibitor-1 (PAI-1) in sepsis. PAI-1 level is increased in patients, and may be used as a predictor of disease severity and all-cause mortality in sepsis.

Low expression levels of human leukocyte antigen D related (HLA-DR) by CD14 leukocytes are increasingly used as a marker of sepsis-induced immunosuppression. Tamulyte et al. report on a prospective cohort assessing monocyte HLA-DR by a point-of-care test. They demonstrate the principle feasibility of the approach, which may help to identify patients with a higher risk of worse outcomes due to sepsis. Excitingly, the development of novel assays for immune profiling patients with severe infections has made huge progress and is anticipated to enable personalized medicine trials in the very near future.

The most widely studied immunomodulatory drugs in sepsis are corticosteroids. A large number of high quality RCTs in critically ill adults are available, as nicely reviewed by Heming et al. The immunomodulatory effects of statins have been recognized for some time. Braga Filho et al. used a mouse model of CLP to demonstrate the positive impact on simvastatin on survival and immune modulation.

CONCLUSIONS

In summary, the knowledge on the pathophysiology of sepsis has expanded in recent years. Many original and review article in this Research Topic describe experimental and clinical studies pointing to new candidates for biomarkers and interventions, which may ultimately pave the way toward personalized care for sepsis. The availability of improved immunophenotyping approaches, coupled with a number of candidate drugs currently under investigation is promising. The marked heterogeneity of sepsis advocates for highly selective interventions, targeted at populations selected for patients who are more likely to respond to, and to benefit from therapeutic interventions. The success of this endeavor will depend on the capability to incorporate effective strategies for patient selection and targeted treatment allocation. As a promising sign, a number of consortia have recently been launched to advance personalized immunotherapy in sepsis. As nicely discussed by Talisa et al. trials using adaptive enrichment and response adaptive randomization may be best suited to tackle the challenges of developing effective treatments for clinical practice. Recently, large international randomized, embedded, multifactorial adaptive platform trials (originally developed in the field of cancer clinical research) have been launched in infectious diseases and hopefully will boost the efficiency and impact of sepsis research. These advances may motivate further personalized medicine trials leading to sustainable outcome improvements for this major killer disease.

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All authors contributed to the article and approved the submitted version.

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The Yin and Yang of Tyrosine Kinase Inhibition During Experimental Polymicrobial Sepsis

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Neutrophils are the first cells of our immune system to arrive at the site of inflammation. They release cytokines, e.g., chemokines, to attract further immune cells, but also actively start to phagocytose and kill pathogens. In the case of sepsis, this tightly regulated host defense mechanism can become uncontrolled and hyperactive resulting in severe organ damage. Currently, no effective therapy is available to fight sepsis; therefore, novel treatment targets that could prevent excessive inflammatory responses are warranted. Src Family tyrosine Kinases (SFK), a group of tyrosine kinases, have been shown to play a major role in regulating immune cell recruitment and host defense. Leukocytes with SFK depletion display severe spreading and migration defects along with reduced cytokine production. Thus, we investigated the effects of dasatinib, a tyrosine kinase inhibitor, with a strong inhibitory capacity on SFKs during sterile inflammation and polymicrobial sepsis in mice. We found that dasatinib-treated mice displayed diminished leukocyte adhesion and extravasation in tumor necrosis factor-α-stimulated cremaster muscle venules *in vivo*. In polymicrobial sepsis, sepsis severity, organ damage, and clinical outcome improved in a dose-dependent fashion pointing toward an optimal therapeutic window for dasatinib dosage during polymicrobial sepsis. Dasatinib treatment may, therefore, provide a balanced immune response by preventing an overshooting inflammatory reaction on the one side and bacterial overgrowth on the other side.

Keywords: sepsis, inflammation, dasatinib, Src tyrosine kinase, leukocyte trafficking

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INTRODUCTION

Sepsis is a life-threatening systemic inflammatory condition which results in shock, multiple organ dysfunction, and eventually death (1, 2). It is characterized by a cytokine storm released from myeloid cells during an inadequate antimicrobial response to invading pathogens (3). Worldwide, more patients die due to sepsis-related complications than of breast and colorectal cancer together (4). The global incidence has been estimated to be 31 million including 6 million fatalities (5).

Neutrophil activation and invasion into inflamed tissue is a critical step in the host's fight against an infection. Under normal circumstances, extravasation of neutrophils is tightly regulated by different receptors and ligands on both the endothelium and neutrophils (6). In order to transmigrate, neutrophils need to roll, adhere, and crawl along the activated endothelium to find a spot for extravasation. Following extravasation, neutrophils release cytokines like interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α to attract more immune cells (7–9). In addition, they start to phagocytose pathogens. In sepsis, pathophysiologic processes are rather caused through an exuberant host response by immune cells against the invading microorganisms than through the direct effects of microbes itself (10). In this respect, a balanced immune response depends on regulatory mechanisms modulating the intensity of the immune response. The Src-family of tyrosine kinases (SFKs) are a group of signaling enzymes with diverse biological effects including, but not limited to, cell proliferation, survival, migration, and metastasis (11–13). SFKs are the largest family of cytoplasmic tyrosine kinases expressed in innate immune cells. The presence of those may vary between innate immune cells, with Hck, Fgr, and Lyn being the most prominently expressed SFKs in monocytes, macrophages, granulocytes, and dendritic cells (14). SFKs bind directly to the cytoplasmatic tail of activated integrins and are responsible for the majority of protein phosphorylations involved in integrin outside-in signaling. Various studies using knockout mice or inhibitors demonstrated the importance of SFKs in host defense and inflammation (15–19), including adhesion and transmigration during leukocyte recruitment (20). Because of these findings, tyrosine kinase inhibitors, originally designed for cancer therapy, have been studied for their role as immune-modulating drugs. Dasatinib, a multi-kinase inhibitor with strong effects on SFKs, acts on both Abl- and Src-family tyrosine kinases (21), and is currently used in patients with chronic myeloid leukemia and acute lymphoblastic leukemia with Philadelphia positive chromosome (Ph+) (22, 23). Besides its effect on malignant cells, dasatinib decreases systemic TNF-α production after LPS injection in a Src and Bruton's tyrosine kinase dependent fashion (24) and reduced lung injury in a dose-dependent manner (25). Additionally, dasatinib treatment reduced chemokine secretion by neutrophils and bone marrow-derived macrophages, suggesting that SFKs are also critical regulators of chemokine secretion in myeloid cells (26). As immune responses to pathogens prevent their dissemination and favor their elimination by the host, there is a concomitant risk of exaggerated immune responses, which may lead to tissue and organ damage. Thus, we hypothesized that immunomodulatory drugs balancing immune responses may be beneficial during systemic severe infection. To test this, we investigated the safety and efficacy of the tyrosine kinase inhibitor dasatinib during inflammation and sepsis. We show that dasatinib diminished the recruitment of leukocytes to the site of inflammation in the inflamed cremaster muscle model. In addition, in a model of polymicrobial sepsis, dasatinib treatment improved survival and sepsis severity in mice and reduced organ damage in a dose-dependent manner with an optimal dose for survival.

MATERIALS AND METHODS

Animals

We used male Swiss Webster (SW) mice (25–30 g) from the Oswaldo Cruz Foundation breeding unit, Rio de Janeiro, Brazil. Animals were lodged at 22°C with a 12-h light/dark cycle and free access to food and water. For *in vivo* cremaster muscle experiments *Lyz2*GFP and *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ (SFK-ko) mice on a C57Bl/6 background were used (27–29). These mice were maintained at the Walter Brendel Center for Experimental Medicine, Ludwig Maximilians Universität, Munich, Germany and accommodated in a barrier facility under SPF conditions. Mice used in the experiment were at least 8 weeks of age and of healthy appearance.

Pharmacokinetic Analysis

Pharmacokinetic evaluations were performed after the second administration of dasatinib (1 mg/kg). The administrations were made at the following time points: 30 min before CLP and 6 h after CLP. Blood samples were drawn at 0.25, 0.5, 0.75, 1, 2, 4, 8, 16, and 23.75 h (**Figure 1C**).

Dasatinib levels in plasma were determined using a validated high-performance liquid chromatography–tandem mass spectrometry method (HPLC–MS/MS). HPLC system (1200 series, Agilent Technologies, Germany) is connected with API 3200 triple quadrupole mass spectrometer (SCIEX, Toronto, ON, Canada) using multiple reaction monitoring (MRM). The MRM transitions monitored were m/z 488.2 → 401.3 for dasatinib, m/z 629.4 → 155.2 for internal standard.

3D Chemotaxis Assay

The analysis of migration in collagen gels was performed in μ-slide chemotaxis chambers (IBIDI, Planegg, Germany). A gel–cell mixture consisting of 3×10^5 neutrophils in 1.5 mg/mL type I rat tail collagen (IBIDI) was applied to the middle channel of the 3D chamber and left at 37°C for 5 min for gelation. After application of 100 nM fMLP for 20 min at 37°C, time-lapse videos were recorded for 10 min using an Axiovert 200 M microscope (Zeiss, Jena, Germany) equipped with a Plan-Apochromat 10×/0.75NA objective, AxioCam HR digital camera, and a temperature-controlled environmental chamber. Migration tracks were analyzed offline with the Image J software. Single cell migration tracks and rose plots were generated using the IBIDI Chemotaxis software.

Intravital Microscopy

We applied intravital microscopy in exteriorized inflamed cremaster muscle venules of *Lyz2* GFP and SFK-ko mice, as

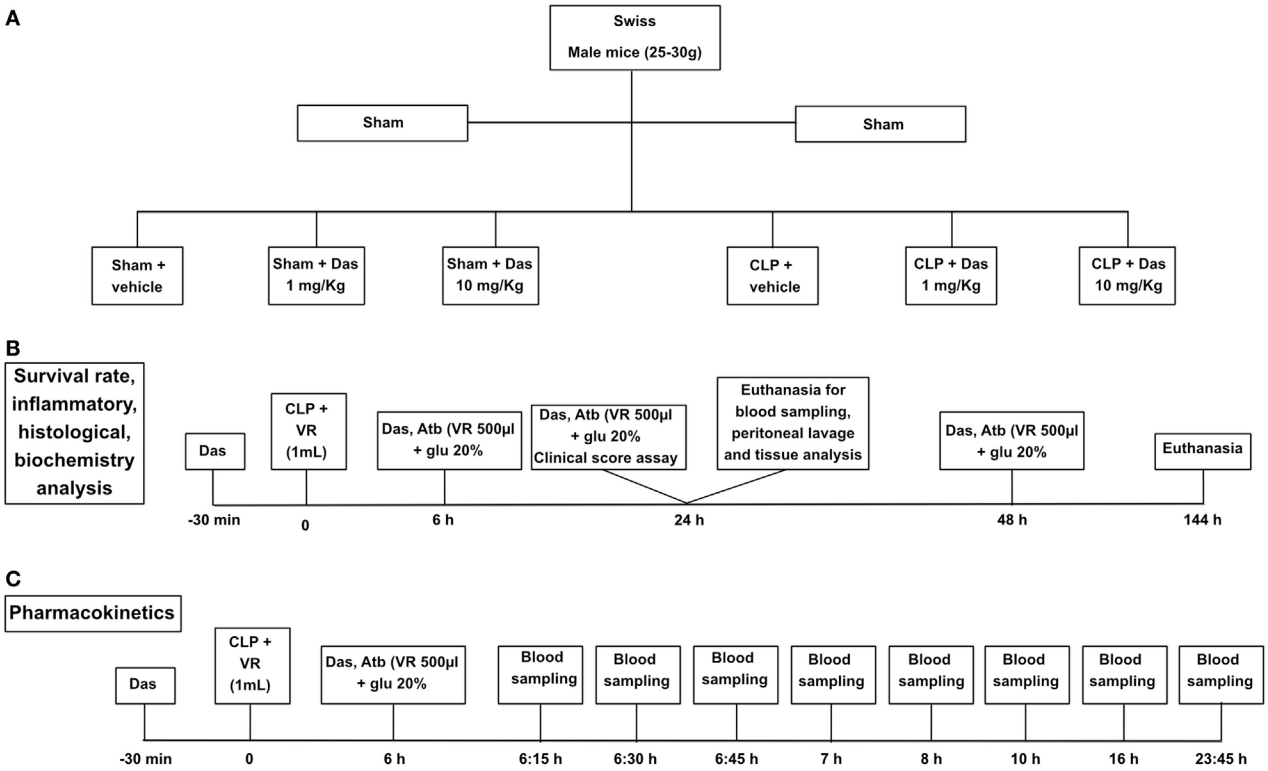

FIGURE 1 | Experimental design. Group designation **(A)**, survival curve and inflammatory parameters **(B)**, and pharmacokinetics **(C)**. Sham, control group; CLP, cecal ligation and puncture; Das, dasatinib; Atb, antibiotic; VR, volemic reposition (500 µL); glu 20%, glucose 20%.

described (30). Briefly, mice were treated with intrascrotal injection of 500 ng TNF-α, 2 h prior to microscopy. Mice were then anesthetized with intraperitoneal (i.p.) injection of ketamine (125 mg/kg body weight, Ketalar; Parke-Davis, Morris Plains, NJ, USA), and xylazine (12.5 mg/kg body weight; Phoenix Scientific, Inc., St. Joseph, MO, USA). Thereafter, mice were placed on a heating pad to maintain body temperature, intubated, and the left carotid artery cannulated for blood sampling and systemic antibody administration. To maintain a neutral fluid balance, mice were given heparinized saline 0.2 mL/h i.v. throughout the experiment. Intravital microscopy was conducted on an upright conventional fluorescence microscope (Olympus BX51WI, Tokio, Japan) with a saline immersion objective (SW40/0.75 numerical aperture, Zeiss, Jena, Germany).

Cremaster Muscle Preparation

The surgical preparation of the cremaster muscle was conducted as described (31). Shortly, after surgically opening the scrotum, the cremaster muscle was exteriorized and spread over a cover glass. The epididymis and testis were gently pinned aside giving full microscopic access to the cremaster muscle microcirculation. Experiments were recorded *via* a CCD camera system (CF8/1, Kappa, Gleichen, Germany) on a Panasonic S-VHS recorder and on hard-drive using virtual dub software. The cremaster muscle was superfused with thermocontrolled (35°C) bicarbonate-buffered saline. Postcapillary venules under observation ranged from 25 to 45 µm in diameter. Blood samples were taken during and after the experiment and WBC/neutrophil counts determined using ProCyte Dx Hematology Analyzer (IDEXX, Westbrook, ME, USA). Venular diameter, venular vessel segment length, and leukocyte rolling velocity were assessed using Fiji software (32). Venular centerline red blood cell velocity in the cremaster muscle preparation was measured during the experiment using a dual photodiode and a digital online cross-correlation program (Circusoft Instrumentation, Hockessin, DE, USA).

In a second set of experiments, the number of transmigrated cells was determined. For this approach, mice were treated as described above. After exteriorization, mouse cremaster muscles were dissected and fixed by 4% PFA (AppliChem GmbH, Darmstadt, Germany). Thereafter, cremaster muscle whole mounts were stained using Giemsa (Merck Millipore, Darmstadt, Germany) and the number of transmigrated cells/mm^2 assessed using a Zeiss Axioskop 40 microscope with an oil immersion objective 100×, 1.25NA (Zeiss, Jena, Germany). Micrographic images are shown using an oil immersion objective 40×, 1.3NA (Zeiss).

Dasatinib Treatment

Lyz2GFP mice received dasatinib (1, 10, or 20 mg/kg) by gavage in a volume of 100 µL/10 g methylcellulose. Control mice received methylcellulose alone. *Swiss* male mice received dasatinib (1 or 10 mg/kg) by gavage in a volume of 100 µL per animal

30 min before, 6 and 24 h after the induction of sepsis. Control animals received DMSO/saline solution (vehicle) same volume of dasatinb treatment. We based our treatment on dasatinib pharmacokinetics data reported by Ref. (33, 34). In acute experiments with end point at 24 h after cecal ligation and puncture (CLP) the animals received only the two first doses of the drugs (**Figures 1A–C**).

CLP Model

Swiss mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg, Cristália) and xylazine (10 mg/kg, Syntec) 10–15 min prior to surgery. The cecal ligation was done below the ileocecal valve and the cecum was perforated four times with an 18G needle. A small amount of fecal material was squeezed from the holes before reinsertion of the cecum in the abdominal cavity. Volemic reposition was made with 1 mL of sterile saline subcutaneously. The animals received meropenem (10 mg/kg) diluted in salina with glucose at 20% intraperitoneally at 6, 24, and 48 h after CLP in 500 µL volume. Sham-operated animals constituted the control and received the same volume reposition and antibiotic treatment administered to CLP animals. After 24 h the animals were submitted to euthanasia using isoflurane (Cristália), and the peritoneal cavity was washed with PBS for colony-forming unit (CFU) analysis and total and differential leukocyte counting (**Figures 1A,B**).

Biochemical Analysis

Mice were kept in a 12-h fasting with water *ad libitum*, and then blood was collected by cardiac puncture. Serum was separated by centrifugation and used for the quantification of albumin, creatinine, alanine, aspartate aminotransaminase. The quantifications were made using the dry chemistry methodology (Ortho Clinical—Johnson & Johnson) for biochemical parameters.

Assessment of Sepsis Severity

At 24 h after CLP, mice were scored for severity of sepsis. In this assessment, higher scores reflect increased severity. Mice were scored based on the following variables: piloerection, curved trunk, alterations in gait, seizures, lethargy, respiratory rate, lacrimation, grip strength, feces alterations, body tone, and body temperature alterations [adapted from Ref. (35, 36)]. Each animal received a total score between 1 and 11 and was ranked as: 1–3 (mild sepsis); 4–7 (moderate sepsis); and 8–11 (severe sepsis). In our experimental conditions, most animals were ranked as moderate sepsis.

Peritoneal Lavage

Briefly, mice were submitted to euthanasia 24 h after surgery using isoflurane (Cristália). The peritoneal cavity was washed with 3 mL of cold sterile saline in the laminar flow cabinet. The peritoneal washes were plated in Difco tryptic soy agar (TSA) (BD) for further analysis of bacterial growth through the count of CFU.

The peritoneal washes were also used for total cell count. Red blood cells were lysed using Turk solution (2% acetic acid) and total cell count was carried out using Neubauer chamber (Neubauer Improved). Differential leukocyte count was performed in cytocentrifuged smears stained with panotic (Laborclin). The supernatant was collected by centrifugation and stored at −20°C for further cytokine quantification.

Cytokine and LTB_4 Measurement

Tumor necrosis factor-α, IL-10, and IL-1β from the supernatant of peritoneal fluid or plasma were measured by enzyme-linked immunoabsorbant assay (ELISA, Duo set kit—R&D systems, Minneapolis, MN, USA) according to the manufacturer's instruction. LTB_4 was measured by enzyme immunoassay (EIA, Ann Arbor, MI, USA) according to the manufacturer's instruction.

CFU Counts

The number of CFU was determined in peritoneal lavage fluid, blood, and other organs that were diluted 1:10,000 and 1:1,000 and incubated under aerobic and sterile conditions on Difco TSA for 24 h at 37°C. The number of bacterial colonies were counted and expressed as CFU/mL.

Histology

Histological analysis of omentum was performed as previously described (37, 38). Briefly, omentums were collected, fixed in 5% buffered formaldehyde and paraffin-embedded. Tissue sections (4 µm thick) were stained with hematoxyline and eosin for histomorphological analysis.

Kidney, Small Intestine, and Liver Tissue Damage

The left kidney and the distal part of the right lobe of the liver were also removed after euthanasia. The tissues were fixed in 5% buffered formaldehyde, paraffin-embedded, and sections (4-µm thick) obtained. Liver sections were stained with hematoxylin–eosin, whereas kidney tissue was stained with periodic acid–Schiff reagent to visualize the basement membrane. 10 to 15 fields per section from random tubular regions of the renal cortex and liver parenchyma were captured at a magnification of 400×. Renal tubular damage was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, and desquamation. A five-point, semi-quantitative, severity-based scoring system was used to assess each lesion parameter, graded as: 0 = normal tissue; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% of examined tissue.

In liver tissue, 10 fields per liver zone (central, lobular, and portal) were captured at a magnification of 400×. The ratio between sinusoidal cells and total cells was computed and expressed as percentage.

Image-Pro Plus 6.3 for Windows (Media Cybernetics, Silver Spring, MD, USA) was used for all analyses.

DNA Measurement

Extracellular DNA was measured as an indicative of neutrophil extracellular trap (NET) formation. The DNA was quantified in the free cell peritoneal lavage fluid by using the Picogreen dsDNA kit (Invitrogen) according to the manufacturer's instructions.

DNAse Treatment

In some experiments, we also treated CLP or CLP+ dasatinib animals with DNase (5 mg/kg dissolved in saline solution enriched

with 2 mM $CaCl_2$, i.p., 1 h after CLP). The CFU was analyzed in peritoneal lavage at an earlier time point (3 h after CLP).

Plasma Non-Esterified Fatty Acid (NEFA) Quantification

Plasma concentrations of the predominant NEFA—palmitic, oleic, linoleic, palmitoleic, and stearic acids—were assessed by a colorimetric assay (Zen-Bio, Inc.) (39).

Phagocytosis Assay

Whole blood was collected from mice receiving dasatinib or vehicle. The assay was performed using the pHrodo *E. coli* bioparticles phagocytosis kit for flow cytometry (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

Data are represented as mean ± SEM and statistically analyzed by analysis of variance (one-way ANOVA) followed by Tukey and Student's *t*-test. Survival curves and comparisons between curves were assessed using the Mantel–Cox log-rank test. $^*P$ values < 0.05 and $^{***}P$ values < 0.001 were considered significant.

RESULTS

Dasatinib Treatment Increased Rolling Velocity and Severely Impaired Neutrophil Adhesion *In Vivo*

Dasatinib, a potent Src family kinase inhibitor is known to modulate immune responses (40). Therefore, we set out to evaluate the effect of dasatinib on leukocyte recruitment in an *in vivo* model of TNF-α (2 h) induced inflammation of the mouse cremaster muscle using *Lyz2*GFP mice. Dasatinib (2, 10, or 20 mg/kg) was given orally 3 h prior to the exteriorization of the cremaster muscle. Observation of leukocyte rolling in cremaster muscle venules revealed a significant increase in the number of rolling leukocytes in the presence of dasatinib (**Figure 2A**). Because dasatinib is a broad-spectrum tyrosine kinase inhibitor, we additionally performed analysis of leukocyte recruitment in $hck^{-/-}$ $fgr^{-/-}$ $lyn^{-/-}$ (SFK-ko) animals. In this model, all neutrophil-specific SFKs are deleted and, therefore, display a positive control for dasatinib specificity. We obtained comparable numbers of rolling cells/min in SFK-ko animals after TNF-α stimulation to 10 and 20 mg/kg dasatinib administration (Figure S1A in Supplementary Material). Because absolute numbers of rolling cells are influenced by changes in WBC count, we determined systemic leukocyte counts for each experiment and detected a dose-dependent increase in WBC counts following dasatinib application (Figure S1B in Supplementary Material). SFK-ko animals also showed an increase in the WBC count, following TNF-α stimulation, indicating that this is an SFK-dependent mechanism. We then assessed leukocyte rolling flux fraction, which is defined by the number of rolling leukocytes/min divided by the WBC count. Interestingly, this normalization reduced the observed increase in rolling (**Figure 2B**), indicating that dasatinib did not alter the relative number of rolling cells, but increased total circulating leukocytes. Next, we analyzed leukocyte rolling velocities and found a significant and dose-dependent increase in rolling velocity in the presence of dasatinib and in the SFK-ko mice (2 mg/kg 10.1 μm/s and 20 mg/kg 15.0 μm/s vs. 6.9 μm/s in WT control mice; **Figure 2C**; Figure S1C in Supplementary Material). This suggests that dasatinib inhibits Src kinase dependent intermediate activation of beta2 integrins, a process known to modulate rolling velocities in inflamed tissues (41). Interestingly, absolute number of adherent leukocytes in TNF-α (2 h) stimulated cremaster muscle venules of dasatinib-pretreated mice showed only minor changes (**Figure 2D**). However, after normalizing the number of adherent cells to changes in WBC revealed a severe and dose-dependent leukocyte adhesion defect (**Figure 2E**). This decrease was also visible in SFK-ko animals (Figure S1D in Supplementary Material). To exclude that these effects were due to changes in surface expression of rolling- and adhesion-relevant molecules, we performed FACS analysis of leukocyte surface molecules (Figure S1E in Supplementary Material). No major differences in surface expression could be detected for CD18, CD11a, CD11b, CD62L, PSGL1, CXCR2, and CD44.

Overall, these findings demonstrate that SFK inhibition by dasatinib significantly increases leukocyte rolling velocity and reduces leukocyte adhesion in inflamed postcapillary venules *in vivo*.

Dasatinib Treatment Strongly Reduced Leukocyte Extravasation

To extravasate into inflamed tissue, leukocytes need to crawl along the endothelial wall to find an appropriate spot for extravasation. We performed time-lapse fluorescence video microscopy in TNF-α (2 h) stimulated cremaster muscle venules and tracked GFP-fluorescent crawling leukocytes in control or dasatinib (10 mg/kg)-treated Lyz2GFP mice. In contrast to previous observations of neutrophil 2D migration in Zigmond chambers (42), dasatinib had almost no detectable effect on 2D neutrophil crawling *in vivo* (**Figure 3A**). No significant differences in crawling velocity or Euclidean distance were observed (**Figure 3B**), indicating that those cells which adhere in the presence of dasatinib display no further migration defect.

The crossing of the vascular wall is the last step in the leukocyte adhesion cascade. To quantify extravasation, we performed Giemsa staining of fixed cremaster muscle tissues of control and dasatinib (10 mg/kg) treated mice after TNF-α stimulation and counted perivascular leukocytes (**Figure 3C**). Quantification of extravasated cells revealed a significant inhibition of leukocyte extravasation by dasatinib compared to control animals (394 vs. 632 cells/mm^2, **Figure 3D**). Likewise in SFK-ko mice, the number of extravasated cells was decreased to a similar degree (430 cells/mm^2). This further strengthens our hypothesis of dasatinib acting mostly on SFKs during leukocyte recruitment. A more detailed analysis of leukocyte subtypes crossing the vessel wall showed that dasatinib mainly inhibited neutrophil extravasation (**Figure 3E**). In contrast to intraluminal crawling, leukocyte migration in the interstitium occurs in a 3D environment and is integrin-independent and eventually also SFK independent. We, therefore, performed *in vitro* 3D migration experiments to investigate the effects of dasatinib on integrin-independent migration. Indeed, analyzing migration of isolated leukocytes

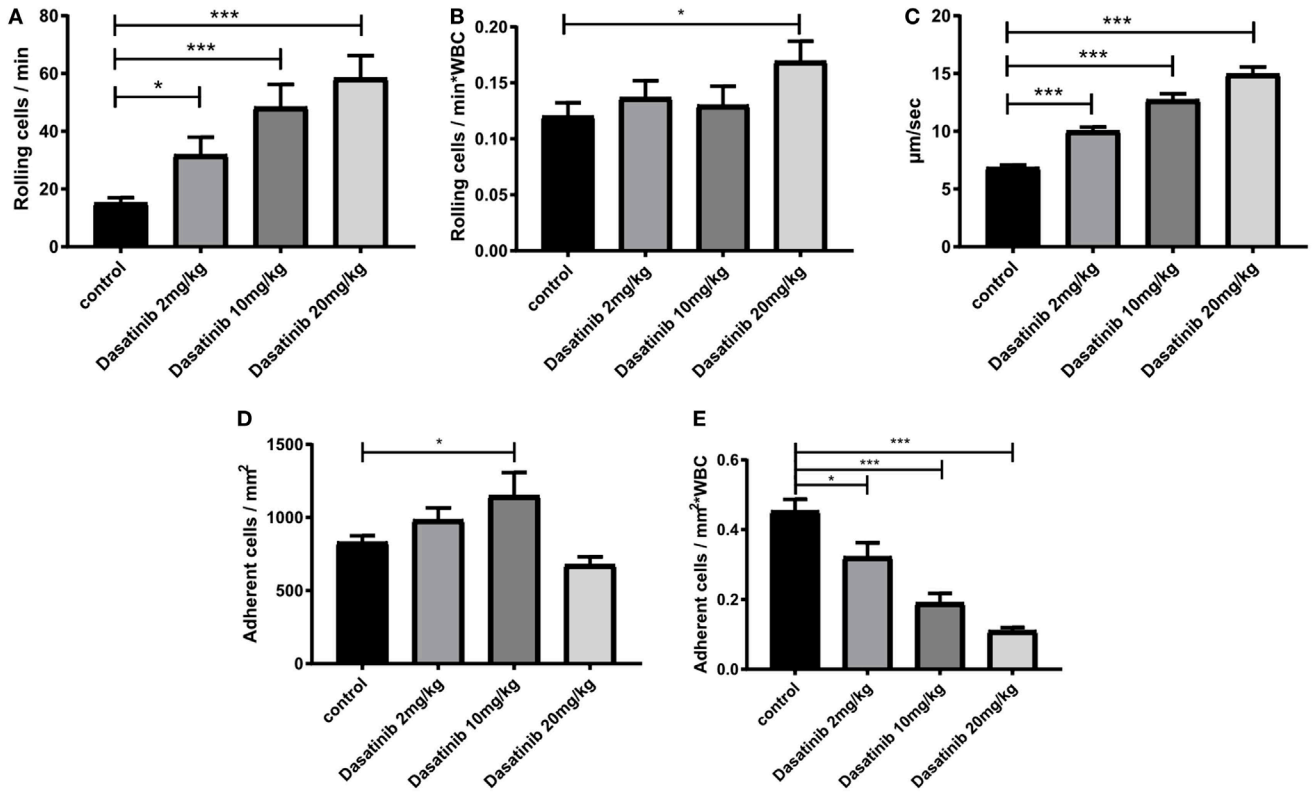

FIGURE 2 | Dasatinib treatment increased rolling velocity and severely impaired neutrophil adhesion *in vivo*. *In vivo* leukocyte rolling was analyzed in rmTNF-α-stimulated venules of mouse cremaster muscles of Lyz^{2}GFP mice pretreated orally with 2, 10, and 20 mg/kg dasatinib in methylcellulose, or with methylcellulose alone (control). Data are presented as mean ± SEM. **(A)** Number of rolling cells/min are shown. **(B)** Rolling flux fraction was calculated by the number of rolling cells/min normalized to the total WBC count. **(C)** Rolling velocities of neutrophils are displayed in micrometer per second. **(D)** Adherent cells were assessed over 1 min of observation. **(E)** Adhesion efficiency was calculated by the number of adherent cells/mm^2 normalized to the total WBC count (*$P < 0.05$ and ***$P < 0.001$).

in a 3D collagen gel matrix revealed no alteration of leukocyte migration behavior in the presence of dasatinib (**Figure 3F**; Figure S1F in Supplementary Material). The Euclidean distance as well as their migration velocity was unchanged. This indicates that SFKs, similar to leukocyte integrins (43), are dispensable for interstitial migration once leukocytes managed to overcome the vascular barrier.

Dasatinib Dose-Dependent Effect on Survival and Severity of Sepsis After CLP

Our *in vitro* and *in vivo* findings described above suggest a potential role of dasatinib treatment on the outcome of sepsis. We, therefore, tested dasatinib administration (1 and 10 mg/kg) in the CLP model of induced sepsis. Our first step was to evaluate the bioavailability of dasatinib by measuring its concentration in the plasma of septic animals. For that, we performed pharmacokinetics analyses and compared the plasma concentration of 1 mg/kg dasatinib after administration to sham or CLP animals (**Figure 4A**). Septic animals had lower peak values of the drug than treated sham animals but their plasma levels remained at pharmacological levels up to 24 h after administration. Of note, plasma concentrations in both animals remained markedly above the concentration (14.9 ng/mL) able to inhibit 90% of phosphorylation of pBCR-ABL protein (34). We next induced polymicrobial sepsis using the CLP model to test for survival and clinical scores in septic mice. Sham-treated animal showed 100% survival rate after 7 days. Following CLP, we observed a survival rate of 50% after 7 days in control animals, with the highest mortality observed between day 1 and 2 after CLP. In contrast, administration of dasatinib at 1 mg/kg protected the animals from lethal sepsis following CLP (**Figure 4B**). Dasatinib at 1 mg/kg administered 30 min before and 6 and 24 h after CLP resulted in an 80% survival rate 7 days after CLP. Interestingly, a higher dose (10 mg/kg) of dasatinib had an opposite effect, with a mortality rate increasing to 85%.

The beneficial effect of low dasatinib doses was also detected in the severity score. As shown in **Figure 4C**, 1 mg/kg dasatinib improved severity scores compared to untreated CLP animals and resulted in only moderate sepsis scores. Again 10 mg/kg dasatinib reversed this effect. Sham-treated animals did not present any sign of disease.

Low Dosage of Dasatinib Decreased Organ Dysfunction in Septic Animals

To evaluate the protective effect of dasatinib treatment in septic animals in more detail, we analyzed the impact of dasatinib

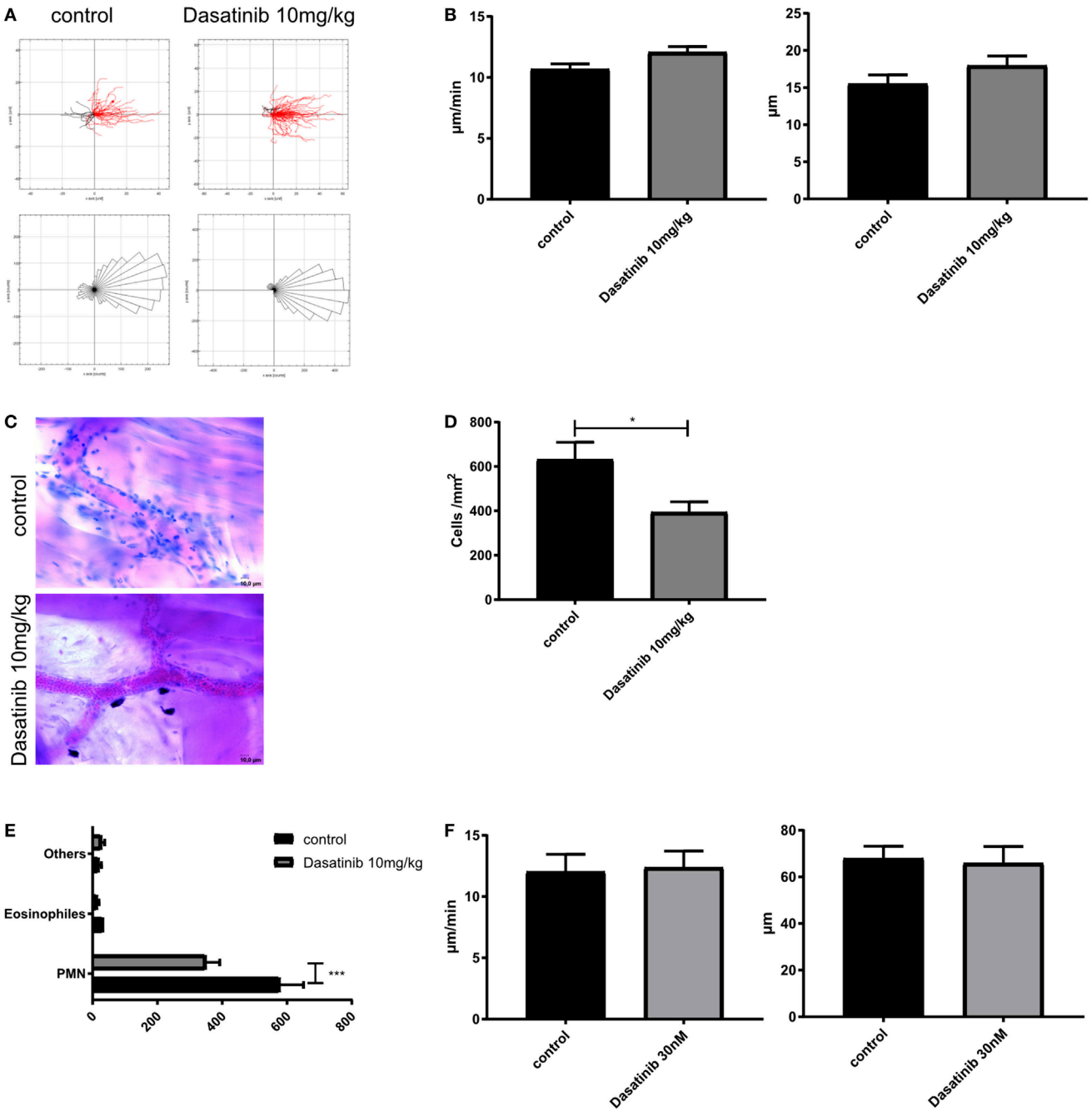

FIGURE 3 | Dasatinib treatment strongly reduces leukocyte extravasation. *In vivo* crawling experiments were analyzed in rmTNF-α-stimulated Cremaster muscle tissue of Lyz^{2}GFP mice, pretreated orally with 10 mg/kg dasatinib in Methylcellulose, or with Methylcellulose alone (control). Time-lapse movies over 15 min were performed and leukocytes visualized by their GFP signal. Extravasated leukocytes were analyzed with Giemsa staining in rmTNF-α-stimulated fixed cremaster muscle tissue of Lyz^{2}GFP mice, treated orally with 10 mg/kg dasatinib in methylcellulose, or with methylcellulose alone. Data are presented as mean ± SEM. **(A)** Representative single cell migration tracks and rose plots for intraluminal crawling are displayed. Red lines indicate migration in, black lines migration against flow direction. At least 80 cells were analyzed for each strain **(B)** Evaluation of crawling velocity and Euclidian distance of crawling cells **(C)** Representative images of cremaster muscle whole mounts after Giemsa staining. Scale bar: 10 μm **(D)** Total number of extravasated cells/mm^2 in muscle tissue in close proximity to a vessel. Star indicates significance over control. **(E)** Differential total cell counts of polymorphonuclear cells, eosinophils, and other cells. **(F)** Evaluation of migration velocity and Euclidian distance of crawling PMNs with or without dasatinib (*$P < 0.05$). Statistical analysis: Student's *t*-test.

treatment in sepsis-induced organ dysfunction. We measured plasma biological markers for kidney (creatinine) and liver (aspartate and alanine aminotransferase), and lipotoxicity (NEFA). All CLP animals displayed significantly increased levels of creatinine, aspartate aminotransferase, NEFA, indicating severe organ damage caused by CLP induced sepsis (**Figures 5A–D**).

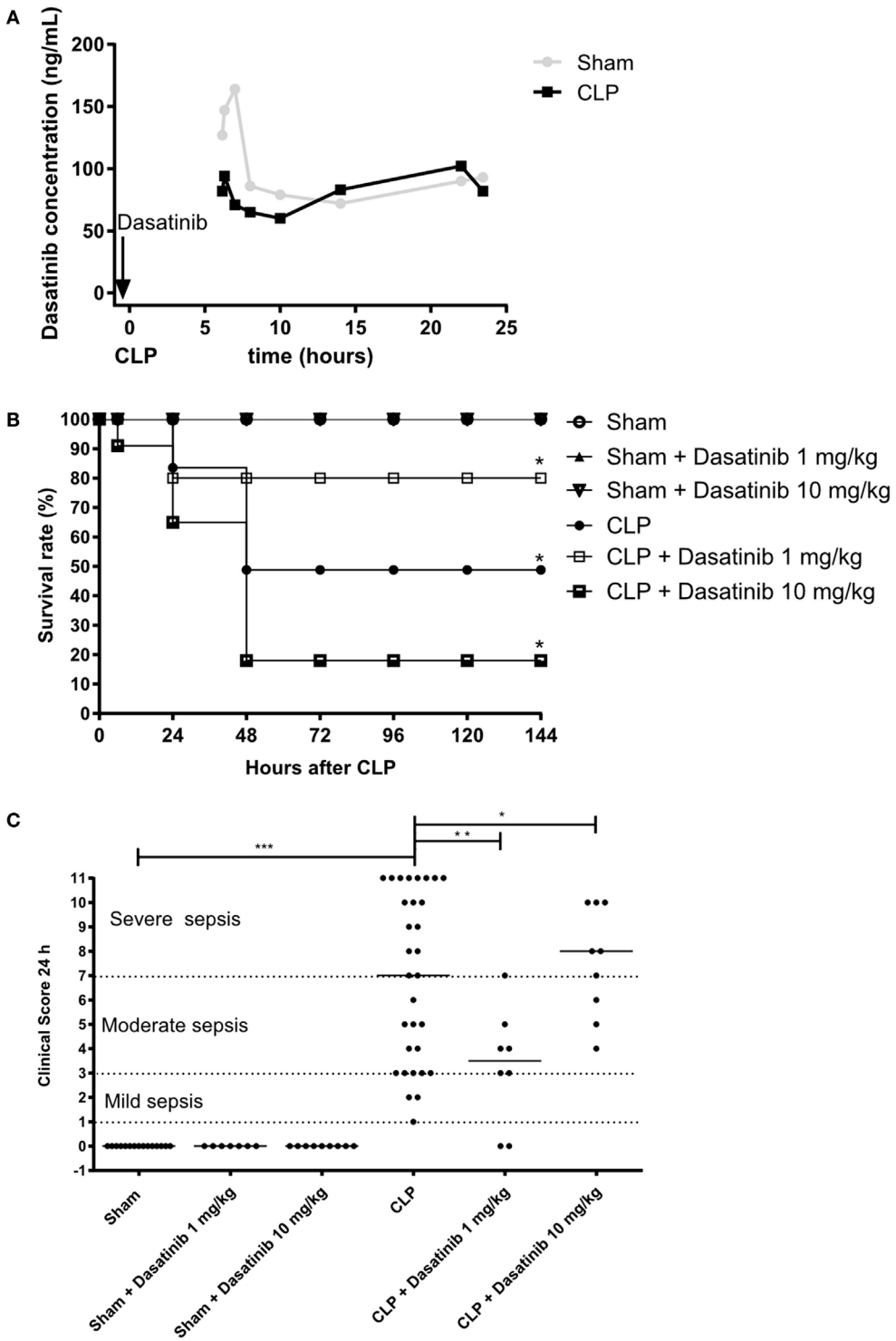

FIGURE 4 | Dose-dependent effect of dasatinib on survival and severity of sepsis after cecal ligation and puncture (CLP). *Swiss* mice were submitted to CLP. Sham-treated animals were used as control. Data are presented as mean ± SEM. **(A)** Dasatinib was given orally 30 min before and 6 and 24 h after CLP. Dasatinib concentration of blood samples taken at indicated timepoints is displayed. **(B)** The survival rate was quantified for 7 days (144 h) in sham mice, untreated animals or each treated with dasatinib at 1 or 10 mg/kg dosage. **(C)** Clinical score was assessed 24 h after CLP. Each dot represents one animal. 1–3 points in the clinical score corresponds to a mild sepsis, 4–7 points corresponds to a moderate sepsis, and 8–11 points corresponds to severe sepsis. The animals were treated with dasatinib 1 or 10 mg/kg orally 30 min before, 6 and 24 h after CLP procedure. At least two independent experiments were performed. Statistical analysis: one-way ANOVA followed by Tukey $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, for **Figure 3B** and Mantel–Cox log-rank test $^{*}P$ values < 0.05, for **Figure 3C**. The number of animals per group range from 3 to 4 for pharmacokinetics, 7 to 31 for clinical score, and 10 to 11 per group from mortality.

Dasatinib treatment at 1 mg/kg lowered these levels in CLP mice indicating that dasatinib can partly rescue sepsis-induced organ damage. Reduced plasma levels of albumin and glucose after CLP could not be rescued by dasatinib (Figures S2A,B in Supplementary Material).

In agreement with systemic biochemical markers of organ dysfunction, histological alterations were detected in the liver (first column), the small intestine (second column), and the kidney (third column) of septic mice, compared to control group (**Figure 5E**). CLP induced liver and kidney steatosis and edema in small intestine villi. However, treatment with 1 mg/kg dasatinib prevented these alterations. Histological changes were scored from 0 (without alterations) to 4 (more extensive lesions) and we could detect an overall decrease in the severity of the organ damage in CLP mice treated with dasatinib to the score levels of CLP mice (**Figure 5F**). In the omentum, we found some neutrophil infiltration due to the inflammatory reaction induced by surgery in sham animals. Dasatinib treatment did not affect omentum morphology or leukocyte infiltration in sham animals (Figure S2C in Supplementary Material). In septic mice, the omentum seems to be liquefied due to the intensity of the acute inflammatory response taking place in the peritoneal cavity. In dasatinib-treated animals, the omentum histology was similar to the sham conditions, reinforcing the protective role of dasatinib in CLP induced sepsis.

Dasatinib Treatment Impaired the Number of Leukocytes in the Peritoneal Cavity and Decreased Concentration of Inflammatory Mediators

We next investigated the effect of dasatinib treatment in leukocyte accumulation and inflammatory mediators in more detail. For this purpose, we analyzed cell accumulation in the peritoneal cavity 24 h after CLP (**Figures 6A–C**). As expected, septic animals had higher numbers of mononuclear cells and neutrophils in the inflamed peritoneal cavity as compared to sham-treated animals (**Figures 6A–C**). Treatment with dasatinib (1 mg/kg) significantly lowered both mononuclear cell and neutrophil accumulation in the peritoneal cavity (**Figures 6B,C**). This finding is in accordance to our previous data of reduced leukocyte extravasation in inflamed cremaster muscle tissue after dasatinib treatment.

Additionally, we analyzed the effect of dasatinib (1 mg/kg) on cytokine, and chemokine production. We measured plasma levels of TNF-α, IL-6, and IL-10 in dasatinib-treated animals 24 h after CLP and compared them to sham-treated mice. Septic animals presented elevated levels of all measured cytokines (**Figures 6D–F**). Animals treated with dasatinib at 1 mg/kg dose presented significantly lower levels of all cytokines indicating a reduced extend of inflammation. We also measured the levels of cytokines, chemokines, and lipid mediators in the peritoneal lavage of septic mice. The levels of TNF-α and IL-6 were increased in septic mice and treatment with dasatinib decreased their levels (**Figures 6G,H**). Dasatinib administration did not affect IL-1β levels (**Figure 6I**). Septic mice also displayed increased peritoneal levels of LTB_4 and CXCL1/KC. Likewise 1 mg/kg dasatinib decreased LTB_4 and CXCL1/KC levels (**Figures 6J,K**), while MCP1 levels remained at the same levels as in the CLP group (**Figure 6L**).

Dasatinib Inhibited Bacterial Growth and Bacterial Spreading in Septic Mice

About 60–70% of patients with sepsis have positive blood cultures, most of them are Gram-negative bacteria (5, 44). In our model, we have a mixed infection with both Gram-negative and Gram-positive bacteria, detected in the peritoneal fluid from septic animals. Interestingly, 1 mg/kg dasatinib significantly reduced CFU counts in the peritoneal fluid (**Figure 7A**). At 10 mg/kg, however, dasatinib-treated CLP animals showed higher CFU numbers compared to CLP (Figure S3A in Supplementary Material). We also evaluated CFU formation in distal organs to assess bacterial translocation and the ability of the organism to fight infection. We could detect high numbers of CFUs in all analyzed organs of CLP animals. Again 1 mg/kg dasatinib successfully prevented bacterial translocation to the blood (**Figure 7B**), lung, spleen, kidney, and liver (Figures S3B–E in Supplementary Material).

Dasatinib Treatment Enhanced Neutrophil Functionality

Neutrophils phagocytose microbes, produce ROS, release antimicrobial factors and form NET as part of their arsenal to fight invading organisms (45). In order to explore why treatment with dasatinib was able to decrease CFU numbers despite reducing the numbers of neutrophils at the site of infection, we investigated the effect of dasatinib on the ability of neutrophils to kill bacteria. To evaluate the effect of dasatinib on NET formation, we measured extracellular double-strand DNA *via* fluorimetry in septic animals with and without dasatinib. As shown in Figure S3F in Supplementary Material, the CLP group showed an increase in extracellular DNA content compared to sham animals. Interestingly, treatment with dasatinib did neither alter extracellular DNA levels nor did the disassembling of NETs by DNAse interfere with the ability of dasatinib to decrease CFU numbers (Figure S3G in Supplementary Material).

Next, we checked the production of nitrite as readout for NO production in the peritoneal cavity after treatment with 1 mg/kg dasatinib. NO and superoxide generate antimicrobial molecules called reactive nitrogen species that act together with ROS in damaging cells and microbes (46, 47). Our results show that dasatinib increased local nitrite production during CLP, which contributes to bacterial clearance (**Figure 7C**). Next we examined the effect of dasatinib treatment on neutrophils phagocytosis. Mice were treated with 1 mg/kg dasatinib and neutrophils phagocytosis was determined in whole blood by flow cytometry. Interestingly, compared to untreated animals, animals treated with dasatinib (1 mg/kg) showed increased neutrophil phagocytosis (**Figure 7D**), suggesting a potential enhancing effect on the ability of neutrophil to clear bacteria. Also here we encountered a dose-dependent effect, because treatment with 10 mg/kg dasatinib decreased the ability of neutrophils to phagocytose.

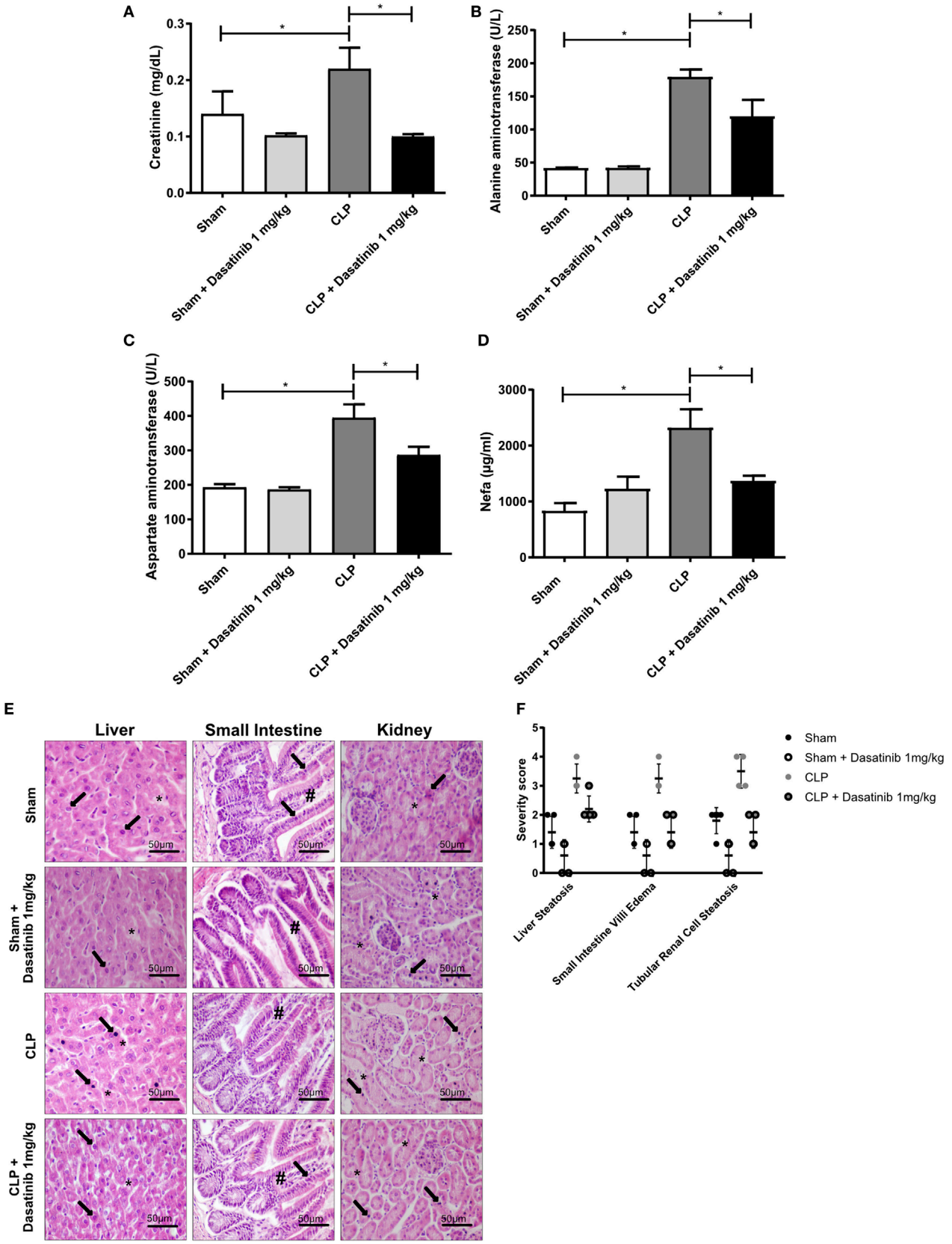

FIGURE 5 | Continued

FIGURE 5 | Low dosage of dasatinib decreases organ dysfunction in septic animals. Swiss mice were submitted cecal ligation and puncture (CLP). Sham-treated animals were used as control. The animals were treated with dasatinib at 1 mg/kg 30 min before and 6 h after CLP. Blood was collected 24 h after CLP procedure, and organs were harvested for HE staining. **(A)** Creatinine, **(B)** alanine, **(C)** aspartate aminotransferase, and **(D)** non-esterified fatty acid (NEFA) were analyzed. Data are presented as mean ± SEM. **(E)** Optical microscopy of liver, small intestine, and kidney. In the CLP group, liver hepatocytes around of centrilobular vein and tubular renal cells exhibit apoptosis (arrows) and diffuse vacuolization in the cytoplasm by accumulation of fat (asterisks), thus characterizing liver and kidney steatosis; the small intestine show apoptosis of enterocytes (arrows) and prominent edema of the villi (#). After dasatinib treatment, the integrity of liver hepatocytes, small intestine villi, and tubular renal cells are restored with reduction in apoptosis and steatosis score similar to Ssam and Sham animals treated with dasatinib. Scale bar is 50 μm. **(F)** Injury score with severity analyses of microscopically visible organ damage. The score ranges from 0 to 4 where 0 means no injury and 4 maximum injuries. Statistical analysis: one-way ANOVA followed by Tukey $^*P < 0.05$. The number of animals per group range from 4 to 10.

DISCUSSION

Sepsis is one of the leading causes of morbidity and mortality in Intensive Care Units, and is associated with increased health-care costs (48, 49). This is complicated by the rise of drug-resistant microorganisms, a growing elderly population, and an increased incidence of immunosuppression (50–54). The failures of anti-toll-like receptor 4 antibody, recombinant activated protein C, and anti-TNF-α therapies in clinical trials require a rethinking of sepsis' pathophysiology and therapeutic strategies (8, 55–60). Systematic approaches, such as presented here, could fuel the discovery of promising immunosuppressive or anti-inflammatory drugs that aim at multifunctional targets such as Src family kinases.

Src family kinases play critical roles in a whole variety of pathologies including cancer; in addition, it was shown that Src is involved in inflammation-related signaling pathways (61). Dasatinib is a type I ATP-competitive protein kinase inhibitor (62). SFK-inhibitors affect signaling pathways and downmodulate the inflammatory response. Nevertheless, the current dose of dasatinib (100 mg daily in human) used to treat some leukemia does not induce severe immunosuppression (63). In the present work, we used lower doses of dasatinib (1 and 10 mg per kg). We chose these doses because initial pharmacokinetic experiments showed that dasatinib at 1 mg per kg yields plasma concentrations that remained above the critical concentration to inhibit SFKs.

It is currently accepted that it is not the insult *per se*, but the host's response, that determines severity and outcome in sepsis (4). Therefore, the immunosuppressive action of dasatinib may affect the response against infectious agents and it is expected that high-dose favors the progression of infection with deleterious effects to the host as shown for *Pneumocystis jiroveci* pneumonia (63). On the contrary, lower doses may modulate the immune response affecting and/or preventing tissue damage resulting from host immune response or cellular hyperactivation. In fact, we show here that dasatinib at 10 mg/kg is deleterious to the host fueling infection progression. In contrast, the lower dose of dasatinib (1 mg/kg) showed promising results improving the animal clinical condition and increasing survival. The higher dasatinib dose may inhibit other kinases and proteins impacting on ability of the host to fight the infection effectively because of its potent anti-inflammatory effect. Lower dose of dasatinib decreases the neutrophil migration but does not abrogate it. So the fewer neutrophils that reach the peritoneum remain effective on killing the bacteria and restrain the infection.

Septic patients present systemic inflammation with exacerbated cytokine production, and increased cell migration to the site of infection or sterile inflammation, as shown with CLP mice in this publication. Trafficking of myeloid leukocytes to the site of inflammation is linked to the generation of an appropriate inflammatory environment (20). In this regard, LTB_4 is a potent chemotactic agent for neutrophils (64). Its levels increase 24 h after CLP and treatment with dasatinib (1 mg/kg) reduced the levels of LTB4. Dasatinib also decreased the levels of CXCL1/KC, another potent chemo attractant to neutrophils. CXCL1/KC is released by resident macrophages and mediates neutrophil accumulation induced by LPS (65).

Src family tyrosine kinases are important components of the signaling pathways initiated by the TLRs (critical for cytokine production) and many cytokines, such as TNF, use Src family kinases in their own signaling pathways (17, 66). In our model, dasatinib reduced the levels of all measured cytokines confirming data from the literature (25, 67), except IL-1β. Impairing TLR4-related signaling pathway inhibits cytokine production including both TNF and IL-10 (68). IL-1β requires the cleavage of the pro-form (pro-IL-1β) by caspase-1 into its biologically active form (69). However, we cannot exclude that IL-1β detected here may reflect the release of already present IL-1β in the cell which would suggest that the release of IL-1β is independent of Src kinases (70) while the other measured cytokines are upregulated by SFK-dependent transcriptional activity, reinforcing the key role of SFK in cytokine production in infectious disease.

Activated neutrophils are able to generate reactive oxygen species, release NETs, increase phagocytosis, and produce nitric oxide (45). We further elucidated the role of tyrosine kinases in these processes and found that dasatinib did not affect NET formation. In contrast, nitric oxide generation was increased in septic mice treated with dasatinib strengthening its potent bactericidal activity at the local level (71, 72).

Phagocytosis is a prime mechanism of bacterial killing. Src-family kinase-deficient leukocytes are less effective than wild-type cells at mediating phagocytosis (73, 74). On the other hand macrophages lacking Src family members Hck, Fgr, and Lyn showed phagocytosis mediated by Fcγ Receptor (75), so phagocytosis obviously can happen independently of neutrophil-expressed SFKs. Interestingly, our experiments revealed that 1 mg/kg of dasatinib increased phagocytotic activity of blood neutrophils compared to control neutrophils, while higher doses (10 mg/kg dasatinib) decreased phagocytosis. The mechanism for this dose-dependent effect of dasatinib on phagocytotic activity in neutrophils is currently unclear and needs further investigations.

Patients with severe sepsis symptoms display metabolic dysfunction with elevated levels of plasma NEFA and lower levels

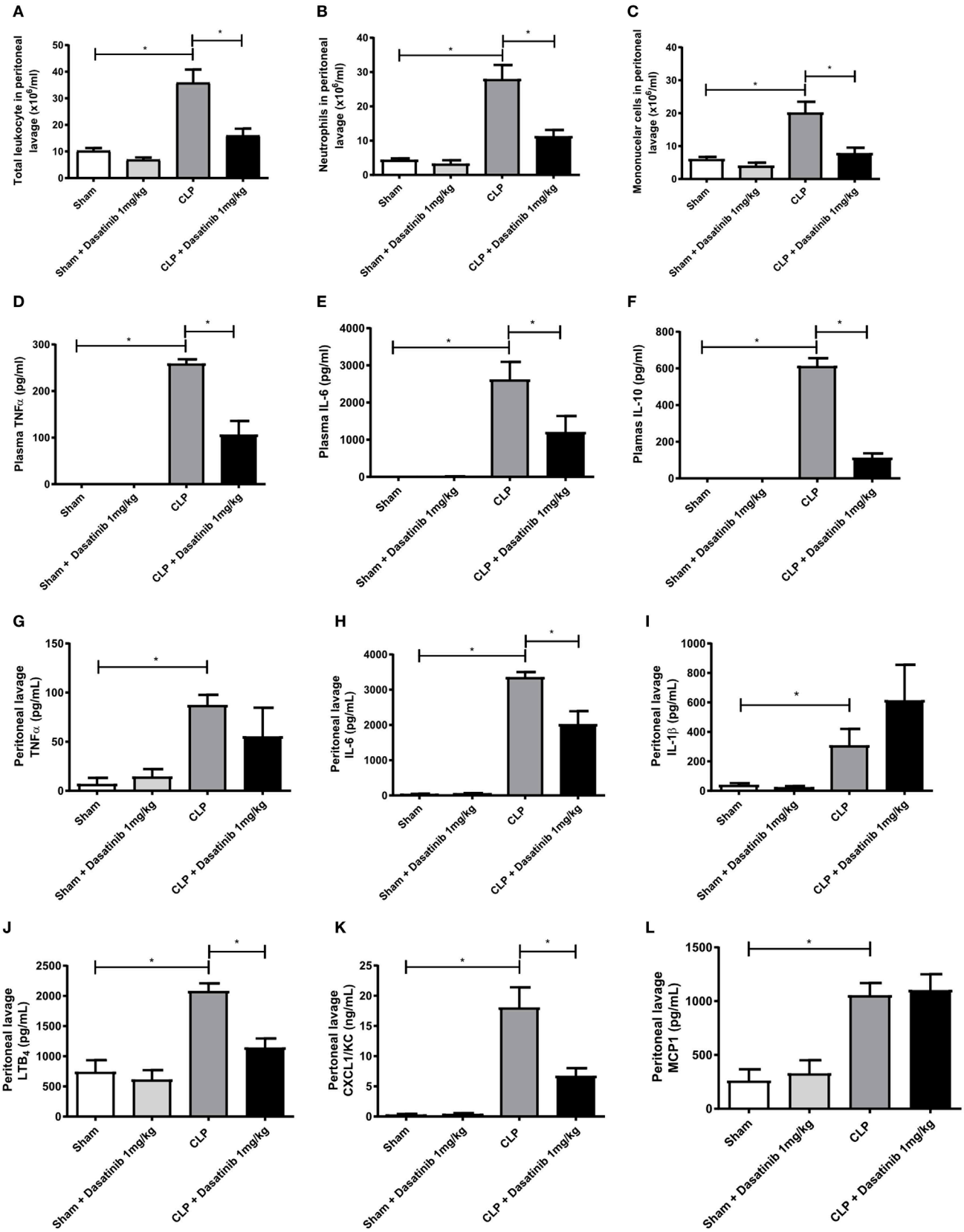

FIGURE 6 | Continued

FIGURE 6 | Dasatinib treatment results in fewer peritoneal leukocytes along with decreased amounts of inflammatory markers. Swiss mice were submitted cecal ligation and puncture (CLP). Sham-treated animals were used as control. The animals received dasatinib at 1 mg/kg 30 min before and 6 h after CLP. Cells were collected 24 h after CLP procedure by peritoneal lavage to assay total and differential counts. Data are presented as mean ± SEM. **(A)** Total leukocyte count, **(B)** mononuclear cell count, and **(C)** neutrophil cell count are shown. Cytokines were measured by ELISA and LTB4 by EIA. **(D–F)** Display plasma levels of tumor necrosis factor (TNF)-α **(D)**, interleukin (IL)-6 **(E)**, and IL-10 **(F)**, **(G–L)** display peritoneal lavage values of TNF-α **(G)**, IL-6 **(H)**, IL-1β **(I)**, LTB4 **(J)**, CXCL1 **(K)**, and MCP1 **(L)** Statistical analysis: one-way ANOVA followed by Tukey $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The number of animals per group range from 6 to 18.

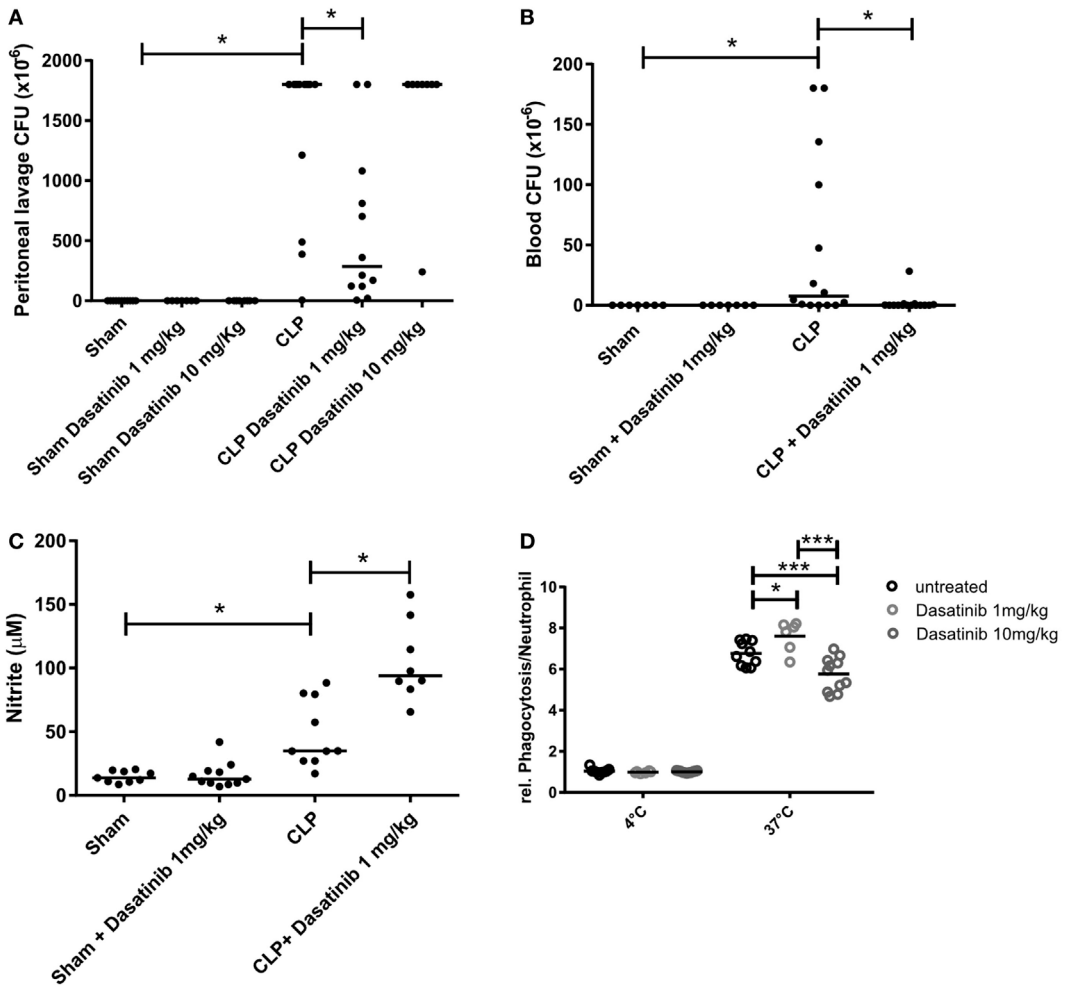

FIGURE 7 | Dasatinib treatment decreased colony-forming unit (CFU) counts and simultaneously enhances neutrophil function. *Swiss* mice were submitted cecal ligation and puncture (CLP). Sham-treated animals were used as control. The animals received dasatinib at 1 mg/kg 30 min before and 6 h after CLP. Data are presented as mean ± SEM. The CFU was determined 24 h after CLP in **(A)** the peritoneal lavage fluid and **(B)** blood. **(C)** Cell-free supernatant from peritoneal lavage of animals was collected 24 h after CLP, and nitrite levels was measured by Griess reaction. **(D)** Relative neutrophil phagocytosis was tested in blood neutrophils from dasatinib or vehicle treated cells using pHrodo *E coli* bioparticles. Statistical analysis: one-way ANOVA followed by Tukey $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The number of animals per group range from 6 to 15.

of albumin (76, 77). NEFA activates TLRs boosting cytokine production (78), induce cell death (79), and inhibit sodium potassium ATPase in several organs, including the lungs (80). In addition, the inhibition of lipogenesis or the increase in lipid oxidation reduces levels of free fatty acids, TNF-α, and IL-6, and reduces liver injury improving survival in sepsis (81, 82). Accordingly, the observed decrease in NEFA levels in the plasma of dasatinib-treated mice might at least in part be responsible for their improved organ function.

Regardless of their clinical potential in septic patients, our study demonstrates that the use of SFK inhibitors needs to be tightly controlled to keep the fine balance between overtreatment with uncontrolled bacterial growth in an immuno-compromised organism and ineffective treatment leading to a hyper-inflammatory response of the host immune system. Keeping this balance at an optimal level will certainly be a challenge and require intensive monitoring. In view of the fact that great efforts have been made to develop tyrosine kinases inhibitors for the therapy

of inflammatory diseases (83), their use might open new doors in modulating the inflammatory response during sepsis and, therefore, improve the outcome of patients suffering from this life-threatening syndrome.

ETHICS STATEMENT

Animal housing conditions and experimental procedures conformed to institutional regulations and were in accordance with the National Institute of Health guidelines on animal care. The Institutional Animal Welfare Committee approved all procedures described here under license number 002-08, LW36/10, and L15/2015. The animal experiments were approved by the Regierung von Oberbayern, Germany (AZ 55.2-1-54-2531-80-76/12). Both Institutions follow the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) originally published in 2010 (84).

AUTHOR CONTRIBUTIONS

Conceptualization: CG-d-A, IR, AS, PR, MS, and HC-F-N. Data curation: CG-d-A, IR, AS, AF, AK, CC, SK, TE, JS, GO, VC, GS, EC, RM, BW, MS, PR, and HC-F-N. Formal analysis: C-G-d-A, IR, AS, AF, AK, and CC. Funding acquisition: HC-F-N, PR, AS, CG-d-A, MS, BW, AM, and IM-P. Investigation: CG-d-A, IR, AS, AF, AK, CC, SK, TE, JS, GS, EC, and RM. Project administration: CG-d-A, IR, AS, CC, PR, BW, PR, MS, and HC-F-N. Supervision: AS, AM, IM-P, PR, MS, and HC-F-N. Validation: CG-d-A, IR, AS, CC, BW, PR, MS, and HC-F-N. Visualization: CA, IR, AS, CC, MS, and HN. Writing—original draft preparation: CG-d-A, IR, AS, MS, and HN. Writing—review and editing: CA, IR, AS, AF, AK, CC, SK, TE, JS, GO, VC, GS, EC, RM, AM, IM-P, BW, PR, MS, PR, and HC-F-N.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00901/full#supplementary-material.

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The Surface-Exposed Protein SntA Contributes to Complement Evasion in Zoonotic *Streptococcus suis*

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Streptococcus suis is an emerging zoonotic pathogen causing streptococcal toxic shock like syndrome (STSLS), meningitis, septicemia, and even sudden death in human and pigs. Serious septicemia indicates this bacterium can evade the host complement surveillance. In our previous study, a functionally unknown protein SntA of *S. suis* has been identified as a heme-binding protein, and contributes to virulence in pigs. SntA can interact with the host antioxidant protein AOP2 and consequently inhibit its antioxidant activity. In the present study, SntA is identified as a cell wall anchored protein that functions as an important player in *S. suis* complement evasion. The C3 deposition and membrane attack complex (MAC) formation on the surface of *sntA*-deleted mutant strain Δ*sntA* are demonstrated to be significantly higher than the parental strain SC-19 and the complementary strain CΔ*sntA*. The abilities of anti-phagocytosis, survival in blood, and *in vivo* colonization of Δ*sntA* are obviously reduced. SntA can interact with C1q and inhibit hemolytic activity *via* the classical pathway. Complement activation assays reveal that SntA can also directly activate classical and lectin pathways, resulting in complement consumption. These two complement evasion strategies may be crucial for the pathogenesis of this zoonotic pathogen. Concerning that SntA is a bifunctional 2′,3′-cyclic nucleotide 2′-phosphodiesterase/3′-nucleotidase in many species of Gram-positive bacteria, these complement evasion strategies may have common biological significance.

Keywords: ***Streptococcus suis*, surface protein SntA, C1q, complement evasion, pathogenesis**

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INTRODUCTION

Streptococcus suis are recognized as an important swine and human pathogen (1). Among the 33 serotypes, *S. suis* serotype 2 (SS2) is the most virulent and prevalent one, which is also an emerging zoonotic pathogen (2). Two large-scale outbreaks of severe human SS2 infection occur in 1998 and 2005 in China causing 229 infections and 52 deaths (3, 4). In 2005, the streptococcal toxic shock like syndrome (STSLS) is first reported to occur in the human. An early burst of inflammatory cytokines could result in the STSLS with death as quickly as 13 h after SS2 infection, and subsequently SS2 breaks through blood–brain barrier (BBB) to cause disease, particularly meningitis (1, 5). Bacterial pathogens evade host innate immune defenses and maintain a high dose in blood causing bacteremia and septicemia. During these processes, the host complement system is an important factor facilitating clearance of bacterial pathogens (6, 7).

The complement system consists of more than 50 plasma and cell surface proteins. As a first line of defense against pathogenic intruders and a mediator between the innate and adaptive immune

response, it plays an essential and efficient role in rapid recognition and elimination for invading pathogens. The complement has three independent but interactive activation pathways: the classical pathway, alternative pathway, and lectin pathway (8). These three different complement pathways are stimulated by different foreign substance through specific recognition molecules (4). All the complement cascades result in the deposition of C3b to amplify the cascades, and mediate phagocytosis and adaptive immune responses by binding to complement receptors; the release of pro-inflammatory anaphylatoxins and chemoattractant C5a and C3a; and formation of membrane attack complex (MAC; C5b-9) then lead to direct lysis of Gram-negative bacteria (9).

Although host complement can rapidly recognized and eliminated foreign microorganisms, it also offers many interference sites that can disrupt this balanced network of protein interactions by complement-binding proteins leading to failure of elimination by host. Complement-binding proteins can be identified from both host and pathogens. These complement evasion mechanisms include (I) recruiting or mimicking of complement regulators; (II) modulating or inhibiting complement by direct interactions; and (III) enzymatic degradation by complement components (4).

C1q is the recognition subunit of C1 complex to trigger the classical complement pathway, following the recognition of IgG or IgM-bearing immune complexes (10). Proteins that interact with C1q have been identified widely in Gram-negative bacteria, such as *Salmonella Minnesota* (11), *Escherichia coli* (12–14), *Klebsiellapneumoniae* (15), *Legionella pneumophila* (16), *Bacillus anthracis* (17), *Moraxella catarrhalis* (12), nontypeable *Haemophilus influenzae* (12), but in Gram-positive bacteria the research is not much, except for Group B *Streptococci* (18, 19), *Streptococcus pyogenes* (20–22) and *Staphylococcus aureus* (23).

In our previous study, surface protein SntA of *S. suis* without any unknown function has been characterized to be a heme-binding protein which involved in the pathogenesis of *S. suis* in pigs. SntA can interact with the host antioxidant protein AOP2 and consequently inhibit its antioxidant activity (24). Complement C1q is identified as another interacting partner of SntA when we screen the SntA binding proteins in the host. In the present study, we demonstrate that SntA is an important player in complement evasion of this important zoonotic pathogen.

MATERIALS AND METHODS

Strains and Culture Conditions

Streptococcus suis serotype 2 strain SC-19 (GenBank accession number: NZ_CP020863.1) used in this study was isolated from a sick pig during the epidemic outbreak in Sichuan province of China in 2005. The *S. suis* strains were grown in tryptic soy broth (TSB; Difco, France) or on tryptic soy agar (TSA; Difco) plates supplemented with 5% newborn bovine serum (Sijiqing, Hangzhou, China) at 37°C. The *E. coli* DH5α and BL21 (DE3) strains were grown in LB broth or on LB agar plates at 37°C. The bacterial strains, plasmids, and primers used in this study are listed in **Table 1**.

TABLE 1 | Bacterial strains, plasmids, and primers used in this study.

Strains, plasmids, and primers	Description and sequence (5′ → 3′)	Source, reference, and products
***Escherichia coli* strains**		
DH5α	Host for cloning vector	Kangwei
BL21 (DE3)	Host for expression vector	Kangwei
***S. suis* strains**		
SC-19	*S. suis* serotype 2, the wild type	(25)
Δ*sntA*	The *sntA*-deleted strain; Em^r	(24)
CΔ*sntA*	Complemented strain of Δ*sntA*; Em^r, Spc^r	This study
Plasmids		
pET28a	Expression vector to produce His-fused proteins; lacZ, $Kana^r$	Novagen
pET28a-SUMO	Expression vector to produce His-and SUMO-fused proteins; lacZ, $Kana^r$	(26)
pQE32	Expression vector to produce His-fused proteins; lacZ, Amp^r	Qiagen
pSET2	*E. coli-S. suis* shuttle vector; Spc^r	(27)
pSET2:*sntA*	pSET2 containing the intact *sntA* gene and its upstream promoter; Spc^r Em^r	This study
Primers		
C1qA_F	GCGTCGACGCTGGCCGTATCCCTG	ORF of *c1qA* gene
C1qA_R	CCAAGCTTTCAGGTGGAGGGGAAG	
C1qB_F	AGAGAACAGATTGGTGGATCCCAGGTCTTCTGCATGCCTGGC	ORF of *c1qB* gene
C1qB_R	CTCGAGTGCGGCCGCAAGCTTTCATGCCTCTGCATCTGGGAAG	
C1qC_F	AGAGAACAGATTGGTGGATCCCAGGACAACTTAAGATGCCATGGG	ORF of *c1qC* gene
C1qC_R	CTCGAGTGCGGCCGCAAGCTTCTAGTTAGGGAAGAGCAAGAAGCCG	
CΔ*sntA*_F	AAAACTGCAGCTCGTTTTTCAAACGAACGTTCAG	*sntA* gene and its upstream promoter
CΔ*sntA*_R	GCGGGATCCTTAATGTTCTTTTTTCTTAGTCCATG	
10065_F	ATGAAAGAAATTAACTACACTGGGGA	ORF of B9H01_10065 gene
10065_R	TTATCCGTTGGTCCTTTTCACTGA	
SntA_F	ATGAATTTTCGTTTCAGTAAGTGTGC	ORF of *sntA* gene
SntA_R	TTAATGTTCTTTTTTCTTAGTCCATGC	
10075_F	ATGAAAAGAATAGGATATGTTTTCATAT	ORF of B9H01_10075gene
10075_R	CTACCAAATATAGGTTCTAATAAAACGG	

Construction of *sntA* Gene Deletion and Complementary Strains

The *sntA*-deleted strain Δ*sntA* was constructed in our previous study (24). To construct *sntA* complementary strain, a DNA fragment containing the entire *sntA* coding sequence and its promoter and terminator was amplified by using primers CΔ*sntA*_F/CΔ*sntA*_R. The amplicon was subsequently cloned into *E. coli-S. suis* shuttle vector pSET2, resulting in the recombinant plasmid pSET2:*sntA*. This plasmid was transformed into the Δ*sntA* strain, and the resulting complementary strain CΔ*sntA* was screened on TSA agar plates supplemented with 100 μg/ml spectinomycin (Spc). The Spc-resistant colonies were verified by PCR and reverse transcription (RT)-PCR analyses by using three pairs of primers SntA_F/SntA_R, 10065_F/10065_R, and 10075_F/10075_R (**Table 1**).

Experimental Infection of Mice

To detect the virulence of *sntA* in *S. suis*, a total of 24 female 5-week-old specific-pathogen-free (SPF) Kunming mice (8 mice per group) were intraperitoneally infected with 2×10^9 colony forming unit (CFU)/mouse of SC-19 and Δ*sntA*. Physiological saline was served as a negative control. Then the morbidity, mortality, and clinical symptoms such as limping, swollen joints, shivering, and central nervous system failure of all the mice were observed for 5 days. To evaluate the effect of *sntA* on survival in blood and organs colonization, competitive colonization experiment of SC-19 and Δ*sntA* was performed. 5-week-old SPF Kunming mice (7 mice per group) were co-infected with SC-19 and Δ*sntA* at the ratio 1:1 (5×10^8 CFU/mouse). Bacterial counts in blood, brain, and lung were collected at 6, 12, 24, and 48 h post infection (hpi). Colonization of bacteria in various tissues were analyzed by serial diluted and plated brain and lung samples after homogenizing, and blood samples on TSA agar plates with (Em^+) and without (Em^c) 100 μg/ml erythromycin (Em) as described previously (28). The number of live bacteria on TSA agar plates with or without Em was calculated as Δ*sntA*, sum of SC-19 and Δ*sntA*, respectively. The number of SC-19 was calculated as $TSA_{Em^-} - TSA_{Em^+}$.

Bactericidal Assays

In human blood and serum killing assays, experiments to evaluate the survival rate of *S. suis* were carried out as described previously (29). Overnight cultures of the *S. suis* strains in TSB were diluted in 1:100 and grown to mid-log phase without agitation at 37°C. The cultures were diluted to 5×10^7 CFU/ml with physiological saline. 50 μl diluted cultures was mixed with 450 μl fresh human blood for human blood killing assay and 20 μl diluted cultures were mixed with 180 μl of normal human serum (NHS) and complement heat inactivated serum by incubation at 56°C for 30 min for human serum killing assay. The resulting mixtures were incubated at 37°C for 30 min. Live bacteria were counted by plating the serial diluted samples on TSA agar plates. The percentage of live bacteria was subsequently calculated as $(CFU_{\text{after incubation}}/CFU_{\text{in original inoculum}}) \times 100\%$.

In PMNs killing assay, PMNs were isolated from heparinized venous blood by human peripheral blood PMN isolation kit (Haoyang, Tianjin, China). Experiment to investigate the survival rate of *S. suis* in PMNs was carried out as described previously (6). PMNs were mixed with bacteria at MOI = 1:10 in RPMI-1640 medium (Hyclone, USA) supplemented with 20% freshly non-immune human serum and incubated at 37°C under 5% CO_2 for 30 min. The percentage of live bacteria was subsequently calculated as $(CFU_{PMN^+}/CFU_{PMN^-}) \times 100\%$.

In phagocytosis assay, the experiment was carried out by our previous study (30). Briefly, the 1.0×10^6 RAW264.7 cells for each well were pooled into 12-well plates. Then *S. suis* strains in mid-log phase were added to the plates (MOI = 10:1) and incubated for 30 min at 37°C to allow cells to be phagocytized. After incubation, ampicillin was applied to plates for 1 h to kill the extracellular bacteria. Then, the cells were lysed with sterile distilled water on ice and the bacterial CFU were counted on TSA agar plates. These data are presented as means ± SDs from three separate experiments.

Complement C3 Deposition and MAC Formation Analysis

C3 deposition and MAC formation assays were performed as previous study (31). Overnight cultures of *S. suis* were diluted in 1:100 in TSB and grown to mid-log phase without agitation at 37°C. Bacteria were collected by centrifugation at 6,000 rpm for 5 min. After two washes, cultures were suspended to 5×10^7 CFU/ml with physiological saline. 300 μl *S. suis* suspensions were mixed with 240 μl freshly undiluted and 1/500 diluted non-immune human serum and incubated at 37°C for 30 min. After two washes, *S. suis* was resuspended in 300 μl anti-C3b monoclonal antibody (20 μg/ml; Abcam, England) to detect C3 deposition and monoclonal anti-C5b-9 antibody (20 μg/ml; Abcam) to detect MAC formation. After incubation for 10 min at room temperature, FITC-conjugate goat-anti-mouse IgG (BD, USA) was used as the second antibody and incubated at room temperature for 10 min. Finally, bacteria were washed and resuspended in 800 μl physiological saline for flow cytometry analysis performed by FACSCalibur (BD). Bacteria were detected by log-forward and log-side scatter dot-plot at middle flow rate. A gating region was set to include the majority of bacteria except debris. 10,000 bacteria/events were acquired and analyzed for fluorescence using log-scale amplifications. C3 deposition and MAC formation capacities were measured by the geometric mean fluorescence intensity (GMFI). GMFI value was measured from three independent experiments performed in duplicate.

SntA Immunodetection

Western-blot analysis was used to determine the location of SntA expressed in the SC-19, Δ*sntA*, and CΔ*sntA* strains. For cell lysate, cell cultures of *S. suis* strains were collected, resuspended with 1 ml bacterial lysis buffer (0.05 M Tris–HCl, 2.5 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100, pH 8.5~9.0) and boiled 20 min to lysis, the resulting cell lysate were used to prepare SDS-PAGE samples. For cell wall proteins, samples were prepared as described previously (32). Briefly, bacterial cultures were collected; washed once in 50 mM Tris–HCl, pH 7.3; resuspended in 1 ml of osmoprotective buffer (50 mM Tris–HCl, pH 7.3, 20% sucrose, 2.5 μM PMSF) supplemented with 175 U/ml mutanolysin (Sigma-Aldrich, Shanghai, IL, USA); incubated at 37°C for

90 min under constant gentle agitation. After centrifugation at 12,000 *g*, 4°C for 15 min, supernatants containing the cell wall proteins were used to prepare SDS-PAGE samples. For secreted proteins, culture supernatants were collected, filtered (Millipore, 0.22 μm, Shanghai, China), and concentrated to 10 mg/ml, and then the proteins were sedimentated with pre-cooled 10% TCA-acetone solution at 4°C overnight. After centrifugation at 12,000 *g*, 4°C for 15 min, washed sediment with pre-cooled 90% acetone supplemented with 10 mM DTT for three times. The last sediment was resuspended with 8 M urea, 2% CHAPS, 10 mM DTT for next step. For all SDS-PAGE samples immunodetection, total proteins were quantified by BCA protein assay kit (Kangwei, Beijing, China), and then 48 μg cell lysate, secreted, and cell wall proteins were applied to 10% SDS-PAGE. Proteins transferred onto PVDF membrane (Millipore, 0.45 μm). Mouse SntA polyclonal antibody (24) was used for SntA immunodetection, and chemiluminescence detection (Bio-Rad, USA) with HRP-conjugate goat anti-mouse IgG (Antgene, Wuhan, China) by MF-Chemi BIS 3.2 (DNR, Israel).

Preparation of Recombinant Proteins

Recombinant SntA protein (Protein ID: WP_012027972.1) was expressed and purified as our previous report (24). To obtain the recombinant proteins of the complement C1q subunits, the cDNAs of C1qA, C1qB, and C1qC were amplified from the total cDNA of porcine lung tissue with primers C1qA_F/R, C1qB_F/R, C1qC_F/R, respectively (**Table 1**). Total cDNA was prepared from total RNA of porcine lung tissue by using RT-PCR. Then the C1qA cDNA was cloned into pQE-32 plasmid (Qiagen, Shanghai, China), while the cDNAs of C1qB and C1qC were cloned into pET-28a-SUMO plasmid (26) to promote soluble expression. The resultant recombinant plasmids were transformed into *E. coli* BL21 (DE3), respectively. The expression of C1qA, C1qB, and C1qC proteins was induced by 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C. The recombinant proteins were purified by using Ni-NTA agarose column (Bio-Rad) and quantified by BCA protein assay kit (Kangwei).

Interaction Between C1q and SntA Proteins

ELISA was used to confirm the interaction between SntA and C1q proteins as described previously (33). For SntA bound assay, the proteins C1q (Quidel, USA), three C1q subunits C1qA, C1qB, C1qC, and BSA were coated to 96-well plates (BIOFIL, Guangzhou, China) with increasing concentrations of 0–7 μg/ml at 4°C for 16 h, respectively. After blocking with blocking buffer (PBS supplemented with 0.05% Tween20 and 1% BSA), 5 μg/ml SntA was poured to each well, and then incubated at 37°C for 1 h. Mouse SntA polyclonal antibody was applied at 37°C for 1 h to test the direct binding capacity. For C1q bound assay, SntA, BSA, and heme were coated to 96-well plates with increasing concentrations of 0–7 μg/ml at 4°C for 16 h, respectively, After blocking with blocking buffer, 5 μg/ml C1q was applied to each well at 37°C for 1 h. Rabbit C1qA polyclonal antibody (Abclonal, Wuhan, China) was applied at 37°C for 1 h to test the direct binding capacity. For inhibition assay, 5 μg/ml human aggregated IgG (Sigma-Aldrich) was coated to plates at 4°C overnight. Human aggregated IgG was prepared by incubating at 63°C for 20 min, immediately placing on ice for 1 h, centrifuging at 16,000 *g* for 5 min and quantification (34). C1q was pre-incubated with increasing concentrations of 0–25 μM SntA, heme, or BSA, and then applied to plates at 37°C for 1 h. Rabbit C1qA polyclonal antibody was applied to plates at 37°C for 1 h to test the indirect binding capacity. All the ELISA assays, the HRP-conjugate goat-anti-rabbit IgG (Antgene), or goat-anti-mouse IgG was used as the second antibody at 37°C for 1 h before chromogenic reaction.

Competitive binding assays were performed as described previously (35). For C1q-IgG binding assay, microtiter plates were pre-coated with 30 μg/ml human C1q overnight at 4°C. After blocking, plates were incubated with the mixtures of 50 μl 2.5 mg/ml human aggregated IgG and 50 μl increasing concentrations of 0–50 μM SntA or BSA in HB^{++} buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$). After incubation for 1 h at 37°C, plates were washed with washing buffer (HB^{++} buffer supplemented with 0.05% Tween20) and developed with HRP-conjugate goat anti-human IgG Fc (1:4,000; Sigma-Aldrich) at 37°C for 1 h before chromogenic reaction. For C1q–Ag–Ab binding assay, microtiter plates were coated with 2.5 μg/ml tetanus toxoid (Millipore) as an antigen for immune complex formation. After blocking, human tetanus toxin immunoglobulin (1:1,000; Hualan Bio, Xinxiang, China) was applied to the tetanus toxoid-coated plates to form immune complexes for 1 h at 37°C. 50 μl 2 μg/ml human C1q were mixed with 50 μl increasing concentrations of 0–50 μM SntA or BSA. The resulting mixtures were applied to each well at 37°C for 1 h. After washed, polyclonal goat antiserum to human C1q (1:5,000; Quidel, USA) was incubated to plates at 37°C for 1 h. HRP-conjugate rabbit anti-goat IgG (Antgene) was used as the second antibody at 37°C for 1 h before Chromogenic reaction. These data are presented as mean ± SD from three separate experiments.

Binding of C1q to *S. suis*

Overnight cultures of the *S. suis* strains in TSB were diluted in 1:100 and grown to mid-log phase without agitation at 37°C. The cultures were washed and resuspended in $DGHB^{++}$ buffer to 5×10^8 CFU/ml. *S. suis* suspensions were incubated with 0, 10, and 20 μg/ml C1q proteins at 37°C for 1 h and the total volume was 100 μl. After incubation and two washes, FITC-rabbit anti-human C1q antibody (Abcam) was applied for 30 min at 37°C. Finally, the bacteria was washed and resuspended in 500 μl PBS for flow cytometry analysis by FACSCalibur. Data from three independent experiments in duplicate were analyzed by Flow Jo 7.6.1 software (33). The assay was repeated three times.

Hemolytic Assay

The hemolytic activity of SntA protein was measured as previous study (33). For the classical pathway, sheep red blood cells (SRBCs) (Baiji, Zhengzhou, China) were washed with pre-cooled $DGHB^{++}$ buffer for three times and diluted to a concentration of 1×10^9 cells/ml. Then the SRBCs solution was mixed with equal volume of ambocepter (1:1,000; Baiji) and rotated at 37°C for 20 min. After two washes, 0.2% NHS was incubated with 0–100 μg/ml SntA at 37°C for 15 min, then the mixtures were incubated with

3×10^8 cells/ml SRBCs at 37°C for 1 h. BSA was used as a negative and the total volume is 150 µl. After centrifugation at 800 *g*, the hemolytic activity was calculated by spectrophotometric measurement of absorbance at 405 nm. For the alternative pathway, rabbit red blood cells (RRBCs) (Baiji) were washed with pre-cooled Mg^{2+}-EGTA-DGHB buffer (4.2 mM Hepes, pH 7.4, 59 mM NaCl, 2.08% glucose, 0.08% gelatin, 7 mM $MgCl_2$, 10 mM EGTA) for three times and diluted to a concentration of 1×10^9 cells/ml. 1.25% NHS was incubated with 0–100 µg/ml SntA at 37°C for 15 min, then the mixtures were incubated with 3×10^8 cells/ml RRBCs at 37°C for 1 h. The hemolytic activity of alternative pathway was determined as the classical pathway. These data are presented as mean ± SD from three separate experiments.

Complement Activation Assay

To detect whether SntA could activate complement directly, complement activation ELISA were performed as described previously (33). 2 µg/ml SntA, 2 µg/ml BSA, 10 µg/ml IgM (Berseebio, Beijing, China) for the classical pathway, 100 µg/ml Mannan (Sigma) for the lection pathway, and 20 µg/ml Zymosan (Sigma) for the alternative pathway were coated to 96-well plates in PBS. The plates were washed with PBS supplemented with 0.05% Tween20 for each step. After blocking with the blocking buffer, 0–7% NHS in $DGHB^{++}$ buffer was applied for classical pathway, 0–7% depleted of protein C1q serum (Quidel) in $DGHB^{++}$ buffer was applied for lectin pathway, and 0–7% NHS in Mg^{2+}-EGTA-DGHB buffer was applied for alternative pathway. After incubation for 1 h, the plates were washed and C3b rabbit polyclonal antibody (1:1,000; Proteintech, Wuhan, China) was poured to plates to detect C3 deposition at 37°C for 1 h. All the ELISA assays, the HRP-conjugate goat-anti-rabbit IgG was used as the second antibody at 37°C for 1 h before chromogenic reaction. These data are presented as mean ± SD from three separate experiments.

RESULTS

SntA Contributes to the Virulence of *S. suis*

Two mouse infection models were performed to investigate the contribution of *sntA* on virulence. The growth curves of *S. suis* presented by OD and CFU were measured before infection in mice. Results showed that no significant differences were observed in SC-19, Δ*sntA*, and CΔ*sntA* in both OD and CFU (Figure S2 in Supplementary Material). First, groups of eight mice were intraperitoneally infected with 2×10^9 CFU/mouse SC-19 and Δ*sntA*. Physiological saline was used as a negative control. Survival rate of infected mice was measured within 5 days post infection (dpi). We observed that four of mice infected with SC-19 died within 24 hpi, another four showed obvious limping, shivering, and central nervous system failure within 5 dpi. By contrast, only two mice infected with Δ*sntA* died within 24 hpi, one mouse died within 36 hpi, and another five mice were survived during the observation within 5 dpi (**Figure 1A**). This revealed that significant difference was observed in survival rate of SC-19 and Δ*sntA* infected mice. Second, groups of seven mice were intraperitoneally infected with the mixtures of SC-19 and Δ*sntA* at the ratio 1:1 (5×10^8 CFU/mouse). Blood and brain, lung colonization were recovered at 6, 12, 24, and 48 hpi, respectively. The efficiency of colonization of SC-19 was much higher at 24 and 48 hpi than that of Δ*sntA* in blood (**Figure 1B**) and lung (**Figure 1D**). In addition, significant differences were observed in brain from 6 to 48 hpi (**Figure 1C**). The results showed that SntA contributed to the survival in blood and the colonization in specific organs.

SntA Possess Obvious Anti-Phagocytic Activity

We constructed a complementary strain of *sntA*, as confirmed by genome PCR (Figure S1A in Supplementary Material) and RT-PCR (Figure S1B in Supplementary Material). To investigate the anti-phagocytosis mediated by SntA, three bacteria killing assays and one phagocytosis assay were performed. In blood killing assay, the survival rate of SC-19 in human blood was 105%, but the absence of *sntA* decreased the survival rate to only 33% (**Figure 2A**). Similar results were observed in mouse blood killing assay (Figure S3A in Supplementary Material). In PMNs killing assay, PMNs supplemented with 20% active human serum were used to test the survival abilities of *S. suis* in human neutrophils. Results revealed that the survival rate of Δ*sntA* was significantly decreased compared to SC-19 and CΔ*sntA* (**Figure 2B**). In phagocytosis assay, RAW 264.7 cells were used to test anti-phagocytic activity of *S. suis*. Results demonstrated that the phagocytic numbers of Δ*sntA* were much higher comparing with SC-19 and CΔ*sntA* (**Figure 2C**). In the human serum killing assay, the active and inactivated human serum were used to investigate the role of complement on *S. suis* clearance. For the *sntA*-deleted mutant strain, only 48% viable Δ*sntA* bacteria survived in freshly active human serum. The presence of *sntA* significantly increased the survival rate of *S. suis* up to 250% (SC-19) and 185% (CΔ*sntA*), respectively. No obvious differences were observed in the survival rates of *S. suis* in inactivated serum (**Figure 2D**). Normal mouse serum was also used in this assay, and similar results were obtained (Figure S3B in Supplementary Material). No significant differences were observed between SC-19 and CΔ*sntA* in all tests. These data demonstrated that SntA was involved in resistance to phagocytosis and the bactericidal activity of blood, PMNs, and NHS dependent on complement. This reveled that SntA could obviously contribute to anti-phagocytosis and this may be involved in complement.

SntA Inhibits the C3 Deposition and MAC Formation on *S. suis*

SntA possessed anti-phagocytosis, but the effect of SntA on complement activation on *S. suis* was still unclear. To investigate that, the C3 deposition and MAC formation assays were performed in both undiluted and 1:500 diluted human serum. Results showed that the viable bacteria number aggregated fluorescence of Δ*sntA* in C3 deposition was obviously increased compared to SC-19 and CΔ*sntA* in undiluted human serum (**Figures 3A,C**). The efficiency of MAC formation was significantly higher comparing with SC-19 and CΔ*sntA* (**Figures 3B,C**) as well. To assess the effect of SntA on complement activation through the classical pathway, 1/500 NHS was used. Results revealed that C3 deposition and MAC formation on surface of Δ*sntA* was significantly higher compared with SC-19 and CΔ*sntA* in 1/500 diluted

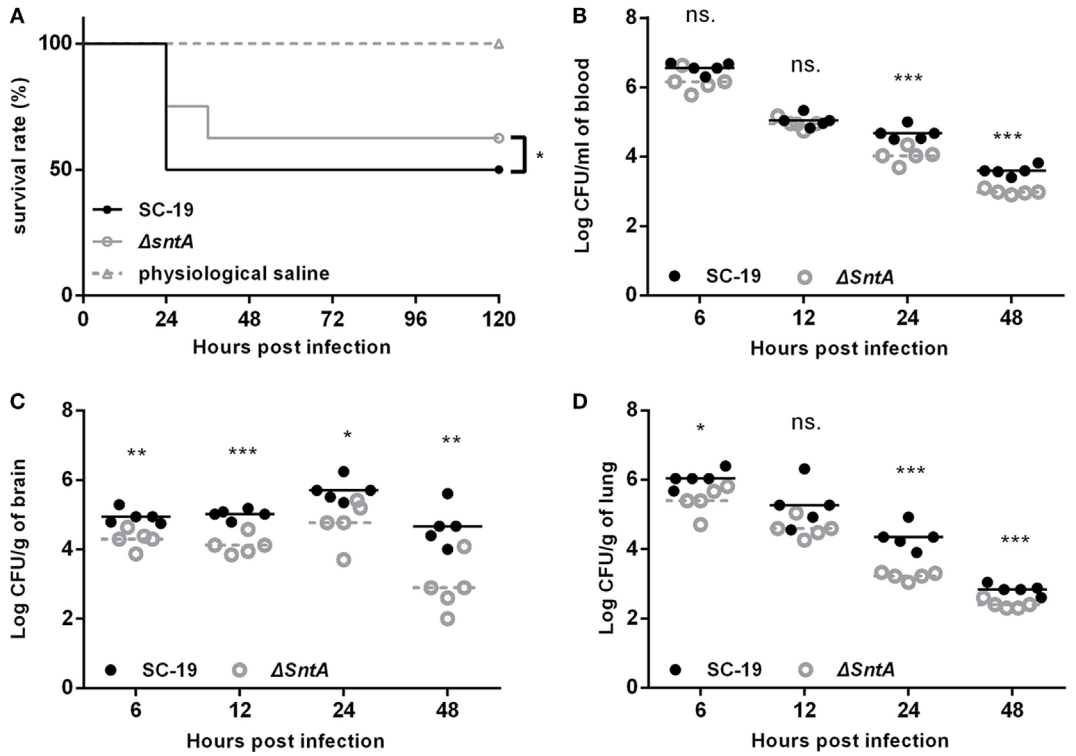

FIGURE 1 | SntA contributes to pathogenesis in mouse infection model. **(A)** Survival curves for mice challenged with *Streptococcus suis*. Groups of eight mice were injected separately and intraperitoneally with 2×10^9 colony forming units (CFU)/mouse of SC-19 and Δ*sntA*. Eight mice inoculated with physiological saline were served as a negative control. Significant difference in survival between different groups was analyzed by Log Rank test for trend. Bacterial loads in blood **(B)**, brain **(C)**, and lung **(D)** were shown. Seven mice per group were intraperitoneally infected with the mixtures of SC-19 and Δ*sntA* at ratio 1:1 (dose of 5×10^8 CFU/mouse). Results were shown as $\log_{10}$ of recovered bacteria counts after deletion of the highest and lowest value (CFU/ml in blood and CFU/g in organs). The statistical significance was shown by asterisks (unpaired *t* test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

human serum (**Figures 3D–F**). No significant differences were observed between SC-19 and CΔ*sntA* in all tests. These assays demonstrated that SntA could inhibit the complement pathway.

SntA Is a Cell Wall Anchored Protein

The cell wall attached protein LPXTG motif region was found at C terminal of amino acid sequence of SntA (**Figure 4A**). To confirm this prediction, western-blot analysis was performed. Results showed that SntA expressed in cell lysate extracted from SC-19 and CΔ*sntA*, but not detected in Δ*sntA*. Cell wall proteins extracted by mutanolysin and secreted proteins sedimentated by TCA-acetone were used to further determine the location of SntA in *S. suis*. SntA was demonstrated to express in cell wall proteins, and not in secreted proteins (**Figure 4B**). These assays indicated that SntA existed in cell wall.

SntA Interacts With Complement C1q

To assess the binding capacity between SntA and C1q, the direct and indirect bound assays were performed. In direct bound assay, C1q could significantly bind with SntA in a dose-dependent manner in both C1q-coated and SntA-coated plates (**Figures 5A,B**). The known C1q-binding molecule heme was used as a positive control and BSA as a negative control. In inhibition assay, both SntA and heme could significantly inhibit the binding capacity between plasma purified C1q and aggregated human IgG on IgG-coated plates, and the inhibitory effect of SntA was much stronger than the positive control heme (**Figure 5C**). Furthermore, three subunits of C1q including C1qA, C1qB, and C1qC could obviously interact with SntA in a dose-dependent manner as well (**Figure 5D**). These results indicated that recombinant SntA could bind to C1q. To investigate whether SntA bound with C1q on bacterial surface, flow cytometry was performed. A dose-dependent increase of C1q-binding capacity was observed on *S. suis*. Compared with SC-19 and CΔ*sntA*, a significant reduction of C1q-binding capacity was observed in Δ*sntA* when 20 μg/ml C1q was added (**Figure 5E**). Taken together, SntA could interact with C1q and act as a C1q ligand on bacterial surface.

SntA Competitively Bind With C1q

The classical pathway is triggered through the recognition of antigen-antibody complex on micro surface by recognition molecule C1q (4). To assess the effect of C1q-binding protein SntA on the recognition step of the classical complement pathway, competitive binding assays were performed. In C1q–IgG binding assay, result showed that SntA strongly decreased the C1q–IgG binding capacity, and 50 μM SntA inhibited the binding capacity down to 3.34% (**Figure 6A**). In C1q–Ag–Ab binding assay, the binding capacity was significantly decreased in a dose-dependent manner and 84.7%

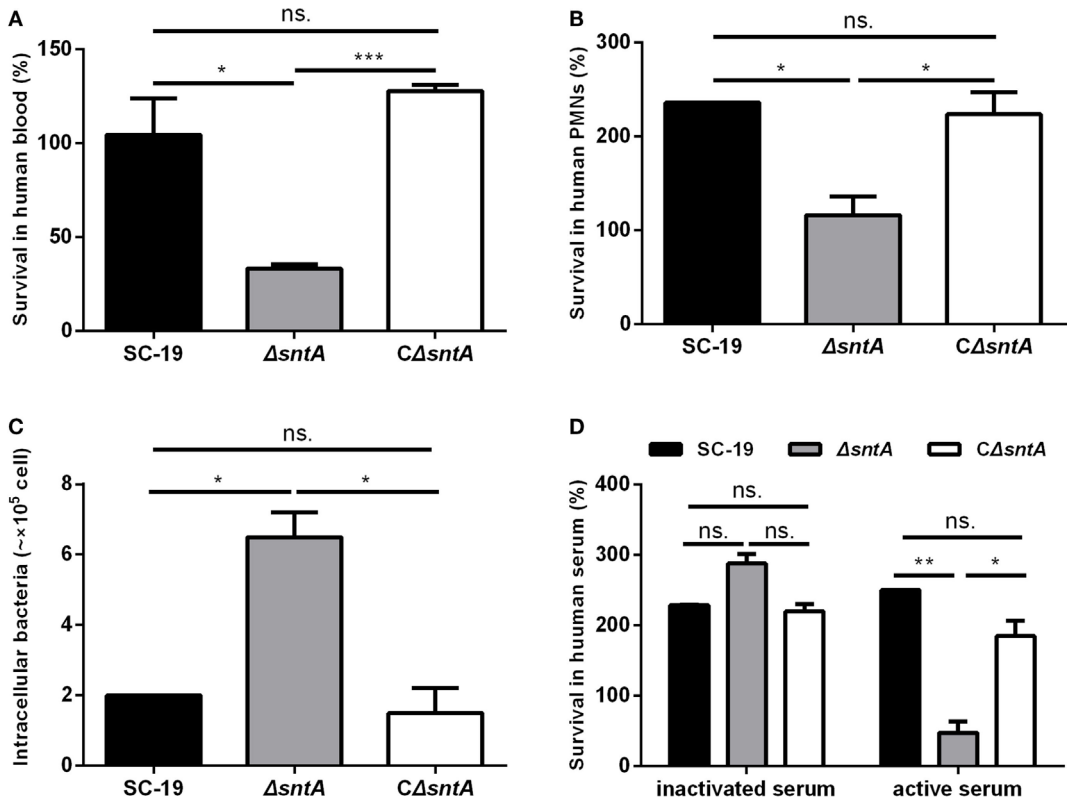

FIGURE 2 | SntA possess anti-phagocytic activity. **(A)** Survival rate of *Streptococcus suis* in human blood. **(B)** Survival rate of *S. suis* in PMNs separated from freshly human blood. **(C)** The numbers of *S. suis* phagocytized in mouse macrophage RAW264.7 cells. **(D)** Survival rate of *S. suis* in human active and inactivated serum. Results were expressed from three independent experiments performed in triplicate. The statistical significance was showed by asterisks (unpaired *t* test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

of it was remarkably inhibited with 50 μM SntA (**Figure 6B**). Results demonstrated that SntA could inhibit C1q–IgG binding and C1q–Ag–Ab binding by competitive interaction with C1q.

SntA Inhibits Hemolytic Activity

SntA inhibited complement pathway, in which pathway SntA acted remain unclear. Hemolytic activity assay is often used to assess the activity of the classical pathway of complement activation with sheep erythrocytes (SRBCs) and of alternative pathway with rabbit erythrocytes (RRBCs) (33). So, to test in which pathway SntA inhibited complement, the hemolytic activity assays were performed. Results showed that SntA remarkably inhibited the hemolytic activity of SRBCs in a dose-dependent manner mediated by the classical pathway in the presence of NHS (**Figure 7A**). No inhibitory effect was observed in the negative control of BSA. However, SntA did not inhibit the hemolytic activity of RRBCs mediated by the alternative pathway (**Figure 7B**). These data revealed that SntA could inhibit the hemolytic activity mediated by the complement classical pathway.

SntA Directly Activates Complement Pathways

To test whether SntA could activate complement directly, the complement activation assays were performed. In the classical pathway, the increasing concentrations of 0–7% NHS were applied to the SntA pre-coated plates to detect C3b deposition with specific C3b polyclonal antibody. IgM acted as a positive control and BSA as a negative control. Significant activation was observed on the SntA pre-coated plates and not on the BSA pre-coated plates (**Figure 8A**). This showed that SntA could activate complement through the classical pathway, although the activation level was lower than the positive control. In the lectin pathway, microtiter plates pre-coated with SntA and positive control Mannan exhibited strong complement activation mediated by the lectin pathway when human serum depleted of C1q was used (**Figure 8B**). Result demonstrated that SntA could activate the lectin pathway directly. In the alternative pathway, NHS in Mg^{2+}-EGTA-DGHB buffer was used to block classical activation pathway and detect complement activation mediated by the alternative pathway. Result showed that no significant activation was observed in the plates pre-coated with SntA, while the positive control Zymosan obviously activated the alternative pathway (**Figure 8C**). Thus, SntA could directly activate the complement by the classical and lectin pathway, not the alternative pathway.

DISCUSSION

Streptococcal toxic shock like syndrome, meningitis, and septicemia are the most important characteristics of the recently

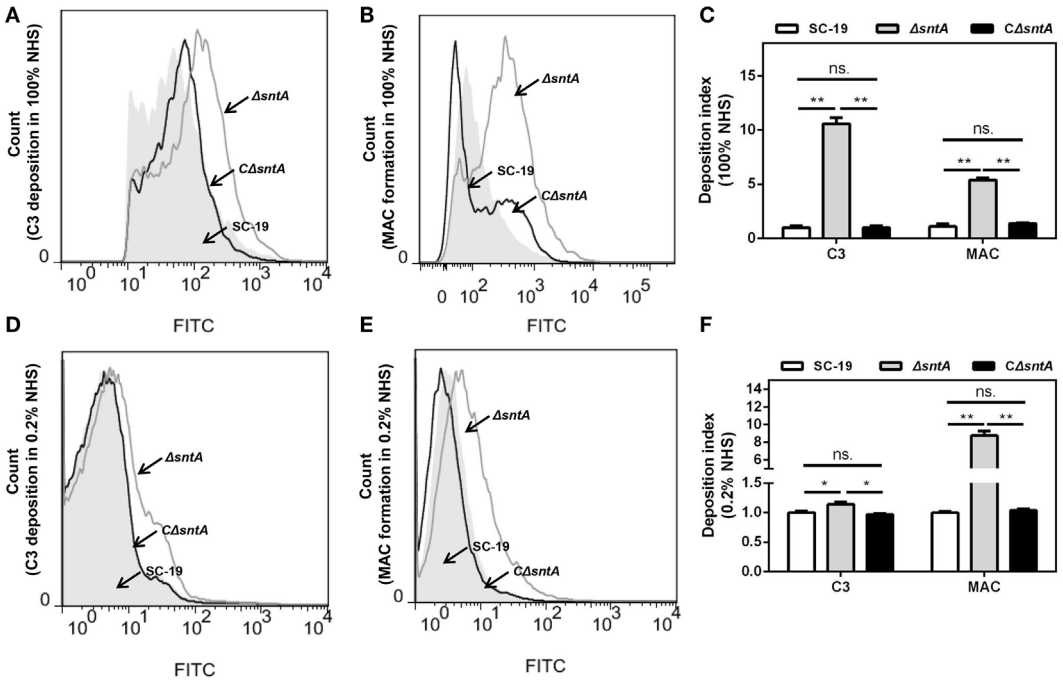

FIGURE 3 | SntA inhibits C3 deposition and membrane attack complex (MAC) formation. **(A–F)** The C3 deposition and MAC formation capacities were presented by count of positive cell aggregated with fluorescence in the presence of complement (100 and 0.2% normal human serum). The representative histogram of flow cytometry from three independent experiments was exhibited **(A,B,D,E)**. Geometric mean fluorescence intensity (GMFI) value was measured from three independent experiments performed in duplicate. The GMFI value of C3 deposition and MAC formation of SC-19 in all tests was set as 1.00, the corresponding Δ*sntA* and CΔ*sntA* values were proportional achieved. The resulting C3 deposition and MAC formation index were presented **(C,F)**. The statistical significance was calculated by two-way ANOVA of Sidak's correct for multiple comparison and shown by asterisks (**$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

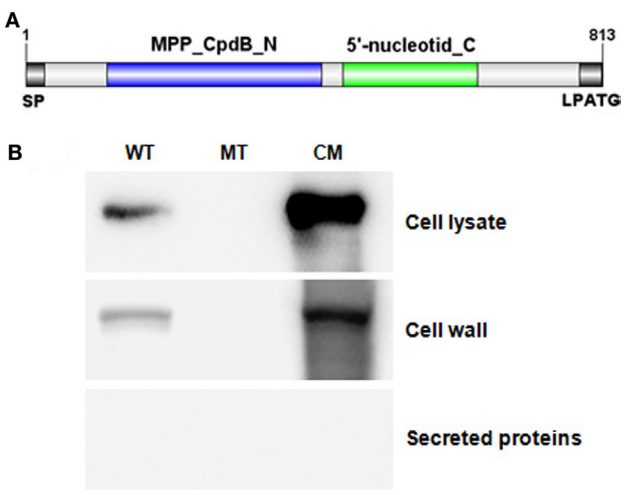

FIGURE 4 | SntA was a conserved cell wall anchored protein. **(A)** Conserved domain of SntA was predicted in NCBI database (https://www.ncbi.nlm.nih.gov/cdd). SntA had two conserved domain: MPP_CpdB_N and 5′-nucleotid_C, and a cell wall attached LPATG motif. **(B)** Western-blot was used to determine surface location of SntA. Cell lysate, cell wall proteins, and secreted proteins were ran 10% SDS-PAGE, transferred onto PVDF membrane and detected by mouse anti-SntA poly-antibody following by HRP-conjugate goat-anti-mouse IgG. WT, SC-19; MT, Δ*sntA*; CM, CΔ*sntA*.

emerging *S. suis* infection, especially in Southeast Asia. During infection, the pathogen must compete with the normal microflora, resist defense mechanisms of the local mucosal immunity, adhere and invade the mucosal epithelial cell barrier, subsequently reach and survive in the blood stream, and finally invade multiple organs including spleen, liver, kidney, and lung, even entry BBB (36). A plenty of factors have been demonstrated to be associated with these processes [reviewed in Ref. (37)]. However, how *S. suis* escape the immune surveillance to invade into blood and most organs is largely unknown. In this study, we found that the surface heme-binding protein SntA of *S. suis* (24) contributes to the resistance of C3 deposition and MAC formation on bacterial surface, the abilities of anti-phagocytosis, survival in blood, and *in vivo* colonization. SntA can interact with C1q, and inhibit the hemolytic activity *via* the classical pathway. SntA could also directly activate classical and lectin pathways, resulting in complement consumption. These two complement evasion strategies may be crucial for the pathogenesis of the zoonotic pathogen *S. suis*.

In our previous study, SntA is identified as a heme-binding protein which can interact with the host antioxidant protein AOP2 and inhibit its antioxidant activity, and thus contribute to the survival and pathogenesis of *S. suis* in pigs (24). This protein is a bifunctional 2′,3′-cyclic nucleotide 2′-phosphodiesterase/3′-nucleotidase, which is distributed in many species of Gram-positive bacteria (24). In the present study, mouse infection model is used to further study the function of SntA involving

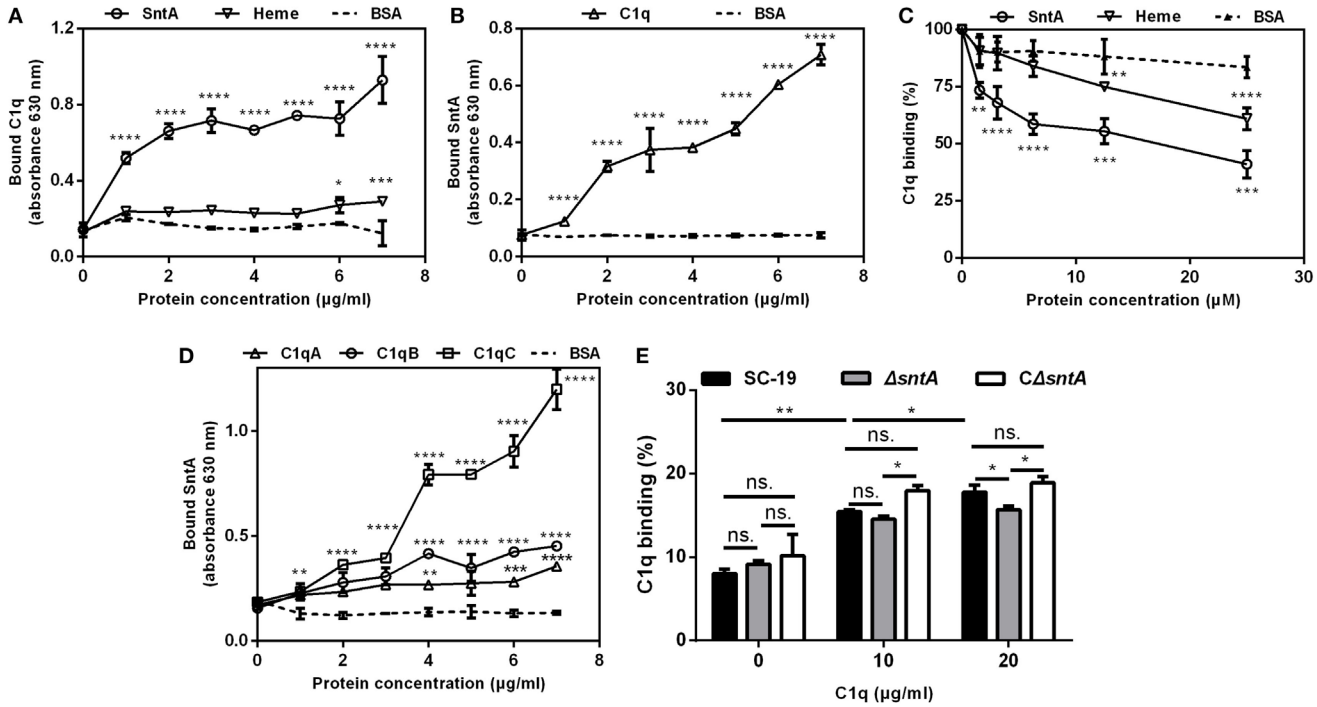

FIGURE 5 | SntA interacts with C1q. **(A)** C1q was applied to SntA-, heme-, and BSA-coated plates to test the C1q bound capacity. **(B)** SntA was applied to C1q- and BSA-coated plates to test the SntA bound capacity. **(C)** SntA, heme, or BSA was premixed with C1q and applied to IgG-coated plates to test the binding capacity between C1q and IgG, which indicating the binding capacity between SntA and C1q. **(D)** SntA was applied to C1qA-, C1qB-, C1qC-, and BSA-coated plates to test the SntA bound capacity. **(E)** Increasing concentrations of C1q were incubated with *Streptococcus suis* to detect the binding capacity by FITC-anti-human C1q antibody. Results were expressed from three independent experiments performed in triplicate. The statistical significance was calculated by two-way ANOVA of Sidak's correct for multiple comparison and shown by asterisks (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

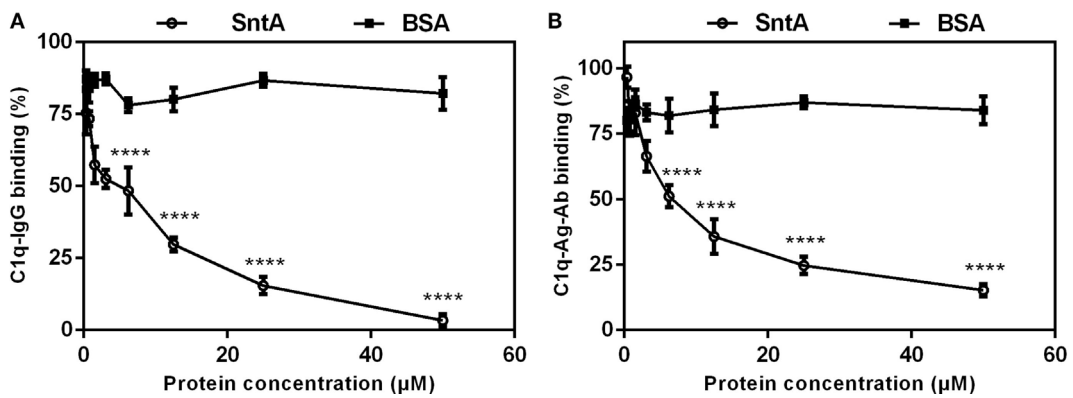

FIGURE 6 | SntA competitively binds with C1q. **(A)** Inhibition of IgG–C1q binding. Increasing concentrations of SntA were premixed with C1q and applied to IgG-coated plates to test the inhibitory capacity of SntA to C1q–IgG binding. **(B)** Inhibition of C1q–Ag–Ab binding. IgG was applied to tetanus toxoid-coated plates for IgG-containing immune complex formation. Increasing concentrations of SntA were premixed with C1q and added to plates to test the inhibitory capacity of SntA to C1q–Ag–Ab binding. BSA was served as a negative control. Results were expressed from three independent experiments performed in triplicate. The statistical significance was calculated by two-way ANOVA of Sidak's correct for multiple comparison and shown by asterisks (****$p < 0.0001$; ns, $p > 0.05$).

in the pathogenesis. Results show that SntA facilitates in *S. suis* survival in blood (**Figure 1B**) and subsequent colonization in some specific organs (**Figures 1C,D**). To entry the bloodstream and spread to distant organs, the Gram-positive pathogens usually utilize opsonophagocytic clearance function of neutrophils and macrophages (38). SntA is demonstrated to contribute to the anti-phagocytosis of *S. suis* mediated by neutrophils and macrophages and the survival in whole blood and serum *in vitro*

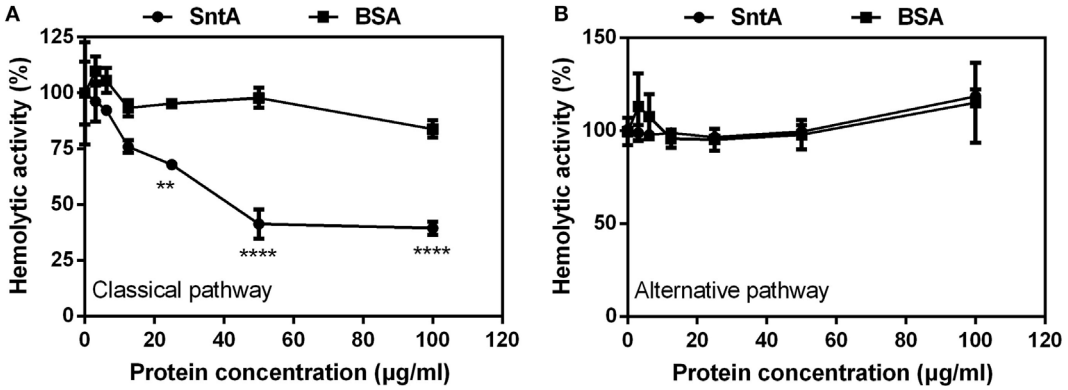

FIGURE 7 | SntA inhibits hemolytic activity. **(A)** To measure the inhibitory effect of SntA on the classical pathway, antibody-coated sheep erythrocytes were subjected to complement attack from normal human serum (NHS) in the presence of the increasing concentrations of SntA. BSA was used as a negative control. The degree of lysis was estimated by measurement of the release of hemoglobin. **(B)** To measure the inhibitory effect of SntA on the alternative pathway, rabbit erythrocytes were subjected to complement attack from NHS in the presence of the increasing concentrations of SntA. BSA was used as a negative control. Cell lysis was measured like **(A)**. The absorbance without inhibitor was set to 100%. Results were expressed from three independent experiments performed in triplicate. The statistical significance was calculated by two-way ANOVA of Sidak's correct for multiple comparison and shown by asterisks (****$p < 0.0001$; **$p < 0.01$; ns, $p > 0.05$).

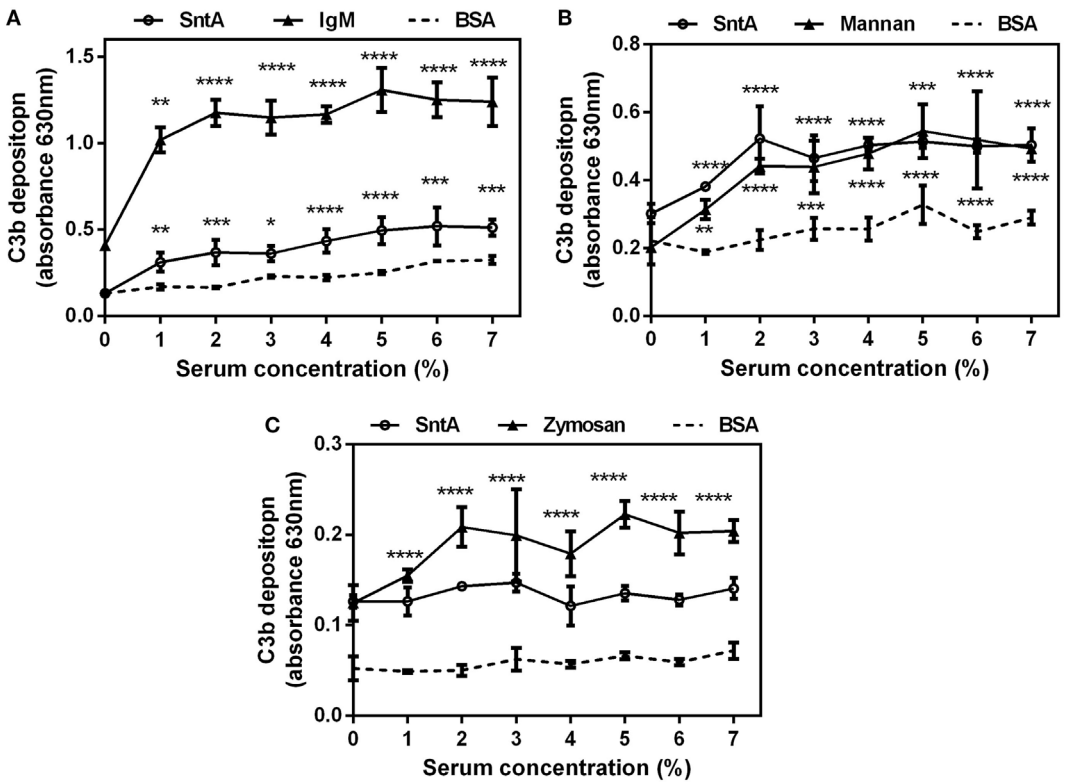

FIGURE 8 | SntA can directly activate the classical and lectin complement pathways. **(A)** Normal human serum (NHS) in $DGHB^{++}$ buffer was applied to the plates pre-coated with SntA, IgM was acted as a positive control and BSA as a negative control to detect C3b deposition. **(B)** Human serum depleted of C1q in $DGHB^{++}$ buffer was applied to the plates pre-coated with SntA, Mannan as a positive control to detect C3b deposition. **(C)** NHS in Mg^{2+}-EGTA-DGHB buffer was applied to the plates pre-coated with SntA, Zymosan as a positive control to detect C3b deposition. Results were expressed from three independent experiments performed in triplicate. The statistical significance was calculated by two-way ANOVA of Sidak's correct for multiple comparison and shown by asterisks (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

(**Figure 2**). The efficient phagocytosis is an important innate immune mechanism for controlling infection of extracellular bacteria such as *Streptococci*, and this process requires serum with intact complement activity (39). Recently, many complement inhibitors target at complements such as FH (29), C1q (23, 40), C3 (41), C4BP (42), mediate complement evasion,

and subsequently contribute to pathogenesis in Gram-positive bacterium. In order to investigate whether SntA involves in the complement evasion of *S. suis*, a series of experimentations are carried out to this end.

First, the complement deposition assays are performed. The C3 deposition and MAC formation on the surface of the mutant strain Δ*sntA* are significantly higher than those on the parental strain SC-19 and complementary strain CΔ*sntA* (**Figure 3**). This indicates that SntA involved in complement activation by *S. suis*. The undiluted and 1:500 diluted human serum (NHS) are used in the complement deposition assays, respectively, C3 deposition and MAC formation on the surface of Δ*sntA* are always significantly higher than those on SC-19 and CΔ*sntA* (**Figure 3**). This suggests that SntA could inhibit both classical pathway and alternative pathway because it has been documented that the classical pathway is activated by using diluted NHS (35), whereas alternative pathway is probably activated by using undiluted NHS in the complement activation assays (30).

Hemolytic assay is often used to assess the activation of the classical pathway by using sheep erythrocytes and the activation of alternative pathway by using rabbit erythrocytes (33). To confirm which complement pathway is inhibited by SntA, hemolytic assays have been performed using recombinant SntA protein. The results show that SntA could inhibit the hemolytic activity of SRBCs, but not of rabbit erythrocytes (**Figure 7**). This indicates that SntA could only inhibit the classical pathway. Furthermore, SntA is confirmed as a cell wall anchored protein that could interact with C1q in a dose-dependent manner (**Figures 4** and **5**). ELISA experiments also suggest that SntA could interact with the three C1q subunits C1qA, C1qB, and C1qC, respectively (**Figure 5**). It is very interesting whether this interaction results in the complement evasion. To this end, the competitive binding assays have been done by ELISAs reported previously (35). The results show that SntA could competitively bind C1q with IgG and Ag–Ab complex (**Figure 6**). Thus, SntA can interact with C1q and inhibit the hemolytic activity mediated by the classical pathway, consequently contribute to complement evasion. Similar inhibitory mechanism has been identified in other Gram-positive strain. *Streptococcus pneumococcal* (33) and *S. aureus* (23) infection. These bacterial proteins interact with C1q and specially inhibit the classical complement pathway, consequently facilitate *pneumococcal* complement escape (23, 33).

Concerning that C1q is a complex of 18 polypeptide chains consisting of six C1qA, C1qB, and C1qC subunits, the binding assays using each subunit could not properly reveal the real interactions between SntA and C1q although it has been reported that the globular heads of the recombinant subunits can assemble to form the high molecular weight oligomers (43). Interestingly, heme has also been demonstrated to inhibit the classical pathway through binding with C1q (35), and SntA is a heme-containing protein (24), it warrants further investigation whether the heme in SntA or SntA as a hemeprotein involves in the complement evasion. To this end, we are trying to obtain the recombinant SntA without heme and holo-form SntA although this is not an easy thing.

To look at whether SntA can directly activate complement pathways, the recombinant SntA protein is subjected to activate the complement C3. The results show that SntA could directly activate both classical and lectin pathways but not alternative pathway (**Figure 8**). This indicates that SntA could mediate complement evasion through complement consumption as well (33). In *S. pneumococcal*, PepO can activate complement through the classical and alternative pathway (33), while anther complement inhibitor phosphoglycerate kinase cannot activate complement (44). Research about complement evasion mediated by complement consumption is not much. The mechanism of this process warrants further study.

In conclusion, *S. suis* cell wall anchored heme-binding protein SntA mediates complement evasion by inhibition of complement activation and complement consumption. The inhibition of complement activation may be *via* the SntA–C1q interaction. These two complement evasion strategies may be crucial for the pathogenesis of *S. suis*.

ETHICS STATEMENT

Animal experiments were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and performed strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei Province, China. Venous blood samples were provided by healthy donors and collected in accordance with the approved guidelines. Approval was obtained from the Institutional Medical Ethics Committee of Huazhong Agricultural University and the healthy donors provided written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

The experiments were performed mainly by SD, some experiment material and data were provided by TX and LY, and some experiments were performed with the assistance of TX, QF, JZ, LC, and JL. SD analyzed the data. The study was conceived and designed by SD and RZ. SD and RZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01063/full#supplementary-material.

FIGURE S1 | Identification of *Streptococcus suis* strains SC-19, Δ*sntA*, and CΔ*sntA*. The *sntA* gene and its flanked genes (B9H01_10065 and B9H01_10075) were amplified by genome PCR **(A)** and reverse transcription-PCR **(B)** using primers SntA_F/R, 10065_F/R, and 10075_F/R, respectively. WT, wild-type strain SC-19; MT, *sntA* gene mutant strain Δ*sntA*; CM, complementary strain CΔ*sntA*.

FIGURE S2 | The growth abilities of *Streptococcus suis* strains SC-19, Δ*sntA*, and CΔ*sntA*. **(A)** Bacterial cell density was measured by spectrophotometer at 600 nm. **(B)** Bacterial colony forming unit count. Results were expressed from three independent experiments performed in triplicate.

FIGURE S3 | SntA possess anti-phagocytic activity. **(A)** Survival rate of *Streptococcus suis* in mouse blood. **(B)** Survival rate of *S. suis* in mouse active and inactivated serum. Results were expressed from three independent experiments performed in triplicate. The statistical significance was showed by asterisks (unpaired *t* test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, $p > 0.05$).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Plasminogen Activator Inhibitor 1 for Predicting Sepsis Severity and Mortality Outcomes: A Systematic Review and Meta-Analysis

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Objectives: Plasminogen activator inhibitor-1 (PAI-1), a crucial regulator of fibrinolysis, is increased in sepsis, but its values in predicting disease severity or mortality outcomes have been controversial. Therefore, we conducted a systematic review and meta-analysis of its predictive values in sepsis.

Methods: PubMed and Embase were searched until August 18, 2017 for studies that evaluated the relationships between PAI-1 levels and disease severity or mortality in sepsis.

Results: A total of 112 and 251 entries were retrieved from the databases, of which 18 studies were included in the final meta-analysis. A total of 4,467 patients (36% male, mean age: 62 years, mean follow-up duration: 36 days) were analyzed. PAI-1 levels were significantly higher in non-survivors than survivors [odds ratios (OR): 3.93, 95% confidence interval (CI): 2.31–6.67, $P < 0.0001$] and in patients with severe sepsis than in those less severe sepsis (OR: 3.26, 95% CI: 1.37–7.75, $P = 0.008$).

Conclusion: PAI-1 is a significant predictor of disease severity and all-cause mortality in sepsis. Although the predictive values of PAI-1 reached statistical significance, the clinical utility of PAI-1 in predicting outcomes will require carefully designed prospective trials.

Keywords: plasminogen activator inhibitor-1, sepsis, mortality, meta-analysis, systematic review

INTRODUCTION

Sepsis, defined as organ dysfunction caused by a dysregulated host response to infection, is a major cause of morbidity and mortality. Sepsis is often complicated by cardiovascular dysfunction, acute respiratory distress syndrome, and/or multiple organ failure (MOF), which leads to severe sepsis (1). In severe cases, septic shock occurs, which is characterized by profound

circulatory abnormalities requiring a vasopressor. Given its clinical importance, extensive investigation has been made on the use of accurate blood biomarkers to predict the severity and mortality in sepsis. Plasminogen activator inhibitor-1 (PAI-1), a crucial regulator of fibrinolysis, has been identified as a potential biomarker. PAI-1 inhibits plasminogen activator, a key enzyme involved in the cleavage of plasminogen to plasmin. This in turn inhibits fibrinolysis, leading to disseminated intravascular coagulation, circulatory hypoperfusion, and organ dysfunction in septic patients.

The major role of PAI-1 in sepsis has subsequently prompted the investigation of PAI-1 as a predictor of disease severity and mortality patients with sepsis. While many studies have explored the role of PAI-1 in this patient subset, few studies have observed differences in PAI-1 levels between sepsis survivors and non-survivors. Moreover, few studies have been conducted to compare PAI-1 levels between patients with septic shock/severe sepsis and those with sepsis alone. This study, therefore, aims to investigate any differences in PAI-1 levels between survivors and non-survivors of sepsis. To achieve this aim, a meta-analysis was performed to systematically evaluate the use of PAI-1 as a biomarker in predicting the severity and mortality of sepsis.

METHODS

Search Strategy, Inclusion, and Exclusion Criteria

PubMed and Embase were searched for studies that investigated the relationship between PAI-1 levels at different degrees of the sepsis syndrome, namely, sepsis, severe sepsis, and septic shock. The two databases were also searched for studies that compared PAI-1 levels in non-survivors and survivors of sepsis. The search terms used were "(mortality or hospitalization or severity) AND (plasminogen activator inhibitor-1 OR SERPINE1) AND sepsis," and the search period was from the beginning of the databases to August 18, 2017, without language restrictions. The inclusion criteria used were a prospective or retrospective cohort study design in humans, and PAI-1 values provided and related to the severity and disease mortality of sepsis patients.

Quality assessment of these studies included in our meta-analysis was performed using the Newcastle–Ottawa Quality Assessment Scale (NOS). The point score system evaluated the categories of study participant selection, comparability of the results, and quality of the outcomes. The following characteristics were assessed: (a) representativeness of the exposed cohort, (b) selection of the non-exposed cohort, (c) ascertainment of exposure, (d) demonstration that outcome of interest was not present at the start of study, (e) comparability of cohorts on the basis of the design or analysis, (f) assessment of outcomes, (g) follow-up period sufficiently long for outcomes to occur, and (h) adequacy of follow-up of cohorts. This scale varied from 0 to 9 stars, which indicated that studies were graded as poor quality if they met <5 criteria, fair if they met 5–7 criteria, and good if they met >8 criteria. The details of the NOS quality assessment are shown in Table S1 in Supplementary Material.

Data Extraction and Statistical Analysis

Using the lists of generated studies from PubMed and Embase, the articles were reviewed to check for compliance with the mentioned inclusion criteria. Out of all of the studies searched, 26 studies contained data suitable for analysis. The data from these articles were entered into a Microsoft Excel file by two independent reviewers. After further screening, 7 more articles were excluded, leaving a total of 19 suitable articles. For this study, the extracted data elements consist of (i) publication details: surname of first author, publication year; (ii) study design; (iii) follow-up duration; (iv) study endpoint; (v) the characteristics of the population including the sample size, gender, age, and cutoff point for PAI-1 levels where available. The mean PAI-1 values in patients with and without septic shock, as well as between non-survivors and survivors were extracted from each study and subsequently pooled into our meta-analysis. For the relationship between PAI-1 levels and mortality, we extracted and analyzed odds ratios (ORs) and 95% confidence intervals (CI) from each study. Hazard ratios were equated as ORs.

RESULTS

A flow diagram detailing the search strategy and study selection process is shown in **Figure 1**. Searches on PubMed and Embase yielded 112 and 251 publications, respectively, of which 19 studies met the inclusion criteria and were included in the final meta-analysis (2–19). The baseline characteristics of these studies are listed in **Table 1**. All studies apart from one were prospective studies. A total of 4,467 patients (36% male, mean age 62 years; mean follow-up duration of 36 days) were analyzed. In terms of assay type, eight articles used an ELISA kit, two used a sandwich ELISA assay, and two used a bead-based multiplexed immunoassay with the Human Cardiovascular-1 Panel. Other assays used included

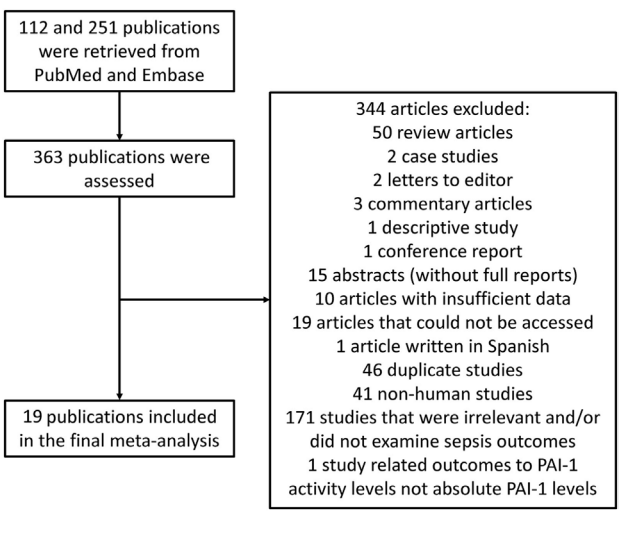

FIGURE 1 | Flow diagram of the study selection process for studies investigating the association between plasminogen activator inhibitor-1 (PAI-1) and outcomes in sepsis.

TABLE 1 | Characteristics of the 19 studies included in the meta-analysis.

First author/year	Population	Assay type	Sample size (*n*)	Age (years)	SD	No. of males	Follow-up (days)	Variables in multivariate model	Reference
Hoshino/2017	Patients with sepsis	STACIA bio-chemical automated clinical testing system (*Mitsubishi Chemical Medience Corp, Tokyo, Japan*)	186	/	/	115	28	Survivors and non-survivors of sepsis	(19)
Koyama/2014	Patients with sepsis in ICU	tPAI latex photometric immuno-assay (*Mitsubishi Chemical Medience, Tokyo, Japan*)	77	69.9	12.9	42	28	Patients with or without septic shock	(14)
Lorente/2014	ICU patients with severe sepsis	ELISA (*American Diagnostica, Inc., Stanford, CT, USA*)	260	59.4	/	170	30	Survivors and non-survivors of sepsis	(15)
Prakash/2015	Sepsis patients with organ failure	Activated human PAI-1 functional assay (*Molecular Innovations, Novi, Mich*)	77	66.2	/	41	3	Survivors of sepsis and septic shock	(18)
Panigada/2015	ICU patients with severe sepsis/ septic shock	ELISA kit (*HYPHEN Biomed France*)	40	61	/	24	1	Survivors of sepsis and patients without septic shock	(17)
Lorente/2014	ICU patients with severe sepsis	ELISA (*American Diagnostica, Inc., Stanford, CT, USA*)	295	60.7	/	100	8	Survivors and non-survivors of sepsis	(16)
Seki/2013	Patients with sepsis	PAI-1 latex immune agglutination test (*Mitsubishi Chemical Medicine Corporation, Tokyo, Japan*)	242	71	/	157	28	Survivors and non-survivors of sepsis, and patients with or without septic shock	(13)
Jalkanen/2012	Mechanically ventilated patients in ICU	ELISA (*BioVendor GmbH, Heidelberg, Germany*)	454	64	/	311	90	Survivors and non-survivors of sepsis	(12)
Perés Wingeyer/2011	Caucasian adults with/without sepsis in ICU	Chromogenic analysis (*Diagnostica Stago, France*)	166	/	/	94	/	Survivors and non-survivors of sepsis	(11)
Schuetz/2011	Patients in A&E with an episode of hypertension	Human cardiovascular-1 panel (*Millipore, Billerica, MA, USA*)	116	60	16.7	67	In hospital only	Patients with or without septic shock	(10)
Mauri/2010	Patients with severe sepsis or septic shock	Sandwich ELISA assay	90	61	15	56	90	Survivors and non-survivors of sepsis	(7)
Tsantes/2010	Patients with severe sepsis or septic shock	Automated coagulation analysis (*Behring Coagulation System, Marburg, Germany*) with reagents (*Berichrom PAI; Dade Behring, Milton Keynes, UK*), protocols from the manufacturer	73	62.5	18.1	46	90	Survivors and non-survivors of sepsis, and patients with or without septic shock	(20)
Shapiro/2010	Patients with clinical suspicion of infection	Human cardiovascular-panel (*Millipore, Billerica, MA, USA*)	221	58	19	106	3	Patients with or without septic shock	(8)
Wagenaar/2010	Patients with severe leptospirosis	ELISA (*HYPHEN Biomed France*)	52	45	/	37	5	Survivors and non-survivors of sepsis	(9)
Wiersinga/2008	Patients with culture proven septic melioidosis	ELISA (*TintElize PAI-1, Biopool, Umea, Sweden*)	34	52	/	26	/	Survivors and non-survivors of sepsis	(6)
Zeerleder/2005	Patients with sepsis and septic shock	ELISA (*Milan Analytica, La Roche, Switzerland*)	42	/	/	32	In hospital only	Patients with or without septic, and patients with septic shock	(5)
Prabhakaran/2003	Patients with acute lung injury and sepsis	Sandwich ELISA (*American Diagnostica, Greenwich, CT*)	26	/	/	14	In hospital only	Survivors of sepsis, and patients with septic shock	(3)
Okabayashi/2004	ICU patients	tPAIc test (*Kokusai-Shiyaku*)	1,789	60.9	6.9	48	/	Survivors and non-survivors of sepsis, and patients with or without septic shock	(4)
Raaphorst/2001	Patients with fever	ELISA	300	/	/	153	28	Survivors and non-survivors of sepsis, and patients with or without septic shock	(2)

the latex agglutination test, latex photometric immunoassay, and other chromogenic analyses (**Table 1**).

PAI-1 for Predicting Disease Severity or Mortality in Sepsis

Eleven studies compared PAI-1 levels between non-survivors and survivors in septic patients. Of these, seven studies reported significantly higher PAI-1 levels in septic patients who died compared with those who survived (**Figure 2**), whereas four studies reported no significant difference between both groups. Nevertheless, PAI-1 levels were significantly higher in non-survivors than survivors (OR: 3.93, 95% CI: 2.31–6.67, $P < 0.0001$). I^2 took a value of 83%, indicating presence of substantial heterogeneity. Sensitivity analysis excluding one study at a time did not significantly affect the pooled estimate (Figure S1 in Supplementary Material). A funnel plot of SE against the logarithm of odds ratio is shown in Figure S2 in Supplementary Material. Begg and Mazumdar rank correlation suggested no significant publication bias (Kendal's Tau value 0.2, $P = 0.39$). Egger's test demonstrated no significant asymmetry (intercept 3.5, t-value 1.7; $P = 0.12$).

Six studies compared PAI-1 levels between patients with severe sepsis and patients with less severe sepsis. Four studies reported significant higher levels in the case of severe sepsis whereas the remaining two studies reported no significant difference between the groups (**Figure 3**). PAI-1 levels were significantly higher in patients with severe sepsis than in those less severe sepsis (OR: 3.26, 95% CI: 1.37–7.75, $P = 0.008$). I^2 took a value of 88%, indicating that substantial heterogeneity was present. Sensitivity analysis excluding one study at a time did not significantly affect the pooled estimate (Figure S3 in Supplementary Material). A funnel plot of SE against the logarithm of odds ratio is shown in Figure S4 in Supplementary Material. Begg and Mazumdar rank correlation suggested no significant publication bias (Kendal's Tau value −0.07, $P = 0.85$). Egger's test demonstrated no significant asymmetry (intercept −4.2, t-value 1.2; $P = 0.28$).

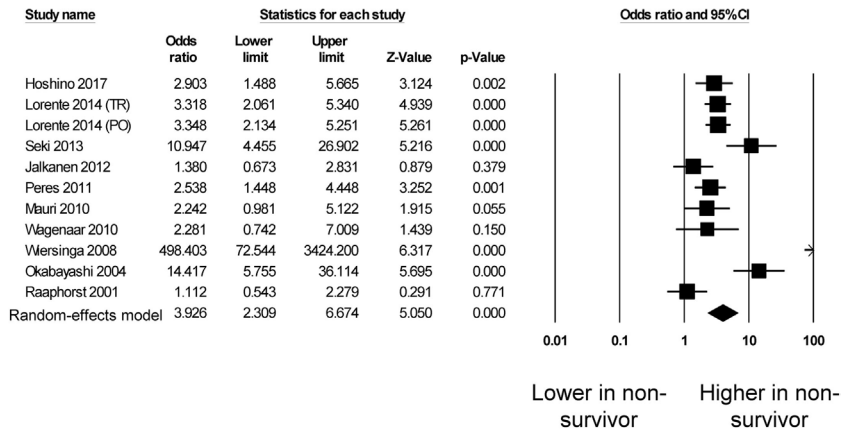

FIGURE 2 | Forest plot comparing plasminogen activator inhibitor-1 (PAI-1) levels between non-survivors and survivors of sepsis.

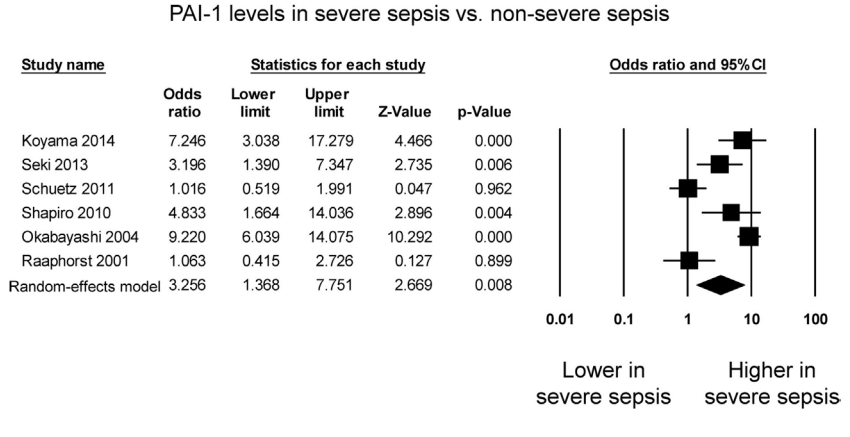

FIGURE 3 | Forest plot comparing plasminogen activator inhibitor-1 (PAI-1) levels between patients with severe sepsis and those with non-severe sepsis.

DISCUSSION

The main findings of this systematic review and meta-analysis are that higher level of PAI-1 are observed in patients with severe sepsis compared with less severe sepsis, and in non-survivors compared with survivors.

Sepsis is a potentially life-threatening condition and is often complicated by MOF involving excessive activation of coagulation (21). Regardless of the inciting pathogen (21, 22), endothelial activation and inflammation are found to be key in the initiation and continuation of host response, which primarily determine clinical outcomes within septic patients (22). Endothelial expression of tissue factor induced by a myriad of pro-inflammatory cytokines leads to subsequent systemic pro-coagulant response and activation of the anti-fibrinolytic pathway (23, 24). With the coagulation cascade inadequately contained by natural anti-coagulant reaction, the shift toward pro-coagulant state results in excessive thrombin generation, microvascular fibrin deposition, and consumption of clotting factors, a process termed disseminated intravascular coagulation, contributing to significant morbidity and mortality secondary to associated MOF and coagulopathy (23, 25). In severe sepsis, fibrinolysis and coagulation inhibitors are depleted, supported by a decrease in major coagulation inhibitors (e.g., PC and anti-thrombin) and plasminogen, along with an increase in PAI-1, our marker of interest, as shown in previous studies (21, 26–27).

Plasminogen activator inhibitor-1, as the primary inhibitor of both tissue-type and urokinase-type plasminogen activators (t-TPA and u-TPA), inhibits fibrinolysis and is associated with various vascular complications. Functionally active at its native conformation upon its release from endothelial cells, PAI-1 exerts inhibitory effects toward u-TPA and t-TPA with a functional half-life of 12 h at 37°C in normal conditions (28). In multiple clinical studies, the increase of PAI-1 was shown to correlate with sepsis severity and mortality (5, 29–31).

However, few studies have demonstrated significant differences of PAI-1 within different patient groups. The findings of this meta-analysis provide further support to the role of PAI-1 to guide clinical management. For example, those patients with initially high PAI-1 levels may be offered a more proactive management, including early ICU admission, initiation of fluid resuscitation, and inotropic support. Its diagnostic and prognostic value in combination with other plasma biomarkers needs to be elucidated in future studies (32–34).

Limitations

We must also acknowledge several limitations in the study. Substantial heterogeneity was observed in three meta-analyses with I^2 larger than 75%. A potential source of this heterogeneity could be due to the differences in study designs, collection times, and quantification assays for PAI-1. Funnel plots also showed significant asymmetry to suggest publication bias. Finally, some studies used univariate analysis, meaning that some data may have some confounding factors. Moreover, it is worth noting that not only is PAI-1 synthesized in a wide range of tissue but also takes different inter-convertible conformations, of which its stability and functional activity widely varies (35–37). Moreover, the influence of PAI-1 on pathophysiology of sepsis may differ across populations, affected by genetic and environmental factors (38–40). Hence, another limitation of using PAI-1 in sepsis lies in the difficulty in interpreting the biomarker level with its biochemical properties taken into account. Further studies on the effects of genetic polymorphism and environmental conditions on the biochemical profile of the biomarker can be conducted to establish the role of PAI-1 in different clinical conditions, particularly sepsis in which deranged homeostasis is evident.

CONCLUSION

A high level of PAI-1 distinguishes non-survivor from survivors for sepsis, consistent with its statistically significant correlation with all-cause mortality. Moreover, higher PAI-1 levels were observed in patients with severe sepsis than those with sepsis. Future prospective studies or trials should focus on the predictive power of combining PAI-1 and existing classic clinical severity scores such as SOFA and APACHE II for guiding clinical management of sepsis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, upon request to the corresponding authors.

AUTHOR CONTRIBUTIONS

TT and WW acquired, analyzed, and interpreted the data. LC, MG, MD, TL, LR, JH, MW, and MC provided important technical and intellectual contents. GT, JW, and SW conceived, designed, and oversaw this study. TT, WW, and GT drafted the manuscript, and all authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01218/full#supplementary-material.

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Arguing for Adaptive Clinical Trials in Sepsis

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Sepsis is life-threatening organ dysfunction due to dysregulated response to infection. Patients with sepsis exhibit wide heterogeneity stemming from genetic, molecular, and clinical factors as well as differences in pathogens, creating challenges for the development of effective treatments. Several gaps in knowledge also contribute: (i) biomarkers that identify patients likely to benefit from specific treatments are unknown; (ii) therapeutic dose and duration is often poorly understood; and (iii) short-term mortality, a common outcome measure, is frequently criticized for being insensitive. To date, the majority of sepsis trials use traditional design features, and have largely failed to identify new treatments with incremental benefit over standard of care. Traditional trials are also frequently conducted as part of a drug evaluation process that is segmented into several phases, each requiring separate trials, with a long time delay from inception through design and execution to incorporation of results into clinical practice. By contrast, adaptive clinical trial designs facilitate the evaluation of several candidate treatments simultaneously, learn from emergent discoveries during the course of the trial, and can be structured efficiently to lead to more timely conclusions compared to traditional trial designs. Adoption of new treatments in clinical practice can be accelerated if these trials are incorporated in electronic health records as part of a learning health system. In this review, we discuss challenges in the evaluation of treatments for sepsis, and explore potential benefits and weaknesses of recent advances in adaptive trial methodologies to address these challenges.

Keywords: sepsis, adaptive clinical trials, Bayesian statistics, platform trials, response adaptive randomization

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INTRODUCTION

Sepsis is a leading cause of critical illness and mortality globally (1, 2). It is a clinical syndrome and defined as a dysregulated host response to infection resulting in organ failure (3). This definition implies that different combinations of host and pathogen characteristics and interactions among them may lead to the same clinical picture. This inherent heterogeneity presents a major challenge to develop treatments and may be an important reason for recent neutral clinical trials. In addition, the traditional sequence to develop new therapeutics by pharmaceutical companies has several limitations that may exacerbate these challenges, and lead to prolonged evaluation periods of up to a decade before results can be operationalized (4). In this review, we discuss challenges in the evaluation of treatments for sepsis, and explore potential benefits of recent advances in adaptive trial methodologies to address these challenges.

CHALLENGES IN THE EVALUATION OF POTENTIAL TREATMENTS

Sepsis Is Extremely Heterogeneous

Several preclinical models suggest that sepsis results from a disproportionate pro-inflammatory response to infection. However, several clinical trials of anti-inflammatory agents in relatively broad patient populations were disappointing (5). Subsequent findings indicated that circulating levels of pro-inflammatory cytokines, such as IL-6 and TNF ranged from 8 to 1,550,000 pg/ml and 7 to 57,000 pg/ml, respectively, in patients with sepsis (6). Thus, the assumption that all patients would benefit equally from anti-inflammatory agents is unlikely. The host immune response during sepsis is complex and dynamic, involving excessive pro-inflammation and immunosuppression, often concomitantly. The balance of pro- and anti-inflammatory responses also evolves over the course of illness, and may be prognostic (7, 8).

A sustained dysregulated immune response may lead to profound alterations in the endothelium and surrounding tissues, including increased leukocyte adhesion, coagulation, and vasodilation, and loss of barrier function, hypoperfusion, and tissue hypoxemia (9). These disruptions and others lead to multisystem organ dysfunction, including acute kidney injury, neurologic complications, acute respiratory distress syndrome (ARDS), hepatic failure, and shock. However, the specific organ systems affected varies between patients. This organ failure is commonly seen against a backdrop of multimorbidity, a condition where two or more comorbidities may exist in a patient. Multimorbidity is observed in more than 30% of ICU patients (10) and further increases clinical heterogeneity. Taken as a whole, such complex variability stemming from these and other sources (e.g., host genetics and microbiologic factors) poses significant challenges to the efficient design and conduct of clinical trials, particularly those testing interventions targeting a specific mechanism. Some subsets of patients may benefit from such an intervention, while others may result in no benefit or even harm. For example, in simulated trials of anti-TNF studied *in silico*, benefit was observed after considering genetic and microbiological factors (11). However, most trials of anti-TNF in humans have ignored these factors.

Biomarkers That Predict Treatment Response Are Unknown Before Initiating a Trial

As discussed above, sepsis pathobiology is complex and evolving. As opposed to other diseases in which the natural history and risk factors are better understood, there are critical gaps in our knowledge of the potential markers of prognosis after sepsis (i.e., prognostic markers) and markers that predict treatment response (i.e., predictive markers). This distinction between prognostic and predictive markers is critical. For example, in a trial of anti-TNF, a group of patients with high levels of IL-6 had a higher mortality rate, but not a higher drug response, suggesting prognostic but not predictive utility as a biomarker (6). In scenarios in which a treatment's effect is meaningfully heterogeneous among the patient population, predictive markers are useful in explaining the sources of this heterogeneity of treatment effect (HTE). Without knowledge of the drivers of HTE, researchers and drug companies either ignore heterogeneity and enroll broadly or take a leap of faith and enroll narrowly based on suspected predictive biomarkers. If the biomarkers are not validated, the latter scenario could lead to exclusion of patients who would have benefited, or inclusion of patients who will not respond to the treatment.

While it is possible that single markers may be identified as sufficiently predictive by themselves, it is also possible that groups of genetic, metabolic, and/or clinical features may often occur together, forming groups or "phenotypes" of patients within the broad umbrella of sepsis that may have similar outcomes or treatment response rates. In sepsis and septic shock, phenotypes have recently been described that are associated with variable risk of mortality and would be considered "prognostic" (12, 13). In ARDS, phenotypes are described and found to respond variably to different fluid and ventilator management strategies (14). As sepsis phenotypes are further described, it will be important to allow for future trials to incorporate possible markers of HTE as efficiently as possible to avoid missing a true drug response by enrolling too broadly or too narrowly.

Optimal Therapeutic Dose and Duration Is Often Poorly Understood

The selection of treatment dosing and duration is often based on limited animal studies and small pharmacokinetic and pharmacodynamics studies in humans, with a focus on evaluating safety (15). Preclinical studies are commonly carried out in simple, often healthy and young, rodent models exposed to a specific endotoxin or using the cecal-ligation and puncture model. These models are criticized for bearing little relation to human sepsis, which occurs in older patients with significant comorbidities and who are often receiving adjuvant support. Furthermore, treatment in the rodent models has typically coincided with the timing of the infectious challenge; in humans, time between treatment and the initial infection is unknown and likely variable (5). The pitfalls of designing a phase 3 study based on an optimistic interpretation of preclinical and traditional early phase designs are suggested in the case of nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA). Promoted by encouraging preclinical data, a phase 2 safety study was conducted and showed a promising trend toward increased survival in the treated group (16). However, a subsequent phase 3 trial using a similar dosing strategy found increased mortality in the treatment arm overall, but significant survival benefit in a group with a relatively low exposure to the drug (17). The investigators concluded that this was likely a result of a relatively high exposure to the drug overall. Other studies suggest that in some cases dosing and duration may interact with each other in complex ways (11). Thus, the impact of dosing and duration, and their interaction, on sepsis outcomes is often poorly understood prior to initiation of phase 3 studies.

Short-Term Mortality Is Insensitive as a Primary Outcome

Short-term, all-cause mortality is a commonly used primary endpoint in phase 2 and 3 trials (18). However, short-term

mortality is declining (19, 20), and those who do not die early often die in the ensuing months or incur considerable morbidity (21, 22). Moreover, pure mortality endpoints are criticized for being insensitive measures of biologic activity, and thus poor tools for use in early phase trials for selection of dosing and duration. Recognizing these shortcomings, there is interest in identifying and validating short-term endpoints that are both more sensitive to treatment effects and good proxies for longer-term patient centered outcomes; one proposed alternative is combination of mortality and organ support duration (23).

NOVEL TRIAL METHODOLOGIES AND THEIR UTILITY FOR EVALUATION OF SEPSIS TREATMENTS

Most sepsis trials use traditional design features, in which all trial parameters are fixed for the duration of the study, including randomization ratios, sample sizes, number of treatment arms, and inclusion/exclusion criteria, among others. These designs have the advantage of optimal statistical power and internal validity when there are only two treatment arms, but this comes at the expense of flexibility should the investigator be interested in testing more complex and potentially numerous hypotheses (24).

By contrast, adaptive designs facilitate the evaluation of several research questions simultaneously and embrace the possibility of emergent discoveries during the course of the trial. During an adaptive trial, updates are made to the design parameters following interim analysis, often conducted several times before the trial's completion. The decision rules dictating which updates can be made are predetermined before initiation to avoid introducing bias (25–27). Below we discuss several features of adaptive designs that could theoretically be used to address key challenges in the evaluation of sepsis treatments (**Table 1**, section A). In addition, we discuss ways in which adaptive designs can potentially accelerate the drug evaluation process (summarized in the **Table 1**, section B).

Bayesian Response Adaptive Randomization (RAR)

Although it is optimal to conduct a two-arm trial using a traditional design, such approaches are inefficient when evaluating more than two treatments against control. Traditional designs typically use the frequentist statistical paradigm, where prior information about efficacy is utilized formally only in the design of a clinical trial (e.g., power calculations), but not during analyses. Alternatively, Bayesian statistical approaches provide a

TABLE 1 | Comparison of traditional and adaptive design features in addressing challenges of sepsis to the evaluation of beneficial treatments (section A), and ways in which common features of each influence the total time spent evaluating treatments (section B).

	Traditional trial designs	Adaptive trial designs
Section A. Challenges of sepsis to the evaluation of beneficial treatments		
High degree of disease heterogeneity as a result of variability among patients (e.g., biochemical and genetic), within patients (e.g., temporal dynamics of immune response), and infection characteristics (e.g., site and pathogen)	Usually test a single drug in a single predefined population; usually use 1:1 ratio of randomization to experimental and control arms	Response adaptive randomization (RAR) enables multiple drugs to be tested in potentially different subgroups based on projected mechanism of action, while preserving efficiency. Randomized, embedded, multifactorial adaptive platform enables recruitment from as broad a population base as possible, necessary for sample sizes to satisfy complex designs testing drugs in multiple subgroups
Specific biomarker profiles may predict treatment response, but the optimal sub-populations are unknown	Usually restricted to a single, predefined population, and as a result, enrollment criteria are often too broad or too narrow	Enrichment designs enable identification of the sub-population in which treatment response is optimized over the course of the trial
Optimal therapeutic dose and duration is often poorly understood	Due to trial inefficiencies, dose selection is often under-studied, potentially including under- or overdosing or using a dose that is constant despite variable patient requirements	Dose-finding designs can use RAR to study optimal dosing while preserving efficiency; can open higher dose arms as evidence in lower doses accumulates in support of efficacy and safety
Short-term mortality is the accepted clinical endpoint, but has been criticized insensitive to possible drug-related changes in morbidity and long-term mortality	A single primary endpoint is usually fixed before the start of the trial	Platform designs can be leveraged to evaluate proxy endpoints over time and feed this information back into the trial by incorporating it into the RAR algorithm
Section B. Major contributors to total time spent evaluating drugs		
Drug evaluation machinery	New study sites, protocols, and designs are usually established anew for each drug	Platform designs can include perpetually active master protocols that facilitate continuous use of existing trial resources on selection of drugs that is periodically updated
Number of drug arms tested simultaneously	Usually one. Traditional trials are most efficient when testing a single drug against placebo. Testing multiple drugs requires larger sample compared with adaptive designs	Multiple drugs can be compared to a single placebo arm while maintaining statistical efficiency using RAR, obviating the need for separate trials
Transitions between phases of the drug evaluation process	Phases are usually carried out one at a time, with sometimes long intervals in between for design and approval of the next phase	Seamless designs consolidate multiple phases into a single protocol that is designed, approved, and executed as a single trial. Sample sizes for component phases can be smaller if efficacy in the final phase is estimated using data from all phases

formal mathematical mechanism for combining prior and current information for use in the design, conduct, and final analysis stages of the trial (28). Adaptive trial designs have been developed under both frequentist and Bayesian paradigms (29).

The Bayesian paradigm provides a natural foundation for statistical tools utilized in many adaptive designs, which involve iteratively updating or "adapting" information gathered during the trial (25). One such tool, RAR, is used to increase efficiency when testing more than one treatment against control. Over the course of the trial, accumulating data are used to adjust the randomization probabilities to preferentially assign future patients to better-performing treatment arms (26). Typically, the first block of patients are randomized to each arm in equal proportion and randomization probabilities for subsequent blocks are calculated based on information accumulated prior to starting the block. A common way of executing RAR is by calculating the Bayesian predictive probability that a given treatment arm will be superior to control in the final analysis. This calculation often requires sophisticated computer simulations, but effectively integrates not only uncertainty about the true drug benefit based on data accumulated so far but also uncertainty about future data that have not yet been observed (30). Unless the predictive probability is too low (i.e., the arm should be dropped), or sufficiently high (the arm may "graduate" to the next phase of testing), the updated randomization probability for the next block of patients is proportional to the predictive probability of success for the treatment relative to control (27). Frequentist adaptive trial designs exist, but are not amenable to RAR.

Implementation of RAR could benefit sepsis trials in several ways. First, it would enable the study of multiple drugs simultaneously in a phase 2 trial, increasing the chances that at least one drug being tested will improve outcomes while reducing the time and costs needed to evaluate them individually by "learning" which ones are superior during the phase 2 trial and will have high likelihood of success in future phase 3 trials. The use of RAR instead of fixed randomization ratios underscores a focus on identifying the best-performing arm, instead of expending resources to rank all arms from worst to best performance. Second, instead of using RAR to assign patients to different arms, phase 2 adaptive trials could test different dosing and/or duration strategies for a single drug to better inform the optimal treatment strategy for phase 3 testing. This approach was implemented in SEPSIS-ACT, an adaptive trial of selepressin dosing strategies in adults with septic shock (31). In this trial, RAR was used to allocate patients to three dose levels until predefined checkpoints for safety and efficacy were triggered. If necessary, a fourth could be introduced based on response to the three doses.

Adaptive Enrichment Designs

Often there is interest in a variety of drugs as well as identifying potential sub-populations within which the drugs are most effective. In a traditional enrichment design, randomization is simply limited to patients with a specific biomarker profile known to be predictive of treatment response. However, we may not know which patient groups may benefit the most from a treatment in sepsis. Using adaptive trial methodologies, it is possible to incorporate putative predictive biomarkers to "learn" the optimal biomarker profile in the case that a meaningful underlying HTE exists (32).

In one approach, the RAR algorithm is used in an adaptive platform trial (see below) of several drugs, where patients are categorized into several candidate predictive biomarker strata before randomization. In the BATTLE I trial, non-small cell lung cancer patients were classified into four candidate strata defined by genomic and expression markers before being randomized to one of four drug regimens. Separately for each stratum, the RAR weights were adjusted as data accumulated to favor assignment of drugs with higher within-stratum response rates (33). The results from BATTLE 1 both confirmed pre-specified hypotheses of treatment efficacy in the presence of individual markers related to the treatments' mechanism of action, and also suggested new treatment–biomarker interactions (34).

An alternative enrichment approach allows for more flexibility in the scenario where candidate predictive biomarkers have not been identified. In this framework, the optimal target population for the experimental treatment is adaptively learned and estimated as a function of baseline covariates (35, 36). Such designs could be useful, for example, to identify the optimal threshold value of a predictive biomarker to use for splitting the patient population into responsive and non-responsive strata (35).

While underlying drug response strata may exist and may be delineated by putative biomarkers, demonstrating this may be difficult in scenarios where treatment effects are relatively homogeneous or when the overall treatment effect is small. Thus, adaptive enrichment strategies present a potential advantage by incorporating mechanisms to adapt to the presence of HTE if evidence for it mounts over the course of the trial.

Seamless Designs

The traditional drug evaluation pipeline is usually segmented into several phases, each involving a brand new trial. To streamline this process and reduce associated time and costs, a number of designs have been developed that combine multiple phases into a single trial, including several within the Bayesian adaptive framework (37). This approach was implemented into the SEPSIS-ACT trial, an adaptive phase 2b/3 trial (31). Part 1 of SEPSIS-ACT uses RAR to "learn" which dosing regimen leads to greatest efficacy, while part 2 is a confirmation stage randomizing 1,000 new patients equally between control and a single treatment arm featuring the dose selected in part 1. Early stopping of part 1 would occur if enough evidence had been obtained to select an optimal dose; otherwise enrollment would continue up to a predetermined maximum sample size. To further increase efficiency, all data from parts 1 and 2 are incorporated in the final analysis. Thus, adaptive seamless designs may lead to more timely conclusions, an advantage which is just as useful for patients and researchers in the case of a truly effective treatment as for a truly ineffective one.

Adaptive Platform Trials

There is significant effort required to launch a trial, including preparing trial documents, identifying sites, initiating the trial, and obtaining regulatory approval. As the name suggests, the adaptive platform trial is capable of being a *platform* for testing experimental treatments in a perpetual manner *via* a common

master protocol, by dropping treatments lacking efficiency and adding new treatments going into the future. They are able to incorporate several design features of adaptive trials, such as RAR, biomarker enrichment, and seamless transitioning, often all in the same design. Currently there are platform trials enrolling patients in oncology (38, 39), infectious diseases (40), neurology (41), and intensive care (42).

In a platform trial, the feedback loop involving collecting data, updating the Bayesian statistical model and updating RAR weights is modified to enable new arms to be added, and old arms to either be dropped or "graduate" to the next phase of testing. A schematic of the platform trial design is shown in the **Figure 1**. I-SPY 2 is a phase 2 platform trial in women with locally advanced breast cancer, and out of eight treatments entered into the trial loop so far, two are considered promising enough to "graduate" out of the trial (43, 44). GBM-AGILE, an inferentially seamless phase 2/3 platform trial in glioblastoma, was designed so that "graduating" treatments are seamlessly transitioned into phase 3 confirmatory testing (39). Both I-SPY 2 and GBM-AGILE incorporate enrichment biomarkers hypothesized to be predictive of response for specific treatment arms.

There is considerable pressure to identify short-term endpoints that can be used to speed the evaluation of treatments by accurately predicting treatment response in terms of a gold-standard endpoint, such as long-term mortality. I-SPY 2 and GBM-AGILE both leverage accumulating data in a continually updated Bayesian longitudinal model to generate predictions of the long-term endpoints for use in updating RAR weights (26, 30, 45). In I-SPY 1, for example, it was found that MRI outcomes within the first few weeks following treatment predicted pathological complete response (pCR) at the time of surgery, about 5 months after treatment (46). Thus, short-term MRI data are used to predict pCR in I-SPY 2 for the purposes of updating the RAR weights months before the actual pCR data are observed, increasing efficiency (38, 46). In GBM-AGILE, useful proxy endpoints are being learned and vetted within the trial, and potentially different endpoints are expected to capture the effects of different treatments (39, 45).

The incorporation of Bayesian models in adaptive sepsis trials could theoretically provide a means of evaluating how changes in short-term endpoints (e.g., 28-day organ failure free days) due to treatment correspond with changes in new long-term endpoints such as quality-adjusted life-years at 6 months.

Embedded, Multifactorial Adaptive Platform Trials

Randomized, embedded, multifactorial adaptive platform (REMAP) trials utilize all of the features of a perpetual adaptive

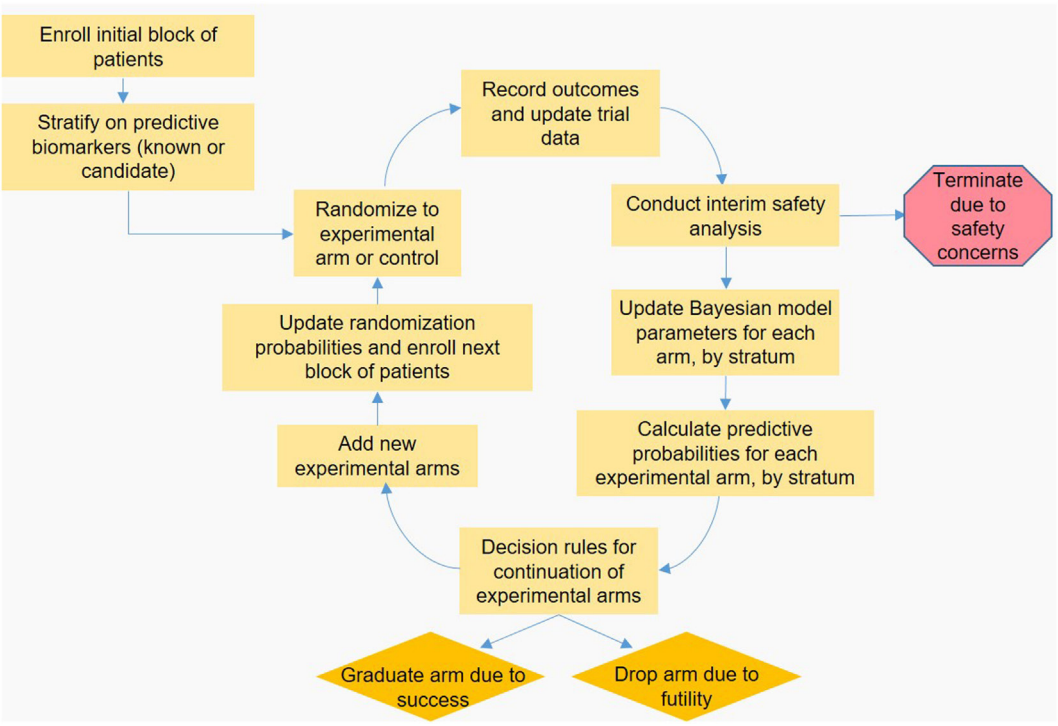

FIGURE 1 | Schematic representation of a hypothetical adaptive platform trial. An initial block of patients is stratified based on known or candidate predictive biomarkers, and then randomized to an experimental or control arm. Once a predefined number of patients is enrolled, outcomes are observed and the data are input to the Bayesian statistical model by arm and stratum, which is used to calculate the predictive probabilities (PP) that each experimental arm will be superior to control in the final analysis. These PP are checked against predefined decision boundaries established so that arms with poor probability of success are dropped, and arms with high probability of success "graduate" to the next phase of testing. Arms with PP that do not require dropping or graduation continue enrolling subjects; arms that are removed may be replaced by new experimental treatments, accrual permitting. Finally, the PP are used to update randomization probabilities used for the next block of patients to be enrolled, and the feedback loop begins anew.

platform trials like I-SPY 2 or GBM-AGILE, the key distinction being that a REMAP trial is executed directly within clinical practice through the electronic medical record [EMR (47)]. A key advantage of embedding trials in clinical care is to create a "learning health system" by enrolling most eligible participants, which increases the speed with which new knowledge is generated and implemented in routine clinical care. In addition, it maximizes internal and external validity, and minimizes operational complexity at the bedside (there is no need to distinguish between trial and non-trial patients, because all patients are trial patients). While screening and recruitment for a REMAP can be conducted by research staff, it is not intended that recruitment should be dependent on research staff because they are typically present during office hours. Thus, REMAP trials may reduce costs.

Most REMAP trials determine the effectiveness of various treatments used in routine clinical care but in a randomized setting. An example is the REMAP-CAP trial being conducted in patients with community-acquired pneumonia severe enough to be admitted to an intensive care unit (42). Upon submitting treatment orders through the EMR, the clinician can choose to instead be randomized the most promising treatment regimens (utilizing RAR weights). To capture the clinical complexity of treatment plans involving combinations of treatments, patients are randomized to multiple sets of treatments within different domains. For instance, different antibiotic regimens and immunomodulatory drugs may be compared in patients with severe pneumonia. This complexity not only requires the use of complex statistical models incorporating interaction terms but also increases penetration of the trial through its embedment in the EMR. Like I-SPY 2 and GMB-AGILE, REMAP-CAP is designed to be perpetual, and as such include mechanisms to incorporate control arms that are updated to incorporate any newly discovered standards of care, e.g., resulting from the trial itself. Although not implemented yet in REMAP-CAP, there is also the capability to incorporate enrichment biomarkers as well. Investigators have recently been funded to launch REMAP initiatives in other conditions, including anti-microbial resistance, cystic fibrosis, hepatitis C, and operative stress in the elderly.

INHERENT CHALLENGES OF ADAPTIVE DESIGNS

Adaptive designs may have many promising features for future trials in sepsis, but they also come with their own challenges. Statistical models and the exploration of operating characteristics are complex and simulation-intensive. Selection of potential trial trajectories is especially important during the simulation process, as an overly narrow set of scenarios may lead researchers to fail to understand the consequences of their design choices. A range of alternative trajectories should be explored by varying important simulation parameters, for example: choice of Bayesian prior (assuming a Bayesian model is used); choice of data model; underlying treatment effects for each arm; proportions of patients within subtypes; accrual rates; and others. For designs relying on simulation-based outcome metrics such as the predictive probability of success, failure to explore sensitivity to modeling assumptions may pose risk to future patients, e.g., if drugs are erroneously selected for graduation. In designs considering many subgroups or combinations of drugs, careful consideration must be taken to craft statistical models with only the necessary complexity to preserve statistical power. In addition, the benefits to trial efficiency of periodically updating RAR weights are somewhat dependent on patient accrual rates; if accrual of data occurs faster compared to ideal updating time, RAR updates may occur based on incomplete follow-up. The complexity of these designs also creates difficulties communicating their features to important stakeholders who may be unfamiliar with them, such as funding agencies, institutional review boards, patients, research journals, and clinicians.

CONCLUSION

Many studies employing Bayesian adaptive trial designs have already led to promising discoveries in several diseases, including the identification of two promising candidate drugs in breast cancer. These studies leverage several key features of adaptive designs, including RAR, enrichment methods, seamless transitioning between trial phases, perpetual platforms for ongoing evaluation of candidate treatments, integration within the EMR, and others. These features are well suited to address the many challenges presented by complex, heterogeneous diseases, yet are rarely utilized in sepsis. Adoption of these designs may aid in the efficient identification of promising treatments for sepsis.

AUTHOR CONTRIBUTIONS

All authors contributed to the editing and preparation of this manuscript.

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IRF5 Is a Key Regulator of Macrophage Response to Lipopolysaccharide in Newborns

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Infections are a leading cause of mortality and morbidity in newborns. The high susceptibility of newborns to infection has been associated with a limited capacity to mount protective immune responses. Monocytes and macrophages are involved in the initiation, amplification, and termination of immune responses. Depending on cues received from their environment, monocytes differentiate into M1 or M2 macrophages with proinflammatory or anti-inflammatory and tissue repair properties, respectively. The purpose of this study was to characterize differences in monocyte to macrophage differentiation and polarization between newborns and adults. Monocytes from umbilical cord blood of healthy term newborns and from peripheral blood of adult healthy subjects were exposed to GM-CSF or M-CSF to induce M1 or M2 macrophages. Newborn monocytes differentiated into M1 and M2 macrophages with similar morphology and expression of differentiation/polarization markers as adult monocytes, with the exception of CD163 that was expressed at sevenfold higher levels in newborn compared to adult M1 macrophages. Upon TLR4 stimulation, newborn M1 macrophages produced threefold to sixfold lower levels of TNF than adult macrophages, while production of IL-1-β, IL-6, IL-8, IL-10, and IL-23 was at similar levels as in adults. Nuclear levels of IRF5, a transcription factor involved in M1 polarization, were markedly reduced in newborns, whereas the NF-κB and MAP kinase pathways were not altered. In line with a functional role for IRF5, adenoviral-mediated IRF5 overexpression in newborn M1 macrophages restored lipopolysaccharide-induced TNF production. Altogether, these data highlight a distinct immune response of newborn macrophages and identify IRF5 as a key regulator of macrophage TNF response in newborns.

Keywords: M1/M2 macrophages, newborns, innate immunity, interferon regulatory factor 5, monocytes, GM-CSF, LPS, tumor necrosis factor

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INTRODUCTION

Despite advances in perinatal care, neonatal infections remain a leading cause of mortality and morbidity worldwide (1–3). The high susceptibility to infection during the neonatal period has been linked to a developing immune system with a limited capacity to mount protective immune responses (4). Indeed, neonatal monocytes and dendritic cells (DCs) exposed to microbial products release reduced amounts of the proinflammatory and T_H1-polarizing cytokines TNF, IFNγ, IL-1β,

and IL-12p70 than adult cells, but similar or even higher levels of the T_H17-polarizing and anti-inflammatory cytokines IL-6, IL-10, and IL-23 (5–7). Yet, uncontrolled inflammatory responses contribute to the pathogenesis of sepsis and septic shock and other conditions associated with adverse outcomes in newborns, such as necrotizing enterocolitis, bronchopulmonary dysplasia, and periventricular leucomalacia (8–11). Attempts at improving the outcome of neonatal sepsis through immune enhancing therapies including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte transfusions, and intravenous immunoglobulins have only yielded a limited benefit (12–14). This underscores our incomplete understanding of how newborns respond to infections, and the need for new therapeutic approaches.

Tissue-resident macrophages are sentinel innate immune cells that display a spectrum of functions and produce a panel of cytokines that orchestrate innate and adaptive immune responses (15, 16). Macrophage activation and function are influenced by signals received from the local environment (17). The functional plasticity of macrophages has given rise to the notion of macrophage polarization, ranging from classically activated proinflammatory M1 macrophages to alternatively activated pro-resolving/anti-inflammatory M2 macrophages (18). The differentiation of monocytes into M1 macrophages is induced by GM-CSF, IFNγ, TNF, and bacterial lipopolysaccharide (LPS) (19–21). M1 macrophages are potent phagocytic cells that produce microbicidal molecules such as reactive oxygen and nitrogen species (ROS and NO) and TNF, IL-1β, IL-6, IL-12p70, and IL-23 (22, 23). In contrast, M-CSF, IL-4, IL-10, IL-13, adenosine, and steroid hormones induce the differentiation of monocytes into M2 macrophages (24). M2 macrophages are involved in resolving inflammation and promote tissue repair and homeostasis. M2 macrophages are characterized by the expression of scavenger receptors (CD36, CD163) and the production of high levels of IL-10 and low levels of TNF, IL-12p70, IL-23, ROS, and NO (25–27).

Monocyte to macrophage differentiation is controlled by the Janus-kinase/signal transducer and activator of transcription (JAK/STAT), MAP kinase (MAPK), and NF-κB pathways (28–31). These pathways activate suppressor of cytokine signaling (SOCS) and interferon regulatory factors (IRFs), leading to M1/M2 macrophage polarization (32, 33). In adults, IRF5, a downstream target of GM-CSF receptor (GM-CSFR), plays a critical role in driving macrophage polarization toward the M1 phenotype (23). However, the response of newborn macrophages to environmental signals driving M1 and M2 polarization and production of proinflammatory and anti-inflammatory cytokines is unknown.

Here, we report that in primary human monocytes exposed to GM-CSF, IRF5 was activated to a lower extent in newborns compared to adults during differentiation into M1 macrophages. Upon TLR4 stimulation, newborn M1 macrophages secreted lower levels of TNF compared to adult macrophages, while the production of other cytokines was not affected. Overexpression of IRF5 in newborn macrophages restored TNF production, suggesting a key role of IRF5 in shaping the distinct immune response of newborn macrophages.

MATERIALS AND METHODS

Subjects and Source of Blood Samples

Umbilical cord blood was collected after delivery of the placenta of 91 healthy term neonates. Peripheral blood was obtained from 71 healthy adult volunteers (age 18–65 years). Monocytes and macrophages from the same subjects (20 newborns and 20 adults) were used for the experiments reported in **Figures 1B** and **2A,C,D**. Macrophages from the same subjects (10 newborns and 10 adults) were used for the experiments reported in **Figures 2B** and **5A–J**. Different sets of newborn and adult donors were used for every other **Figures 1A,C**, **3A,B**, **4A,B**, **5A–J**, and **6A–C**. Blood was collected in heparinized tubes (10 U/ml). Our study was approved by the Cantonal Human Research Ethics Committee of Vaud (CER-VD, Lausanne, Switzerland).

Cells and Reagents

Mononuclear cells were isolated by Ficoll Hypaque (GE Healthcare) gradient density centrifugation. Monocytes were extracted from blood mononuclear cells by positive selection using magnetic microbeads coupled to anti-CD14 antibodies (Miltenyi Biotec) (34–36). Purity assessed by flow cytometry was >95%. Viability determined by trypan blue exclusion was >95%. Monocytes were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FCS (GE Healthcare) and GM-CSF (50 ng/ml) (Peprotech) or M-CSF (50 ng/ml) (Peprotech) for 1 week to induce M1 or M2 macrophages, respectively. Ultrapure *E. coli* O111:B4 LPS was purchased from List Biological Laboratories. Polyclonal and monoclonal antibodies (pAbs and mAbs) used for flow cytometry, Western blotting and cytometry by time of flight (CyTOF) are described in Table S1 in Supplementary Material. Unless specified otherwise, all other reagents were obtained from Sigma-Aldrich.

RNA Analyses

RNA was extracted, reverse transcribed, and used in real-time PCR as described (37). The primers (5′–3′ sequences, sense and antisense) used for amplification were: HPRT, GAA CGTCTTGCTCGAGATGTG and CCAGCAGGTCAGCAAA GAATT; CD14, CGCCCTGAACTCCCTCAAT and CTTGG CTGGCAGTCCTTTAGG; TLR4, AGTTTCCTGCAATGGAT CAAGG and CTGCTTATCTGAAGGTGTTGCAC; CD64, TG CCACAGAGGATGGAAATG and CTGGAGGCCAAGCAC TTGA; IRF4, AATCCTCGTGAAGGAGCTGA and GTAGAT CGTGCTCTGGCACA; SOCS2, GGATGGTACTGGGGAAGT ATGACTG and AGTCGATCAGATGAACCACACTGTC; SOCS3, GCTCCAAGAGCGAGTACCAG and CTGTCGCGG ATCAGAAAGGT; TNF, CAGAGGGCCTGTACCTCATC and GGAAGACCCCTCCCAGATAG. Gene-specific expression was normalized to the expression of HPRT and was expressed in arbitrary units (A.U.).

Flow Cytometry Analyses

Mononuclear cells and macrophages were stained using mAbs (Table S1 in Supplementary Material) as described (38). Thirty thousand events were acquired with a LSR-II flow cytometer

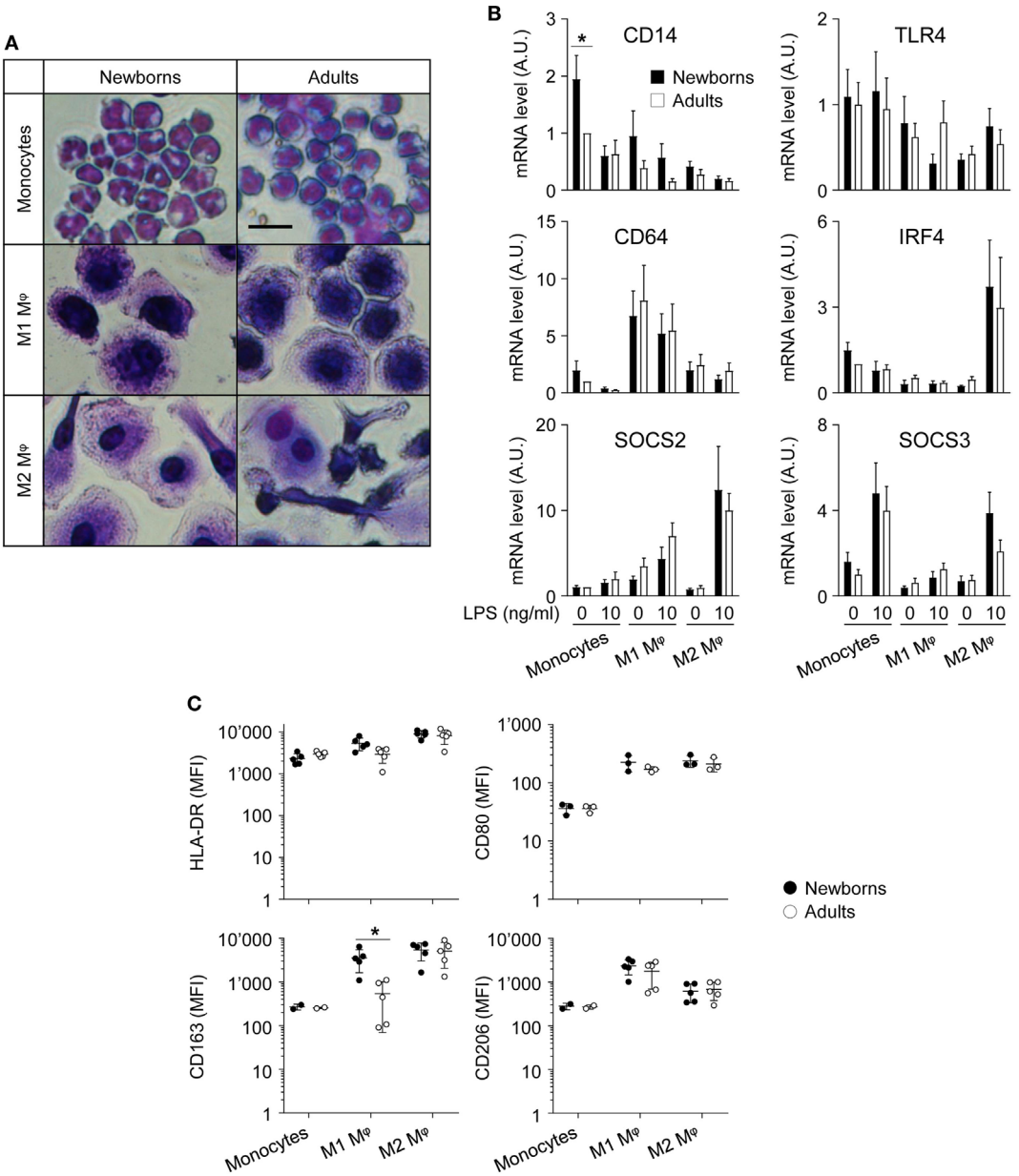

FIGURE 1 | Adult and newborn monocytes differentiate into M1 or M2 macrophages following exposure to GM-CSF or M-CSF. Freshly isolated monocytes were cultured for 7 days with GM-CSF and M-CSF (50 ng/ml) to induce M1 and M2 macrophages. **(A)** Hematoxylin and eosin staining of monocytes and M1 and M2 macrophages. Scale bar = 30 μm. Data are representative of results obtained from five newborns and five adults. **(B)** CD14, TLR4, CD64, SOCS2, and IRF4 mRNA expression levels in newborn (black bars) and adult (white bars) monocytes and M1 and M2 macrophages were measured by RT-PCR. Data are means ± SEM from eight newborns and eight adults. **(C)** HLA-DR, CD80, CD163, and CD206 mean fluorescence intensity in newborn (black circles) and adult (white circles) monocytes and M1 and M2 macrophages was analyzed by flow cytometry in three to five healthy newborns and adults. Each dot represents one subject. CD163 and CD206 were not detected in monocytes from 2/5 newborns and 2/5 adults. Means ± SEM are presented. *$P < 0.05$.

(BD Biosciences). Data were analyzed using the BD FACSDiva™ software (BD Biosciences).

Cytokine Measurements

Cytokine concentrations in cell-culture supernatants were measured by ELISA (BD Biosciences, for TNF, IFNγ, IL-1β, IL-6, IL-8, and IL-10) or by the Luminex technology (Affymetrix eBioscience, for IL-12p70, IL-20, IL-23, and IL-27).

CyTOF Analyses

Monocytes were exposed for 0, 15, 30, 60, or 120 min to GM-CSF and fixed with formaldehyde at a final concentration of 1.5%.

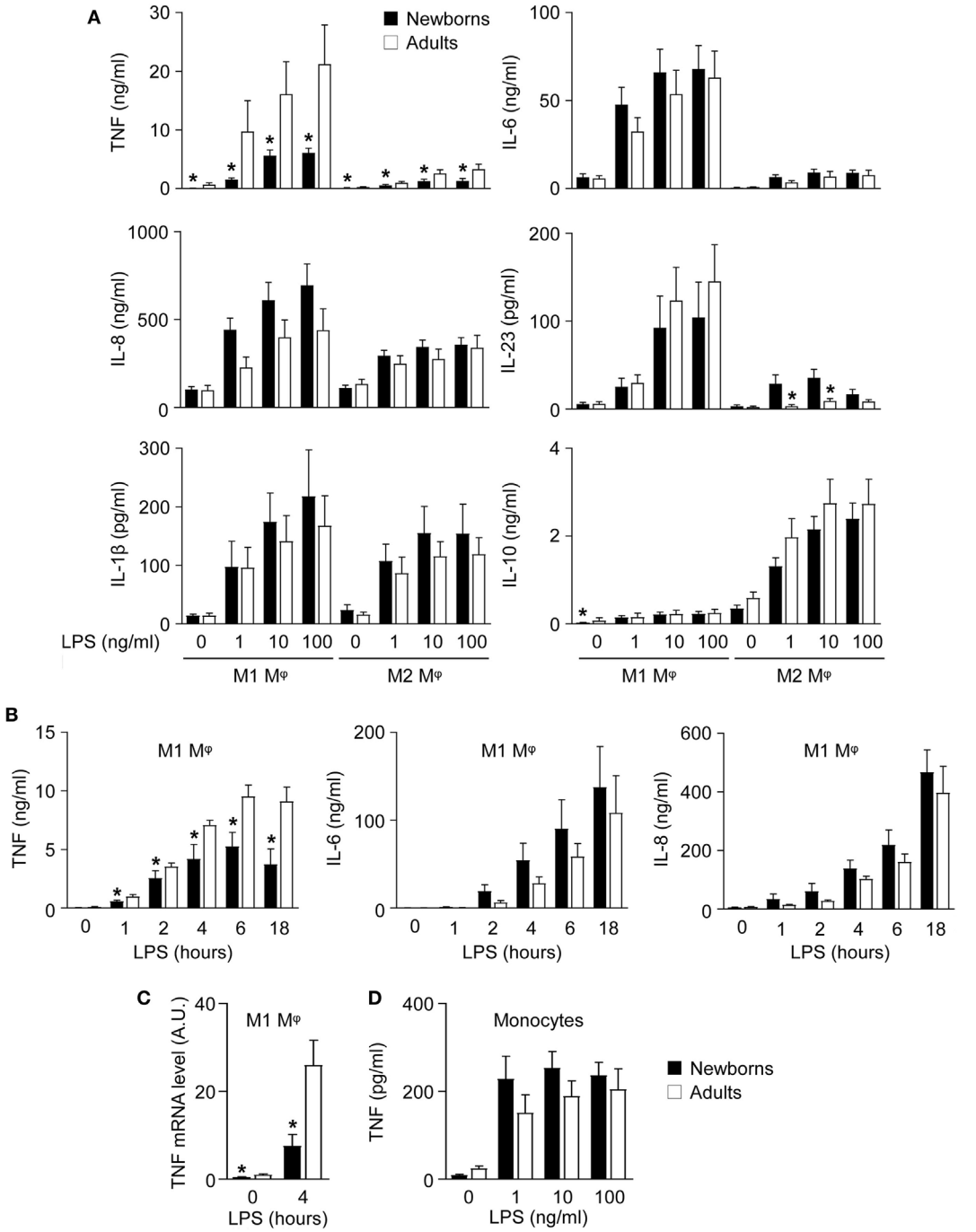

FIGURE 2 | Newborn M1 macrophages secrete reduced amounts of TNF after lipopolysaccharide (LPS) stimulation. Newborn (black bars) and adult (white bars) monocytes were cultured for 7 days with GM-CSF (50 ng/ml) and with M-CSF (50 ng/ml) to induce M1 **(A–C)** and M2 macrophages **(A)**. Macrophages **(A–C)** and monocytes **(D)** were stimulated with 0–100 ng/ml LPS. **(A)** Cytokine levels were measured in cell culture supernatants collected after 20 h. Data are means ± SEM from 20 (TNF, IL-6, IL-8, IL-1β, and IL-10) or 10 (IL-23) newborn and adult subjects. **(B)** TNF, IL-6, and IL-8 levels were measured in cell culture supernatants collected after 0–18 h. Data are means ± SEM from eight newborns and six adults. **(C)** TNF mRNA expression levels in M1 macrophages exposed for 0 and 4 h to 100 ng/ml LPS were measured by RT-PCR. Data are means ± SEM from 10 newborns and 9 adults. **(D)** TNF concentrations in cell culture supernatants of monocytes exposed for 20 h to 0–100 ng/ml LPS. Data are means ± SEM from 20 newborns and 10 adults. *$P < 0.05$.

Cells were stained using an anti-CD14 mAb conjugated with the Fluidigm MaxPar conjugation kit (Fludigm). Cells were washed with Cell Staining Media and PBS, fixed with 2% formaldehyde, and bar-coded using Scn-Bn-EDTA-palladium barcode reagents (39). After barcoding, cells were pooled, permeabilized for 30 min at −20°C using 100% methanol, washed twice with 6 ml

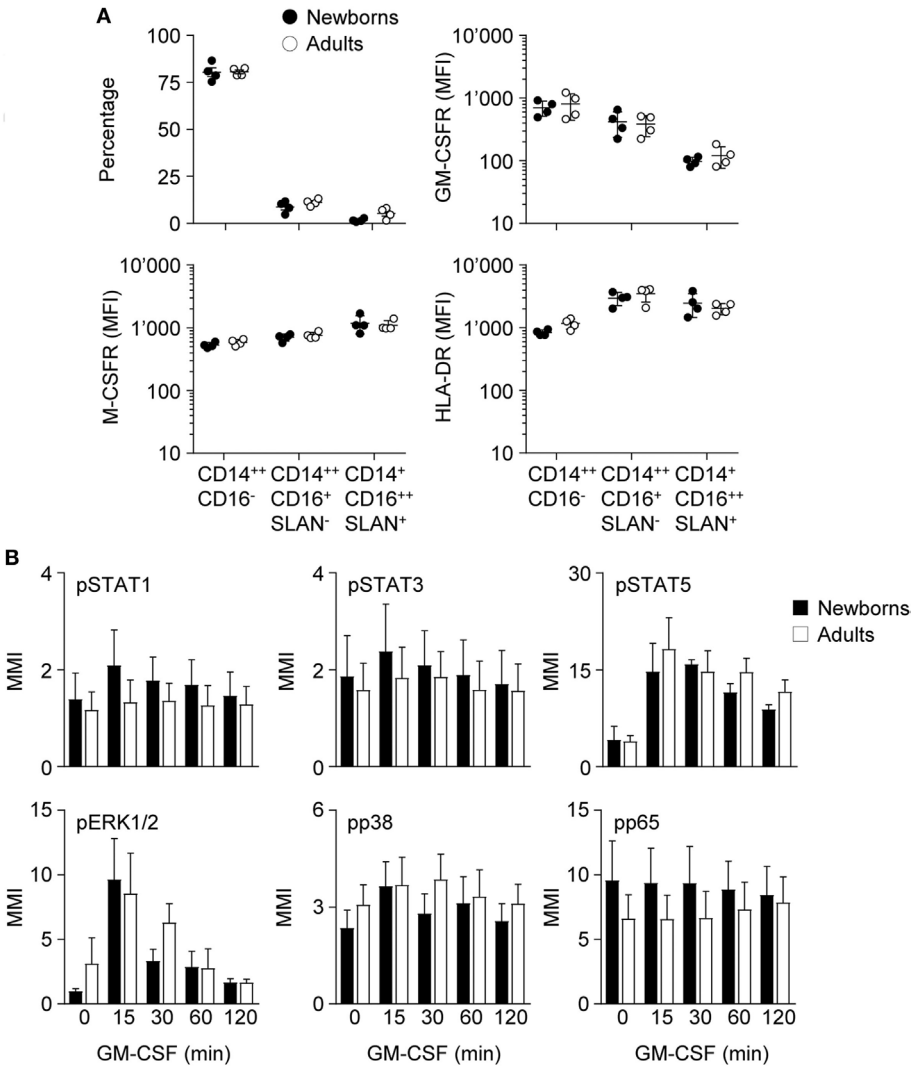

FIGURE 3 | Proportions of monocyte subpopulations, expression of GM-CSF receptor (GM-CSFR), M-CSFR and HLA-DR and activation of intracellular signaling pathways by GM-CSF in newborn and adult monocytes. **(A)** Percentages of classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}SLAN^{-}$, and non-classical $CD14^{+}CD16^{++}SLAN^{+}$ subpopulations and mean fluorescence intensity of GM-CSFR, M-CSFR, and HLA-DR in newborn (black circles) and adult (white circles) monocytes were determined by flow cytometry. Each dot represents one healthy subject. Means ± SEM are depicted. **(B)** Nuclear levels pSTAT1, pSTAT3, pSTAT5, pp38, pERK, and pNF-κBp65 in newborn (black bars) and adult (white bars) monocytes exposed for 15–120 min to 50 ng/ml GM-CSF were analyzed by CyTOF. Mean magnetic intensities were determined. Data are means ± SEM from six newborns and six adults.

Cell Staining Media containing 0.3% saponin, and incubated for 30 min with mAbs directed against intracellular targets. Finally, cells were incubated overnight at 4°C in intercalation solution (PBS, 0.3% saponin, 1% formaldehyde, 125 nM Cell-ID Intercalator-Ir, Fluidigm) before acquisition on a CyTOF 1 upgraded to a CyTOF 2. Individual data files were concatenated, normalized, and deconvoluted as described (40) and were analyzed using Cytobank (Cytobank Inc.).

Western Blot Analyses

Whole cellular extracts and cytoplasmic and nuclear extracts were prepared as described previously (34). Equal amounts of protein extracts were electrophoresed through SDS/PAGE. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were incubated with Abs (listed in Table S1 in Supplementary Material) directed against NF-κBp65, IκBα, total and phosphorylated p38, ERK1/2, and JNK MAPKs, MAP kinase phosphatase-1 (MKP-1), IRF5, IRF8, total and phosphorylated Akt, GAPDH, β-actin, and TATA-binding protein. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary Abs (Pierce Biotechnology Inc.). Signals were revealed using enhanced chemiluminescence detection (GE Healthcare). Images were recorded using a Fusion Fx system (Viber Lourmat).

Adenovirus Transduction

IRF5-encoding and control empty adenoviral vectors (Applied Biochemical Materials Inc.) were amplified in HEK-293 cells (ATCC

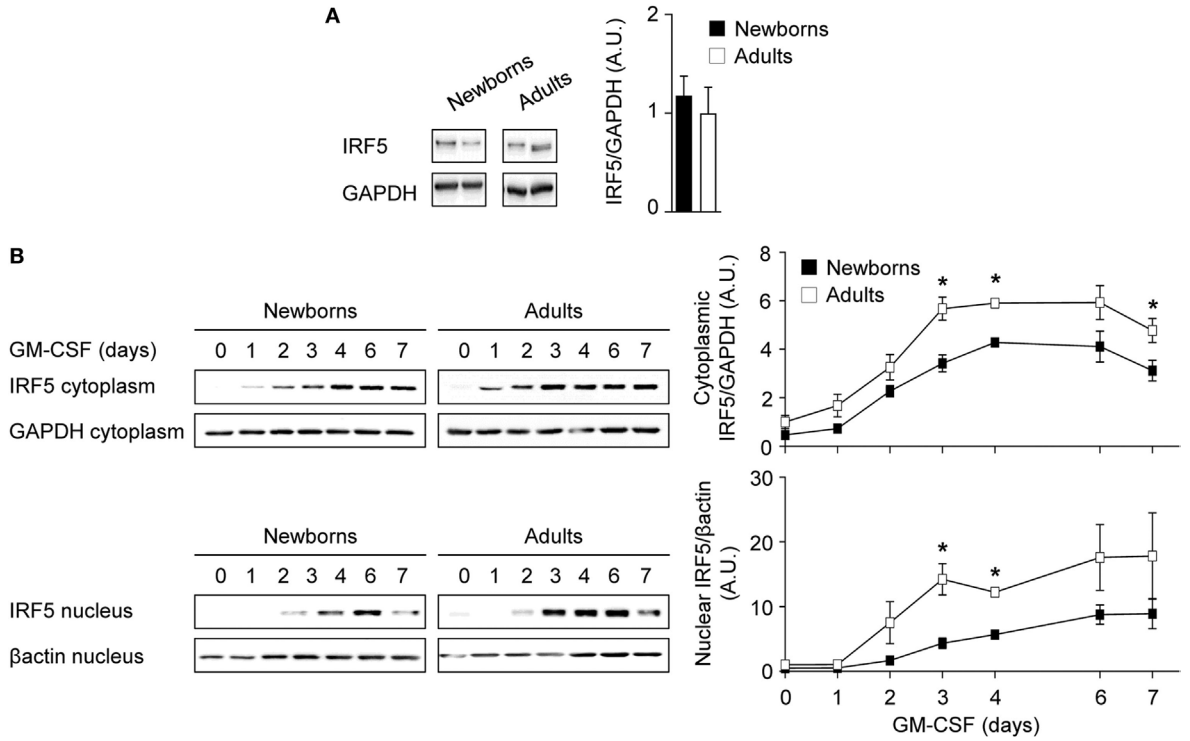

FIGURE 4 | Reduced cytoplasmic and nuclear IRF5 levels during GM-CSF-induced monocyte to macrophage differentiation in newborns. **(A)** Intracellular IRF5 levels in freshly isolated newborn (black bars) and adult (white bars) monocytes. Data are means ± SEM from 10 newborns and 10 adults. **(B)** Cytoplasmic and nuclear IRF5 levels in newborn (black) and adults (white) monocytes cultured for 0–7 days with 50 ng/ml GM-CSF. IRF5 levels were analyzed by Western blotting (left panels) and quantified by imaging (right panels). Data are means ± SEM from nine newborns and nine adults. *$P < 0.05$.

CRL-1573) and stored at −80°C in 10% glycerol. Macrophages were transduced with the adenoviral preparations (50 µl for 10^5 cells, 1 ml for 2.5×10^6), and used 24 h later for functional studies.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation analyses were performed according to the manufacturer's recommendations (MAGnifiy Chromatin Immunoprecipitation System, Thermo Fisher). Briefly, 1×10^6 M1 macrophages were fixed with 1% formaldehyde. Chromatin was sheared by 16 cycles of 30-s pulse/30-s rest with an amplitude of 14% using an Ultrasonic Liquid Processor (Branson). Chromatin was incubated overnight at 4°C with 5 µg of antibodies directed against IRF5 (Cell Signaling Technology), or RNA polymerase II (Pol II, Table S1 in Supplementary Material), or with control IgGs (provided in the kit). Real-time PCR was performed with a 7500 Fast Real-Time PCR System using the SYBR Kapa Fast Mix (Sigma-Aldrich). The following sense and antisense primers (5′–3′ sequences) were used for amplification: TNF, TGCTTGTTCCTCAGCCTCTT, and TCACCCATCCCATCTCTCTC.

Statistical Analyses

Statistical analyses were performed using PRISM (Graphpad Software Inc.). Data are expressed as means ± SEMs. Comparisons between the different groups were performed by two-two-tailed *t* tests. Findings were considered statistically significant when $P < 0.05$.

RESULTS

Newborn and Adult Monocytes Differentiate into M1 and M2 Macrophages in Response to GM-CSF and M-CSF

The differentiation and polarization of freshly isolated newborn and adult monocytes into M1 and M2 macrophages following 7 days of culture with recombinant GM-CSF and M-CSF were analyzed by hematoxylin and eosin staining (**Figure 1A**) and by measuring the expression of maturation/differentiation markers by RT-PCR (CD14, CD64, SOCS2, SOCS3, and IRF4; **Figure 1B**) and flow cytometry (HLA-DR, CD80, CD163, CD206; **Figure 1C**). Monocytes and M1 macrophages from healthy term newborns and adult volunteers showed a round shape, while M2 macrophages displayed a more elongated shape, consistent with the expected phenotype (41, 42). No difference in morphology and viability (91 ± 1 versus 95 ± 1% and 91 ± 1 versus 94 ± 1% for newborn and adult M1 and M2 macrophages, respectively) was noticed between newborn and adult cells.

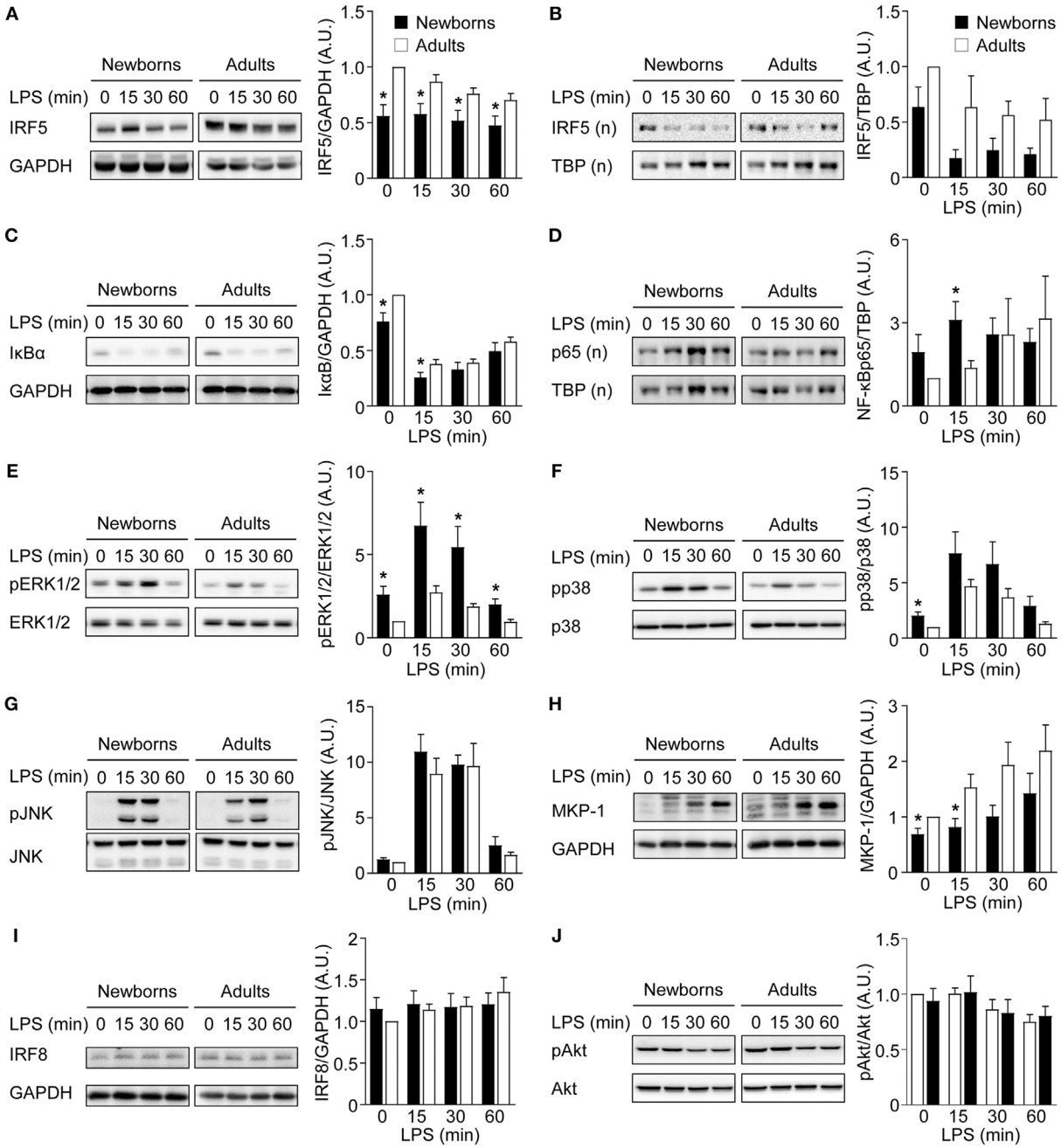

FIGURE 5 | Reduced IRF5 expression levels in newborn M1 macrophages. Newborn (black bars) and adult (white bars) M1 macrophages were stimulated with 100 ng/ml lipopolysaccharide for 0–60 min. IRF5 **(A,B)**, IκBα **(C)**, NF-κBp65 **(D)**, phosphorylated and total ERK1/2 **(E)**, p38 **(F)** and JNK **(G)**, MAP kinase phosphatase-1 **(H)**, IRF8 **(I)**, and phosphorylated and total Akt **(J)** were analyzed by Western blotting using total **(A,C,E–J)** or nuclear **(B,D)** cellular extracts and quantified by imaging. Values were normalized to those obtained from resting adult M1 macrophages set at 1. Data are means ± SEM from 8 to 10 newborns and 8 to 10 adults. *$P < 0.05$.

CD14 mRNA levels were higher (2.1- to 4.7-fold) in monocytes than in macrophages (**Figure 1B**), as anticipated (43). Unexpectedly, CD14 was more expressed (1.9-fold) in newborn than in adult monocytes. When compared to monocytes, TLR4 was expressed at lower levels in M2 macrophages (2.4- to 3.2-fold), while the M1 marker CD64 was enriched (5.2- to 8.1-fold) in M1 macrophages, and the M2 markers SOCS2 and IRF4 were enriched (5.1- to 8.1 and 3.6- to 4.8-fold) in LPS-stimulated M2 macrophages (44–47). SOCS3, a gene implicated in the repression of the M1 phenotype (48), was expressed at lower levels in M1 macrophages than in monocytes. Newborns and adult cells expressed similar levels of TLR4, CD64, SOCS2, SOCS3, and IRF4.

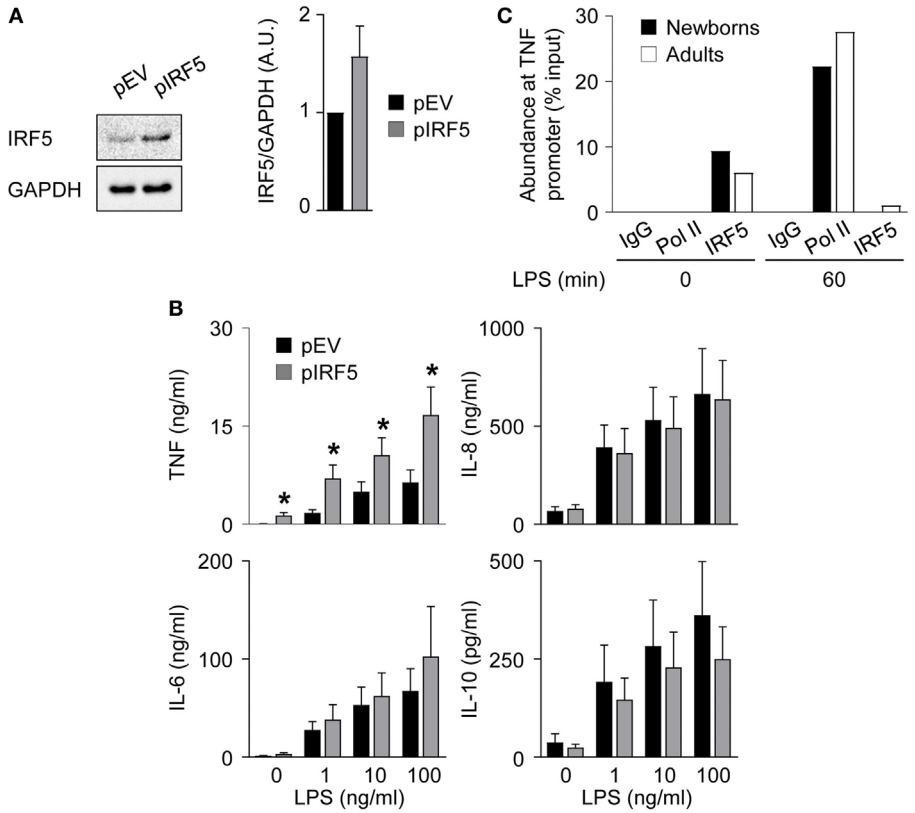

FIGURE 6 | IRF5 overexpression restores TNF secretion by newborn M1 macrophages. Newborn M1 macrophages were transduced with empty control (pEV) (black bars) and IRF5-expressing (gray bars) adenoviral vectors **(A,B)**. **(A)** IRF5 levels were analyzed by Western blotting (left panel) and quantified by imaging (right panel). **(B)** Newborn M1 macrophages were stimulated with 0–100 ng/ml lipopolysaccharide (LPS). TNF, IL-6, IL-8, and IL-10 concentrations were measured in cell culture supernatants collected after 20 h. Data are means ± SEM from 20 (TNF) or 10 (IL-6, IL-10, and IL-8) newborns. *$P < 0.05$. **(C)** The recruitment of RNA polymerase II (Pol II) and IRF5 to the TNF promoter in M1 macrophages before and 1 h after stimulation with 100 ng/ml LPS was assessed by chromatin immunoprecipitation. Data from one experiment representative of two experiments are presented as the percentage input relative to genomic DNA set at 100%.

HLA-DR, CD80, CD163, and CD206 were expressed at similar levels by newborn and adult monocytes (**Figure 1C**). The mean fluorescence intensity (MFI) of each of the molecules increased, albeit to different extents, in M1 and M2 macrophages (MFI fold increase versus monocytes: HLA-DR: 2.3–3.9; CD80: 6.2–6.6; CD163: 33–50; CD206: 5.5–16.5). HLA-DR and CD80 MFI were similar in newborn and adult M1 and M2 macrophages. CD163 was previously reported as an M2 marker in adults (41, 49, 50). However, in newborns, CD163 MFI strongly increased in both M1 and M2 macrophages compared to monocytes (33- and 50-fold). CD206, an M2 polarization marker at the transcript level (27), was more expressed in GM-CSF than M-CSF-derived macrophages by flow cytometry (41, 49). Accordingly, CD206 MFI was higher in newborn and adult M1 macrophages than in M2 macrophages, without noticeable difference of expression between newborns and adults. Overall, following exposure to GM-CSF and M-CSF, newborn monocytes differentiated into cells adopting morphological features and expressing markers of M1 and M2 macrophages similar to adult cells, with the exception of CD163 that was expressed at higher levels in newborn than adult M1 macrophages.

TLR4-Mediated TNF Secretion Is Selectively Reduced in Newborn M1 Macrophages

Functional studies were performed to compare the capacity of newborn and adult M1 and M2 macrophages to secrete proinflammatory and anti-inflammatory cytokines in response to TLR4 stimulation. In response to LPS, newborn and adult M1 macrophages secreted higher levels of TNF, IL-6, IL-8, and IL-23 and lower levels of IL-10 than M2 macrophages (**Figure 2A**). IL-1β secretion was similar between M1 and M2 macrophages. IFNγ, IL-12p70, IL-20, and IL-27 were undetectable.

Interestingly, newborn M1 macrophages secreted 3- to 6-fold less TNF (6.1 versus 21.2 ng/ml using 100 ng/ml LPS), while they produced IL-1β, IL-6, IL-8, IL-10, and IL-23 in the same range as adult M1 macrophages. Reduced TNF secretion was detected as early as 1 h following LPS stimulation (**Figure 2B**) and was associated with lower TNF mRNA expression in newborn M1 macrophages (**Figure 2C**).

We then evaluated whether the diminished TNF secretion by newborn M1 macrophages was also present in monocytes

(**Figure 2D**). LPS-induced TNF secretion was much lower in monocytes than in macrophages and was similar in newborn and adult monocytes. These findings suggested that GM-CSF triggered a different response in newborn and adult cells resulting in a specific reduction of TNF production by newborn M1 macrophages. This was unlikely to be due to gender differences, as macrophages from males released similar amounts of TNF compared to macrophages from females, both in newborns and adults.

Reduced IRF5 Activation During Monocyte to Macrophage Differentiation in Newborns

As a first approach to decipher the impact of GM-CSF on macrophage differentiation, we analyzed the expression CSF receptors and the activation of downstream signaling pathways in monocytes. Classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}SLAN^{-}$), and non-classical ($CD14^{+}CD16^{++}SLAN^{+}$) monocyte subsets (51, 52) were equally distributed in newborns and adults and expressed similar levels of GM-CSFR, M-CSFR, and HLA-DR (**Figure 3A**; Figure S1 in Supplementary Material).

Binding of GM-CSF to the GM-CSFR initiates the JAK/STAT, MAPK, and NF-κB intracellular signaling pathways and activates IRF5 in DCs (53). The pathways activated by GM-CSF in newborn and adult monocytes were investigated by mass cytometry. Exposure of newborn and adult monocytes to GM-CSF increased the phosphorylation of STAT5 (3.5- to 4.6-fold) and ERK1/2 (2.8- to 9.9-fold), but not that of STAT1, STAT3, p38, and NFκBp65 (**Figure 3B**). No difference was detected between newborns and adults.

IRF5 is a downstream target of GM-CSFR signaling and plays a key role in M1 polarization (23). IRF5 is activated by phosphorylation, leading to its dimerization and nuclear translocation to promote the expression of immune response genes (54). Intracellular levels of IRF5 were similar in newborn and adult monocytes (**Figure 4A**). We then quantified IRF5 in cytoplasmic and nuclear fractions obtained from monocytes exposed for 0–7 days to GM-CSF (**Figure 4B**). Cytoplasmic levels of IRF5 started to rise at day 1, peaked at day 3–6, and declined at day 7, while nuclear levels of IRF5 increased from day 2 to day 7. Cytoplasmic levels of IRF5 were 1.4- to 2.3-fold higher in adults than in newborns at days 1–7, while nuclear levels were 2.0- to 4.5-fold higher in adults from day 0 to day 7. Of note, IRF5 was detected at 15–20 lower levels in the nucleus than in the cytoplasm at day 7. Thus, during GM-CSF-induced monocyte to M1 macrophage differentiation, IRF5 was expressed at lower levels and translocated to the nucleus to a lower extent in newborn than in adult cells, a difference that might well explain the reduced expression of TNF in newborn M1 macrophages.

Reduced IRF5 Expression in Newborn M1 Macrophages

To further characterize the mechanisms underlying M1 macrophage polarization, the expression of IRF5 and IRF8, another transcription factor implicated in M1 polarization (55), and the activation of NF-κB, MAPK, and Akt signaling pathways were analyzed in GM-CSF-induced M1 macrophages exposed to LPS for 0, 15, 30, and 60 min. Cytosolic IRF5 levels were lower in newborn than in adult M1 macrophages before and following LPS exposure (**Figure 5A**). Nuclear IRF5 levels decreased following LPS stimulation and were lower in newborns than in adults at all time points, although differences were not statistically significant (**Figure 5B**). Newborn M1 macrophages expressed lower levels of cytosolic IκBα and higher levels of nuclear NF-κBp65 before and 15 min after LPS stimulation (**Figures 5C,D**). Phosphorylation of ERK1/2 and p38, but not of JNK, was higher in newborn M1 macrophages at baseline and upon LPS stimulation (**Figures 5E–G**). In line with these findings, expression of MKP-1/dual specificity phosphatase (DUSP1), a DUSP that inactivates ERK1/2 and p38, was reduced in newborn M1 macrophages (1.4- to 1.9-fold less at baseline and 15 min after LPS stimulation; **Figure 5H**). No difference in IRF8 expression was noticed between newborns and adults (**Figure 5I**). Phospho-Akt levels were not affected by LPS stimulation in newborn and adult M1 macrophages (**Figure 5J**). Combined altogether, and considering that NF-κB and MAPK signaling pathways were not impaired in newborns, our data pointed toward IRF5 as a possible regulator, which decreased expression in newborn M1 macrophages could be involved in a selectively reduced TNF production.

IRF5 Overexpression Restores TNF Secretion in Newborn M1 Macrophages

To investigate the relationship between lower levels of IRF5 and reduced TNF secretion in newborn M1 macrophages, we transduced newborn M1 macrophages with an IRF5 expressing adenoviral vector. Transduction increased IRF5 expression 1.6-fold (**Figure 6A**) and markedly increased (2.1- to 4.0-fold) TNF secretion, while it did not affect IL-6, IL-8, and IL-10 secretion (**Figure 6B**). Next, we examined the recruitment of IRF5 and RNA Pol II to the TNF promoter by ChIP. IRF5 binding was detected in unstimulated M1 macrophages and strongly decreased 1 h after exposure to LPS in both newborns and adults (**Figure 6C**). LPS stimulation for 1 h led to the recruitment of RNA Pol II to the TNF promoter in newborn and adult M1 macrophages. In summary, a selective increase in LPS-induced TNF production following IRF5 overexpression in newborn M1 macrophages strongly suggests an important role for IRF5 in shaping the TNF response in newborns. Yet, the mechanism of action of IRF5 might be independent of its recruitment to the *TNF* promoter following LPS stimulation.

DISCUSSION

We report that monocyte-derived M1 macrophages from newborns exhibit a strongly reduced capability to release TNF upon TLR4 stimulation, while the production of other cytokines is at similar levels as in adults. IRF5 is a key factor shaping this important functional characteristic of newborn macrophages (**Figure 7**).

Studies in mice, rats, and monkeys have described organ, tissue, and species-specific phenotypic and functional differences between newborn and adult macrophages. Globally, newborn

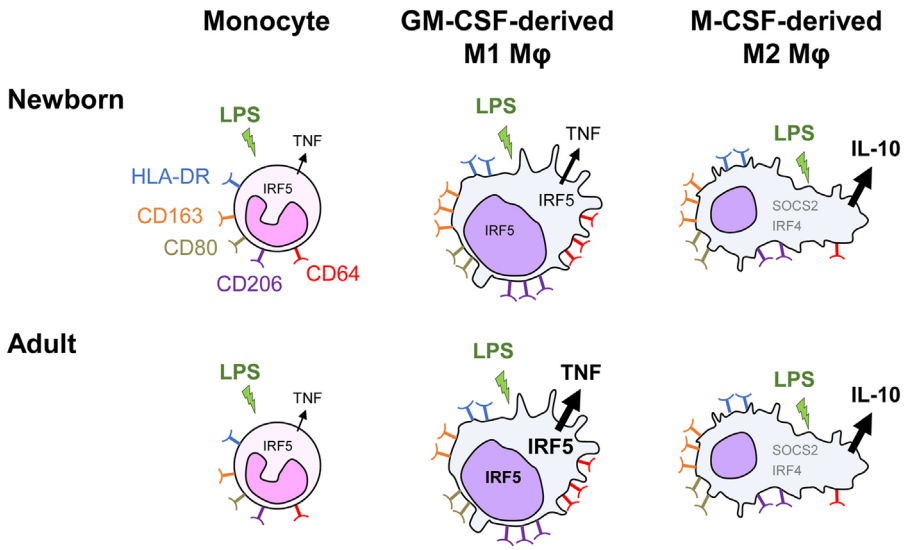

FIGURE 7 | Distinct TLR4 response in neonatal monocyte-derived macrophages. Exposure of newborn and adult monocytes to GM-CSF and M-CSF drives their differentiation into macrophages having an M1 (CD64^{high} and CD206^{high} round cells) and an M2 (SOCS2^{high}, IRF4^{high} and CD163^{high} elongated cells) phenotype. Macrophages express high levels of membrane-bound HLA-DR, CD80, CD163, and CD206, with newborn M1 macrophages expressing higher levels of the hemoglobin scavenger receptor CD163 than adult M1 macrophages. Following TLR4 stimulation, newborn monocytes secrete TNF to a similar level as adult monocytes. M1 macrophages secrete higher levels of TNF but low levels of IL-10, whereas M2 macrophages secrete low levels of TNF but high levels of IL-10. TNF secretion is threefold to sixfold lower in newborn than in adult M1 macrophages, while the production of other cytokines (IL-1β, IL-6, IL-8, and IL-23) is at similar levels. IRF5, a transcription factor implicated in M1 polarization, increases in the cytoplasm and in the nucleus during monocyte to M1 macrophage differentiation. Both cytoplasmic and nuclear levels of IRF5 are lower in newborn than adult M1 macrophages. IRF5 overexpression in newborn M1 macrophages restores LPS-induced TNF production in newborn M1 macrophages.

macrophages display reduced capacities to kill bacteria (56–59) and to produce proinflammatory cytokines (59–62) while they release anti-inflammatory cytokines at the same levels as adult macrophages (61–63). Previous studies in humans have investigated mixed populations of umbilical cord blood mononuclear cells or monocyte-derived cells, without a phenotypic characterization of differentiated cells (64–67). Cord blood-derived macrophage-like cells have a reduced capacity to kill group B *Streptococcus* and *Candida*, and release lower amounts of TNF, IL-1β, IL-6, and IL-12 in response to LPS (64, 68).

M-CSF is constitutively expressed by several cell types including fibroblasts, endothelial cells, stromal cells, and osteoblasts (69). Besides promoting survival, proliferation, and differentiation of bone marrow progenitors and monocytes, steady-state expression of M-CSF contributes to polarize macrophages toward an M2 phenotype (70). GM-CSF is expressed at low levels in the circulation and in tissues at homeostasis and plays a critical role in the terminal differentiation and functions of alveolar macrophages (71). Inflammation and infections trigger the production of GM-CSF by endothelial cells, fibroblasts macrophages, T cells, mast cells, and natural killer cells. GM-CSF drives M1 polarization, which is essential to mount efficient antimicrobial responses. Morphological and phenotypical analyses confirmed that newborn monocytes differentiate into cells adopting features of M1 and M2 macrophages, similar to adult cells. Uniquely, CD163 was strongly upregulated by both M1 and M2 macrophages in newborns, while this molecule is commonly used as an M2 marker in adults [(41, 49, 50) and our data]. Reduced activation of IRF5 in newborns might be implicated as IRF5 downregulates CD163 expression in adult macrophages (23). CD163 is a scavenger receptor involved in the clearance of free hemoglobin (72). During the neonatal period, high expression of CD163 in both M1 and M2 macrophages could be relevant, since newborn infants have an elevated turnover of erythrocytes under physiologic conditions and are prone to hemolysis during infection (8, 73, 74).

The lower capacity of newborn M1 macrophages to release TNF is most likely acquired during the process of monocyte to macrophage differentiation. Indeed, newborn monocytes released similar levels of TNF as adult monocytes under the experimental conditions used in the present study. Clearly, newborn M1 macrophages are not globally defective in TLR4 signaling, considering that TLR4 expression, NF-κBp65 nuclear translocation, ERK1/2 phosphorylation and MKP-1 expression, and production of IL-1β, IL-6, IL-8, and IL-23 are not diminished in newborn M1 macrophages.

IRF5 regulatory axis shapes the phenotype of newborn macrophages and plays an important role in systemic inflammation (54), as IRF5-deficient mice are protected from LPS-induced systemic inflammation and autoimmune diseases (75). Freshly isolated newborn monocytes expressed IRF5 to a similar extent as adult monocytes but had reduced expression and nuclear translocation of IRF5 when cultured with GM-CSF. Moreover, adenoviral-mediated IRF5 overexpression in newborn M1 macrophages restored TLR4-mediated TNF secretion, while it did not impact IL-6, IL-8, and IL-10 production, indicating that

IRF5 might play a key role in the selective reduction of TNF secretion observed in newborn macrophages. In contrast, germline deletion of IRF5 impairs LPS-induced production of Th1/Th17 cytokines in mice (75), and IRF5 overexpression in adult human macrophages increases expression of TNF, IL-1β, IL-12p70, and IL-23 and reduces secretion of IL-10 (23). These data suggest that IRF5 has a broader impact on cytokine production in adult than in newborn cells. Further studies will be required to define whether IRF5 differential expression impacts on immune functions besides TNF production in newborns.

Following exposure of macrophages to LPS, IRF5 is recruited to regulatory elements of the *TNF* gene and stimulates transcription (75, 76). In adult M1 macrophages, NOD2 stimulation triggers an IRF5-dependent activation of MAPKs, NFκB, and Akt2, increasing TNF, IL-1β, and IL-12 production (77). However, in our study, LPS stimulation did not increase IRF5 expression, nuclear translocation, and recruitment to the TNF promoter in M1 macrophages. Moreover, NF-κB and MAPKs signaling pathways were not impaired in newborn M1 macrophages, and Akt was not activated following LPS stimulation. Chromatin remodeling is implicated in monocyte to macrophage differentiation and macrophage polarization (78, 79), and histone acetylation and methylation are regulators of *TNF* gene expression (80). Further studies will be required to address whether GM-CSF induced a specific epigenetic reprogramming in newborn monocytes making newborn M1 macrophages less prone to transcribe *TNF* in response to TLR4 stimulation. It will be also important to define whether posttranscriptional modifications of IRF5 required for optimal *TNF* transcription are reduced in newborn macrophages.

Previous studies have identified reduced activation of IRF family members as mechanisms underlying the limited capacity of neonatal DCs to mount proinflammatory responses. Lower IRF3 activity in newborn monocyte-derived DCs in response to TLR4 stimulation is associated with reduced expression of IFN-β, IL-12p70, and the IFN-inducible chemokines CXCL9, CXCL10, and CXCL11 (5). Moreover, the limited production of typeI/III IFNs by newborn plasmocytoid DCs exposed to herpes simplex virus-1 is linked to a reduced nuclear translocation of IRF7 (81). Combined altogether, these studies put forward a major role of IRFs in shaping the unique characteristics of newborn myeloid cells.

Our findings recognize characteristics of newborn macrophages that could be relevant to the vulnerability to infections observed during the neonatal period. Indeed, TNF is an early response cytokine that plays a crucial role in recruiting innate immune cells to sites of infection and promoting microbicidal activities. However, during established infections, excessive levels of TNF participate to the dysregulated immune responses that contribute to the pathogenesis of sepsis (74). Moreover, inflammation can cause considerable damage to developing organs, resulting in death or long-term disability (10). Thus, lower production of TNF by newborn macrophages exposed to microbial products could be advantageous to limit inflammatory responses during postnatal colonization of the skin and gastrointestinal tract and to reduce organ dysfunction and damage during systemic infection. The observation of a selective reduction in TNF secretion by newborn macrophages, while activation of major signaling pathways and production of other cytokines is maintained, supports the concept that immune responses are highly regulated to meet the specific requirements of early life. While we focused on differences between the developing neonatal immune system and the fully developed adult immune system, the absence of data from children is a limitation.

In summary, we identified distinct characteristics of the monocytic lineage in newborns that show limited IRF5 activation during monocyte to macrophage differentiation, and a specific reduction of TNF production upon TLR4 stimulation in M1 macrophages. These observations are relevant in the context of neonatal inflammation and infection and may provide a new potential target for immune modulating therapies during the neonatal period.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Swiss Ethics Comittees on research involving human subjects. The protocol was approved by the Cantonal Human research Ethics Committee of Vaud (CER-VD, Lausanne, Switzerland). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

EG and AS designed the study and wrote the first draft of the manuscript. AS and MW performed experiments. JH performed CyTOF studies. AS, JH, MP, TC, TR, and EG analyzed and interpreted the data. All the authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01597/full#supplementary-material.

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Immune Effects of Corticosteroids in Sepsis

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Sepsis, a life-threatening organ dysfunction, results from a dysregulated host response to invading pathogens that may be characterized by overwhelming systemic inflammation or some sort of immune paralysis. Sepsis remains a major cause of morbidity and mortality. Treatment is nonspecific and relies on source control and organ support. Septic shock, the most severe form of sepsis is associated with the highest rate of mortality. Two large multicentre trials, undertaken 15 years apart, found that the combination of hydrocortisone and fludrocortisone significantly reduces mortality in septic shock. The corticosteroids family is composed of several molecules that are usually characterized according to their glucocorticoid and mineralocorticoid power, relative to hydrocortisone. While the immune effects of glucocorticoids whether mediated or not by the intracellular glucocorticoid receptor have been investigated for several decades, it is only very recently that potential immune effects of mineralocorticoids *via* non-renal mineralocorticoid receptors have gained popularity. We reviewed the respective role of glucocorticoids and mineralocorticoids in counteracting sepsis-associated dysregulated immune systems.

Keywords: glucocorticoids, mineralocorticoids, NF-κB, animal models, clinical trials, septic shock, sepsis, organ function

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INTRODUCTION

Sepsis is defined by a life-threatening organ dysfunction resulting from deregulated host response to invading pathogens (1). The host–pathogen interaction in sepsis is associated with an excessive response of the innate immune system leading to systemic inflammation and organ failure (2). This excessive inflammatory response coexists with compensatory anti-inflammatory signaling (3). An initial immune response occurs after recognition of pathogen- or damage-associated molecular patterns by specific cellular receptors, leading to cellular activation and systemic inflammation (4, 5). In practice, patients with sepsis may present with a hyperimmune response, typically around the time of admission when the infectious process is not fully under control, or with an immune suppression state, which tends to occur at a later time (3). The resolution of inflammation is also an active process, partly mediated by lipid mediators such as eicosanoids, which exhibit pro-resolving proprieties, and lead to tissue reparation (6). Then, knowing the time course of the immune response to sepsis is likely a key factor for the success of immunomodulatory interventions (7). Sepsis is a leading cause of mortality and morbidity, with annual prevalence of sepsis estimated at 31.5 million and the annual number of deaths at 5.3 million, worldwide (8, 9). The incidence of sepsis is steadily rising (10). Approximately half of sepsis survivors suffer from physical and psychological sequel, directly impacting their quality of life (11, 12). Treatment

of sepsis is based on source control and organs support (13). Corticosteroids are produced by the adrenal glands lying at the superior pole of the kidneys. Corticosteroids are synthesized by adrenal cortical cells from esterified cholesterol and possess four carbon rings. Each step of corticosteroid biosynthesis is controlled by a specific enzyme (**Figure 1**). Corticosteroids are divided into mineralocorticoids, which preferentially affect salt and water balance while glucocorticoids preferentially affect sugar metabolism and sex hormones. The adrenal cortex is divided into the zona glomerulosa, the outermost layer beneath the capsule, which secretes mineralocorticoids, the zona fasciculata, which secretes glucocorticoids, and the innermost layer, the zona reticularis, which secretes sex hormones. We will hereafter describe the biological effects of glucocorticoids and mineralocorticoids, without further mentioning sex hormones. Corticosteroids are commonly categorized according to their glucocorticoid and mineralocorticoid power, relative to hydrocortisone (**Table 1**). Glucocorticoids exhibit immune-modulating proprieties, in part through interaction with NF-κB (14). Thus, glucocorticoids have been used to treat patients with severe infections for more than 50 years. Much less information is available regarding immune effects of mineralocorticoids, yet the combination of fludrocortisone to hydrocortisone significantly reduced mortality from septic shock (15, 16). We herein reviewed the immune effects of glucocorticoids and mineralocorticoids that may be relevant to the management of sepsis.

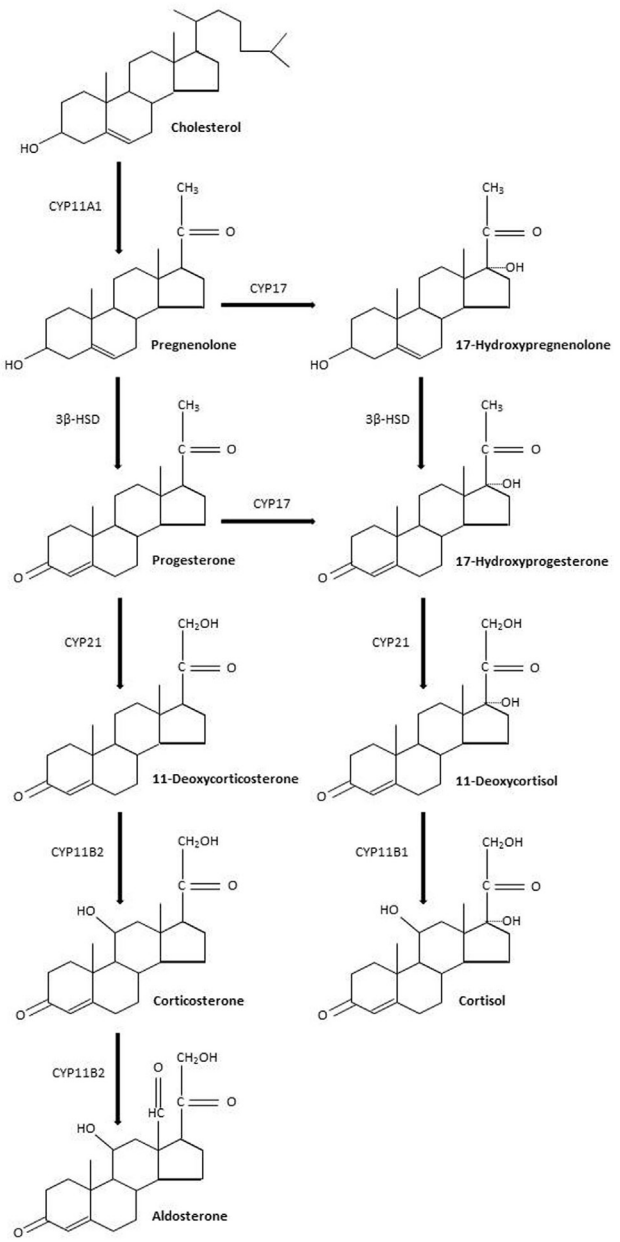

FIGURE 1 | Corticosteroids biosynthesis. Abbreviations: CYP11A1, cholesterol desmolase; 3β-HSD, 3β hydroxysteroid dehydrogenase; CYP17, steroid 17α-hydroxylase; CYP21, steroid 21-hydroxylase; CYP11B2, aldosterone synthase; CYP11B1, steroid 11β-hydroxylase.

IMMUNE-MODULATING EFFECTS OF CORTICOSTEROIDS

Glucocorticoids

Molecular Mechanisms of Action

Glucocorticoids have anti-inflammatory effects through the production of anti-inflammatory proteins and inhibition of pro-inflammatory proteins. Glucocorticoids bind to a specific intracellular receptor, the glucocorticoid receptor (GR). The GR is a transcription factor belonging to the nuclear receptor superfamily encoded on chromosome 5q31-31 (17). Glucocorticoid-regulated transcription factors are 94-kDa proteins, composed of several specific domains. A ligand-binding domain made up of 12 α-helices is involved in the recognition and binding of corticosteroids, a DNA-binding domain composed of two zinc fingers for interaction of the hormone–receptor complex with specific DNA sequences, and a trans-activating domain for binding of transcriptional factors (18). Unbound GR located in the cytoplasm of almost all cells, are stabilized by chaperone proteins such as heat-shock proteins 70, heat-shock protein

TABLE 1 | Relative potencies of natural and synthetic steroids.

Compound	Glucocorticoid activity	Mineralocorticoid activity
Natural steroids		
Cortisol	1	1
Corticosterone	0.3	15
Aldosterone	0.3	3,000
Deoxycorticosterone	0.2	100
Synthetic steroids		
Cortisone	0.8	0.8
Fludrocortisone	10	125
Prednisone	4	0.8
Prednisolone	4	0.8
Methylprednisolone	5	0.5
Betamethasone	25	0
Dexamethasone	25	0

Glucocorticoid and mineralocorticoid activity of natural and synthetic steroids, relative to cortisol.

90 (Hsp90), and immunophilin (19). Upon binding with glucocorticoids, the GR dissociates from chaperone proteins and translocates into the nucleus. Within the nucleus, homodimers of the glucocorticoid–GR complex interact with specific DNA sequences (glucocorticoid responsive elements) of the regulatory region of target genes (**Figure 2**). The expression of genes modulated by the hormone–GR complex occurs through chromatin remodeling (20, 21). Chromatin consists of nucleosomes; DNA associated with core histone proteins. Quiescent genes are composed of tightly wound DNA around histone proteins, hampering the ability of RNA polymerases to bind to DNA and to produce mRNA. Core histones may be acetylated, modifying the structure of nucleosomes, loosening the chromatin, and ultimately enhancing gene expression. Transcription factors such as NF-κB activate histone acetyltransferases (HATs), leading to acetylation of core histones. By contrast, histone deacetylases (HDACs) induce a tightening of the chromatin, repressing target genes expression. Activated GR inhibit HATs and activate HDACs, overall repressing the expression of pro-inflammatory genes. For instance, the expression of the IRF3 transcription factor, implicated in interferon production and viral protection, is downregulated by glucocorticoids (22, 23). The GR–glucocorticoid complex also inhibits the production of pro-inflammatory proteins by sequestration of NF-κB within the cytosol (24). NF-κB is implicated in the production of pro-inflammatory cytokines (25, 26). In a resting state, inactive NF-κB is bound to IκBα. Upon cellular activation, NF-κB and IκBα dissociate and NF-κB translocates into the cellular nucleus. Glucocorticoids increase the expression of the inhibitory protein IκBα, thereby sequestering NF-κB (27). Glucocorticoids also induce the expression of glucocorticoid-induced leucine zipper (GILZ) which inhibits NF-κB (28) as well as the anti-inflammatory protein MAP kinase phosphatase 1, which inhibits nuclear translocation of transcription factor GATA-3 implicated in Th2 type cytokine expression (29). In addition, glucocorticoids promote the production of annexin 1, which inhibits the expression of phospholipase A2. Phospholipase A2 catabolizes the production of arachidonic acid-derived elements, including prostaglandins and leukotrienes, which are implicated in pain and inflammatory responses (26). Annexin 1 is also implicated in the resolution of inflammation as well as in the phagocytosis by macrophages of apoptotic neutrophils (25).

The genomic effects of glucocorticoids take place only after several hours, following nuclear translocation of activated GR and gene regulation (30). This latency is explained by the time needed for mRNA production, protein synthesis, and transport (31).

Effect of Glucocorticoids in Health

Glucocorticoids suppress the production of acute phase reactants and of chemokines implicated in leukocyte chemo-attraction (32, 33), thereby reducing leukocytes migration into inflamed

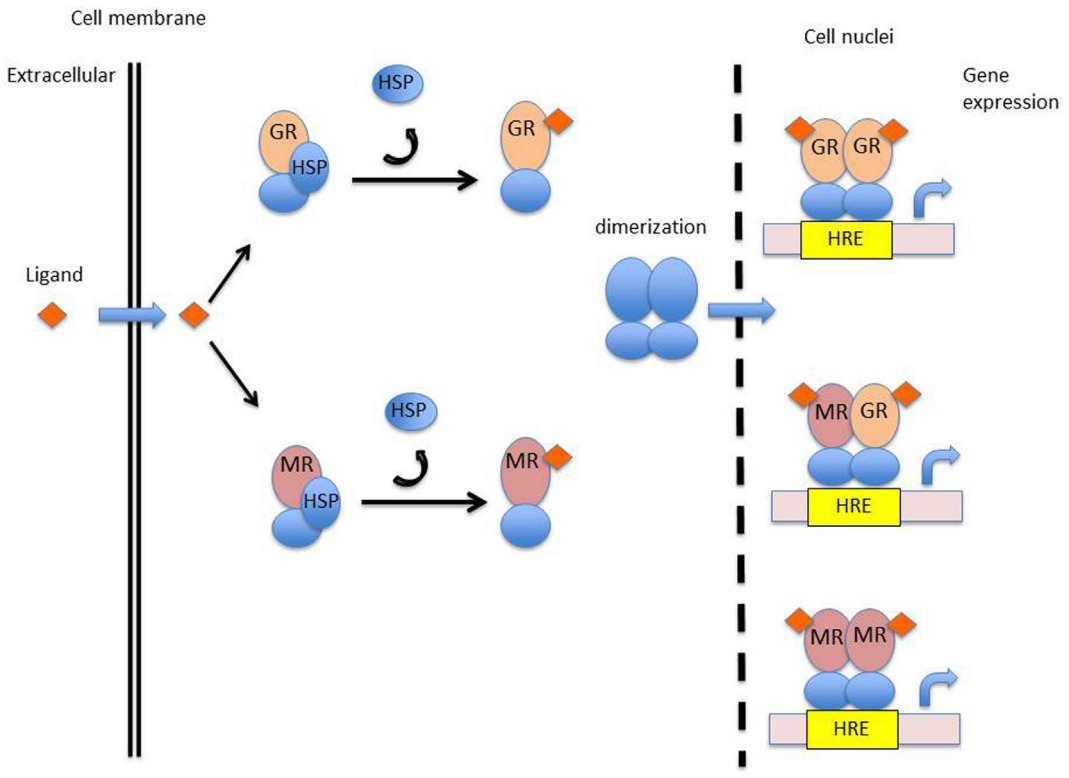

FIGURE 2 | Mechanism of action of corticosteroids. Abbreviations: GR, glucocorticoid receptor; MR, mineralocorticoid receptor; HSP, heat-shock protein; HRE, hormonal response element.

areas. Glucocorticoids suppress the expression of endothelial-leukocyte adhesion molecule 1, intracellular adhesion molecule 1 (ICAM-1), and vascular adhesion molecule 1 (VCAM-1) opposing to leukocytes trafficking through the endothelium (34, 35). Glucocorticoids affect both the innate and adaptive arms of the immune system. Target immune cells include (1) myeloid cells; macrophages, monocytes, dendritic cells (tissue-resident DCs, migratory DCs, and plasmacytoid DCs), as well as granulocytes and (2) lymphocytes, including CD8, T helper 1 (Th1), Th2, and Th17 as well as Treg and B cells (14). Broadly speaking, glucocorticoids repress the maturation, differentiation and proliferation of leukocytes of all subtypes.

Glucocorticoids attenuate fever by reducing monocytes and macrophages production of interleukin (IL)-1, TNF, IL-8, and MCP-1 (36). Glucocorticoids reduce the number of monocytes/macrophages, dendritic cells, and eosinophil and basophil granulocytes (37). Despite reducing the number of monocytes/macrophages, the capacity of glucocorticoid-treated macrophages for phagocytosis seems unaltered or even improved (38). Neutrophil granulocytes are not affected by the increased apoptosis induced by glucocorticoids, possibly because of the specific production of an inactive isoform of the GR (GR-beta) (39). Indeed, after treatment by glucocorticoids, the number of circulating neutrophil granulocytes increases, through an increased release by the bone marrow associated with increased demargination. However, these leukocytes may be functionally less efficient. Glucocorticoid-treated circulating polymorphonuclear leukocytes exhibit decreased levels of L-selectin receptors (40). Dexamethasone induces the expression by polymorphonuclear leukocytes of a decoy receptor for IL-1 (41). Glucocorticoids stabilize the lysosomal membranes, greatly reducing the amount of proteolytic enzymes released by lysosomes. Dendritic cells treated by glucocorticoids produce increased levels of the anti-inflammatory cytokines IL-10 and TGF-β (42). Glucocorticoids reduce the membrane expression of MHC class II and Fc receptors (43, 44) and suppress antigen presenting to T cells (45).

Activation, proliferation, and production of immunoglobulins by B cell lymphocytes are depressed by glucocorticoids (46, 47). They deplete thymic stroma cells and T cells by apoptosis (48–50). Circulating T-cell numbers are reduced with a shift from a pro-inflammatory Th1 phenotype to an anti-inflammatory Th2 phenotype (51–54). Glucocorticoids suppress the production by lymphocytes of the pro-inflammatory cytokines IL-2, IL-4, IL-5, IL-13, and INF (55, 56).

Mineralocorticoids

Molecular Mechanisms of Action

While the immune effects of glucocorticoids have been extensively investigated, those of mineralocorticoids have only recently gained attention. The biological activity of mineralocorticoids is mediated by interaction with a specific intracellular receptor, the mineralocorticoid receptor (MR). The MR is a transcription factors belonging to the nuclear receptor superfamily, encoded on chromosome 4, in the q31.1 region (57, 58). The structure of the mineralocorticoid-regulated transcription factor is highly similar to that of the GR and displays several specific domains, including a ligand-binding domain, a DNA-binding domain, and a trans-activating domain. The amino acid sequence of the DNA-binding domain of the GR and MR are approximately 94% similar, indicating that these two receptors may recognize and bind similar DNA sequences. The activated MR regulates the expression of a set of genes within target tissues, in a similar way to that of the activated GR. Unbound MR are stabilized in the cytoplasm by Hsp90. Activated MR will shed their chaperone proteins and translocate into the nucleus, form dimmers, and go on to recognize specific hormone recognizing elements of the DNA (**Figure 2**) (59). There is evidence that MR and GR may form functional heterodimers with specific properties (60, 61).

Surprisingly, cortisol and aldosterone bind the MR with equal affinity, indicating that the MR does not specifically recognize mineralocorticoids over glucocorticoids (57). However, the transcriptional response of the MR in response to aldosterone is approximately 100-fold higher than cortisol (62). Corticosteroid specificity is in part due to the activity of the type 2 isoenzyme of the 11β-hydroxysteroid dehydrogenase (11βHSD2), found in the kidney and in the colon and located near the MR. 11βHSD2 metabolizes glucocorticoids into an inactive derivative, cortisone. Since mineralocorticoids are unaffected by 11βHSD2 they are therefore able to interact with the MR (63). Therefore, the role of glucocorticoid binding of MR in non-epithelial tissues, which are devoid of 11βHSD2, is raised. The MR has several isoforms, some of which are able to bind both glucocorticoids and mineralocorticoids, while other isoforms bind exclusively mineralocorticoids (64). The density of GR and MR varies from one tissue to another. For instance, MR expression is higher than GR expression in the central nervous system; MR and GR are similarly expressed in the cardiovascular system while GR expression is higher than MR expression in the immune system (65). Finally, GR and MR also differ in their capacity to inhibit AP-1-mediated gene activation (66).

Effect of Mineralocorticoids on the Immune System in Health

Mineralocorticoid receptors play specific roles depending on their tissue expression. MRs located in the kidneys and the colon are implicated in NaCl reabsorption and K^+ secretion (67, 68), where NaCl reabsorption is mediated by serum and glucocorticoid-induced kinase (SGK1), GILZ protein, and the epithelial sodium channel (69). Mineralocorticoid stimulation promotes the expression by endothelial cells of the VCAM-1, ICAM-1, and P-selectin membrane receptors, implicated in the adhesion of leukocytes to endothelial cells (70). In endothelial cells, mineralocorticoids also induce the production of reactive oxygen species *via* the activation of NADPH oxidase and Rac1 (71). In the brain, MRs are specifically located in the limbic system and are implicated in learning and memory (72). MRs are expressed in monocytes and macrophages (73), dendritic cells (74), and neutrophils (75). MR signaling in myeloid cells induces a pro-inflammatory response (76, 77). Indeed, macrophages exposed to mineralocorticoid agonists undergo a M1 type pro-inflammatory polarization associated with an increased production of TNF-α and of reactive oxygen species (78–80).

In microglial cells, which are resident macrophages of the central nervous system, aldosterone activation induces an increased production of TNF-α and IL-6 in response to lipopolysaccharide stimulation (81). By contrast, MR knockout macrophages or macrophages treated by MR antagonists exhibit a M2 anti-inflammatory polarization (78). Mineralocorticoid agonists induce the activation of the mitogen-activated protein kinase pathway in dendritic cells, leading to the secretion of IL-6 and TGF-β1 (74). Mineralocorticoids indirectly lead to an increase in platelet cytosolic calcium concentrations, leading to platelet activation, thrombin formation, and platelet procoagulant activity (82, 83). Indeed, platelet cytosolic calcium entry is upregulated by the serum- and glucocorticoid-inducible kinase isoform SGK1, which is upregulated by mineralocorticoids (84). SGK1 upregulates IL-17-producing CD4+ helper T cells (Th17 cells). Th17 cells are dependent on IL-23 expression; SGK1 ensures the proper expression of the IL-23 receptor (85).

Mineralocorticoid receptor activation indirectly affects T lymphocyte phenotype. Indeed, dendritic cells activated by mineralocorticoid agonists impose a pro-inflammatory Th17 phenotype on CD4 T cells (74). Aldosterone stimulates IL-1β secretion by macrophages through NF-κB signaling and reactive oxygen species generation. Aldosterone also increases the expression of NLRP3, implicated in the formation of inflammasone and mature IL-1β in human peripheral blood mononuclear cells (86). Infusion of aldosterone in rodents results in elevated plasma IL-1β levels (86). Human blood mononuclear cells exposed to MR antagonists produce less cytokines, including TNF, IL-1α, IL-2, IL-6, INFγ, and GM-CSF (87). Aldosterone and MR agonists promote myocardial and kidney fibrosis (88, 89). Fludrocortisone, at high doses administered *in vivo*, paradoxically exhibits anti-inflammatory properties. Fludrocortisone inhibits histamine release by basophils (90) and IL-1 production by lung fragments (91).

IMMUNOMODULATION IN SEPSIS

Glucocorticoids

Animal Studies

The expression of the GR is upregulated in LPS-stimulated mouse macrophages and in mouse models of sepsis (92–94) (**Table 2**). However, others have shown that GR expression and protein levels decreased following a TNF challenge (95). The binding capacity of the GR decreases after an endotoxin challenge (94). The binding capacity of the GR may be altered through the action of nitric oxide (96, 97). LPS challenge in GR knockout mice induces higher mortality than in control animals (98). The endothelial GR regulates NF-κB in a model of endotoxin-induced sepsis (99). The deletion of the GR from endothelial cells, through the activation of NF-κB, is associated with higher mortality, higher nitric oxide levels, and higher levels of pro-inflammatory cytokines (TNF-α and IL-6) (99). In small animals with sepsis, high doses of dexamethasone and methylprednisolone significantly prolong survival (100–102). High doses of methylprednisolone continuously administered in a canine model of endotoxin shock also improve survival (103).

TABLE 2 | Main effects of glucocorticoid or mineralocorticoid administration during sepsis.

Compound	Glucocorticoid	Mineralocorticoid
Small animal	– Improves survival (100–102)	– Restores the expression of the mineralocorticoid receptor (104) – Reduces the levels of plasma histamine, plasma serotonin, blood bradykinin, and plasma catecholamine concentration (105) – Improves the hemodynamic response (106) – Reduces mortality (107–109)
Large animal	– Improves survival (103)	– Improves survival and the hemodynamic response (110, 111) – Lowers IL-6 levels (111)
Man	– Reduces plasma levels of TNF-α (112) – Reduced levels of E-selectin sE-selectin (113, 114) – Decreases the number of eosinophils (115) – Decreases levels of phospholipase A (116) – Decreases levels of nitrite/nitrate, IL-6, IL-8, and markers of neutrophil activation (decreased expression of CD11b, CD64, and neutrophil elastase) (116) – Lowers whole blood production of IL-1 and IL-6 (113, 116–119) – Monocyte mHLA-DR levels are depressed (113, 118) – Decreases monocyte production of migration inhibitory factor (120) – Decreases the binding capacity of glucocorticoid receptor in neutrophils (121) – Improves the hemodynamic response and survival (122)	– Improves the hemodynamic response in the most severe subgroup of a pediatric population (123)

Studies in Humans

When LPS is administered to healthy subjects, the concomitant administration of hydrocortisone reduces plasma levels of TNF-α (112). The levels of E-selectin and of the soluble form of the receptor sE-selectin are also reduced following hydrocortisone therapy in sepsis (113, 114). During septic shock, hydrocortisone decreases the number of eosinophils (115), circulating levels of phospholipase A (116), serum levels of nitrite/nitrate, IL-6, IL-8, and markers of neutrophil activation (decreased expression of CD11b, CD64, and neutrophil elastase). Moreover, hydrocortisone lowers *ex vivo* whole blood production of IL-1 and IL-6 in response to LPS (113, 116–119). In hydrocortisone-treated patients with septic shock, monocyte mHLA-DR levels are depressed, while the

capacity for phagocytosis of monocytes increases (113, 118). Glucocorticoids also attenuate LPS-stimulated monocyte production of migration inhibitory factor (120). In neutrophils of hydrocortisone-treated sepsis patients, the binding capacity of GR for glucocorticoid is reduced (121).

Clinical Trials

Short courses of high dose methylprednisolone or dexamethasone do not significantly reduce mortality and may even harm patients with sepsis (124–127). The introduction of the concept of sepsis-associated relative adrenal insufficiency in the 90s, led physicians and trialists to consider using prolonged courses of low doses of hydrocortisone (128). Several small size trials found that 200–400 mg of hydrocortisone per day for more than 3 days improved cardiovascular function in sepsis (117, 129, 130). GERINF05 was the first phase 3 trial that tested a prolonged course (7 days) of low to moderate doses (200 mg/day) of corticosteroids in septic shock with evidence of relative adrenal insufficiency (15). This trial found a significant improvement on survival and cardiovascular function in patients with septic shock and non-responders to a 250 µg ACTH test (Delta cortisol <9 µg/dl) (15). The CORTICUS trial could not reproduce the survival benefit of corticosteroids found in GERINF05 while confirming the benefit on cardiovascular homeostasis and organs function (131). A meta-analysis of the use of corticosteroids in sepsis published in 2015 concluded that corticosteroids reduced 28-day mortality, increased the rate of shock reversal, without increasing the risk of infection (122). More recently, the ADRENAL trial found no significant survival benefit from a continuous infusion of hydrocortisone in patients with septic shock (132). Nevertheless, the trial found that, when compared with placebo, hydrocortisone fasten the resolution of shock, shortened the duration of mechanical ventilation, and reduced the requirement for blood transfusion (132). In keeping with GERINF05 observations, the APROCCHSS trial found that the combination of hydrocortisone to fludrocortisone significantly reduced 90-day mortality (16). Likewise, corticosteroids hastened the resolution of shock and organs failure without causing major adverse events.

Mineralocorticoids

Animal Studies

In models of chronic cardiovascular diseases, aldosterone is associated with increased vascular and cardiac oxidative stress, inflammation, and fibrosis (133). In models of acute cardiovascular diseases, both exogenous aldosterone and overexpression of the MR increased blood pressure (134, 135). Aldosterone plays a role in salt appetite (136), and in coping behavior (137). MRs, contrary to GRs, when activated, play a neural antiapoptotic role by differentially influencing genes of the bcl-2 family (138). Similar to the renal epithelium, aldosterone may also favor the clearance of alveolar fluid by type II epithelial cells (139). By extrapolation, these responses may be of benefit during sepsis. However, very little is known about the MR or its regulation during sepsis. In small animals, sepsis is associated with downregulation of the MR in endothelial cells (92, 104), which is restored by supplementation by exogenous MR (104). In endotoxin shock, aldosterone reduces plasma levels of histamine, serotonin, bradykinin, and catecholamine (105). MR agonists reduce mortality in endotoxin-challenged small animals (107–109). In large animal sepsis models, aldosterone levels correlate with the severity of shock and with mortality (140), and mineralocorticoid supplementation improved survival and hastened shock reversal when administered prior to a bacterial challenge (110). The early administration of glucocorticoids plus mineralocorticoids improves the outcome in large animals with sepsis. In the sickest animals, glucocorticoids plus mineralocorticoids treatment hastened shock reversal and lowered plasma IL-6 levels (111).

Clinical Studies

The plasma concentrations of aldosterone were found to be unexpectedly low in meningococcal sepsis compared with ICU admissions for other reasons (141). Lower than expected aldosterone levels have also been reported in adults' septic shock (142, 143). Inappropriately low aldosterone levels during septic shock were associated with increased ICU length of stay, an increased incidence of acute kidney failure (144), and increased mortality (145). Low aldosterone levels occurred despite high renin levels, suggesting impaired adrenal synthesis of aldosterone. In addition, a subset of patients with sepsis did not increase aldosterone levels in response to ACTH stimulation (146). Mineralocorticoid levels were found to correlate with IL-6 levels in meningococcal sepsis (147). The expression of the MR is downregulated in human endothelial cells exposed to TNF-α (104). The MR agonist fludrocortisone is administered orally, and there is no currently available intravenous formulation. The pharmacokinetics of fludrocortisone were assessed in healthy subjects (148), and in septic shock patients (149). However, there are still too few data on the direct effects of mineralocorticoids in patients with sepsis. A retrospective study in a pediatric population found that hydrocortisone combined with fludrocortisone was associated with shorter duration of vasopressor support in the most severe patients (123). Finally, it remains unclear whether or not part of the survival benefit from corticosteroids observed in the GERINF05 (15) and APROCCHSS (16) trials are directly related to fludrocortisone.

Future research should start with an individual patient data meta-analysis of all trials comparing hydrocortisone and/or fludrocortisone against placebo. Then, next trial should be designed as a two-by-two factorial placebo-controlled trial comparing hydrocortisone versus fludrocortisone versus hydrocortisone plus fludrocortisone (150).

CONCLUSION

In sepsis, there is sufficient evidence from animals and humans studies to support that glucocorticoids modulate innate immunity to promote the resolution of inflammation and organs failure. Much less is known about the immune effects of mineralocorticoids though increasing evidence from laboratory investigations suggested that they might favorably impact the outcome from

sepsis. So far, survival benefits in adults with septic shock have been shown only for the combination of hydrocortisone plus fludrocortisone and not from the administration of hydrocortisone alone.

AUTHOR CONTRIBUTIONS

DA has conceived the manuscript. All the authors have contributed to the literature search and writing of the manuscript.

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REVIEW
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Precision Immunotherapy for Sepsis

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Decades of sepsis research into a specific immune system-targeting adjunctive therapy have not resulted in the discovery of an effective compound. Apart from antibiotics, source control, resuscitation and organ support, not a single adjunctive treatment is used in current clinical practice. The inability to determine the prevailing immunological phenotype of patients and the related large heterogeneity of study populations are regarded by many as the most important factors behind the disappointing results of past clinical trials. While the therapeutic focus has long been on immunosuppressive strategies, increased appreciation of the importance of sepsis-induced immunoparalysis in causing morbidity and mortality in sepsis patients has resulted in a paradigm shift in the sepsis research field towards strategies aimed at enhancing the immune response. However, similar to immunosuppressive therapies, precision medicine is imperative for future trials with immunostimulatory compounds to succeed. As such, identifying those patients with a severely suppressed or hyperactive immune system who will most likely benefit from either immunostimulatory or immunosuppressive therapy, and accurate monitoring of both the immune and treatment response is crucial. This review provides an overview of the challenges lying ahead on the path towards precision immunotherapy for patients suffering from sepsis.

Keywords: sepsis, hyperinflammation, immunoparalysis, immunosuppressive therapy, immunostimulatory therapy, biomarkers, precision medicine

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INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). It is the number one cause of death in the Intensive Care unit (ICU), and the worldwide incidence of sepsis is estimated to exceed 30 million cases per year (2, 3). Despite advances in ICU management and goal-directed interventions in the last decades, sepsis mortality rates remain as high as 30% (4). In the Western world alone, annually an estimated 6 million people die of sepsis, representing more deaths than lung, breast and colon cancer together (5). In addition, it is also one of the most expensive conditions encountered in hospitals, with annual costs exceeding 20 billion dollars in the US alone[1]. Despite these alarming facts, sepsis remains a relatively neglected condition that is unknown to the general public.

[1] *Agency for Healthcare Research and Quality Healthcare Cost and Utilization Project Statistical Brief No 160.* Available online at: https://www.hcup-us.ahrq.gov/reports/statbriefs/sb160.pdf. 2013; August.

In May 2017, the WHO adopted a resolution aimed to improve the prevention, diagnosis, and management of sepsis[2], illustrating the urgency of the problem. The search for a specific immune system-targeting adjunctive therapy has dominated the sepsis research field for more than 4 decades, with the disappointing result of dozens of negative trials and not a single adjunctive treatment in current clinical use. Experts agree that this is not due to the fact that the drugs tested are ineffective *per se*, but rather to the inability to restrict treatment to selected patient groups that may actually benefit from a specific type of therapy (6–9). Not surprisingly, it is therefore also agreed upon that precision medicine is imperative for future trials to succeed, but accurate and reliable immunomonitoring is currently not a reality (6, 8–10). For new therapies, it is paramount that we do not make the same mistake that was previously made for immunosuppressive treatments by advocating the use of compounds in all sepsis patients. Instead, we should only target patients who are truly hyperinflamed with immune suppressive drugs and immunoparalyzed patients with immunostimulatory compounds to avoid unnecessary risks of side effects in patient groups with a lower chance of a therapeutic effect and increase the chances of success in patient groups with a higher likelihood of benefit. In addition to immunomodulatory therapy, advocating personalized medicine is also relevant for other promising sepsis treatments, for instance those targeting the well-described metabolic dysfunction observed in these patients. However this is beyond the scope of this review. This review discusses whether we are targeting the right pathophysiological immunological mechanisms and emphasizes the challenges that lie ahead on the path towards precision immunotherapy for septic patients.

Hyperinflammation in Sepsis

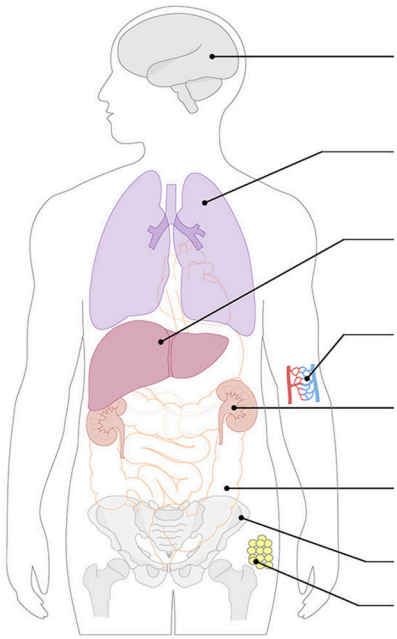
The proinflammatory response in sepsis is directed at eliminating invading pathogens and involves leukocyte activation, cytokine production, reactive oxygen species and protease release, and complement and coagulation activation (9). An overzealous hyperactive proinflammatory response may exert detrimental effects for the host by eliciting high fevers, hypotension, tachycardia, tachypnoea, coagulation disorders and organ failure, the latter resulting from collateral tissue damage. Examples of deleterious effects of hyperinflammation in various tissues are illustrated in **Figure 1**. Pulmonary hyperinflammation may lead to the development of acute respiratory distress syndrome (ARDS), a life-threatening condition characterized by unexplained respiratory failure through hypoxemia with bilateral infiltrates of noncardiac origin (11). In the circulating blood compartment hyperinflammation alters coagulation, which may result in a relatively uncommon but fulminant phenomenon called disseminated intravascular coagulation (DIC) (12), which is characterized by simultaneous widespread microvascular thrombosis and profuse bleeding. Capillary leak and development of interstitial oedema are unfortunately very common problems resulting from a systemic hyperinflammatory state, and have widespread cardiovascular effects such as hypotension, tachycardia and in severe cases even septic shock which results in more tissue damage. In the acute phase, hyperinflammation in the brain may cause headaches, nausea, apathy, somnolence or delirium. Furthermore it is suggested that long-term effects such as cognitive decline and behavioral changes can also be attributed to a hyperinflammatory state (13). Other organs that are often affected by hyperinflammation include the kidneys (acute kidney injury which frequently requires renal replacement therapy), the intestines (paralytic ileus), and the liver (liver failure with liver test abnormalities and altered glycemic control).

From the 1970s until the turn of the century, it was commonly assumed that sepsis mortality resulted exclusively from this overzealous pro-inflammatory response. As a consequence, therapeutic research in the sepsis field was solely focused on dampening or preventing excessive inflammation. However, all clinical trials to date investigating immunosuppressive therapy in sepsis, including anti-endotoxin (signaling) molecules, TLR-receptor antagonists, anti-cytokine therapies (e.g., anti-TNF-α, IL-1RA etc.), and high dose corticosteroids(14–24), have convincingly demonstrated that inhibition of the immune response exerts no beneficial effects in an unselected heterogeneous group of sepsis patients.

Paradigm Shift in the Understanding of Sepsis: The Detrimental Role of Sepsis-Induced Immunoparalysis

In the last decade a paradigm shift in our understanding of the immune derangements in sepsis has taken place (25). It is increasingly recognized that, for many patients not excessive immune activation, but rather immunosuppression, also known as "sepsis-induced immunoparalysis," is the overriding immune dysfunction associated with high mortality and morbidity (7, 25–29). The capacity of circulating leukocytes to release proinflammatory cytokines is impaired during immunoparalysis and apoptotic immune cell death is profoundly increased. In contrast to necrotic cell death, which generally causes stimulation of the immune system and enhanced defense against microbes, apoptotic cell death results in anti-inflammatory cytokine production, and cellular anergy (30–32). The majority of cells lost through apoptosis in septic patients are lymphocytes (33) and low absolute lymphocyte counts were shown to be associated with mortality (34). Sepsis-induced immunoparalysis renders patients unable to clear their primary infection and more likely to develop secondary infections with opportunistic bacteria or fungi (35) later on. This is in line with data that the vast majority of septic patients do not die from the initial pro-inflammatory hit, but at a later time point from secondary or opportunistic infections in an immunosuppressed state (35–38). More precisely: approximately a quarter of sepsis patients die within 4 days. Of the remaining three-quarter that survives, one third regains immunocompetence and the mortality in this group is 10%. Two-thirds develops immunoparalysis accounting for 65% of total mortality (10). The

[2]Available online at: https://www.global-sepsis-alliance.org/news/2017/5/26/wha-adopts-resolution-on-sepsis. WHA resolution sepsis.



Compartment	Cells of interest	Clinical consequences of	
		Hyperinflammation	Immunoparalysis
Brain	*microglia astrocytes neurons*	*headache, nausea apathy, somnolence delirium cognitive decline behavioural changes*	*unknown, potentially brain-protective*
Lungs	*alveolar macrophages epithelial cells, endothelial cells, neutrophils*	*dyspnoea lung oedema ARDS*	*increased susceptibility to secondary pulmonary infections*
Liver	*Kuppfer cells, LSEGs stellate cells, hepatocytes MDSC, neutrophils lymphocytes, platelets*	*jaundice toxin and drug accumulation metabolic disturbances coagulation disorders*	*unknown*
Blood	*neutrophils, monocytes dendritic cells, lymphocytes platelets*	*coagulation disorders hypotension and oedema septic shock*	*increased susceptibility to secondary infections*
Kidney	*renal dendritic cells, tissue-resident macrophages, lymphocytes, mast cells, RTEC*	*oliguria, anuria fluid retention toxin and drug accumulation*	*unknown*
Intestines	*epithelial cells, neutrophils, intestinal dendritic cells, macrophages, M-cells, lymphocytes, microbiome*	*ileus deteriorating nutrional status gut failure (need for TPN)*	*unknown*
Bone marrow	*HSPCs*	*unknown*	*unknown*
Adipose tissue	*adipose tissue macrophages*	*unknown, potentially sustained low-grade inflammation leading to metabolic changes*	*unknown*

FIGURE 1 | Clinical consequences of hyperinflammation and immunoparalysis to selected tissues. ARDS, acute respiratory distress syndrome; LSEC, liver sinusoidal endothelial cells; MDSC, myeloid-derived suppressor cells; RTEC, renal tubule epithelial cell; M-cell, microfold cell; TPN, total parenteral nutrition; HSPC, hematopoietic stem and progenitor cells.

development of immunoparalysis appears to start simultaneously with the proinflammatory response. The clinical relevance of immunoparalysis is further illustrated by several observational findings: (i) in patients who died from sepsis or septic shock, a continuous septic focus was observed in 63 of the 71 patients (89%) who were treated with antibiotics for more than 7 days (39), (ii) during the late phase of sepsis, infections due to opportunistic bacteria increase from 9 to 18% and Candida-infections from 13 to 30% (35), and (iii) reactivation of latent viruses was found in 43% of critically ill patients (40). Of interest, detection rate of positive viral PCR results increased with ICU length of stay and was associated with the development of fungal and opportunistic bacterial infections. Importantly, Epstein Barr virus and CMV PCR-titers in patients with a bacterial sepsis was similar to those reported in stem-cell and organ transplant patients, indicative of clinically relevant immune suppression. These recent observational insights indicate that sepsis-induced immunoparalysis accounts for the majority of sepsis-related deaths. Although the importance of immunoparalysis is increasingly recognized (28), there is still lack of consensus that immunosuppression is a clinically important phenomenon (41, 42). Nevertheless, combined with the many disappointing trials on immunosuppressive strategies, appreciation of the detrimental role of sepsis-induced immunoparalysis (7, 25–29, 43) has led the sepsis research field to focus more and more on ways to restore the suppressed immune response through immunostimulatory treatment (7, 25, 28, 29). This concept is appealing and the treatments under investigation are most likely effective to reverse immunoparalysis. However, in light of previous sepsis trials, only patients with proven immunoparalysis should receive immunostimulatory treatment to avoid unnecessary risks and increase the chances of successful trials. However, methods to identify patients with immunoparalysis and subsequent therapeutic reversal of immunoparalysis are still in their infancy.

Biomarkers to Stratify the Immune Status in Sepsis Patients

Many cytokines, chemokines or other proteins have been studied as potential biomarkers to characterize a hyperinflammatory state in sepsis patients. Three pro-inflammatory cytokines, namely tumor necrosis factor (TNF), interleukin-1β (IL-1β) and interleukin-6 (IL-6) play a pivotal role in the initial response of the innate immune system to injury or infection. These three cytokines are, among others, crucial for activation of endothelial cells, recruitment of leukocytes to the site, generation of fever and other systemic symptoms, production of acute phase reactants and induction of a shift in cell production in the bone marrow (44). Nevertheless neither TNF or IL-1β have emerged as reliable biomarker for hyperinflammation in sepsis patients, potentially because they are elevated only for a very short period of time in the initial phase of sepsis, when patients may not yet have been admitted to the ICU. IL-6 has been most extensively studied as a potential biomarker, with the advantages of being elevated for a longer period of time

and the availability of commercial bedside immunoassays (45). Elevated levels of IL-6 in septic patients have been shown to be associated with increased mortality (46, 47). However this illustrates prognostic rather than diagnostic value and like most cytokines, IL-6 is not specific for sepsis as increased levels are observed in many inflammatory conditions. Moreover the chemokines interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) have been shown to be superior to IL-6 for diagnosis of sepsis (48) and prediction of sepsis mortality (49) respectively. The acute phase protein CRP has a high sensitivity for detection of early onset sepsis (50), but its low specificity is a major drawback for its use as a biomarker to stratify the immune status in patients with sepsis. Procalcitonin (PCT) is elevated in patients with invasive bacterial infections (51). However it remains to be determined whether the detection of bacteraemia with PCT can accurately distinguish patients in a hyperinflammatiory state from patients with immunoparalysis. Research on monocyte activation markers as potential biomarkers of a hyperinflammatory state in sepsis has identified a possible role for the soluble form of the receptor for advanced glycation end-products (sRAGE). This molecule may be considered as a receptor for danger associated molecule patterns (DAMPs) and elevated levels were shown to be associated with poor survival in severe sepsis (52). Despite the numerous laboratory options illustrated above no accurate single biomarker for hyperinflammation in sepsis is currently used in clinical practice. As an alternative, a more clear clinical example for a severe hyperinflammatory state is the macrophage activation syndrome (MAS). This is defined as a fulminant cytokine storm concurrent with hepatosplenomegaly, liver dysfunction, hyperferritaemia, pancytopenia, and disseminated intravascular coagulation (53). MAS is a serious complication of sepsis and the clearly overriding hyperinflammatory state in these patients, may provide opportunities for targeted application of anti-inflammatory therapies, as discussed elsewhere in this review.

Identification of patients with immunoparalysis is currently based on HLA-DR expression on circulating monocytes and, to a lesser degree, cytokine production of leukocytes stimulated *ex vivo* with lipopolysaccharide (LPS, endotoxin) (54–57), although the accuracy of these markers still lacks solid evidence. There are no data on the predictive value on the individual patient level, meaning that precision immunostimulatory treatment for sepsis patients may not be feasible using the markers that are currently advocated. The inability to restrict immunostimulatory treatment to those patients who will actually most likely benefit from it may result in another series of failed clinical trials, because beneficial effects in immunoparalyzed patients will be offset by possible harmful effects of these compounds in immunocompetent patients. Several other markers of immunoparalysis are proposed (25), based on the increasing knowledge of the pathophysiology of immunoparalysis. These include expression of inhibitory receptors like programmed death-1 (PD-1) and its ligand PD-L1 (58), cytotoxic T lymphocyte antigen-4 (CTLA-4) (59), and B and T lymphocyte attenuator (BTLA) (60), molecules that play a role in the exhaustion of lymphocytes. Furthermore, several immunosuppressive lymphocyte subpopulations (including T-regulatory cells) have been identified in patients suffering from immunoparalysis (61). Moreover, epigenetic changes were shown to be involved in immunoparalysis (54), revealing that TLR-induced chromatin modifications are responsible for transient silencing of tolerizable (T) genes (including those encoding proinflammatory mediators), and for priming of non-tolerizable (NT) genes (including those encoding antimicrobial peptides). The T genes are transiently inactivated to prevent pathology associated with excessive inflammation, while the NT genes remain inducible to provide continuous protection from infection and tissue repair. In addition, it was demonstrated that negative TLR regulators such as IRAK-M and SHIP-1, might also participate in the development of immunoparalysis (62). However, the role of these relatively recently discovered mechanisms in the pathogenesis of immunoparalysis is currently unclear and clinical application as a biomarker is not yet feasible.

Understanding what causes the contrasting hyperinflammatory and immunoparalyzed phenotypes observed in sepsis—in other words: why do some patients exhibitit a prevailing hyperinflammatory response while others display immunoparalysis—would greatly aid biomarker discovery and development. To the best of our knowledge, this is currently unknown and a multifactorial etiology is likely, including host-related factors such as age, gender, comorbidities, (epi)genetic predisposition, microbiome composition, expression levels of pattern-recognition receptors (PRRs), and release of DAMPs as well as pathogen-related factors such as the type of pathogen, its virulence and load, and quorum sensing.

Whether it concerns hyperinflammation or immunoparalysis, it appears implausible that a single marker can act as a reliable tool to guide immunomodulating therapy since biomarkers are often related to one or a limited number of pathophysiological mechanisms/pathways, while it has become clear that multiple pathways are activated or inhibited at the same time in sepsis. Therefore, it appears likely that a panel of markers reflects the immune status of the sepsis patient more accurately.

Compartmentalization of the Immune Response

At this moment, plasma markers or expression of molecules in/on circulating immune cells are used to identify the immune status of sepsis patients. However, due to compartmentalization of the immune response and temporal differences in the immune response between compartments, the phenotype of blood leukocytes may not always be reflective of the current immune status. There are several observations in support of this notion. For instance, leukopenia is associated with a more pronounced cytokine response in animal models of sepsis (63), indicating limited importance of blood immune cells in producing inflammatory mediators that are important for host defense. Furthermore, the compartmentalized nature of the immune response is supported by several results obtained by our group in the human endotoxemia model. In this model endotoxin [also known as lipopolysaccharide (LPS)] is administered to healthy volunteers. Numerous studies have established that the immune response to LPS captures many hallmarks of the immune response observed in sepsis, including a phase of immune

suppression. The latter phenomenon is known as "endotoxin tolerance," and characterized by a blunted inflammatory response upon subsequent LPS challenges *in vivo* and *ex vivo* (64). In keeping with the overlap between LPS-elicited effects and clinical sepsis, endotoxin tolerance bears many similarities to sepsis-induced immunoparalysis, including decreased cytokine production by circulating leukocytes and attenuated mHLA-DR expression (25, 27, 43). Therefore, endotoxin tolerance has been used in translational research to model and investigate treatments for sepsis-induced immunoparalysis, both *in vitro* (65) and *in vivo* in humans (43). Strikingly, however, animal studies have shown that despite a diminished immune response indicating endotoxin tolerance, pathogen clearance and survival upon a live bacterial challenge were improved in mice pre-treated with LPS or other TLR ligands (66–69). It is unknown whether these counterintuitive findings of an enhanced defense against pathogens concurrent with endotoxin tolerance are also present in humans, which should be an important focus for future studies. Nevertheless, using the human endotoxemia model we have demonstrated profound differences in *ex vivo* and *in vivo* endotoxin tolerance kinetics. Both mHLA-DR expression (43) and *ex-vivo* cytokine production by stimulated leukocytes (70–72) are suppressed rapidly in the first hours after endotoxin administration. The same studies show that these markers normalize quickly afterwards and are restored within 24 h after endotoxin administration, indicating that functionality of circulating leukocytes can quickly recover in the absence of ongoing inflammation. In contrast, the *in vivo* response 1 to 2 weeks after the first endotoxin administration is still severely blunted with an attenuation of pro-inflammatory cytokine levels of approximately 60% compared to the first endotoxin challenge (43, 70). From these observations it could be concluded that parameters measured in the blood compartment may not reflect the responsiveness of the immune system as a whole, and that immune cells in other compartments than the blood may likely better reflect the *in vivo* immune status at a given moment in time. It is currently unknown whether this discrepancy between the immune status of cells within the blood compartment and the responsiveness of the *in vivo* immune system is of clinical importance in sepsis patients, but tissue resident macrophages and not circulating immune cells appear to be predominantly responsible for the innate immune response in sepsis. The relevance of organ-specific immunology and possible consequences of a hyperinflammatory or immune-suppressed state in several tissues (e.g., lungs, brain, adipose tissue, and bone marrow), are graphically presented in **Figure 1** and outlined below.

The lungs likely represent a highly relevant compartment in the context of sepsis-induced hyperinflammation and immunoparalysis. An exaggerated pro-inflammatory response in the lungs may result in the life-threatening condition of ARDS, requiring invasive mechanical ventilation and complex respiratory therapy (73). In contrary, an immunosuppressed response as observed in immunoparalysis increases the susceptibility to secondary pulmonary infections. Along these lines, hospital-acquired pneumonia represents the secondary infection with the highest incidence observed in patients that recovered from their initial sepsis (42, 74, 75). Alveolar macrophages (AMs), as the first line of defense in the lungs, play a key role in host defense. Whereas previous work has demonstrated that AMs display a primed rather than tolerant phenotype shortly (1.5–6 h) after LPS administration in healthy volunteers (76), small studies in septic patients point toward a tolerant phenotype of AMs (77, 78). This may indicate that AMs switch from an initially primed phenotype to a tolerant immunosuppressed phenotype at later stages of disease progression, but these dynamics have yet to be unraveled.

The brain was long regarded as an immune privileged organ. The last decades of research have shown an important immunological role of the brain in "non-immunological" diseases like dementia, or psychiatric diseases. The so-called microglial cells represent the resident macrophage population in the brain, and account for 5–20% of the total glial cell population of the brain. Research in healthy humans (79), animal data (80) and post-mortem brain tissue of patients suffering from severe systemic inflammation (81, 82) show that systemic inflammation is a strong trigger that activates the resident microglia (macrophages) of the brain. During systemic inflammation, systemic inflammatory cytokines can enter brain tissue due to a disrupted blood-brain barrier, but also through several parts of the brain that lack a blood-brain barrier and directly activate microglial cells. As a result, systemic inflammation may result in an exaggerated neuroinflammatory cascade, which disrupts normal homeostasis and cell function and may lead to neuronal cell loss and cognitive deterioration (83, 84). Neuroinflammation is thought to contribute to both acute sepsis-associated encephalopathy, as well as long-term cognitive impairment following critical illness. Previously, research into immune responses of the brain during systemic inflammation was impossible in living patients as the brain is a body compartment not accessible for immunological research purposes without using the invasive procedure of brain biopsies. Recently, several innovative nuclear imaging tracers have been developed that can quantitatively measure microglial activation *in vivo*, by targeting the mitochondrial 18 kDa translocator protein (TSPO). Systemic inflammation evoked during experimental human endotoxemia was demonstrated to induce a 30–60% increase in microglial activation in healthy volunteers 3 h after administration of endotoxin (79). However, patients suffering from sepsis often develop more prolonged periods of systemic inflammation and a subsequent immunosuppressive state. The longitudinal effects of endotoxemia and systemic inflammation on tissue resident macrophage activation in the brain are unclear. A recent study in mice showed that repeated subjection to systemic inflammation on consecutive days induced a brain-specific training effect initially, followed by reduced immunological response (immune tolerance) of the brain after successive stimuli (85). In addition, a recent human study in prostate surgery patients found decreased microglial activity, measured by reduced microglial nuclear ligand binding, 3–4 days postoperatively compared to baseline (86). These results are the first indications that innate immunity responses in the brain show signs of immunoparalysis, coinciding with *ex vivo* and *in vivo* peripheral immunoparalysis.

Recent studies have demonstrated that microbial components can directly interact with hematopoietic stem and progenitor cells (HSPCs) in the bone marrow via Toll-like receptors (TLRs) expressed on HSPCs (87, 88). Microbial sensing by HSPCs during infection may therefore influence hematopoietic cell division and differentiation (88), and may ultimately impact the efficacy of host defenses during infection. Moreover, septic patients also exhibit reduced expression of HLA-DR in the myeloid lineage of the bone marrow (89). To date, the effects of systemic inflammation on HSPCs and their role in development and maintenance of hyperinflammatory responses or immunoparalysis in humans are unknown and represents an exciting field for further study.

Adipose tissue is increasingly recognized as an immunological organ, containing substantial amounts of adipose tissue macrophages (ATMs) (90). Advances on the interplay between metabolic and immunological research suggest an important role of the immune system in metabolic conditions such as obesity and diabetes mellitus (91). Lipids are important signaling moieties for both immune responses and metabolic regulation. Lipid infusion *in vivo* activates TLR4 signaling in adipocytes and macrophages and enhances inflammatory gene expression in adipose tissue (92) which adds to systemic insulin resistance. During obesity, many immune cells infiltrate in adipose tissue and promote a low-grade chronic inflammation (91). Conversely is the role of adipose tissue during systemic inflammation (e.g., sepsis) less well studied and human studies concerning the dynamics of immune suppression/tolerance in ATMs are completely lacking.

Future studies to characterize the immune response in other body compartments are ongoing and necessary to further characterize the *in vivo* immune status. These studies will improve the insight in the mechanistics of the immunological response during sepsis. It needs to be acknowledged that guiding immunotherapy in clinical practice based on other compartments than blood will however not be readily applicable, since tissue-resident immunological markers are not easily harvested or measured.

Novel Treatment Modalities in Sepsis

As mentioned earlier, previous therapeutic strategies for sepsis patients have virtually exclusively focused on blocking inflammation early in the course of sepsis. It has become clear that a considerable proportion of sepsis patients do not die from an overwhelming immune response and that suppressing the immune system is not an effective strategy when applied to all sepsis patients. Nevertheless, it is conceivable that a subgroup of hyperinflamed patients may have benefited from immunosuppressive therapy if they had been treated according to their immune status. For example, the phase 3 trial evaluating the immune suppressant IL-1 receptor antagonist (anakinra), performed more than 20 years ago, revealed no effect on mortality in severe sepsis patients (21). However, a *post-hoc* analysis of this study published many years later (93) demonstrated a significantly lower mortality in a subgroup of patients with MAS, which represented approximately 6% of the enrolled sepsis patients. Although no definitive conclusions can be drawn from this *post-hoc* analysis, it does suggest that a specific subgroup of patients in a hyperinflammatory state could benefit from immune suppressive therapy. Another argument that therapy aimed at inhibition of the immune response should not be discarded as of yet comes from a meta-analysis of 17 randomized controlled trials (almost 9,000 patients) evaluating the effects of anti-tumor necrosis factor alpha (TNF-α) therapy on mortality in severe sepsis. Despite negative results in each individual study, the pooled odds ratio showed a significantly reduced 28-day all cause mortality (94).

In the last decade, several immunostimulatory treatments have shown promise in preclinical, as well as in case series and/or small clinical studies (58, 95–104). Granulocyte-macrophage colony stimulating factor (GM-CSF) and interferon-gamma (IFNγ) are the most extensively investigated immunostimulatory agents in sepsis. These compounds are potent stimulators of myeloid cell function and they potentiate antigen presentation capabilities through increasing mHLA-DR expression on and pro-inflammatory cytokine production by monocytes (105, 106). A biomarker-guided (inclusion criterion mHLA-DR>8000 monoclonal antibodies per cell) randomized controlled trial comparing GM-CSF to placebo showed that GM-CSF was safe and effective in restoring monocytic immunocompetence. Exploratory endpoints suggested that treated patients had shorter duration of mechanical ventilation and a more swift decrease of disease severity scores (95). Although a meta-analysis of 4 RCT's did not reveal a beneficial effect of GM-CSF on 28-day mortality, patient numbers were probably too small for evaluation of this endpoint (107). Treatment with IFNγ resulted in increased mHLA-DR expression and restored TNFα production in a human endotoxemia study, while further attenuating production of the key anti-inflammatory cytokine IL-10 (43). Furthermore, IFNγ showed promising results in several small case series, for instance in patients suffering from opportunistic infections not responding to regular treatment (97). Targeting lymphocyte loss with apoptosis inhibitors has shown potential in animal studies (108–110) but could theoretically result in uncontrolled cell growth and organ injury as consequence of neutrophil accumulation in tissues. Recently, the IRIS-7 randomized controlled phase 2 trial was published, in which 27 patients with septic shock were treated with recombinant human IL-7 or placebo (111). In this trial, severe lymphopenia was used to identify immunosuppressed patients. The anti-apoptotic and lymphocyte function-enhancing cytokine IL-7 was well tolerated and reversed sepsis-induced lymphopenia in these patients. Naturally, statistical power to demonstrate clinically relevant treatment effects was inadequate. Another attractive immunostimulatory target is blockade of programmed death-1 (PD-1) or its ligand PD-L1. The PD-1 system is upregulated in sepsis patients (37) and inhibition of the interaction between PD-1 and its ligands promotes immune responses and antigen-specific T-cell responses. In recent years, positive responses to anti-PD-L1 therapy was demonstrated in the field of oncology and given the many similarities between the immunosuppressive mechanisms in cancer and sepsis, this could be promising for the potential of PD-L1 antagonism in sepsis-induced immunoparalysis. Unfortunately, a large multicenter trial was

TABLE 1 | Examples of immunotherapy in sepsis.

	Mechanism of action	Summary of evidence
IMMUNOSUPPRESSIVE COMPOUNDS		
anti-TNFα (various)	Blocks pro-inflammatory effects of TNFα	- Individual studies: no beneficial effects (94) - Meta-analysis: reduced 28-day mortality, OR = 0.91 [95% CI 0.83–0.99] (94)
IL-1RA (anakinra)	Blocks IL-1 receptor → inhibits downstream pro-inflammatory effects	- Study in unselected population of severe sepsis patients: no effect on mortality (21) - *Post-hoc* analysis in subgroup of hyperinflamed patients with macrophage activation syndrome: lower mortality (93)
IMMUNOSTIMULATORY COMPOUNDS		
GM-CSF	Enhances antigen presenting capacity and pro-inflammatory cytokine production	- Meta-analysis: no effect on 28-day mortality in sepsis patients (probably underpowered) (107) - Biomarker-guided study (based on mHLA-DR expression): restoration of monocytic immunocompetence, shorter duration of mechanical ventilation, and more swift improvement of disease severity scores as exploratory endpoints (95)
IFN-γ	Enhances antigen presenting capacity and pro-inflammatory cytokine production	- Human endotoxemia model (mimicking sepsis-induced immunoparalysis): increased mHLA-DR expression, restored TNFα production and further attenuated IL-10 production (43) - Case series in patients suffering from opportunistic infections not responding to regular treatment: increased mHLA-DR expression and cytokine production by *ex vivo*-stimulated leukocytes (97)
Recombinant human IL-7	Reduces apoptosis and enhances lymphocyte function	- Phase 2 trial in septic shock patients with severe lymphopenia: safe, well-tolerated and reversal of lymphopenia (111)
anti-PD-(L)1	Inhibits PD-1-PD-L1 interaction → reduces apoptosis and promotes T-cell responses	- Preclinical data in sepsis models: promising results (e.g., prevention of sepsis-induced depletion of lymphocytes, increased TNF-α and IL-6 production, decreased IL-10 production, enhanced bacterial clearance, improved survival (102) - Clinical data in the oncology field: effective, especially in advanced melanoma and non-small cell lung cancer. - No clinical trials in sepsis patients yet.

TNFα, tumor necrosis factor alpha; IL1RA, Interleukin-1 receptor antagonist; IL-1, interleukin-1; GM-CSF, granulocyte-macrophage colony stimulating factor; IFNγ, interferon gamma; IL-7, interleukin-7; anti-PD-L1, programmed death-1 ligand antagonist; OR, odds ratio.

recently aborted by the sponsor due to other priorities ("CA209-9FH, a randomized, double-blind, placebo-controlled, parallel-group study to evaluate the efficacy and safety of nivolumab in adults with sepsis"). **Table 1** summarizes the mechanism of action and the (clinical) evidence thus far of the immunomodulatory compounds described in this paragraph.

Fortunately, a number of clinical trials investigating these and other immunostimulatory treatments are currently underway or planned, illustrating the current interest and relevance of this type of treatment for sepsis[3]. However, these either do not use markers to enrich their patient population, or use biomarkers of which the accuracy and robustness has not been sufficiently demonstrated.

Summary and Future Directions

This review highlights the current challenges we face toward precision immunotherapy for patients suffering from sepsis. The urgent need for a patient-tailored approach in sepsis treatment is clear, as 40 years of undirected sepsis trials have not resulted in a single adjunctive therapy in current clinical use. Clearly, we need tools to determine whether hyperinflammation or immune suppression is the overriding immune dysfunction in a specific patient. The paradigm shift in the understanding of the pathophysiology of sepsis has resulted in increased interest for promising immunostimulatory therapies, but it is key to identify and select the appropriate patient population who may most likely benefit from these compounds. So far, current circulating biomarkers measured in blood have not been found sufficiently robust for use in clinical practice. From a pathophysiological perspective studies into other body compartments are warranted to increase our understanding of the *in vivo* immune response in patients with sepsis, but these insights will not be easily translated in feasible methodology for clinical practice. As such, finding a reliable biomarker to classify and monitor the overall immune response in patients with sepsis and to guide and personalize immunotherapy remains a holy grail.

[3]Clinicaltrials.gov registration no's: NCT02797431, NCT02576457, NCT02361528, NCT02867267.

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GENERAL COMMENTARY
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Commentary: Precision Immunotherapy for Sepsis

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A Commentary on

Precision Immunotherapy for Sepsis
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We read with interest the review article published in *Frontiers in Immunology* by Peters van Ton et al., who suggest that precision immunotherapy might benefit organ failure and reduce the mortality of sepsis patients. Sepsis is still an enormous health-threat worldwide and mortality rates are still very high despite recent advances in early recognition. In that sense, we agree with Peters van Ton and colleagues, a therapy that will reduce mortality and morbidity in sepsis patients is urgently needed. The question is; will that be immunotherapy?

More than a century ago bacterial infection was recognized as the cause of sepsis. When antibiotics did not save all patients, the logical step was to suspect a failing immune system. The immune system is clearly an early and active responder during organ failure in sepsis but there is more to the host response than just a derailed immune system. What clinical evidence really supports the immune system as the key "organ" causing death in sepsis patients?

As the authors state, decades of failed clinical trials focusing on immunomodulating therapies have not resulted in a new therapy for sepsis. Back in 1996, Roger Bone discussed the first failed clinical trials and already suggested then, that the model of persistent, uncontrolled inflammation was inaccurate (1). He pleaded that we should learn from our mistakes and emphasized the necessity to examine all of the physiological responses the body was capable of mounting, not simply the most or least severe (1). Instead, his advice was largely ignored, and a further 25 years of clinical trials targeting the hyperinflammatory response in sepsis patients ensued; primarily driven by the hope of finding the "magic bullet," but also fueled by the pharmaceutical industry. At that time, Bone mentioned that the anti-inflammatory response had, for the most part, been ignored. However, in recent years it has gained increasing attention mainly due to the many clinical trials that were unable to improve patient outcome. Sepsis induced-"immunosuppression" or "immunoparalysis" is now thought to be one of the main drivers of mortality and morbidity in patients, which has led to the birth of immunostimulatory compounds as a potential new therapy.

However, we fear that switching from taming an overactive immune system to stimulating a depressed system, or even regulating the immune system on-demand will end up in new disappointments. Moreover, recent evidence suggests that it's not *either-or*, patients can become hyperinflammed and immunosuppressed concurrently (2), questioning the usefulness and safety of immunostimulatory compounds. We therefore agree with the authors, identifying patients that might benefit from immunostimulatory compounds and those that might not is extremely crucial.

Many believe that patients succumb to sepsis as a result of secondary infection due to immunosuppression and the inability to fight infection. Although this might be the case for some patients, it certainly doesn't hold true for all patients with sepsis. Van Vught et al., showed in their study that only 13.5% of all sepsis ICU-admissions developed secondary ICU-acquired infection.

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Despite these patients having a higher disease severity score at admission the contribution of secondary infection on overall mortality was low (3). The authors also refer to studies showing that pre-exposure to bacterial products resulted in improved clearance and survival upon rechallenge with live bacteria (4, 5). Moreover, a causal relationship between immune suppression and mortality from ICU-acquired secondary infection has not yet been reported.

The authors also highlight an important concept, it is currently unknown whether the immune status of organs in septic patients is comparable to the immune status within the blood compartment. This knowledge is clinically important, since as the authors state, tissue resident macrophages and other cells appear to be primarily responsible for the innate immune response in sepsis rather than the circulating immune cells. Whether treatment with immunostimulatory compounds results in further organ dysfunction is also currently unknown. One might imagine that treatment with immunostimulatory compounds that promote systemic TNFα release within the blood compartment of immunosuppressed patients may be beneficial if they need to fight secondary infection (6, 7), but it may also result in detrimental cellular responses resulting in further organ function deterioration.

Expecting immunotherapy to diminish organ failure in all patients with sepsis is unrealistic. Recent studies have identified sepsis endotypes with different clinical and molecular profiles (8, 9). The host-response to sepsis therefore differs per patient implying the need for different treatment strategies rather than the traditional "one-size-fits-all" approach. These types of studies should be embraced since this will unquestionably promote recognition of specific groups for precise therapy as well as advancing better clinical trial design.

There is also a danger that the immunotherapy hype will overshadow investment into other promising therapies targeting other aspects of organ failure. This would be unfortunate since some patients will benefit from immunotherapy whereas others may benefit from a different type of treatment, or more likely, a combination of different therapies. In that sense, we really have to broaden our view and support translational research aimed at further elucidating the other "host-responses" and mechanisms that may be mediating organ failure in patients with sepsis, in order to uncover treatment strategies other than immunotherapy.

AUTHOR CONTRIBUTIONS

JZ and JM: conceived, drafted, and wrote this commentary; MvM provided important intellectual contribution. All authors read and approved the submitted version.

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Therapeutic Potential of the Gut Microbiota in the Prevention and Treatment of Sepsis

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Alongside advances in understanding the pathophysiology of sepsis, there have been tremendous strides in understanding the pervasive role of the gut microbiota in systemic host resistance. In pre-clinical models, a diverse and balanced gut microbiota enhances host immunity to both enteric and systemic pathogens. Disturbance of this balance increases susceptibility to sepsis and sepsis-related organ dysfunction, while restoration of the gut microbiome is protective. Patients with sepsis have a profoundly distorted composition of the intestinal microbiota, but the impact and therapeutic potential of the microbiome is not well-established in human sepsis. Modulation of the microbiota consists of either resupplying the pool of beneficial microbes by administration of probiotics, improving the intestinal microenvironment to enhance the growth of beneficial species by dietary interventions and prebiotics, or by totally recolonizing the gut with a fecal microbiota transplantation (FMT). We propose that there are three potential opportunities to utilize these treatment modalities over the course of sepsis: to decrease sepsis incidence, to improve sepsis outcome, and to decrease late mortality after sepsis. Exploring these three avenues will provide insight into how disturbances of the microbiota can predispose to, or even perpetuate the dysregulated immune response associated with this syndrome, which in turn could be associated with improved sepsis management.

Keywords: microbiota, sepsis, pathogenesis, therapeutics, probiotics and synbiotics, fecal microbiota transplantation

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INTRODUCTION

Sepsis is a highly heterogeneous and multifaceted syndrome that is caused by a dysregulated host response to an infection (1, 2). A disproportionate pro-inflammatory response to invasive infection was once believed to be the main pathophysiological paradigm of sepsis syndrome. However, recent studies have shown that patients with sepsis suffer from both sustained excessive inflammation and immune suppression, with a failure to return to normal homeostasis (3). Despite our increased understanding of the underlying pathophysiology of the syndrome, targeted therapies aimed at modifying the disrupted host response in patients with sepsis have been unsuccessful to date (4). Treating physicians are limited to supportive care and antibiotics as the disorder continues to drain billions of dollars and contributes to an estimated 5 million deaths worldwide each year (5, 6).

Therefore, new tools in the management of sepsis are highly warranted.

In tandem with the advances of our understanding on the pathophysiology of sepsis, tremendous strides have been made in understanding the pervasive role of the gut microbiota in both health and disease states. Pre-clinical work has shown that a diverse and balanced gut microbiota is able to enhance host immunity to both enteric and systemic pathogens, and that disturbance of this balance potentially leads to increased susceptibility of sepsis (7, 8). Of interest, a handful of pioneering studies have shown that the composition of the intestinal microbiota is severely affected by sepsis, but the short- and long-term clinical consequences of these disturbances remain largely unknown (9–12). In this Review, we aim to provide an overview of the mechanisms through which the microbiota contributes to host defense in sepsis, and how these insights could be of relevance to this multi-facetted syndrome. Better insights into the drivers of microbiota-mediated host resistance could provide novel preventive and therapeutic strategies against sepsis.

MICROBIOTA-MEDIATED HOST RESISTANCE

The intestinal microbiota is a complex ecosystem that consists of trillions of bacterial cells that have evolved with its hosts over millions of years (13). In the last decade, it has been revealed that these microbial communities are involved in a wide of array of functions, such as the digestion of food, production of hormones, and the development and maturation of the immune system (14–16). In addition, it has been shown that a state of disturbance of the intestinal microbiota, otherwise known as dysbiosis, plays an important role in increasing susceptibility to infectious diseases (17, 18). Several mechanisms have been uncovered on how the intestinal microbiota contributes to protection against enteric pathogens, such as *Clostridium difficile* (19). For example, intestinal microbes directly outcompete pathogens for nutrients, possess the capacity of producing antibacterial peptides, modify bile salts to render them harmful to other microorganisms, as well as drive increased mucus production and intestinal epithelial integrity. In addition, gut bacteria contribute to resistance to enteric pathogens by inducing the production of antibacterial factors by epithelial cells, and enhancing humoral responses against invading pathogens (19).

Recent insights have revealed that the microbiota also modulate systemic immunity (**Figure 1**). A 2016 hallmark study involving 500 healthy human volunteers linked the gut microbiota to inflammatory cytokine production capacity using *ex vivo* stimulation of whole blood and peripheral blood mononuclear cells: the observed inter-individual variation in cytokine responses was significantly correlated with the composition and function of the microbiota (20). Moreover, in healthy subjects in which the microbiota is disrupted by broad-spectrum antibiotics, systemic mononuclear cells produced lower levels of tumor necrosis factor (TNF)-α after *ex vivo* stimulation with lipopolysaccharide (LPS) (21). Of interest however, microbiota disruption by broad-spectrum antibiotics did not affect systemic innate immune responses in a human endotoxemia model, perhaps due to redundancies in the human immune response (22).

Numerous murine models have shown the existence of so called "gut-organ axes" such as the "gut-lung axis", and the "gut-brain axis." Besides cytokines, communication in these axes is probably mediated by microbe-associated molecular patterns (MAMPs), such as LPS, peptidoglycan and flagellin, as well as by microbiota-derived metabolites that are able to translocate from the gut into the systemic circulation, where they have the potential to modulate immune cells to enhance regulatory or pro-inflammatory responses (23, 24). In this way, intestinal bacteria can even direct the influx of immune effector cells into distant organs. For example, in newborn mice, exposure to gut bacteria increased homing of group 3 innate lymphoid cells (ILC3) from the gut mucosa toward the lung, which increased resistance against pneumonia (25). Other studies have shown that systemic exposure of microbiota-derived ligands increases the activity of alveolar macrophages and bone marrow derived neutrophils, which enhances the killing of Gram-positive and Gram-negative pathogens in the lung (26–28).

Similar priming mechanisms by toll-like receptor (TLR) ligands have shown to induce protection against respiratory viruses and fungi (29, 30). For example, neomycin-sensitive commensal bacteria induce the expression of messenger RNA (mRNA) for pro–IL-1β and pro–IL-18, which enhances the clearance of influenza virus in a T helper cell type 1, Cytotoxic T cell, and Immunoglobulin A (IgA) dependent manner (31). These findings were recently confirmed by Rosshart and colleagues, who developed a model of microbiota reconstitution from mice that were caught in the wild into genetically identical laboratory mice (32). Recolonization with a "natural" microbiota increased survival following influenza virus infection, which was attributed to a decreased pro-inflammatory response and a pronounced IL-10- and IL-13-mediated anti-inflammatory phenotype (32).

It has been shown that short-chain fatty acids (SCFAs), products of fiber fermentation by anaerobic bacteria, possess strong immunomodulatory properties through activation of certain G-protein-coupled receptors (GPRs), such as GPR41 and GPR43 (14, 33). In addition, the SCFAs butyrate and propionate facilitate the generation of extrathymic regulatory T cells, which have a key role in limiting inflammatory processes (34). Administration of butyrate also has the potential of restoring interleukin (IL)-10 levels in the lung by inhibiting histone deacetylase in myeloid-derived suppressor cells (MDSCs), which in turn reduces persistent lung inflammation during murine *K. pneumoniae* infection (35). Similar findings were observed in a cohort of allogeneic stem cell recipients, as a higher representation of butyrate-producing bacteria in the gut was associated with ameliorated respiratory viral infections (36). Besides short-chain fatty acids, it has also been found that desamonityrosine (DAT), a degradation product of flavonoids that is produced by *Flavonifractor plautii*, increases survival in mice infected with influenza through augmentation of type I interferon (IFN) signaling (37).

The immunomodulatory role of the microbiota extends beyond the lung, as presence of microbiota-derived LPS leads

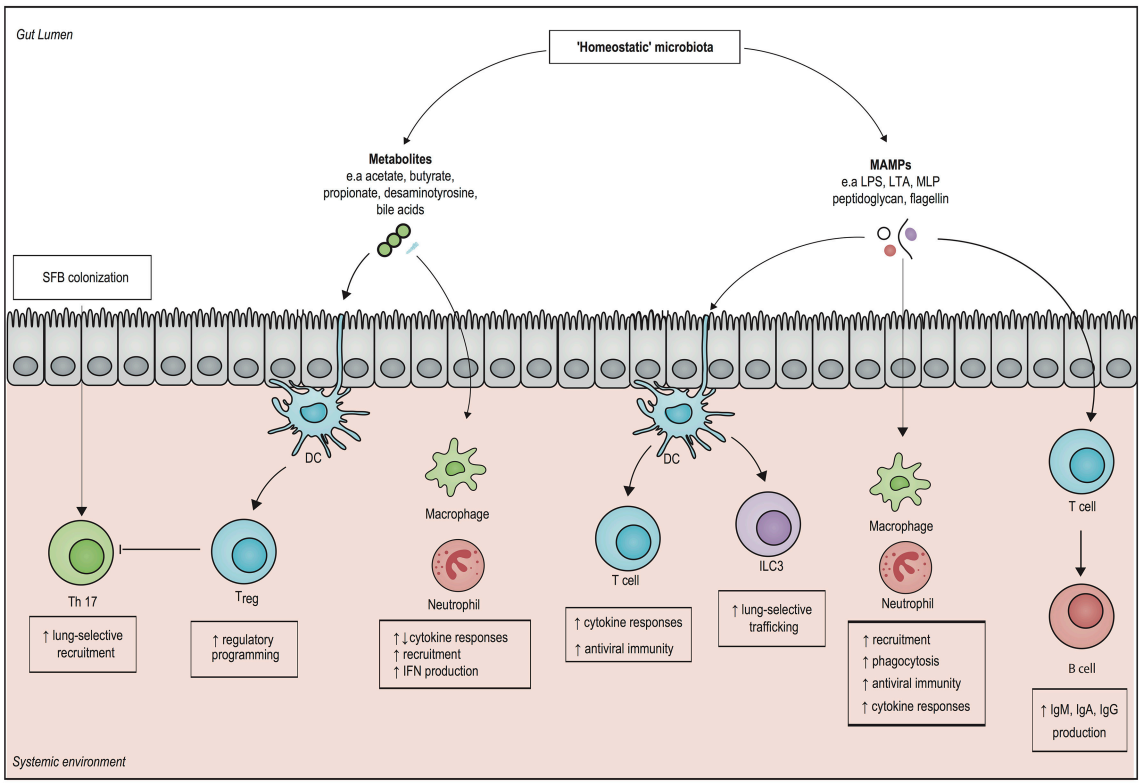

FIGURE 1 | Overview of systemic immunomodulatory mechanisms associated with the microbiota. Structural components of gut microbiota, otherwise known as microbe-associated molecular patterns (MAMPs), can elicit a systemic pro-inflammatory response by activating pattern recognition receptors of both the innate and the adaptive immune system. Microbial metabolites, such as short chain fatty acids (SCFAs) modulate epigenetic changes in host leukocytes, which can induce both pro- and anti-inflammatory responses. The presence of the SCFAs butyrate and propionate drives the generation of regulatory T cells (T_{reg}), which dampen inflammation. In addition, the gut metabolite desaminotyrosine enhances clearance of respiratory viruses by inducing type 1 interferon (IFN) responses. Direct interactions with epithelial cells by segmented filamentous bacteria (SFB) can enhance mucosal immunity by upregulating T helper 17 (Th 17) cells in both in the gut and in the lung. It is important to realize that our knowledge on microbiota-derived host-resistance is fragmented and it remains to be determined how these individual mechanisms fit in an overarching framework of systemic immunity. DC, dendritic cell; ILC3, type 3 innate lymphoid cell; Treg cell, regulatory T cell; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, immunoglobulin M; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MLP, murein lipoprotein.

to increased recruitment of bone marrow-derived neutrophils, which enhances clearance of blood-borne pathogens, such as *Escherichia coli* and *K. pneumoniae* (38). Additionally, outer membrane components of Gram-negative bacteria in the gut, such as murein lipoprotein (MLP) and LPS, protect against experimental sepsis by modulating serum levels of Immunoglobulin G (IgG) and IgM, in a T cells and Toll-like receptor 4 on B cells-dependent manner (39, 40). These findings have recently been supplemented by Wilmore and colleagues (41), who have shown the that enriching the microbiota with members of the Proteobacteria phylum led to T cell-dependent increases in serum IgA levels by IgA-secreting plasma cells in the bone marrow. The resulting serum IgA bound specifically targeted a restricted number of pathogens that translocate from the gut into the systemic circulation, which increased resistance to sepsis (41). Despite the abovementioned indications that the microbiota is involved in the systemic host-defense against a wide variety of pathogens, further mechanistic studies are needed to provide overarching pathways that can be fitted into the framework of host-defense against sepsis.

CAUSES AND CONSEQUENCES OF DYSBIOSIS IN SEPSIS

It has been shown that patients with sepsis have a profoundly distorted composition of the intestinal microbiota (9–12). In general, the diversity of the microbiota in patients with sepsis decreases rapidly upon hospital admission, a finding that becomes even more pronounced in later stages of their hospitalization (11). An extensive overview of the potential effects of sepsis on the intestinal microbiota has recently been published elsewhere (42). In short, these shifts in microbiota composition can be partially explained by clinical interventions, such as (par)enteral feeding, mechanical ventilation, as well as the omnipresent administration of proton pump inhibitors, opioids, vasopressors and—above all—antibiotics (42–44). Moreover, patients with sepsis have impaired gastrointestinal motility and diminished intestinal epithelial integrity, leading to a loss of "beneficial" anaerobic bacterial families, such as *Lachnospiraceae* and *Ruminococcaceae*, which further impairs intestinal epithelium function and allows for the expansion and

potential translocation of aerobic opportunistic pathogens (45–47). The effects of sepsis on the microbiota extend beyond the gastrointestinal tract and can also influence the composition of for instance the lung and skin microbiota (42).

Several researchers have hypothesized that these shifts in microbiota composition potentially predispose patients to a state of immunosuppression (21, 48). There has also been renewed attention to the theory that systemic translocation of opportunistic gut bacteria increases the risk of organ failure (49). For example, the severity of acute kidney injury during sepsis can be ameliorated by administration of the three SCFAs acetate, propionate, and butyrate, which epigenetically attenuate the inflammatory process and reduce subsequent damage (50, 51). In addition, reconstitution of mice with the commensal *E. coli* O21:H+ induces signaling of the IGF1/PI3K/Akt pathway, which in turn prevents muscle wasting triggered by sepsis (52). Studies from the previous century showed that translocation of intestinal bacteria seems to be also associated with the development of acute respiratory distress syndrome (ARDS) (53, 54). Dickson and colleagues have recently found support for these findings by revealing that during murine sepsis and in human patients with ARDS, lung communities are enriched by bacteria that have translocated from the intestine. The presence of these communities, such as *Bacteroides spp*, is correlated with the intensity of systemic inflammation (55). Finally, preliminary research in mice and patients who died of sepsis suggests that bacterial translocation could also be associated with acute neuro-inflammation in sepsis (56), which aligns with the notion that gut microbes play a role in regulating the central nervous system (57). These studies provide clues that the extreme shifts observed in sepsis patients are not merely markers of illness severity, but also potentially contribute to worse outcome. However, human translational studies are lacking, so our current understanding of the short- and long-term consequences of ICU-related dysbiosis in clinical practice is limited (42). Further studies are needed to confirm that disturbances of the microbiota truly contribute to organ failure, and that dysbiosis is not merely a reflection of the profound dysregulation that is present during sepsis.

POTENTIAL FOR MODULATION OF THE MICROBIOTA IN THE MANAGEMENT OF SEPSIS

Harnessing the immunomodulatory properties of the microbiota could provide an attractive preventive and therapeutic opportunity for sepsis. Modulation of the microbiota has been studied extensively outside of the sepsis arena, and predominantly consists of either resupplying the pool of beneficial microbes by administration of probiotics, improving the intestinal microenvironment to enhance the growth of beneficial species by dietary interventions and prebiotics, or by totally recolonizing the gut with a fecal microbiota transplantation (FMT). We propose that, within the course of sepsis, three therapeutic approaches exist that, when exploited, could potentially improve prevention and management of the disorder.

Avenue 1: Targeted Microbiota Modulation for At-Risk Patients, Prior to Sepsis Development

The abovementioned pre-clinical observations demonstrating a link between dysbiosis and increased risk of sepsis have now gained some preliminary footing in human studies. For example, patients undergoing allo-HCT who develop antibiotic-induced dysbiosis have a 5- to 9-fold increased risk of bloodstream infection and sepsis (58). In line with these findings, a retrospective cohort of over 10.000 elderly patients in the United States showed that hypothesized instances of dysbiosis were associated with a more than 3-fold increased incidence of a subsequent hospitalization for sepsis (7). And, expanding on these findings, Baggs et al. recently showed that exposure to longer durations of antibiotics, additional classes of antibiotics, and broader-spectrum antibiotics during hospitalization are each associated with dose-dependent increases in the risk of subsequent sepsis (59). This association was not found for other causes of hospital readmissions, suggesting that the association between antibiotic exposure and subsequent sepsis is related to microbiome depletion, not merely illness severity (59).

Probiotics have been evaluated for preventing nosocomial infection in many smaller studies, and meta-analyses suggest that probiotics are safe and effective at preventing infection in both post-operative and mechanically ventilated patients (60, 61). However, the small size of the individual studies, as well as the variable type and dose of probiotic therapy limit strong conclusions. A large-scale study of probiotics is underway in Canada to test the benefit of probiotics for preventing ventilator-associated pneumonia (clinicaltrials.gov: NCT02462590). Excitingly, in a recent randomized, double blind, placebo-controlled trial of 4,556 healthy, term infants in rural India, administration of an oral synbiotic preparation (*Lactobacillus plantarum* plus a fructooligosaccharide) resulted in a 40% relative risk reduction for lower respiratory tract infections, sepsis, and death (62). The study was stopped early due to overwhelming benefit, and the authors have asserted that with a single investment of only 27 dollars, one case of neonatal sepsis could be prevented, which would be an unparalleled breakthrough in decreasing the worldwide incidence of sepsis. However, other studies that assessed the role of probiotics in other populations, such as pre-term and underweight children, showed no differences in sepsis incidence and mortality, indicating that the potential effects of microbiota restoration are not uniformly conserved across populations and settings (63, 64). Therefore, large stratified cohorts that collect data before the potential onset of sepsis are warranted to help pinpoint in which context gut commensals are able drive protection against sepsis. Recognizing these drivers of protection could help develop means of targeted microbiota restoration in order to decrease the incidence of sepsis.

Avenue 2: Addressing Dysbiosis During the Course of Sepsis

Based on the abovementioned findings that probiotics and synbiotics decrease the incidence of infections complications

at the ICU, a strong interest exists to investigate the use of these agents to improve outcome in patients with sepsis (60, 61). However, implementation of probiotic treatment in sepsis has been limited due to a lack of consensus with regard to the optimal choice of probiotic species and dosages, as well as lingering concerns for patient safety (65, 66). Recently, FMT has been used successfully in four cases of therapy-resistant sepsis and diarrhea (67–69). These initial studies describe that recolonization of the intestinal microbiota via FMT during sepsis could counterbalance dysbiosis, induce recovery of gut microbial barrier, which in turn could potentially improves the outcome of sepsis on the ICU. However, the first clinical studies investigating this modality on a larger scale have yet to commence. Other studies propose that treatment with microbiota-targeted metabolites, such as butyrate or other SCFAs, could be used as more stable immunomodulators than their bacterial counterparts (70). However, at the moment, surprisingly few studies have investigated the use of microbiota-targeted therapies during sepsis (45). These studies are essential to define the efficacy, safety and potential benefit of these therapies in the management of the disease.

Avenue 3: Rapid Microbiota Restoration in Sepsis Survivors

Pre-clinical models have shown that a state of immunosuppression can be maintained long after recovery from sepsis (71, 72), which is thought to predispose sepsis survivors to infections and increased mortality up to 2 years following sepsis discharge (73–75). Indeed, recent studies have shown that 20% of the patients who survive sepsis has a late death—predominantly caused by infections, respiratory failure, and aspiration pneumonitis—that cannot be attributed to prior health status and comorbidities (76). These findings have two implications. First, the general dysregulation that occurs during sepsis is profound and its consequences are long lasting. Second, as late death after sepsis cannot be attributed to previous health status, it suggests that targeted interventions can be designed to reverse this hypothesized state of immunosuppression and decrease mortality after sepsis (76). Given the potential role of dysbiosis in maintaining immunosuppression, restoration of gut commensals after sepsis discharge by probiotic administration in order to reduce late infections and subsequent mortality warrants further research. Another concept within the scope of microbiota restoration after sepsis discharge consists of microbiota auto

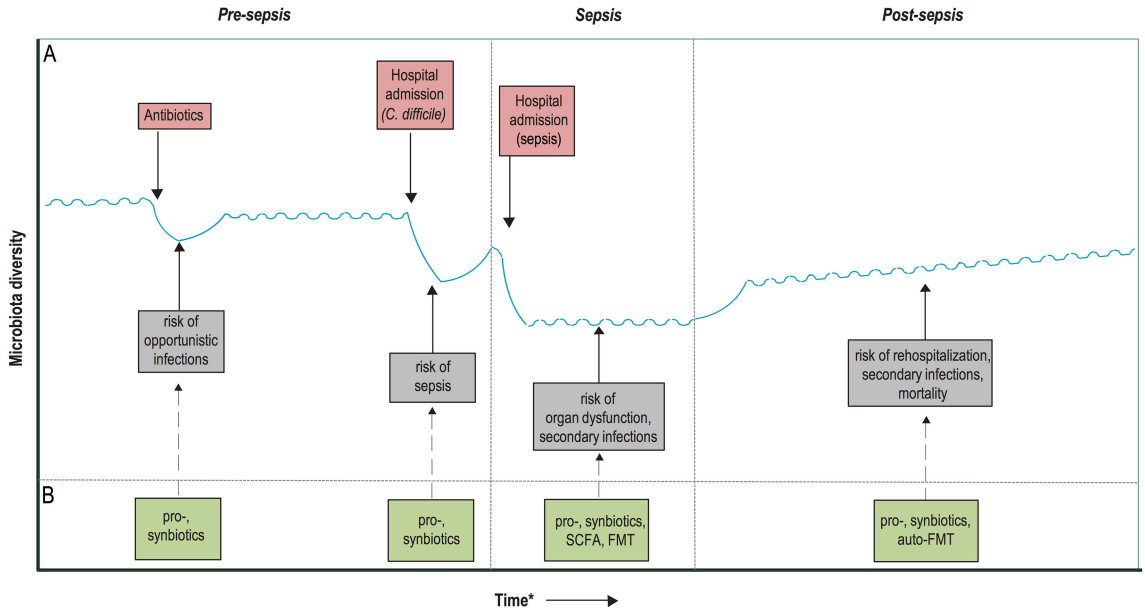

FIGURE 2 | Timeline of potential microbiota-associated interventions prior to, during and post-sepsis. **(A)** Hypothetical diagram of the associations between the risk of dysbiosis and sepsis development and outcome. Dysbiosis occurs due to the administration of antibiotics and/or hospitalizations, recover quickly upon hospital discharge or cessation of antibiotic treatment, but it often takes long for the microbiota to recover entirely. These periods of dysbiosis predispose to the development of opportunistic infections, which in turn further worsens the state of dysbiosis and predispose to sepsis development. During sepsis, the microbiota composition is extremely hampered, which has been associated with an increased risk of secondary infections, immunosuppression and potentially even organ dysfunction. Recolonization of a homeostatic microbiota is slow upon hospital discharge and sepsis recovery, which might contribute to prolonged immunosuppression, rehospitalization due to infections and increased mortality. **(B)** Probiotics and synbiotics have the potential to enhance microbiota colonization in neonates, as well as accelerate the recovery of the microbiota after periods of dysbiosis, which in turn could provide protection against the development of sepsis. During the course of sepsis, microbiota disruption could be ameliorated by the administration of pro- and synbiotics, which potentially reduces the occurrence of secondary infections. In addition, future treatment with fecal microbiota transplantation (FMT) or targeted restoration with microbiota-associated metabolites, such as short-chain fatty acids (SCFAs), could reduce the risk of prolonged immunosuppression and organ dysfunction. Finally, microbiota restoration after sepsis recovery could be accelerated with pro- and synbiotics, or by an autologous FMT. *Time depiction is schematic.

banking, which encompasses the storage of feces of patients in an ambulatory setting (77). These samples could subsequently be used in an autologous FMT in order to restore the microbiota after periods of dysbiosis, in this case after sepsis discharge. Of interest, the efficacy of auto-FMT for the prevention of infections after a period of dysbiosis is currently studied within a cohort of patients receiving allo-HCT (clinicaltrials.gov: NCT02269150). A schematic overview of the proposed therapeutic avenues in the prevention and management of sepsis is depicted in **Figure 2**.

THE ROAD AHEAD

Our understanding on how disturbances of the microbiota can predispose to, or even perpetuate the dysregulated immune response associated with sepsis is fragmented. The more we know about these collections of micro-organisms that inhabit our body compartments, the more we realize how delicate the mechanisms are through which these microbes contribute to homeostasis. Moreover, micro-organisms that reside in other body compartments than the gut, such as the skin and the lung, are likely to play an equally important but underexplored role in baseline immune function (42, 78, 79). To add an additional layer of complexity; emerging data indicate that eukaryotic viruses regulate, and are in turn regulated by the host and other microbial constituents that inhabit the intestine (80, 81). These co-inhabitants consist not only of bacteria, but also bacteriophages, helminths, and fungi, which influence each other through a series of processes termed "transkingdom interactions." This holistic view of the human microbiota represents an uncharted paradigm that will need to be further evaluated in the context of sepsis.

In general, large human cohort studies that document microbiota composition, prior to, during and after an episode of sepsis are needed to identify those commensals that protect against sepsis vs. those that are potentially associated with increased susceptibility and worse outcome. These insights could allow us to identify bacterial groups that are associated with immunological resilience, which could be harnessed as potential biomarkers of susceptibility to sepsis or adverse outcome. In parallel, mechanistic animal studies—that should include the use of older and "dirtier" mice (32, 82, 83) and better mimic the clinical scenario by using antibiotics and other treatments often given in sepsis (84)—are needed to disentangle potential mechanisms that drive the phenotypes seen in the human situation. These combined efforts have recently lead to the successful identification and development of new next-generation probiotics that are able to selectively treat specific pathogens, such as *C. difficile* and vancomycin resistant *Enterococcus* (VRE) (85, 86). Despite the significant challenges ahead, we foresee that further implementation of microbiota-targeted therapies could improve sepsis management and prevention.

DISCLOSURE

The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs or the US government.

AUTHOR CONTRIBUTIONS

BH, WW, and HP equally contributed to the write-up of the manuscript. BH drafted the figures and performed the literature search.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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"Real-Time" High-Throughput Drug and Synergy Testing for Multidrug-Resistant Bacterial Infection: A Case Report

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Antibiotic management of infections with multidrug-resistant organisms (MDRO) represents a complex clinical challenge. We report here the first patient with a severe MDRO infection managed with assistance of a novel "real-time" 3-day high-throughput screen (HTS) that allowed screening of 9 drugs in 14 combinations in 2,304 total samplings. Identified synergies were used to modify patient therapy with the goal of reducing drug-induced toxicity. The desired clinical outcome was achieved on the HTS-informed therapeutic regimen, supporting the utility of HTS technology to expand standard antimicrobial susceptibility testing.

Keywords: multidrug-resistant organisms, *Klebsiella pneumoniae*, real-time drug test, antibiotic combination therapy, drug repurposing screen

BACKGROUND

Infections with multidrug-resistant organisms (MDRO) have emerged as a worldwide health crisis with approximately two million cases and 23,000 deaths in the U.S. annually (1). Rapid identification of effective drugs is key to patient outcome (2). However standard susceptibility testing by broth microdilution, disk diffusion, gradient diffusion and traditional automated instrument systems are suitable for testing only ~20 antibiotics with limited capacity for testing drug combinations, despite routine use of combination therapy in these situations (3). Recently, while performing high-throughput (HTS) antibiotic screening for MDROs in the research setting, we encountered a severely ill patient with a complex MDRO infection. We successfully applied this highly-automated quantitative technology in miniaturized 1,536-well plate format (4) to patient-derived bacterial isolates and obtained results within a clinically-actionable time frame (in our case, a 3-day turnaround time for the primary drug screen). While this approach does not replace traditional microbiological laboratory methods, the results exemplify how HTS can rapidly identify alternative patient-specific drug combinations, providing empiric data on a wide range of theoretical treatment options for these more challenging MDRO cases.

In the primary compound screen, the bacterial strains (KP11, Ec1A, and Ec2B) were prepared and antibacterial growth assays were performed as previously described (4). Briefly, 2.5 μl TSB medium was dispensed into each well of 1,536-well plates followed by addition of 23 nl compound and 2.5 μl/well of bacterial culture with a final dilution of 1:500. After incubation for 7 h at 37°C,

5% CO2, the plates were measured for absorbance (OD600). The lead compounds were confirmed using the broth microdilution assay. The lead compounds against KP11 were also evaluated in a broth microdilution analysis according to the methods recommended by the CLSI. Briefly, a standardized inoculum was prepared by diluting the overnight culture to an optical density 625 nm (OD625) of 0.1 (equivalent to a 0.5 McFarland standard). The suspended inoculum at 1×10^6 colony forming units per milliliter (CFU/ml) in 100 μl was added into each well of a 96-well plate containing 100 μl of test compound in Mueller Hinton Broth (MHB). The plates were incubated for 24 h at 37°C and microbial growth with each test compound and combination was determined by measuring the optical density at 625 nm and visually by scoring the plates ± for bacterial growth.

CASE REPORT

The patient was a 16 year-old Kenyan male with severe aplastic anemia resulting in transfusion dependence. He sought care in India where he was treated with horse anti-thymocyte globulin (h-ATG) and cyclosporine. The patient was unresponsive to treatment and had several hospitalizations for disease-related complications. During this time he accumulated multiple risk factors for MDRO carriage including frequent antibiotic and healthcare exposure. He was transferred to the NIH for enrollment in a research study involving a potential haplo-cord transplant, but arrived septic with vancomycin-resistant *Enterococcus fecium* (VRE) and MDR *E. coli*-positive blood cultures. The source was identified as a large superinfected presacral hematoma, thought to have resulted from chronic rectal tube trauma. Given the patient's pressor requirement, severe pancytopenia and advanced debilitation, deep surgical resection of the infected hematoma was deemed impractical. The clinical strategy shifted to gaining sufficient control of the infection to enable hematopoietic reconstitution via stem cell transplant.

Expanded susceptibility testing for the two MDR *E. coli* isolates from the blood showed highly resistant organisms with *in vitro* susceptibility to colistin/polymyxin B and tigecycline only. Borderline susceptibility to imipenem was detected in one of the two isolates. Three MDR isolates detected on peri-rectal screening (one isolate of *Klebsiella pneumoniae*, two isolates of *E. coli*) showed susceptibility to colistin/polymyxin B, tigecycline and ceftazidime-avibactim. The VRE isolate showed susceptibility to daptomycin and linezolid. Consequently, the patient was treated with an antibiotic regimen that included daptomycin, imipenem, ceftazidime-avibactim, colistin and tigecycline. This combination was formulated to accommodate the differing antibiotic sensitivities among the gram-negative isolates and to apply aggressive pressure to a large inoculum of polymicrobial MDROs poised to continue seeding the patient's bloodstream. In this setting of extensive rectal fistulization and severe immunocompromise, antifungal therapy was added empirically.

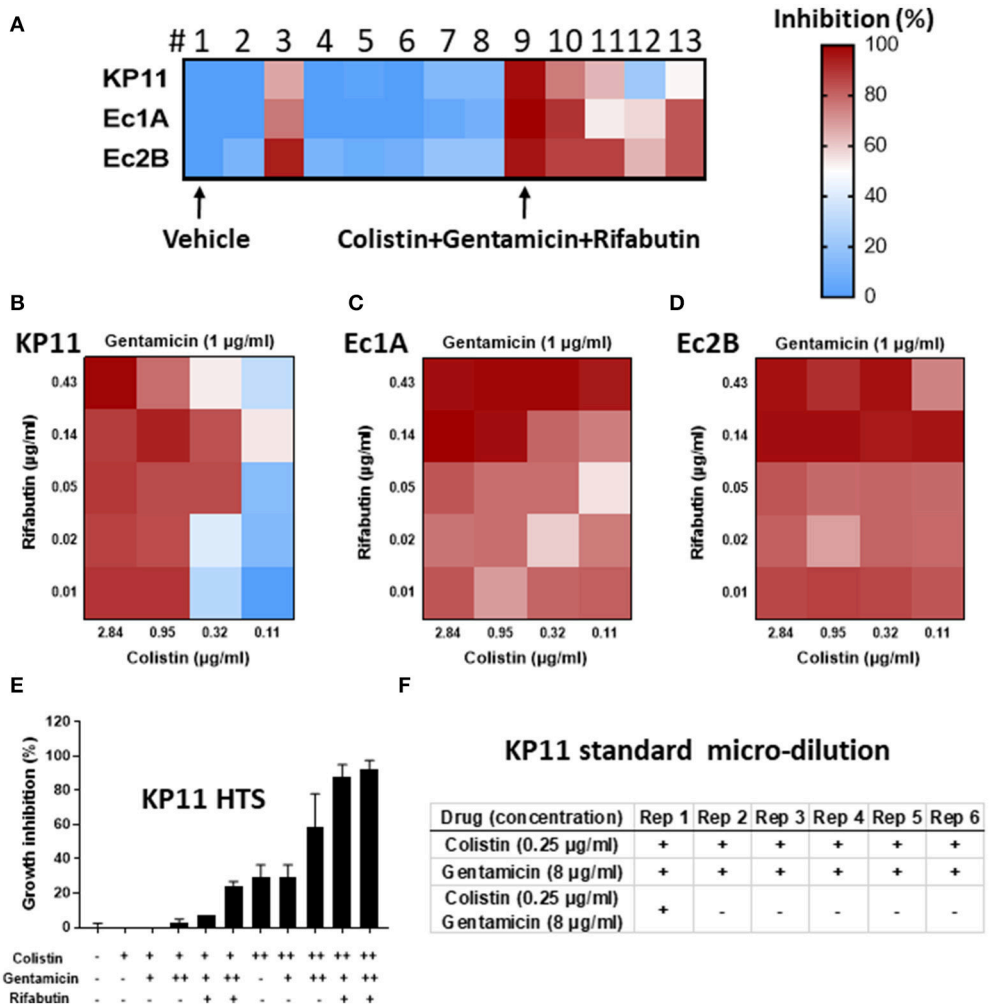
Following initiation of antibiotics the patient's fever defervesced, his blood cultures cleared and his hemodynamic instability improved. However, a direct hyperbilirubinemia developed between Week 1 and Week 3 of admission, reaching a peak total bilirubin level of 26.3 mg/dl. Transaminases remained normal. No signs of cholestatic obstruction were observed by imaging. There was concern that imipenem may be contributing to cholestasis as has been reported previously for beta-lactam antibiotics, particularly carbapenems (5). However, there was also concern that discontinuation of imipenem could compromise control of the patient's MDROs. The preservation of hepatic function being imperative, it was decided that the patient would be trialed on a carbapenem-sparing regimen.

To identify the most suitable alternative antibiotic combinations, a "real-time" HTS combinational drug screen was performed. Three MDROs were isolated from the patient on admission: one *Klebsiella pneumoniae* (KP11) and two *Escherichia coli* (Ec1A and Ec2B). These strains were tested for sensitivity to a total of 8 drugs (gentamicin, colistin, rifabutin, imipenem, ceftazidime, meropenem, tigecycline, and auranofin) in 9 unique combinations and 14 total combinations. Each sample was tested in quadruplicate, amounting to a total of 2,304 samples which were run on three 1,536-well plates using clinically-relevant drug concentrations (**Figures 1A–D**). None of the tested drugs was able to completely suppress the growth of all three strains as monotherapy. Notably, colistin was the only solo drug that mediated >50% inhibition of each strain. It exerted a greater inhibitory effect on Ec1A and Ec2B than KP11. The other antibiotics demonstrated negligible activity against these three strains (<20% inhibition).

For the three-drug combinations, #9 (gentamicin+colistin+rifabutin) successfully suppressed > 90% growth of all three strains (**Figure 1A**). #10 (colistin+imipenem+rifabutin) and #11 (meropenem+tigecycline+colistin) were less effective, inhibiting 54–90% of all three strains. In the four-drug combination tests, #12 (meropenem+tigecycline+colistin+ceftazidime) and #13 (meropenem+tigecycline+colistin+rifabutin) inhibited 22–83% of all three strains.

Next, we studied the three-drug combination (gentamicin+colistin+rifabutin) against KP11 at various drug concentrations (**Figure 1E**). Colistin was tested as a single drug at 0.5 or 2 μg/ml. Either 1 or 4 μg/ml gentamicin was added into colistin as two-drug combinations. 0.06 μg/ml rifabutin was added into the mixture of colistin and gentamicin to form three-drug combinations. Addition of 4 μg/ml gentamicin into 2 μg/ml colistin improved growth inhibition from 29 ± 7% to 59 ± 20%. However, growth inhibition was not enhanced with addition of 1 μg/ml gentamicin, suggesting dose-dependent synergy. Addition of 0.06 μg/ml rifabutin into 4 μg/ml gentamicin and 2 μg/ml colistin further improved growth inhibition to 93 + 5%.

Verification of the inhibitory effect of these drug combinations in an accredited clinical laboratory by traditional methods could not be performed in real-time, prior to the decision about therapy, due to time constraints. The additional testing was performed retrospectively to validate the selected drug combinations for this report. Colistin and gentamicin as single agents and in combination with each other were tested against KP11 in the broth microdilution assay recommended by the CLSI. Neither 0.25 μg/ml colistin nor 8 μg/ml gentamicin



Drug (concentration)	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
Colistin (0.25 μg/ml)	+	+	+	+	+	+
Gentamicin (8 μg/ml)	+	+	+	+	+	+
Colistin (0.25 μg/ml) Gentamicin (8 μg/ml)	+	-	-	-	-	-

FIGURE 1 | "Real-time" high-throughput drug and combination testing against multidrug-resistant organisms (MDRO). A total of 8 drugs and 14 drug combinations (in 2,304 samples) were tested against three MDRO *K. pneumoniae*-KP11, *E. coli*-Ec1A and *E. coli*-Ec2B. **(A–D)** Growth inhibition of KP11, Ec1A and Ec2B by individual drugs (1 vehicle; 2 gentamicin; 3 colistin; 4 rifabutin; 5 imipenem; 6 ceftazidime; 7 meropenem; 8 tigecycline) and drug combinations (9 colistin+gentamicin+rifabutin, 10 colistin+imipenem+rifabutin, 11 meropenem+tigecycline+colistin, 12 meropenem+tigecycline+colistin+ceftazidime, 13 meropenem+tigecycline+colistin+rifabutin) in high-throughput bacterial assay. Colors represent drug potency (% of growth inhibition). Vehicle control was normalized as 0% inhibition and no bacterium was normalized as 100% inhibition. $n = 4$, mean ± SEM. **(E)** Growth inhibition of KP11 by combinations of colistin, gentamicin, and rifabutin in high-throughput bacterial assay. Vehicle (–), colistin at 0.5 μg/ml (+) or 2 μg/ml (++), gentamicin at 1 μg/ml (+) or 4 μg/ml (++), or rifabutin at 0.06 μg/ml were tested at indicated conditions. $n = 3$, mean ± SEM. **(F)** Growth inhibition of KP11 by colistin, gentamicin, and the combination of colistin and gentamicin in standard micro-broth assay. 100 μg/ml meropenem (–) or vehicle alone (+) were included as control. + was visual bacterial growth. – was no visual bacterial growth. Rep, repeat. $n = 6$.

as single therapy was fully inhibitory. The combination of 0.25 μg/ml colistin and 8 μg/ml gentamicin was inhibitory in all but one of six replicates (**Figure 1F**). The significance of this finding is unclear but may be related to the poor reproducibility of colistin, which is well known to interfere with methods of MIC testing (6). Additionally, a relatively low density of MDRO was inoculated in the HTS assay, while a higher density of MDRO was inoculated in the broth microdilution assay. This may contribute to the differing degree of inhibition observed for colistin between these two methods.

Infection with MDROs in this case proved difficult to treat due to the extensive drug resistance and the potential liver toxicity. We continued colistin as the "backbone" of the antibiotic regimen based on the initial MIC data generated by conventional susceptibility tests and successful clinical microbiological control. Imipenem was discontinued and replaced with piperacillin-tazobactam plus gentamicin, the former to maintain broad spectrum coverage for the infected hematoma and the latter for synergy based on the HTS data. Rifabutin was eschewed secondary to an adverse side effect profile and problematic drug-drug interactions. Beginning 2 days after the change

in antibiotic therapy and continuing over the next 3 weeks, total bilirubin levels steadily declined to 2.2 mg/dl and the MDR *E. coli* and *K. pneumoniae* remained controlled with negative blood cultures, which allowed reinstatement of the patient's aplastic anemia therapy that included eltrombopag (contraindicated with severe liver toxicity) prior to transplant. The patient subsequently received a haplo-cord transplant but died 4 months after presentation due to disseminated infection with a resistant *Scopulariopsis* mold. MDROs did not appear to be a source of systemic infection at the time of his death. The patient's direct hyperbilirubinemia was, in the end, ascribed to imipenem toxicity given the tight temporal correlation between increasing/decreasing bilirubin levels and imipenem exposure, as well as the lack of convincing evidence for other etiologies.

DISCUSSION

Multidrug-resistance in bacteria has risen markedly, limiting effective treatment options (7). Combination antibiotic therapy has shown benefit in severe MDRO infections, reducing mortality and potentially reducing further development of resistance (8). Expanded susceptibility testing including broad-based screening for synergistic antibiotic combinations may assist clinicians in identifying not only the optimal first-line antibiotic regimen but also alternative regimens when drug interactions or toxicities occur, as was the case for this patient. This report presents a research protocol for high-throughput susceptibility testing to identify effective *in vitro* drug combinations within a clinically-actionable time frame. This model has the potential for widespread implementation at many hospitals where these infections arise and are treated, particularly the 77 academic universities or hospitals within the US that already possess HTS capability (9).

The data here are limited in that they utilize *in vitro* combination therapy testing which may lead to discrepant results (10) and lack controlled clinical trial data to evaluate effects on patient outcomes. For example, while cystic fibrosis patients are among the populations that stand to benefit the most from validated synergy testing, only one controlled clinical trial assessing its efficacy has been published (11). Although it did not find evidence of benefit from synergy testing compared to conventional susceptibility testing, a recent Cochrane Review on the subject (which identified only one trial—the trial referenced above—which was suitable for inclusion in its analysis) lamented the extreme paucity of evidence available for assessment (12). A multi-center randomized controlled study is currently underway to assess the benefits of colistin in combination with carbapenems vs. colistin alone based on promising *in vitro* synergy data; however, this will only indirectly address the question of whether combination susceptibility testing has clinical utility (13). Clearly more study is required in this area as combination therapy will only become more common in the foreseeable future.

The other conclusion to be drawn from this case is that HTS technology has developed to the point of becoming tractable for a wide range of applications. With its miniaturized format and automated workflow, the expanded susceptibility testing performed in the case requires relatively scant raw materials and human labor on a per-assay basis. Therefore, if data on pathogen sensitivity to combinations of antimicrobials are determined to have clinical value, HTS will serve as a powerful vehicle for its actualization.

ETHICS STATEMENT

Written informed consent was obtained from the patient representative for publication of the aforementioned data. All patients cared for in the NIH Clinical Center are in IRB approved research protocols with signed consents.

AUTHOR CONTRIBUTIONS

PW, WZ, and WS: Conception and design of the work; WS, SH, MX, RC: Data collection; WS, SH, WZ, and PW: Data analysis and interpretation, manuscript writing, and critical revision of the article; WS, SH, MX, RC, WZ, and PW: Approval of the final version of the article.

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Prophylactic Treatment With Simvastatin Modulates the Immune Response and Increases Animal Survival Following Lethal Sepsis Infection

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Chronic use of statins may have anti-inflammatory action, promoting immunomodulation and survival in patients with sepsis. This study aimed to analyze the effects of pretreatment with simvastatin in lethal sepsis induced by cecal ligation and puncture (CLP). Male *Swiss* mice received prophylactic treatment with simvastatin or pyrogen-free water orally in a single daily dose for 30 days. After this period, the CLP was performed. Naïve and Sham groups were performed as non-infected controls. Animal survival was monitored for 60 h after the CLP. Half of mice were euthanized after 12 h to analyze colony-forming units (CFUs); hematological parameters; production of IL-10, IL-12, IL-6, TNF-α, IFN-γ, and MCP-1; cell counts on peritoneum, bronchoalveolar lavage (BAL), bone marrow, spleen, and mesenteric lymph node; immunephenotyping of T cells and antigen presenting cells and production of hydrogen peroxide (H_2O_2). Simvastatin induced an increase in survival and a decrease in the CFU count on peritoneum and on BAL cells number, especially lymphocytes. There was an increase in the platelets and lymphocytes number in the Simvastatin group when compared to the CLP group. Simvastatin induced a greater activation and proliferation of CD4+ T cells, as well as an increase in IL-6 and MCP-1 production, in chemotaxis to the peritoneum and in H_2O_2 secretion at this site. These data suggest that simvastatin has an impact on the survival of animals, as well as immunomodulatory effects in sepsis induced by CLP in mice.

Keywords: simvastatin, prophylactic treatment, sepsis, immunomodulation, macrophages, hydrogen peroxide, cytokines

INTRODUCTION

Sepsis is defined as a complex systemic inflammatory response caused by an uncontrolled infection with activation of both pro- and anti-inflammatory responses. The development and progression of sepsis are multifactorial, affecting the cardiovascular, neuronal, endocrine, and immune systems (1, 2). It is the second most frequent cause of death among patients hospitalized in UTIs and the 10th most frequent cause of mortality in general (3).

Clinical manifestations of sepsis such as fever, hypercoagulation, and peripheral hypotension are derived from the release of inflammatory cytokines, such as IL-1β, IL-6, IL-17, TNF-α, and anti-inflammatory cytokines: IL-4, IL-10, IL-13, TGF-β (4).

Severe sepsis and septic shock are the leading causes of morbidity and mortality in hospitalized patients and immunosuppressed individuals (5). Usually, patients are treated with antimicrobials that can cause cardiovascular and renal changes, with serious side effects (6). In addition, the worsening of the condition is very rapid, and there is no adequate therapeutic response. For these reasons, developing new treatment strategies for these patients has become necessary.

In this context, statins emerge as a potential therapy, since it was shown that patients who used statins were less likely to develop sepsis (7). Statins are medications widely used to treat hypercholesterolemia by reducing the serum concentration of low-density lipoprotein (LDL) cholesterol. In enzymatic terms, statins act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) preventing the conversion of HMG-CoA to mevalonic acid, which is a cholesterol precursor (8).

The use of statins reduces the expression of adhesion molecules, both in monocytes and neutrophils, and in endothelial cells, with a consequent decrease in the migration of polymorphonuclear leukocytes to tissues (9). In addition, they decrease the synthesis of acute-phase proteins, such as C-reactive protein, which is used as an inflammatory and cardiovascular risk marker, and reduce pro-cytokine secretion of inflammatory agents, such as IL-1β and IL-6, without TNF-α influences (9, 10).

Niessner et al. (11) reported that statins play a key role in modulating leukocyte and monocyte functions, reduce oxidative stress, and improve endothelial function and platelet activity. Simvastatin also reduced the systemic response to endotoxin administration and decreased the expression of Toll-like receptors, which play a key role in sepsis.

Another important factor in the inflammatory context is that statins increase the expression of endothelial nitric oxide synthase (eNOS), which is essential for adequate endothelial function. This may be instrumental in restoring the balance between eNOS and inducible nitric oxide synthase (iNOS), which is disrupted in sepsis, and is an important factor in the genesis of septic shock (9). In sepsis experiments, simvastatin proved to be effective in hemodynamic stabilization in mice with sepsis induced by cecal ligature and puncture (CLP). In addition to stabilizing hemodynamics, these animals showed improved responses to beta-adrenergic vasopressin drugs (dobutamine), significantly increasing their blood pressure, with decreased polymorphonuclear cell adhesion to the previously activated endothelium (12).

Despite this evidence, little is known about the immunological mechanisms involved in this apparent protective effect of simvastatin in the context of sepsis, considering both lymphocyte and macrophage activation, as well as about its possible effects on the inflammatory process and on the various metabolic and organic dysfunctions accompanying sepsis, which led to the present study.

MATERIALS AND METHODS

Animals and Treatment

In this study, 12-week-old male Swiss mice were used. The animals were obtained from the Central animal house of the Federal University of Maranhão (UFMA) and were maintained with water and food *ad libitum*. All procedures were evaluated and approved by the Research Ethics Committee of the Federal University of Maranhão (Protocol: 012975/2008-43).

The animals were separated into 4 groups (10 animals/group). In the control group (CLP group), 200 μL of apyrogenic water was administered orally in a single daily dose. In the Simvastatin group, 200 μL of simvastatin (40 mg/kg) was administered orally in a single daily dose. The treatments lasted 30 days and the dosage used was chosen according to Winkler et al. (13). Two additional groups were performed as negative controls, the Naïve group, which received no procedures and the Sham group, which was operated but had no perforations in the cecum.

Cecal Ligature and Puncture (CLP)

After 30 days of pretreatment with simvastatin or water, the animals underwent CLP. Polymicrobial sepsis was induced using the CLP method described by Benjamim et al. (14), with modifications in the anesthetic used. Initially, the mice were anesthetized with 25 mg/kg ketamine hydrochloride and 20 mg/kg xylazine hydrochloride according to Machado et al. (15). Then, a laparotomy was performed, and the cecum was mobilized, ligated below the cecal valve, and punctured 10 times with an 18-gauge needle to induce lethal sepsis. The cecum was placed back into the peritoneal cavity, and the abdomen was closed in two layers. Saline (0.5 mL/10 g body weight) was given subcutaneously to CLP animals for fluid resuscitation. After 12 h of CLP, half of the mice were euthanized with an overdose of anesthetic (150 mg/kg ketamine hydrochloride and 120 mg/kg xylazine hydrochloride) and another half of the animals were maintained alive to evaluate the lifespan ($n = 5$). The mortality of the animals was recorded every 12 h. The Sham group was submitted to all the procedures with exception of the perforations.

Evaluation of Hematological Parameters

To determine the hematological parameters, 100 μL of blood was collected by retrorbital route, 12 h after the CLP. Blood was stored in 1.5 mL tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. An automated hematology analyzer (Poch-100iV Diff, Sysmex Corp) was used. The following parameters were analyzed: red blood cells, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red-cell distribution width (RDW), and number of total leukocytes, neutrophils, lymphocytes, monocytes, and platelets.

Bronchoalveolar Lavage

The trachea of the animals was exposed, fitted with a cannula, and 1 mL of cold PBS was injected in the bronchoalveolar space using a syringe. After a short massage on the chest, the

solution was aspirated at least three times. To determine the total number of cells in the bronchoalveolar lavage, cell suspensions were stained with crystal violet (0.05%) in 30% acetic acid at a ratio of 9:1. Cells were counted in a Neubauer chamber using an optical microscope (×400). For differential counting, slides were prepared using a cytospin (800 rpm/3 min) and then were fixed and stained using an Instant-Prov Kit (Newprov, Pinhais, Brazil).

Colony-Forming Unit (CFU) Determination

The mice were killed 12 h after the CLP. The skin of the abdomen was cut open on the midline after thorough disinfection and without injury to the muscle, and the peritoneal cavity was washed with 2 mL of sterile phosphate buffered solution (PBS). Aliquots of serial log dilutions of the obtained peritoneal fluid were plated on Mueller-Hinton agar dishes (Difco Laboratories, Detroit), colony-forming units (CFU) were counted after overnight incubation at 37°C, and the results were expressed as the number of CFU per peritoneal cavity.

Collection and Counting of Cells From the Peritoneal Lavage and Lymphoid Organs

The animal's peritoneal cavity was washed with 5 mL sterile PBS. After abdominal wall excision, cell suspensions were obtained by aspiration using a syringe and needle, transferred to conical-bottom polypropylene tubes, and maintained in an ice bath at 4°C until the cells were counted. After collection of the peritoneal lavage, the spleen, and mesenteric lymph nodes were collected, weighed, and crushed. The femur was perfused with 1 mL PBS to obtain bone marrow cells. For total cell number counting, 90 μL of each cell suspension was fixed and stained with 10 μL 0.05% crystal violet in 30% acetic acid. The cells were counted using a Neubauer chamber with the aid of an optical microscope at 400× magnification (16). Differential peritoneal cell counts were determined using the cytospin system (800 rpm/3 min), fixed, and stained with the Instant-Prov kit (Newprov, Pinhais, Brazil). The percentage of cell subpopulations was calculated based on the count of 100 cells and transformed in absolute number based on the total count.

Phenotype Characterization of Leukocytes

The phenotypes of cells from the mesenteric lymph node were characterized using commercial monoclonal antibodies (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Two panels of antibodies were used, one for adherent cells, including anti-CD14 (FITC) and anti-IA/IE (PE), and the other for non-adherent cells, anti-CD3 (FITC), anti-CD4 (PE), and anti-CD8 (PerCP). After the acquisition of 10,000 events in a FACSCalibur flow cytometer, the obtained data were analyzed using FlowJo software.

Quantification of Cytokines

The cytometric bead array (CBA) technique was used for the quantification of TNF-α, MCP-1, IL-6, IL-10, IL-12, and IFN-γ in serum, as described by Maciel et al. (17), using the mouse inflammation cytokine kit (Becton Dickinson Biosciences, San Jose, CA, EUA).

Determination of Hydrogen Peroxide Release (H_2O_2)

To evaluate H_2O_2 release, a horseradish peroxidase-dependent phenol red oxidation microassay was used (18, 19). In this assay, two million peritoneal cells were suspended in 1 mL freshly prepared phenol red solution that consisted of ice-cold Dulbecco's PBS containing 5.5 mM dextrose, 0.56 mM phenol red (Sigma), and 8.5 U/mL horseradish peroxidase type II (Sigma). One hundred microliters of the cell suspension was added to each well and incubated in the presence or absence of 10 ng phorbol myristate acetate (PMA) (Sigma), for 1 h at 37°C in a humid atmosphere containing 5% CO_2 and 95% air. The plates were centrifuged once at 150 × g for 3 min and the supernatants were collected and transferred to another plate. The reaction was stopped with 10 μL 1N NaOH. The absorbance was measured at 620 nm with a microplate reader (MR 5000, Dynatech Laboratories Inc., Gainesville, VA, USA). Conversion of absorbance to μM H_2O_2 was done by comparison to a standard curve obtained with known concentrations of H_2O_2 (5–40 μM).

Statistical Analysis

Results were expressed as the mean ± standard deviation. The normality of data was evaluated by the D'Agostino-Pearson test. Statistical analysis was performed using ANOVA followed by Student's *t*-test for parametric data and the Mann-Whitney for non-parametric data, using Graph Pad Prism software, version 6.0. The differences were considered to be significant when $p \leq 0.05$. The lifespan of the mice was demonstrated using the Kaplan-Meier curve, and the log-rank statistical test was applied to compare the curves. All experiments were repeated at least two times.

RESULTS

Prophylactic Treatment With Simvastatin Increases the Survival of Animals With Sepsis

Control animals (CLP group) died within 48 h. However, it was only after 30 h that the first death occurred in the simvastatin group. In this group, over 80% of the animals survived up to the 50 h follow-up. The last death of the simvastatin group occurred at 60 h (**Figure 1**). The animals from the Sham group remained alive until the last day of observation.

Simvastatin Induces a Reduction in the Number of CFU at the Focus of the Infection

Pretreatment with simvastatin induced a reduction in the bacterial counts in the peritoneum, the focus of the infection when compared to the CLP group (**Figure 2**). At the Sham group no CFU was detected in the peritoneum.

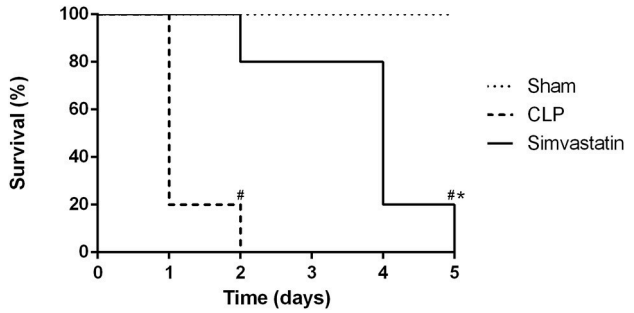

FIGURE 1 | Survival curve of animals with sepsis. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. Each group consisted of 5 animals. The groups were clinically evaluated for the number of deaths after 12 h of CLP at regular 12 h intervals for 60 h. The Sham group was submitted to all the procedures with exception of cecum perforation. $^{*}p < 0.05$ compared to CLP group. $^{\#}p < 0.05$ when compared to the Sham group.

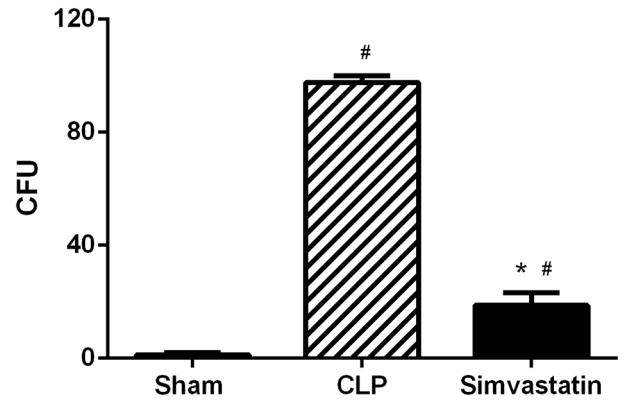

FIGURE 2 | Effect of pretreatment with simvastatin on the number of CFU in peritoneum. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. Each group consisted of 5 animals. After 12 h of CLP, aliquots of the peritoneal lavage were diluted and plated on Mueller-Hinton agar and incubated at 37°C for 18 h for counting of CFU. The data are represents as mean ± S.D. of CFU × 10^4. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation. $^{*}p < 0.05$ compared to CLP group. $^{\#}p < 0.05$ when compared to the Sham group.

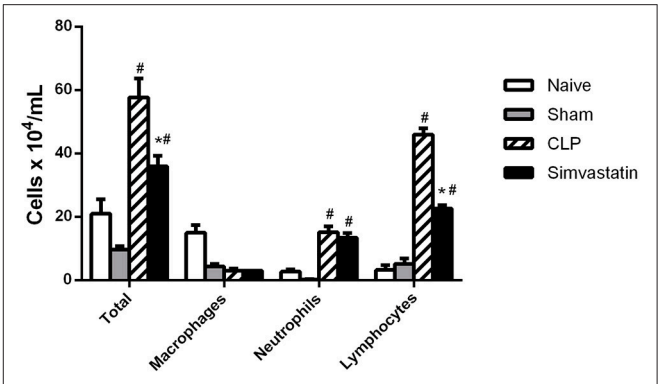

FIGURE 3 | Effect of pretreatment with simvastatin on the total and differential counts of Bronchoalveolar lavage cells (BAL). Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. The total and differential count of the bronchoalveolar cells was made 12 h after the induction of sepsis by the CLP model. The results represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation. $^{*}p <$ 0.05 compared to the CLP group. $^{\#}p < 0.05$ when compared to the Naïve and Sham groups.

TABLE 1 | The effect of oral use of simvastatin in hematological parameters in septic mice.

Hematological parameters	Naive	Sham	CLP	Simvastatin
Hematocrit (%)	50.8 ± 0.5	46.9 ± 1	46.2 ± 0.9[a]	45.3 ± 1.1
Hemoglobin (g/dL)	15.0 ± 0.2	15.1 ± 0.4	13.8 ± 0.2	13.8 ± 0.5
Erythrocytes ($\times 10^6$/dL)	10.0 ± 0.3	10.9 ± 0.2	9.1 ± 0.2	9.5 ± 0.4
Platelets ($\times 10^3$/dL)	1286.0 ± 218.7	1650.0 ± 397.3	556.2 ± 52.5$^{\#}$	832.8 ± 77.5$^{\#*}$
Leukocytes ($\times 10^3$/dL)	3.1 ± 0.2	2.2 ± 0.29	8.4 ± 0.6$^{\#}$	8.0 ± 0.9$^{\#}$
Neutrophils ($\times 10^3$/dL)	1.8 ± 0.1	1.5 ± 0.3	3.3 ± 0.6$^{\#}$	3.0 ± 0.5$^{\#}$
Lymphocytes ($\times 10^3$/dL)	1.2 ± 0.2	0.8 ± 0.2	4.9 ± 0.2$^{\#}$	5.6 ± 0.4$^{\#*}$
Monocytes ($\times 10^3$/dL)	0.05 ± 0.02	0.1 ± 0.02	0.1 ± 0.0	0.2 ± 0.1

[a] *The results are presented as the mean ± SEM; $^{*}p < 0.05$ when compared to the CLP group; $^{\#}p < 0.05$ when compared to the Naïve and Sham groups.*

Simvastatin Inhibits the Recruitment of Cells Into the Bronchoalveolar Space

Considering that pulmonary inflammation is one of the causes of death in sepsis, we investigated the leukocyte distribution in bronchoalveolar lavage. CLP and Simvastatin groups showed increased recruitment of inflammatory cells, especially neutrophils and lymphocytes, to the BAL when compared to Naïve and Sham groups. However, the treatment with simvastatin reduced this leukocyte infiltrate, specially the number of lymphocytes when compared to CLP group. On the other hand, Simvastatin did not affect the migration of macrophages and neutrophils when compared to CLP group (**Figure 3**).

Simvastatin Induces Increased Lymphocytes and Platelets in the Blood

No significant differences between Naïve and Sham groups were observed in blood analysis. However, CLP and Simvastatin groups showed a significant decrease in the number of platelets and an increase in the number of leukocytes, specially lymphocytes, when compared to Naïve and Sham groups. The number of platelets in the Simvastatin group was increased when compared to the CLP group, but it was still smaller than the Sham group. The treatment with simvastatin also increased the number of lymphocytes in the blood when compared to the CLP group (**Table 1**).

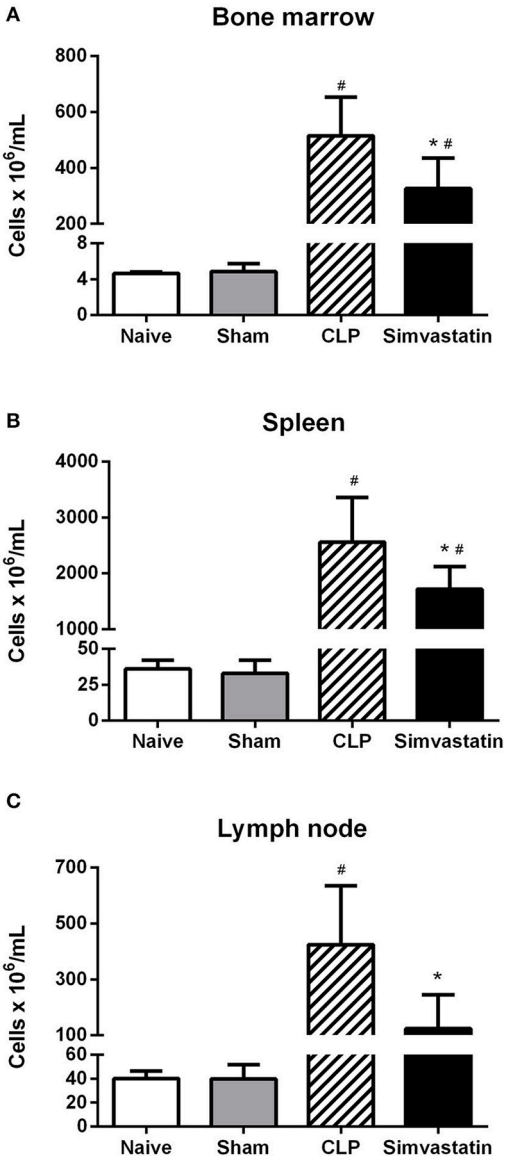

FIGURE 4 | Effect of pretreatment with simvastatin on the total cell count of bone marrow, spleen, and lymph node in septic mice. The animals received water or simvastatin orally for 30 days when CLP was induced. After 12 h of CLP, the bone marrow **(A)**, spleen **(B)**, and lymph node **(C)** were collected and quantified. The results represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation. *$p < 0.05$ compared to control group. #$p < 0.05$ when compared to the Naïve and Sham groups.

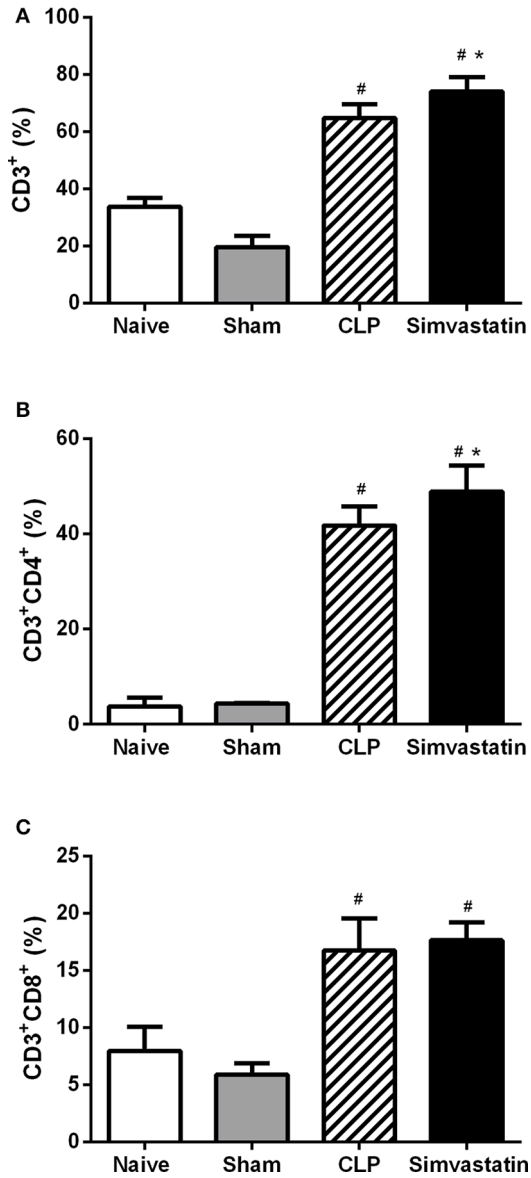

FIGURE 5 | Effect of simvastatin pretreatment on T cells populations in the lymph node. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. Immunophenotyping was performed 12 h after the induction of lethal sepsis by the CLP model. **(A)** % of T lymphocytes; **(B)** % of T-helper lymphocytes; **(C)** % of T-cytotoxic lymphocytes. The results are expressed as percentage values. These values represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation.* $p < 0.05$ compared to the CLP group. #$p < 0.05$ when compared to the Naïve and Sham groups.

Effects of Pretreatment With Simvastatin on the Total Cell Count of Bone Marrow, Spleen, and Mesenteric Lymph Node

Bone marrow, spleen and mesenteric lymph node cells were also collected to investigate whether simvastatin could induce any changes in lymphoid cells. The results from Simvastatin and CLP groups were similar and always high than those counts observed in the Naïve and Sham groups (**Figure 4**).

Pretreatment With Simvastatin Induces an Increase in the Percentage of Helper T Lymphocytes and the Expression of Class II MHC in Animals With Sepsis

Control group showed an increased percentage of T lymphocytes when compared to Naïve and Sham groups. Treatment with

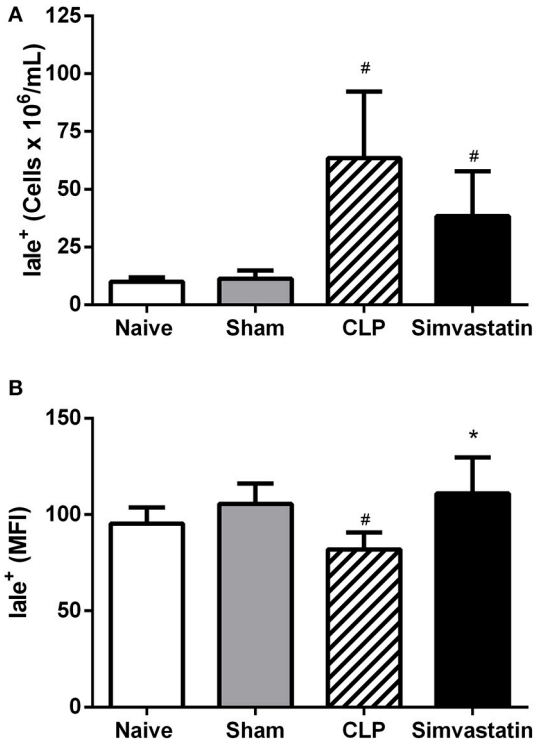

FIGURE 6 | Effect of simvastatin pretreatment on the number of antigen presenting cells in the lymph node. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. Immunophenotyping was performed 12 h after the induction of lethal sepsis by the CLP model. **(A)** % of antigen presenting cells (Ia-Ie+); **(B)** The mean fluorescence intensity (MIF). These values represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation. $^{*}p < 0.05$ compared to the CLP group. $^{\#}p < 0.05$ when compared to the Naïve and Sham groups.

simvastatin increased the percentage of both total T lymphocytes (**Figure 5A**) and helper T lymphocytes (CD3+CD4+) (**Figure 5B**) but did not affect the percentage of cytotoxic T lymphocytes (CD3+CD8+) when compared to Control group (**Figure 5C**).

The presence of antigen-presenting cells (APCs) was also investigated by the expression of class II MHC molecules (Ia-Ie marker). The CLP group showed an increase in the number of APCs (IaIe+) and a decrease of Class II MHC expression (MFI) when compared to Naïve and Sham groups (**Figures 6A,B**). Simvastatin treatment did not change the number of APCs (IaIe+), however there was an increased expression of class II MHC molecules in APC from this group (**Figure 6B**) when compared to the CLP group.

Pretreatment With Simvastatin Induces an Increase in Inflammatory Cytokines in Animals With Sepsis

The CLP induced an increase of all the cytokines when compared to Naïve and Sham groups. Simvastatin treatment increased IL-6 and MCP-1 production (**Figures 7A,B**), without significant differences in TNF-α, IFN-γ, and IL-10 expression (**Figures 7C–E**) when compared to CLP group. The IL-12 was not detected.

Effect of Pretreatment With Simvastatin on the Phenotypic and Functional Characteristics of Peritoneal Cells

In the peritoneal cavity, CLP induced an increase of inflammatory cell influx when compared to Naïve and Sham groups. Moreover, the total cell count was significantly increased in the Simvastatin group (**Figure 8A**). Analyzing the cell populations, there were an increased number of neutrophils and lymphocytes compared to the controls, with no difference in the number of macrophages (**Figure 8A**).

The activation of the peritoneal cells was evaluated by the production of NO and hydrogen peroxide. The NO production was not detected neither in the peritoneum not in the lymph node cells cultures. However, there was an increase of both spontaneous and PMA-stimulated H_2O_2 release in by peritoneal cells from Simvastatin group when compared to CLP group (**Figure 8B**). The Naïve and Sham groups had no production of NO and H_2O_2.

DISCUSSION

In this work, it has been demonstrated that the use of simvastatin for 30 days protects animals from lethal sepsis and increases the survival of animals. This effect is related to the decrease of CFUs in the infectious focus and pulmonary infiltrate, inhibition of peripheral thrombocytopenia, increase in the percentage of helper T lymphocytes and expression of MHC class II molecules in macrophages, as well as of microbicidal mediators produced by the phagocytic cells.

Considering that the animals from both groups developed clinical signs of sepsis, the great benefit that chronic lipid-lowering therapy had on the animals was evident, since the animals from the simvastatin group died later than those in the control group. These results are in agreement with Ando et al. (20), who used animals with sepsis induced by intraperitoneal injection of lipopolysaccharide (LPS) and demonstrated an increase in survival in mice treated with cerivastatin compared to the control group. In another study, Ajrouche et al. (3) compared 105 patients who had been treated with statins with 246 patients who did not use lipid-lowering drugs and showed a decrease in mortality and a reduction in C-reactive protein levels. Similarly, Ou et al. (21) observed that patients who chronically used high-potency statins (rosuvastatin 10 mg, atorvastatin 20 mg, and simvastatin 40 mg) had an additional survival benefit when compared to chronic low-dose statins and, especially, when compared to the control group.

The increase in survival observed in the Simvastatin group may be associated with a decrease in the number of CFUs at the focus of the infection (peritoneum), which could be related to a microbicidal effect of the drug used or to its effect stimulating host microbicidal mechanisms. However, the possibility of an

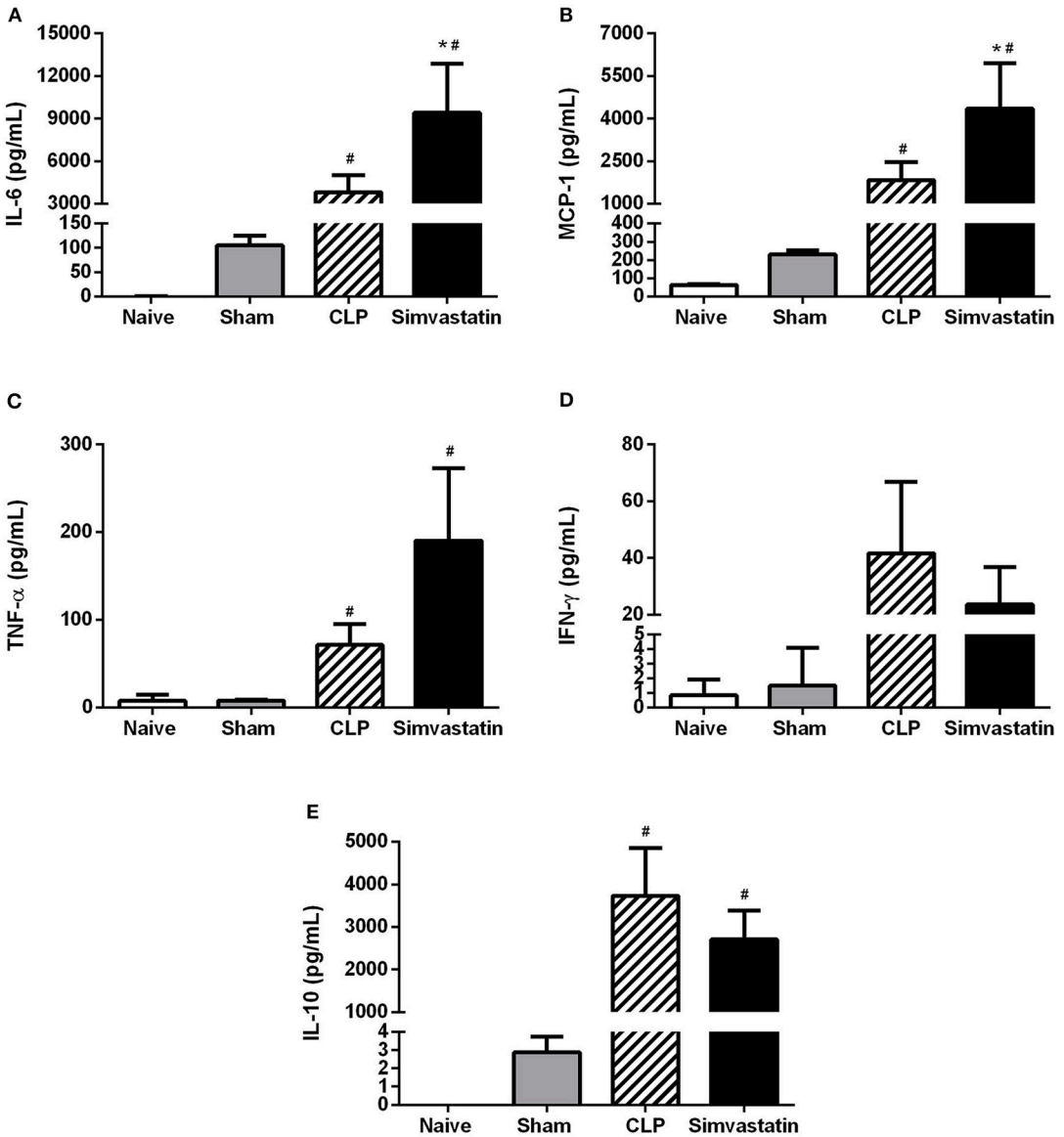

FIGURE 7 | Effect of simvastatin pretreatment on serum cytokines. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. IL-6 **(A)**, MCP-1 **(B)**, TNF-α **(C)**, IFN-γ **(D)**, and IL-10 **(E)** dosing was done 12 h after the induction of lethal sepsis by the CLP model. These values represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation. $^{*}p < 0.05$ compared to the control group. $^{\#}p < 0.05$ when compared to the Naïve and Sham groups.

antimicrobial effect of statin, as described previously (22, 23), can be disproven since there were no differences in CFU number in blood cultures and BAL (data not shown), suggesting that its effectiveness could be due to the immune activation at the site of infection.

In infection, resident macrophages are able to phagocytose microorganisms and to secrete IL-1 and TNF-α. These cytokines, in turn, stimulate the recruitment of neutrophils, monocytes and T lymphocytes to the site of infection. This process is mediated by selectins, integrins, and chemokines (10, 24). In fact, simvastatin induced an increase in the number of cells recruited to the infectious focus, especially related to neutrophils and lymphocytes. However, the opposing effect was observed at the lung, since occurred a decreased infiltration of inflammatory cells in the BAL in the simvastatin group, with a smaller number of lymphocytes. These data are in agreement with Merx et al. (12), who demonstrated a reduction in leukocyte chemotaxis in inflammatory processes after the use of statins. These authors collected mononuclear cells from animals treated with simvastatin or untreated controls, and submitted them to cystine pre-stimulated endothelial cell adhesion assays. After 5 min and under physiological conditions of flow, the results showed a reduced amount of monocytes in the CLP+ simvastatin group adhered to the endothelial surface when compared to the control.

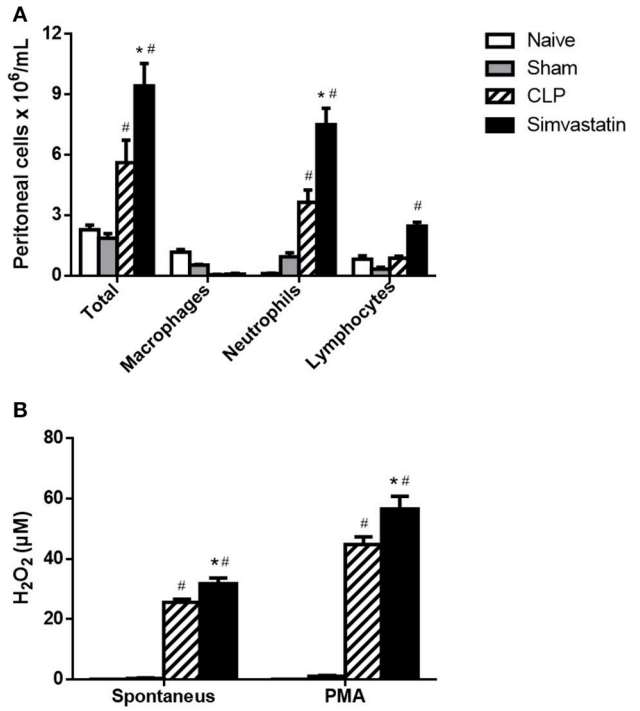

FIGURE 8 | Effect of simvastatin pretreatment on the recruitment and activation of peritoneal cells. Animals received water (CLP group) or simvastatin (Simvastatin group) orally for 30 days when CLP was induced. **(A)** Total and differential cell counts were made 12 h after the CLP. **(B)** Release of H_2O_2, spontaneous and induced by PMA, into the peritoneum of animals with sepsis induced by CLP. The results represent the mean ± S.D. of 5 animals per group. The Naïve group received no procedures and the Sham group was submitted to all the procedures with exception of cecum perforation.*$p < 0.05$ compared to the CLP group. #$p < 0.05$ when compared to the Naïve and Sham groups.

Likewise, Almog showed decreased expression of selectins and integrin ligands in endothelial cells in the presence of statins (9). It is important to note that pulmonary inflammation is a frequent lethal complication of sepsis. Thus, a reduction of this cellular infiltrate at this site is a beneficial effect of simvastatin and could, at least partially, explain the increase of survival.

Despite the decrease in cellular recruitment to the bronchoalveolar space, simvastatin induced an increase in the percentage of CD4+ T lymphocytes in the lymph node, suggesting a greater proliferation and activation of these cells, what could justify the increase of lymphocytes in the blood. This increase may be also related to the increased expression of class II MHC molecules in the host cells, since the presentation of antigens via this molecule is a condition for the activation and proliferation of helper T cells. This result partially disagrees with the studies of Sun and Fernandes (25) and Ghittoni et al. (24) that demonstrated that statins have the ability to decrease the expression of class II MHC molecules on the cell surface of antigen-presenting cells, as well as to decrease the migration of these cells to the sentinel lymph node.

In addition to the increased expression of MHC class II molecules, there was a significant increase in the serum concentration of IL-6 and MCP-1, without significant differences in IL-10, IFN-γ, and TNF-α. Sun and Fernandes (25) already showed an increase in the mRNA expression for IL-6 and MCP-1 induced by lovastatin in LPS-stimulated dendritic cells. These results suggest that the effect of simvastatin on the increase of survival is not related to its anti-inflammatory activity, but to a modulation of immune response directed to antimicrobial action.

Finally, to investigate whether a greater cellular activation occurred at the focus of the infection that justified the reduction of the CFUs and consequent increase in the survival of the animals, the recruitment of cells to the peritoneal cavity and the microbicidal capacity of these cells by the production of H_2O_2. The animals from the simvastatin group presented significantly increased values of H_2O_2 in the peritoneum, the focus of the CLP infection. Macrophages and neutrophils are known to contain enzyme-rich cytoplasmic granules, one of which is NADPH oxidase or phagocyte oxidase. This converts molecular oxygen into superoxide anions, free radicals of oxygen and hydrogen peroxide; all are microbicidal agents in both phagolysosomes and extracellular microorganisms (26). In this study, it was observed that the peritoneal phagocytes from the animals of the simvastatin group secreted an increased amount of hydrogen peroxide compared to the control animals. This difference became more important when such cells were stimulated by PMA. The microbicidal potential of these cells is even more evident when the smaller number of cells that migrate to the peritoneum as a function of simvastatin are observed, i.e., fewer cells migrating to the peritoneum, but with a greater microbicidal capacity. This fact, together with a decreased number of CFUs in the peritoneum and an increased number of inflammatory cells in the peritoneal lavage, show that this statin is able to exert immunomodulatory effects on the peritoneum.

Altogether, the data indicate that pretreatment with simvastatin was able to increase the survival of animals with sepsis induced by CLP, because of its ability of improve the immune response, specially the microbicidal activity, the macrophage ability to present antigens to T cells and also, the immunomodulatory properties related to inflammatory cytokines. Considering the increase of class II MHC molecules expression, hydrogen peroxide secretion and inflammatory cytokines, is reasonable to suppose that the simvastatin could be directing the macrophages to a M1 polarization in the time evaluated in this model. Moreover, it is important to mention that immunomodulatory effect of simvastatin cannot be considered the only mechanism associated to the increase of survival since Merx et al. (12) have demonstrated that the improvement in hemodynamic functions played a major role in the survival benefit of simvastatin pre-treatment in mice with sepsis.

It is important to emphasize that simvastatin is widely used to control hypercholesterolemia and hypertriglyceridemia, and since its pharmacokinetics and pharmacodynamics are already widely known, the use of this drug as an adjuvant in the treatment of sepsis could be a good alternative to control the manifestations

associated with sepsis and change the poor prognosis of sepsis and its related conditions.

ETHICS STATEMENT

All performed procedures were authorized by the National Council for Animal Control and Experimentation (CONCEA) and approved by the Animal Use Ethics Committee of the UFMA under protocol number 23115. 012975/2008-43.

AUTHOR CONTRIBUTIONS

JB, CR, TF, MM, and FN conceived and designed the experiments. JB, CR, TF, LT, DS, DC, and JN performed the experiments. JB, AA, DC, JN, LS, and FN analyzed the data. FN and CS contributed reagents, materials, and analysis tools. JB, AA, MM, LS, RG, CS, and FN wrote the paper.

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REVIEW
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Epidemiology and Immune Pathogenesis of Viral Sepsis

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Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Sepsis can be caused by a broad range of pathogens; however, bacterial infections represent the majority of sepsis cases. Up to 42% of sepsis presentations are culture negative, suggesting a non-bacterial cause. Despite this, diagnosis of viral sepsis remains very rare. Almost any virus can cause sepsis in vulnerable patients (e.g., neonates, infants, and other immunosuppressed groups). The prevalence of viral sepsis is not known, nor is there enough information to make an accurate estimate. The initial standard of care for all cases of sepsis, even those that are subsequently proven to be culture negative, is the immediate use of broad-spectrum antibiotics. In the absence of definite diagnostic criteria for viral sepsis, or at least to exclude bacterial sepsis, this inevitably leads to unnecessary antimicrobial use, with associated consequences for antimicrobial resistance, effects on the host microbiome and excess healthcare costs. It is important to understand non-bacterial causes of sepsis so that inappropriate treatment can be minimised, and appropriate treatments can be developed to improve outcomes. In this review, we summarise what is known about viral sepsis, its most common causes, and how the immune responses to severe viral infections can contribute to sepsis. We also discuss strategies to improve our understanding of viral sepsis, and ways we can integrate this new information into effective treatment.

Keywords: viral sepsis, epidemiology, immune pathogenesis, herpes simplex virus, human enterovirus, human parechovirus, influenza virus, dengue virus

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DEFINITION AND EPIDEMIOLOGY OF VIRAL SEPSIS

Definition of Viral Sepsis

Sepsis is a complex syndrome of physiological and pathological abnormalities resulting from infection (1). The pathophysiology of sepsis is not fully understood, making it difficult to give an unambiguous and comprehensive definition of sepsis. The Third International Consensus Definitions Task Force (1) advocated a new definition of sepsis and septic shock in 2015. Sepsis should be defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection," whereas septic shock is defined as "a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone." This version of the definition is designated as "Sepsis-3," while the previous versions are "Sepsis-1" and "Sepsis-2," proposed in 1991 (2) and 2001 (3), respectively.

The main differences between Sepsis-3 and previous versions are that Sepsis-3 eliminated the terms sepsis syndrome and severe sepsis, and introduced a new definition of sepsis, which is more comparable to the older definition of severe sepsis. In addition, the systemic inflammatory response syndrome (SIRS) criteria, which were the essential elements of Sepsis-1 and Sepsis-2, are no longer used to define sepsis, but they still play a role in the recognition of infection and warrant early intervention for possible sepsis (1, 4). Sepsis-3 provides a more specific and universal definition for sepsis, which would improve clinical management and facilitate epidemiological surveys. It also has better predictive ability for in-hospital mortality (1).

However, Sepsis-3 is not designed for paediatric populations, which carry a high burden of sepsis (5). The current, widely-used consensus definition of paediatric sepsis, proposed in 2005, was still built on the SIRS criteria (6). It has been shown to lack specificity and perform poorly in identifying children at high risk of mortality from infection (7–9). In addition, its feasibility and applicability when applying to a non-intensive care setting or low- and middle-income countries remain questionable (9). Some evidence has suggested it may be useful to apply the Sepsis-3 definition to paediatric populations (8, 10–12). However, many children with severe viral respiratory tract infections (e.g., bronchiolitis) fulfil the criteria for viral sepsis, but generally clinicians would not regard them as "septic," highlighting the difficulty in providing a robust definition of viral sepsis (13). Therefore, there is an urgent need to convene a consensus task force and design a paediatric definition (9, 14).

Although bacterial or fungal infections are commonly attributed as the cause of sepsis, sepsis is infrequently attributed to viral infections. In some cases, viral sepsis is regarded as virus-induced direct tissue or cell damage (e.g., influenza virus-induced pulmonary epithelial damage) instead of systemic dysregulation caused by virus. However, the above-mentioned consensus definitions of sepsis, either for adult (1) or paediatric (6) populations are not pathogen-specific, so the same definitions should also apply to viral infection. Therefore, in this review article, viral sepsis is defined as life-threatening organ dysfunction due to a dysregulated host response to viral infection in both adult and paediatric populations. Viral infection can be diagnosed by associated clinical presentations plus positive results of culture, antigen detection, molecular detection (e.g., polymerase chain reaction, PCR), serology, histopathology or immunohistochemistry (15). Viral sepsis should always be considered in septic patients lacking evidence of bacterial, parasitic or fungal infection, and laboratory tests for viruses should be arranged accordingly. In the following sections, we will review the current evidence available about the epidemiology, aetiology, immune pathogenesis, and potential treatments of viral sepsis.

Burden of Viral Sepsis

Most of the available large-scale epidemiological studies on sepsis were based on the Sepsis-2 criteria. Severe sepsis defined by Sepsis-2 (3) is similar to the definition of sepsis according to the Sepsis-3 criteria (1). Therefore, where available, we will use the data on severe sepsis from studies based on Sepsis-2 to represent the epidemiological data on sepsis, defined by Sepsis-3.

In general, the incidence and severity of sepsis are climbing over time, whereas sepsis-associated mortality is declining (16–19). A recent systematic review and meta-analysis (17) reported that the global incidence and mortality of hospital-treated sepsis in adult populations were 270 per 100,000 person-years and 26%, respectively, in the last decade. Extrapolating these figures translated to global estimates of 19.4 million sepsis cases and 5.3 million deaths annually. Another global study (5) found that the incidence and mortality of sepsis in paediatric populations (between 4 weeks and 20 years of age) were 22 per 100,000 person-years and 9–20%, respectively; the incidence and mortality of neonatal sepsis (Sepsis-2 definition) were 2,202 per 100,000 live births and 11–19%, respectively. Extrapolation of the data resulted in global estimates of 3 million sepsis episodes in neonates and 1.2 million episodes in paediatric populations annually. Infection-related mortality outside the neonatal period has been falling, but these sepsis episode rates emphasise the importance of focus on tackling sepsis in the first 4 weeks of life.

Both studies (5, 17) indicated several limitations about the epidemiological surveys on sepsis. Firstly, there was no population-level data available from low-income countries, which represent 87% of the world's population (17) and bear a huge burden of infectious diseases and sepsis (20). Therefore, these figures were likely to be underestimates. Secondly, the lack of a universal and specific sepsis definition and severity criteria leads to substantial heterogeneity in case definitions among studies. Lastly, studies may use different denominators to calculate the incidence and mortality (e.g., person-years, specified populations, live births). These hurdles make meta-analysis more challenging and susceptible to bias.

On top of these limitations, most epidemiological studies on sepsis either excluded cases of viral origin or did not specify the proportion of viral sepsis. Organisms that contribute to sepsis can be identified in 59–69% of septic patients (i.e., documented sepsis), with bacteria usually accounting for more than 70% of the documented sepsis cases (21–23). Viruses only contribute ∼1% of the documented sepsis cases in some studies (22, 23). However, this figure likely understates the prevalence of viral sepsis for several reasons. A recent Southeast Asian prospective study (24), using a predefined set of laboratory tests (including PCR tests for multiple viruses), demonstrated that viruses accounted for 76 and 33% of the documented sepsis cases (Sepsis-2 definition) in paediatric (excluding neonates) and adult populations, respectively. The most common viruses identified were dengue viruses (27%), followed by rhinovirus (23%), influenza viruses (14%), and respiratory syncytial virus (12%). Although the study was only conducted in tropical middle-income countries and not using the Sepsis-3 definition, it still provides direct evidence that viral sepsis may be underdiagnosed if diagnostic tests for viruses are not performed.

However, the identified viruses could be the single causative agent of sepsis (e.g., dengue), a contributor to secondary bacterial sepsis (e.g., influenza and staphylococcal sepsis) (25), coinfection of unknown significance (e.g., rhinovirus), prolonged or persistent shedding of a previous infection (e.g., adenovirus)

(26), an "innocent" latent infection (e.g., Epstein-Barr virus) or a false positive result. This also needs to be taken in the clinical context. For example, a profoundly immunosuppressed child with respiratory symptoms and a high rhinovirus viral load in blood would suggest rhinovirus as the cause of the sepsis. However, a previously well child with purulent meningitis and rhinovirus in a nasal swab would not. This uncertainty is another major obstacle to studying the epidemiology of viral sepsis, particularly in cases where bacterial infection is also documented or the identified virus does not usually cause fulminant disease. What role the identified virus plays in a septic patient is still an area of debate.

Similarly, another prospective study demonstrated that a third of adult patients requiring intensive care for severe pneumonia had viral infections, detected by a predefined array of diagnostic tests (including PCR tests for multiple viruses) (27). Pneumonia is the most common clinical syndrome in patients with sepsis (21, 22, 28). Moreover, another prospective study found that patients with culture-negative sepsis had significantly lower levels of procalcitonin than those with documented sepsis (21). It has been shown that elevated procalcitonin levels are more likely to be seen in bacterial infections than viral infections and may be used to differentiate infections caused by bacteria and viruses (29, 30). Therefore, these studies suggest that viruses may cause more sepsis cases. The exact incidence of viral sepsis remains to be elucidated.

The World Health Organization and the World Health Assembly have listed sepsis as one of the global health priorities for the following years (31). They also recognized the importance of understanding the epidemiological burden of sepsis. In order to obtain a comprehensive picture of the burden of sepsis, there is an urgent need to understand the epidemiology of viral sepsis.

AETIOLOGY OF VIRAL SEPSIS

Almost any virus can cause viral sepsis in susceptible populations (24). Herpes simplex virus (HSV) and enteroviruses are the most common viral causes of neonatal sepsis (32), while enteroviruses and human parechoviruses (HPeVs) are the most common causes of viral sepsis in young children (33). In addition, influenza viruses are not only a major cause of severe infections and deaths among children younger than 5 years of age, older adults, pregnant women and immunosuppressed individuals (34), but can also lead to substantial morbidity and mortality in older children and adults in other age groups (35). Furthermore, dengue viruses are a leading cause of sepsis in some tropical countries (24). We will review the characteristics and epidemiology of these prominent causes of viral sepsis in the following section.

Herpes Simplex Viruses

HSV is one of the leading causes of neonatal sepsis (32). In neonates, HSV can cause three types of disease: skin, eye and mouth disease, encephalitis, and disseminated disease (36). Disseminated HSV disease is the most severe form of HSV infection, with a case fatality rate of as high as 29% (29). Patients with lethargy, severe hepatic dysfunction or delayed treatment have higher mortality (29). HSV can also cause fulminant hepatitis in non-neonatal populations, typically without obvious cholestasis (37). Both disseminated HSV disease and fulminant HSV hepatitis have a clinical presentation of viral sepsis, involving hepatic dysfunction, respiratory failure, disseminated intravascular coagulopathy, and haemodynamic instability (36, 38). In the absence of skin lesions, disseminated HSV disease and HSV hepatitis are difficult to differentiate clinically from sepsis caused by other pathogens (36, 38).

Studies have reported various incidence rates of neonatal HSV infection, ranging from 8 to 60 per 100,000 live births, with disseminated HSV disease accounting for 25% of cases (39). Some viral factors are associated with viral sepsis. Firstly, a large inoculum of HSV may increase the risk of viral sepsis (38). Secondly, it has been shown that maternal genital HSV type 1 (HSV-1) infection has a higher probability of transmission to neonates during labour than HSV type 2 (HSV-2) infection (40). However, HSV-2 accounts for a higher proportion of neonatal central nervous system (CNS) and disseminated HSV diseases although HSV-1 causes about 60% of cases of neonatal HSV infection (41). This also explains why HSV-2 is associated with higher morbidity and mortality (39). Furthermore, neonates born to mothers with newly-acquired genital HSV infection near term are at greater risk for neonatal HSV infection than those born to mothers with reactivated genital HSV infection (42).

Human Enteroviruses

Enterovirus is a genus of viruses of the family *Picornaviridae*, which have been shown to cause sepsis in immunodeficient and paediatric populations (43). Enteroviruses are the causative agents of a broad range of clinical conditions including aseptic meningitis and myocarditis, although many cases are asymptomatic or benign (44). There are about 10–15 million symptomatic enteroviral infections in the United States per year (43) with a disproportionately high number of infections occurring in neonates (11.4–11.6% compared with the average yearly birth cohort percentage of 1.5%) (45, 46). The enteroviruses that have the highest association with sepsis are coxsackievirus and echovirus (47); these viruses primarily cause sepsis in neonates (47). In contrast, enterovirus A71 can lead to viral sepsis in children beyond the neonatal period (48, 49), predominantly in children younger than 2 years of age (50).

Risk factors for neonatal enteroviral infection include exposure to maternal secretions or blood during delivery, maternal infection just before or at delivery, and a lack of previous maternal infection by the infecting serotype, resulting in low maternal antibody levels against that serotype of virus. The majority of severe neonatal enteroviral infections occur between days 3 and 5 of life, suggesting that the acquisition is generally in the perinatal period and is preceded by maternal infection (51).

Human Parechoviruses

HPeVs are also frequently associated with sepsis in paediatric and immunodeficient populations. HPeVs have previously been defined as a subset of the genus *Enterovirus* but were eventually re-classified as their own genus of viruses after sequencing revealed them to be unrelated to enterovirus. Antibodies against

HPeVs are present in the cerebrospinal fluid (CSF) of up to 99% of the population (52), with HPeV type 3 (HPeV3) (the most common cause of HPeV sepsis) being present in 10% of the population studied in the Netherlands and 13% in Finland (52). HPeVs are the second most common cause of viral sepsis in young children after enteroviral infections (33). HPeV infections are often asymptomatic or present with very mild symptoms, although severe infections can have symptoms ranging from sepsis and sepsis-like illnesses to viral meningitis and encephalitis (33). White matter abnormalities on magnetic resonance imaging have been found in cases of HPeV encephalitis, which may play a role in the development of sequelae (53–55). The development of white matter abnormalities and sequelae seems to show little association with short term outcomes (54). Clinical presentations of HPeV infections are similar to those of enteroviral infections, are clinically indistinguishable and require serology or PCR to discriminate between them. HPeV3 is the HPeV most commonly associated with severe disease, with other HPeVs known to cause severe disease only rarely (56, 57).

Licensed specific antiviral therapy is not available for HPeV or enterovirus infections, despite their relatively high incidence in neonatal encephalitis and systemic infections (58). This presents a promising target for future research, and a tangible way to reduce the incidence of neonatal viral sepsis and associated infant mortality globally.

The addition of a PCR test for HPeVs to the re-analysis of 761 banked CSF samples from children presenting with sepsis found a 31% increase in detection of a viral cause of sepsis in these cases (33), with HPeV being found in 0.4–8.2% of neonates presenting with sepsis, depending on the year, with an overall detection rate of 4.6%. It is thus likely that viral sepsis caused by HPeVs is frequently underdiagnosed.

Influenza Viruses

Influenza A and B viruses cause seasonal epidemics and out-of-season outbreaks worldwide (34). Influenza sepsis can present as severe pneumonia, acute respiratory distress syndrome (ARDS), myocarditis or encephalopathy. The estimated annual incidence proportions of influenza virus infection are 5–10% in adults and 20–30% in children (34). A modelling study demonstrated that seasonal influenza epidemics account for an estimated 290,000–650,000 respiratory deaths annually, with 10,000–110,000 occurring among children younger than 5 years of age (59). The true mortality attributable to influenza viruses must be higher because the figures do not include deaths from other causes, such as circulatory deaths (59), which also make up a large proportion of influenza virus-associated deaths (60). Approximately 60% of mortality from seasonal influenza occurs in people older than 65 years of age (59), while 80% of the non-survivors in the 2009 influenza H1N1 pandemic were people younger than 65 years of age (61). The age shift may be explained by some level of immunity to H1N1 strains in people born before 1957, when H1N1 strains widely circulated and had not been replaced by the H2N2 pandemic strain (35). In addition, a study (62) found high titres of low-avidity, non-protective immunoglobulin G against the viral H1 antigen in severely ill middle-aged adult patients. Pulmonary immune complex deposition and complement activation were also observed. This provides evidence that immune complex-mediated disease may be part of the pathogenesis of severe pneumonia in middle-aged patients, which also contributes to the age shift.

The pathogenesis of influenza virus infection depends on viral virulence and host responses (63). Host responses will be discussed in the next section. The crucial site for influenza virus infection that leads to severe pneumonia is the alveolar epithelium (64). Haemagglutinins (HAs) of different strains of influenza viruses have varied tropism for the airway epithelium. For example, seasonal influenza viruses bind preferentially to the epithelium in the upper airway and bronchi and, to a lesser extent, to the alveolar epithelium (65, 66). By contrast, HA of the 2009 pandemic influenza A (H1N1) virus attaches to both type 1 and type 2 pneumocytes, while HA of the avian influenza H5N1 virus primarily binds to type 2 pneumocytes (64). The second determinant of viral virulence is the viral polymerase complex, which is associated with different levels of viral replication and cytokine production in the infected epithelial cells (65). Therefore, the differing tropism, along with the varied degrees of viral replication and cytokine production, induces various extents of cell death and in part explains the differences in pathogenicity between different strains (63, 67).

Dengue Viruses

Dengue is a common viral infection in tropical countries and has the capacity to cause viral sepsis. Dengue viruses are currently considered the most important and widespread virus spread by mosquitos (68). Over 50% of the world's population live in areas where dengue infection occurs (68). Estimates of yearly dengue infections range from 50 million up to 400 million (68). Dengue viruses are members of the family *Flaviviridae*, with four distinct serotypes; serotypes 1, 2, 3, and 4 (69). Infection with any of these serotypes gives full protection against that serotype; however, after infection with one serotype, infection with any of the others can result in an enhanced and more severe form of the disease (70). All serotypes of dengue viruses have been implicated in severe dengue (68).

One study in Thailand found that ∼14% of patients diagnosed with sepsis (Sepsis-2 definition) tested positive for dengue viruses upon re-analysis of banked serum samples by PCR (71). Of the patients who were diagnosed with dengue by PCR from banked serum samples five had died, of which four had been diagnosed with sepsis but not dengue infection. It was suggested that it may prove beneficial to increase testing for dengue viruses in patients presenting with sepsis to ensure the patient receives appropriate treatment (71).

Table 1 summarises the clinical syndromes, epidemiology and risk factors of sepsis caused by these viruses and adenovirus. While being the most commonly detected viruses causing sepsis, they are far from the only ones. Other viruses, such as chikungunya virus (84), hantavirus (85), coronaviruses (86), Ebola virus (87), and Lassa virus (88), among many others, are also major contributors to viral sepsis across the globe. Due to the limited data on many of these viruses, the immune responses against them and their pathogenesis are poorly understood. In addition, these viruses do not occur at a high enough incidence in

TABLE 1 | Summary of the clinical syndromes, epidemiology and risk factors of sepsis caused by different viruses.

	Clinical syndromes	Epidemiology	Risk factors for sepsis	References
HSV	Disseminated disease Hepatitis	Neonatal disseminated disease Incidence: 2–15 per 100,000 live births Mortality: up to 29%	Newly-acquired maternal genital infection near term HSV-2 infection (compared with HSV-1 infection)	(29, 38, 39)
Enterovirus	Sepsis-like illness Myocarditis Encephalomyelitis Pulmonary oedema or haemorrhage	Incidence: 37% of young infants (<90 days of age) with sepsis[a] and without signs of localised infection Mortality of neonatal enteroviral sepsis: up to 42%	Lack of maternal antibodies Maternal infection just before or at delivery Neonatal infection with echovirus 6, 9, 11, 19 or coxsackievirus B2–B5 Enterovirus A71 infection in young children	(29, 48, 72, 73)
HPeV	Sepsis-like illness Meningoencephalitis	Incidence: 15% of young infants (<90 days of age) with sepsis[a] and without signs of localised infection	HPeV3 infection (compared with infection with other types of HPeV)	(33, 73)
Influenza virus	ARDS Myocarditis Encephalopathy	Incidence: 1 million cases of severe respiratory infections in children <5 years of age worldwide annually Mortality: 290,000–650,000 respiratory deaths worldwide annually (all age groups)	People of extreme age (<5 or >65 years) Immunosuppression Pregnancy Influenza A (H3N2) virus infection (compared with influenza A (H1N1) or B virus infections)	(34, 59, 74, 75)
Dengue virus	Severe dengue[b] Dengue shock[b]	Incidence: 58–96 million symptomatic dengue infections with 250,000–500,000 progressing to severe disease worldwide annually; 8% of sepsis cases[c] in Southeast Asian Mortality: 9,000–24,000 deaths worldwide annually	Previous dengue infection (with a different serotype)	(20, 24, 70, 76–79)
Adenovirus	Disseminated disease Meningoencephalitis Severe pneumonia	Disseminated disease in children Incidence: 2.5% of adenovirus infection Mortality: 55%	Immunosuppression (particularly allogeneic HSCT) Young children Infection with adenovirus serotypes 3 and 7	(80–83)

[a] *Sepsis was defined according to age-specific criteria, Rochester criteria and Yale observation scale.*
[b] *2009 WHO revised dengue case classification.*
[c] *Sepsis was defined by the Sepsis-2 definition.*
HSV, herpes simplex virus; HPeV, human parechovirus; ARDS, acute respiratory distress syndrome; HSCT, haematopoietic stem cell transplants.

populations in high-income countries to gain significant research funding. This may change in future as more effective treatments are discovered for more frequently occurring infections and less common diseases become more attractive to research.

SUSCEPTIBLE POPULATIONS

Neonates and young children (89), pregnant women (90), older adults (89), and immunosuppressed individuals (91) are especially susceptible to severe infections and sepsis. Here, we will review current evidence regarding the immunological characteristics of these susceptible populations that predispose them to severe infections, especially viral sepsis.

Neonates and Young Children

The immature and naïve immune system of neonates predisposes them to infection with intracellular pathogens and sepsis (92, 93). One of the most remarkable features of the neonatal innate immune system is the bias in favour of type 2 helper T (T_H2)-cell responses, which results in reduced secretion of pro-inflammatory cytokines, such as interleukin (IL)-12, tumour necrosis factor (TNF), interferon (IFN)-γ, and IL-1β, which together with immature innate immunity allows pathogens to replicate and spread more easily (93, 94). In contrast, neonatal monocytes and antigen-presenting cells display preserved or even enhanced Toll-like receptor (TLR)-mediated production of some cytokines (e.g., IL-6, IL-10, and IL-23) (93). In addition, studies have shown that neonates can experience highly exaggerated inflammatory responses through some pathways, such as the TLR2 pathway, in response to specific antigens (95, 96). These exaggerated responses may play a role in the development of sepsis in response to viral infections.

There are also other features of the neonatal immune system that increase susceptibility to severe viral infection. Firstly, neonatal monocytes have decreased expression of the major histocompatibility complex (MHC) class II, which leads to impaired antigen presentation (93). Secondly, neonatal dendritic cells have a reduced production of TNF and type I IFNs, impaired upregulation of CD80 and CD86 co-stimulatory molecules, and reduced stimulation of T cell proliferation, all of which can contribute to a decreased ability to clear viruses (93). In addition, neonates have low levels of complement

components (93); complement is responsible for antibody-independent opsonization and lysis of pathogens and plays a role in the activation and enhancement of the adaptive immunity against infections (97). Furthermore, there are both quantitative and qualitative defects in neonatal neutrophils (93). For example, lower levels of neutrophils in stress situations, such as sepsis, are seen in neonates. The qualitative deficiencies in neonatal neutrophils include impairment of adhesion, migration, chemotaxis and amplification. These defects lead to a reduced ability to clear viruses and other pathogens. Lastly, the naïve, immature adaptive immunity of neonates, together with the lack of pre-existing immunological memory, increases their susceptibility to various pathogens and severity of infection (94).

It is worth noting that susceptibility to sepsis persists beyond the neonatal period (i.e., 4 weeks of age). Young children also bear a substantial burden of sepsis, with the peak incidence of 516 per 100,000 population in the infant group (Sepsis-2 definition) (5). Likewise, hospitalisation rates for viral infections, such as influenza (98) and respiratory syncytial virus infection (99), are highest in children younger than 2 years of age. In addition, children with underlying diseases (e.g., bronchopulmonary dysplasia, congenital heart diseases, neurological disorders) are at greater risk of developing severe viral infections and sepsis (98, 100, 101).

Pregnant Women

Pregnant women are another population at greater risk of viral sepsis than the general population. There have been several reports on maternal sepsis caused by influenza, herpes simplex, varicella-zoster, and chikungunya viruses, among others (102–106). Recognition of the problem of influenza related mortality in late pregnancy has led a number of countries to introduce routine influenza vaccination in pregnancy. According to the Global Burden of Disease Study, there were an estimated 17,900 deaths from maternal sepsis and other infections globally in 2015, accounting for 6.5% of the total deaths from maternal disorders (20). The incidence of maternal sepsis is around 41–49 per 100,000 pregnancies with a mortality rate of 1.8–4.5% in the United Kingdom (105) and the United States (107). An increasing trend in the incidence and mortality of maternal sepsis has been seen in the recent decades (108). However, these studies did not report the proportion of women with viral sepsis.

The maternal immune system is complicated and delicately modulated. It is tolerant to paternal antigens and the "allogeneic" foetus, while efficient at identifying and defending against invading pathogens to protect the mother and the foetus (109). The immunological characteristics during pregnancy depend on the stage of gestation and the area of focus. For example, a pro-inflammatory [type 1 helper T (T_H1)-biased] status with high levels of IL-6, IL-8, and TNF-α is seen in pregnant women during the first trimester of pregnancy, which is critical for embryo implantation, placentation and initial foetal growth. Following this, pregnant women develop a more anti-inflammatory (T_H2-biased) status with increased levels of prostaglandin E_2, IL-4, and IL-10 while the foetus grows rapidly. Before labour, the immune system shifts back to a pro-inflammatory (T_H1-biased) status, which helps parturition (110). Additionally, the epithelial cells of the reproductive tract are down-regulated with low levels of IL-1β, IL-8, and IL-6 in cervical fluid (109). Pregnant women also encounter reduced levels of immunoglobulin G (111) and a decreased number of helper T lymphocytes (111) throughout pregnancy.

The maternal immune system is not yet understood completely. However, we do know that it is constantly changing, and not just universally suppressed. The unique immune profiles result in different responses to pathogens, which may make pregnant women more susceptible to some pathogens depending on the stage of pregnancy (110). In addition, the immune response originating from the placenta also influences the maternal immune response to microorganisms. For example, an insult such as a subclinical viral infection of the placenta can affect the maternal immune system and increase the maternal susceptibility to various pathogens, including viruses (112).

Older Adults

Older adults (>60 years old) are a population at a significantly greater risk of sepsis from all causes than the general population (113). Older adults were found to have an incidence of sepsis of 26.2 cases per 1,000, which is considerably higher than the 3.0 cases per 1,000 observed in the general population (114). There are many reasons for this increased susceptibility, including a higher likelihood of co-morbidities, prolonged hospitalisation times, generally weaker immune responses, and immunosenescence (115). Viral sepsis in older adults presents one of the most serious upcoming global health problems, as the global population of older adults is set to overtake the "young" population by 2050. It is therefore important to understand the unique conditions within older adults to better facilitate the development of suitable treatments. Many of the factors involved in the increased susceptibility of older adult populations to sepsis also increase the susceptibility to viral infection, and thus viral sepsis.

Comorbidities for sepsis and severe viral infection such as diabetes mellitus (116), renal failure (117, 118) chronic obstructive pulmonary disease (117, 119), heart conditions (120), and obesity (120, 121) are much more prevalent in older adult populations. There are many other comorbidities in older adults that can increase the risk of viral sepsis. These often are associated with immunosuppression [e.g., renal failure (122)].

Immunosenescence is the gradual deterioration of the immune system brought on by advancing age, which increases susceptibility to both viral infections, and the development of viral sepsis. It is characterised by a decrease in the function of phagocytes (123–125), antigen presentation (124) and lymphocytes (126, 127), as well as decreased cellular replication (128, 129) and ability to respond to cytokine stimulus (130). Older adults also experience persistent T cell exhaustion in part due to constant low-level inflammation, thought to be caused by accumulation of self-debris brought on by a decrease in the ability to clear them (131). This process, often called "inflammaging" is characterised by elevated baseline levels of the cytokines IL-6, IL-1, and TNF-α (130, 132). Another factor contributing to

T cell exhaustion in older adults is the prolonged length of inflammatory states after infections (133) which can result in decreased T cell replication and inhibition of co-stimulation by antigen presenting cells. This results in a decreased ability to effectively respond to infection, allowing viral infections to easily evade eradication by the immune system and develop into serious systemic infections.

Immunosuppressed Individuals

The number of immunosuppressed hosts has grown considerably over the last decades due to the widespread use of cytoablative chemotherapy, monoclonal antibodies and immunomodulatory agents for neoplastic and autoimmune diseases, the epidemic of human immunodeficiency virus (HIV) and increasing numbers of haematopoietic stem cell transplants (HSCT) and solid organ transplants (SOT). The clinical picture of sepsis in immunosuppressed hosts is usually diminished or non-specific, making it difficult to diagnose or distinguish from other non-infectious causes, such as transplant rejection (91, 134). Thus, infection and sepsis continue to be the major cause of morbidity and mortality in immunosuppressed hosts (91, 135). A multicentre, prospective study (135) in the United States showed that 42% of HSCT recipients had viral infections at some point post-transplant (median follow-up, 413 days), and infection accounted for 21% of deaths. However, the authors did not specify the mortality rate caused by viral infections.

Individuals with neutropenia or taking corticosteroids mainly have impaired innate immunity, while transplant recipients primarily have defects in adaptive immunity (91, 136). Similar to immunocompetent hosts, many viruses are able to cause sepsis in immunosuppressed hosts, but some are of particular concern. For example, HSCT and SOT recipients are at high risk of infection with cytomegalovirus (CMV), other herpesviruses and respiratory viruses (e.g., adenovirus, influenza virus, parainfluenza virus and respiratory syncytial virus) (136). In transplant recipients, the timing of viral infections varies according to the types of transplant, antimicrobial prophylaxis received, and other host and donor factors, but they are most likely to occur 1 month after transplantation when defects in cell-mediated immunity dominate or graft-versus-host disease occurs (134, 137). By contrast, the risk of infection with CMV, other herpesviruses, and respiratory viruses may be lower in neutropenic hosts, patients with HIV infection and individuals taking corticosteroids than in transplant recipients (136).

Primary immunodeficiency is another special category of diseases comprising at least 200 genetic disorders of variable severity (138) and affecting more than six million people worldwide (139). This population is at substantial risk of disseminated viral infection and sepsis. Young children with inborn errors in signalling pathways upstream of the production of type I IFNs are at higher risk of developing life-threatening viral infection (140). For example, signal transducer and activator of transcription 1 (STAT1) or nuclear factor (NF)-κB essential modulator (NEMO) deficiency leads to lethal HSV disease and various other severe viral infections (140–142). Deficiency in interferon regulatory factor 7 also leads to more severe viral infections due to decreased downstream IFN signalling (143). Other disorders that have been demonstrated to have an effect in viral infections are the IFITM3 SNP rs12252, which affects CD8+ T cell numbers (144), and variations in receptor components such as the IFIH1 receptor, which decreases downstream signalling to IFNs (145). In addition, patients with severe combined immunodeficiency have major defects in B and T lymphocyte development, facing a substantial risk of infection caused by a wide variety of pathogens (e.g., fulminant adenovirus and HSV infections) (146, 147).

THE IMMUNOLOGY OF VIRAL SEPSIS

Normal Immune Responses to Viral Infection

Pattern recognition receptors (PRRs) are responsible for the initial detection of viruses (148). They can recognize pathogen-associated molecular patterns (PAMPs) (e.g., viral RNA and DNA) and damage-associated molecular patterns (DAMPs) (e.g., host DNA and proteins) (148). There are several families of PRRs, such as TLRs, cytosolic RNA sensors [e.g., retinoic acid–inducible gene (RIG)-I and melanoma differentiation–associated gene 5 (MDA5)] and cytosolic DNA sensors (e.g., absent in melanoma 2, IFN-γ-inducible protein 16, and cyclic GMP-AMP synthase) (149). When encountering pathogens, PRRs play a critical role in the activation of innate immune responses and the recruitment of leucocytes (148, 149).

First of all, the innate responses stimulate the production of pro-inflammatory cytokines and have an immediate antiviral effect on preventing virus spread and replication, which is mainly exerted by type I IFNs (150, 151). Furthermore, PRRs can trigger the development of virus-specific adaptive immunity (e.g., cytotoxic T lymphocytes, antibodies) to clear viruses and virus-infected cells (152). Lastly, PRRs can induce the secretion of anti-inflammatory cytokines such as IL-10 and IL-13, which help to resolve the pro-inflammatory state and promote tissue repair (149, 153, 154). The normal immune responses to viral infection are summarised in **Figure 1A**.

Hyper-Inflammatory Phase of Viral Sepsis

Although pro-inflammatory cytokines are essential to mediate innate immunity, they (particularly IL-6) can cause host damage (149). The release of DAMPs from the damaged tissues and cells can further stimulate PRR signalling and lead to a chain reaction culminating in viral sepsis if the infection is not cleared (155).

Herpes Simplex Viruses

TLR2 and TLR9 comprise the major PRR signalling pathways activated in response to HSV infection (96, 156). Many of the clinical features of viral sepsis caused by HSV can be attributed to exuberant responses induced through TLR signalling (95). It has been demonstrated that levels of IL-6 are negatively associated with the survival of HSV encephalitis (157). Neonatal cord-blood cells mount higher levels of pro-inflammatory cytokines (IL-6 and IL-8) when challenged with HSV than adult blood cells (95). Therefore, HSV causes a higher ratio of IL-6 to TNF in neonates, which contributes to severe inflammation and the development of sepsis (93).

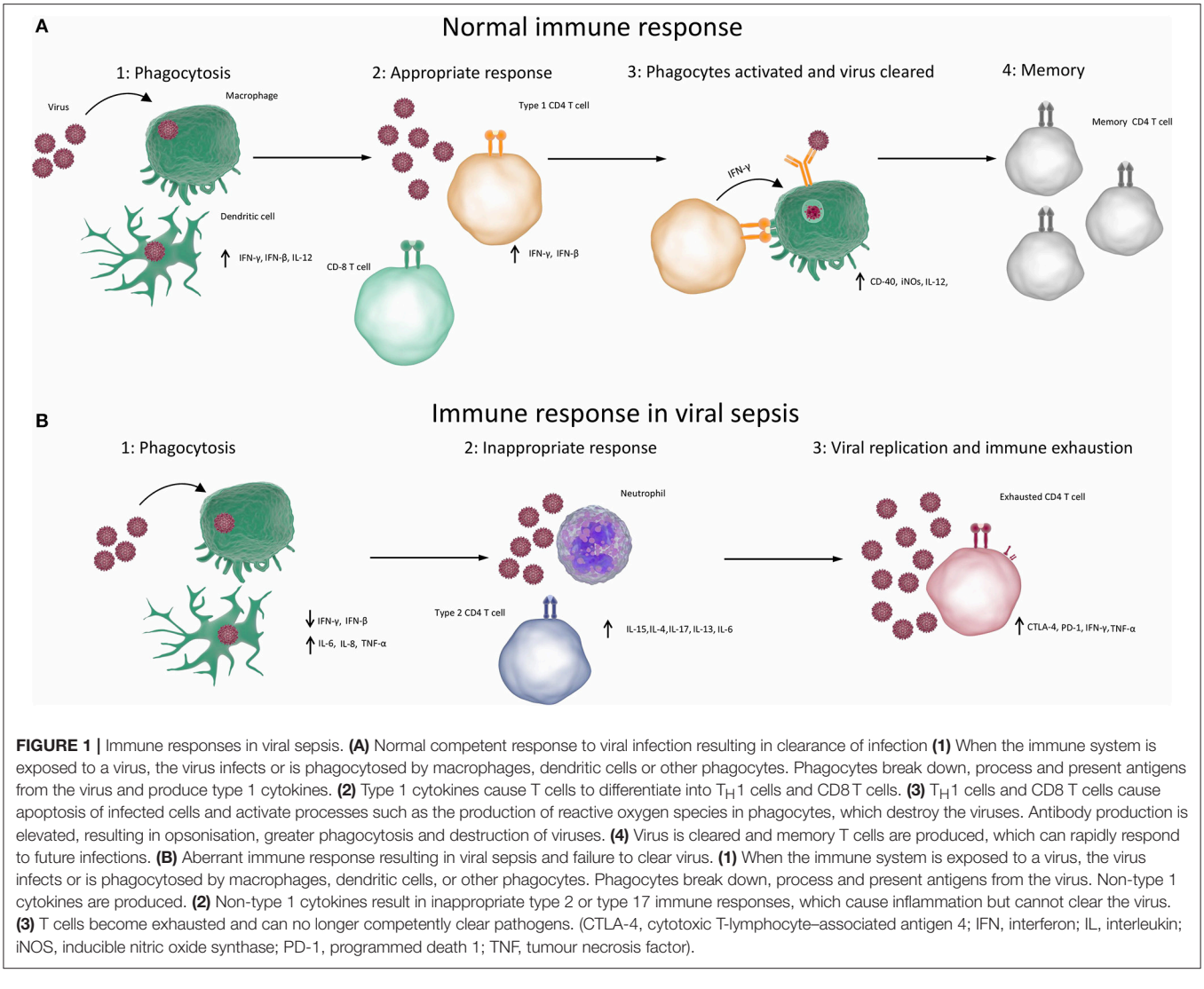

FIGURE 1 | Immune responses in viral sepsis. **(A)** Normal competent response to viral infection resulting in clearance of infection **(1)** When the immune system is exposed to a virus, the virus infects or is phagocytosed by macrophages, dendritic cells or other phagocytes. Phagocytes break down, process and present antigens from the virus and produce type 1 cytokines. **(2)** Type 1 cytokines cause T cells to differentiate into T_H1 cells and CD8 T cells. **(3)** T_H1 cells and CD8 T cells cause apoptosis of infected cells and activate processes such as the production of reactive oxygen species in phagocytes, which destroy the viruses. Antibody production is elevated, resulting in opsonisation, greater phagocytosis and destruction of viruses. **(4)** Virus is cleared and memory T cells are produced, which can rapidly respond to future infections. **(B)** Aberrant immune response resulting in viral sepsis and failure to clear virus. **(1)** When the immune system is exposed to a virus, the virus infects or is phagocytosed by macrophages, dendritic cells, or other phagocytes. Phagocytes break down, process and present antigens from the virus. Non-type 1 cytokines are produced. **(2)** Non-type 1 cytokines result in inappropriate type 2 or type 17 immune responses, which cause inflammation but cannot clear the virus. **(3)** T cells become exhausted and can no longer competently clear pathogens. (CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; PD-1, programmed death 1; TNF, tumour necrosis factor).

Dysregulated secretion of pro-inflammatory cytokines in response to HSV infection also induces the production of high mobility group box 1 protein (HMGB1) from injured cells (158). HMGB1 is a nuclear protein and regulates DNA transcription. HMGB1 can mount a pro-inflammatory cytokine response to pathological levels and lead to the release of cytochrome c (158). It has been demonstrated that the peak of HMGB-1 comes before the peak of cytochrome c in a clinical case of neonatal disseminated HSV disease (158). Cytochrome c subsequently activates caspase-3 and caspase-7, resulting in extensive apoptosis (149). Apoptosis is responsible for the development of multiorgan dysfunction in septic patients (159).

Human Enteroviruses

Human enterovirus infections are characterised by a type I IFN response, induced by PRRs that respond to RNA viruses. Enterovirus detection has been found to primarily involve the TLR and RIG-I-like receptor (RLR) signalling pathways. In particular TLRs 3 (160), 7 (161), 8 (162), and 9 (163) have been implicated in the innate response to enteroviruses. The RLR most commonly associated with enterovirus infection is MDA5. MDA5 is involved in detecting intracellular RNA viruses (164) and has been found to play a small role in the development of the innate immune response to enterovirus infection (165). Although other PRR signalling cascades are likely to have a role in sensing enteroviruses, this role has not yet been characterised. Human enteroviruses employ a diverse range of strategies to evade the immune response and replicate. These strategies are critical to the ability to cause viral sepsis, as they allow the virus to replicate sufficiently to cause significant inflammation. Some broad strategies of immune evasion used by enteroviruses are interference with innate immune signalling by either interfering with or avoiding initial PRR recognition [poliovirus can subvert MDA5 signalling and induce apoptosis of innate immune effector cells (166)] or interfering with the downstream cytokine signalling pathways. There is evidence that enterovirus A71 can interfere with the IFN signalling pathway at several points (167). These immune evasion mechanisms have been shown to have

a direct impact on survival in mouse models, with blockage of type I IFN pathways in enterovirus A71 infections resulting in increased mortality and viral load (168). Severe infections with coxsackievirus have been associated with host expression of the decay-accelerating factor (DAF) and coxsackievirus and adenovirus receptor (CAR), which have been shown in mouse models to facilitate coxsackievirus infection of neural stem cells (169, 170). DAF and CAR are cell surface receptor proteins involved in the complement pathway (171) and cell adhesion (172), respectively. The role of DAF in disease is not known (170, 173). CAR functions as a receptor which can be used by coxsackieviruses to enter the cell (170). The immune response to enteroviral infections is varied and strain specific; however, a general strong pro-inflammatory response involving IL-1α, IL-1β and TNF-α, alongside an increase in the expression of innate immune receptors for double stranded RNA is observed in most cases (174). These responses alongside virally induced lytic cell death can result in extensive necrosis, further compounding the inflammatory response and potentially leading to conditions of sepsis (174). Interestingly, some strains of enterovirus, such as echovirus-9, seem to cause a particularly high degree of necrosis in pancreatic β islet cells, resulting in a strong correlation between enteroviral infections and diabetes (174).

Human Parechoviruses

HPeV3 infection has been shown to initiate distinct innate immune responses in CNS infections to those of enteroviral infections despite their similar clinical presentations (175). Enteroviral responses are generally stronger; levels of almost all cytokines are higher in enteroviral responses than in HPeV infections. HPeV infections also do not demonstrate the strong type I IFN responses generally induced by RNA viruses. In addition, HPeV infections demonstrate a lower level of IL-6 expression than both enterovirus infections and controls, suggesting some method of viral immune evasion (175), resulting in these muted immune responses. The markedly lower innate immune cytokine responses induced by HPeV3 infection are interesting, as HPeV3 has a similar clinical presentation to that of many enteroviral infections, raising questions about disease pathogenesis and viral classification. It has also been shown that HPeV is detected by TLRs 7 and 8 (176) which activate the type I IFN pathway (177). There is evidence that HPeVs (in particular HPeV type 1) employ methods to dampen type I IFN signalling (178) in a cell type dependent manner. Treatment of these cells with type I IFN, however, was not found to inhibit viral infection of these cells. It could be interesting to explore the relevance of this evasion mechanism in the context of a systemic infection. The immune response to HPeV infection is not as well-characterised as that of enteroviral infection; however, certain constants, such as the suppression of type I IFNs as an immune evasion mechanism, indicate that type I IFN pathways are important in the resolution of these infections. There are few data analysing cytokine populations of these infections in the context of sepsis, so while it is possible to characterise the immune responses involved in these infections, it is not known if these hold true in viral sepsis.

Several reasons have been proposed for why HPeV3 is more likely to cause severe infections than other HPeVs. It has been observed that HPeV3 lacks a sequence motif present in other HPeVs that is thought to play a role in viral use of integrins as host cell receptors, suggesting that the virus may be exploiting a different receptor to enter cells. This may allow it to more easily access host cells and thus replicate to a higher degree than other HPeVs (179). It has also been noted that HPeV3 is not efficiently neutralised by antibodies naturally generated against it, while HPeV type 1 was efficiently neutralised, which may explain why HPeV3 infections tend to be more severe. Maternal antibodies would be insufficient to neutralise the virus in a neonate, resulting in more severe, prolonged infections (180).

Influenza Viruses

Recognition of influenza virus is primarily through TLR3 (181), TLR4 (182), TLR7 (183), and RIG-I (184), which signal through NF-κB to induce subsequent immune responses (185). The polymorphisms of these receptors in different individuals in part determine their susceptibility to, and the severity of, influenza virus infection (186). The disruption of the alveolar epithelial-endothelial barrier, leading to pulmonary oedema and further respiratory insufficiency, is essential to the development of severe pneumonia and ARDS caused by influenza virus (64). Influenza virus first infects alveolar epithelial cells rather than alveolar endothelial cells, which are usually the primary target for bacteria-induced ARDS (64, 187). Influenza virus can cause apoptosis of the epithelial cells by the upregulation of the *Fas* gene via activating protein kinase R (188). In addition, the infected epithelial cells produce a broad range of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8, CCL5 (RANTES), and CXCL10 (IP-10) (64). These cytokines can damage the epithelial-endothelial barrier through mechanisms that are not yet fully understood (64). Studies have demonstrated that IL-1β and TNF-α can cause decreased activity of amiloride-sensitive epithelial sodium channels, one of the key ion channels that clear alveolar fluid (189). TNF-α has also been shown to be able to disrupt the tight junctions between epithelial cells (190).

Some of the cytokines that are produced by epithelial cells can cause the recruitment and extravasation of monocytes and neutrophils by direct chemotaxis or upregulating adhesion molecules (e.g., P-selectin, E-selectin) on the endothelial cells (64). Newly-recruited neutrophils and macrophages, derived from monocytes, can produce a wide variety of pro-inflammatory cytokines, reactive oxygen species and nitric oxide (by inducible nitric oxide synthase), all of which further damage the barrier (64). These cytokines can also recruit more neutrophils and monocytes into the alveolar lumen (64). It has also been shown there is a positive correlation between the concentration of neutrophils in bronchoalveolar lavage fluid and the severity of ARDS (191). In addition, macrophages produce IFN-β in a protein kinase R- and NF-κB-dependent fashion and express TNF-related apoptosis-inducing ligand (TRAIL) by the stimulation of IFN-β (192). The interaction between TRAIL and death receptor 5 on epithelial cells is another mechanism for the apoptosis of epithelial cells (64).

Dengue Viruses

The innate immune response to dengue infection is primarily characterised by an IL-8 and type I IFN response activated by TLR3 after the virus is degraded by endosomal acidification (193). Dengue virus also activates the TLR7, TLR8, RIG-I and MDA5 signalling pathways, which signal through type I IFNs (194–196). There is also evidence that dengue virus may signal through TLRs 2 and 4, and that this may contribute to the pathogenesis and extreme inflammation of severe dengue (197). Type I IFN induction is important for a successful immune response to dengue virus (198). Many of the documented immune evasion mechanisms employed by dengue virus involve the inhibition of type I IFN signalling by some method. Dengue virus has been demonstrated to inhibit IFN production by a RIG-I dependent mechanism (199). Inhibition of IFN production contributes to the ability of dengue virus to replicate and spread, and thus to cause severe systemic infections and viral sepsis. Dengue virus can also cause an antibody enhanced form of disease when a patient is exposed to a different serotype of dengue virus than the one with which they were initially infected (70). Enhanced dengue disease involves the same immune responses as the non-enhanced form of disease, with a key difference being the presence of cross-reactive antibodies, which do not neutralise the pathogen but instead allow the virus to replicate within Fc receptor containing cells, resulting in a more severe infection (200).

Immune Suppressive Phase of Viral Sepsis

Figure 1B summarises the aberrant immune responses in viral sepsis. It has become apparent in recent years that the immunosuppression that results from sepsis may contribute more to mortality than the initial hyper-inflammatory response. A more immunosuppressive genotype in patients with sepsis is correlated with increased mortality (201, 202). It has also recently been reported that the majority of deaths in adults [68%] that occur during sepsis from all causes happen on the third day or later (203). 20.4% of these deaths could be attributed to nosocomial infections. Independent predictors of third day or later death were corticosteroid treatment, no identification of the pathogen (203), and age. These risk factors differ in paediatric populations, with most deaths occurring within 48 h of presentation (204, 205). Most deaths in children occurred due to refractory shock (204). This suggests that in adults most sepsis related deaths are not due to an initial overpowering immune response, rather due to an inability to control infections that result in excessive pathogen proliferation and inflammation, whilst children are less able to survive an initial fulminant infection.

The initial inflammatory immune response that characterises viral sepsis is usually followed by a period of immune suppression. This phenomenon is characterised by decreased function in both innate and adaptive immunity, with common features including increased expression of negative co-stimulatory molecules and decreased expression of positive co-stimulatory molecules, T cell exhaustion, apoptosis of effector cells, increased regulatory T cell expression and higher numbers of myeloid derived suppressor cells (206, 207). This can result in increased infections from secondary pathogens, but also the reactivation of dormant infections, and natural microbiota becoming pathogenic (206). In particular, the reactivation of herpesviruses such as CMV and HSV have been found to occur in 33 and 21% of immunocompetent patients with severe infections requiring hospitalisation, respectively (208, 209). Due to the inability of the immune system to adequately control or eradicate these infections they can often result in severe disease (210), tissue damage, and even death. Mortality in sepsis is often caused by serious secondary infection after the initial inflammation has already passed. Immune suppression is a common feature of persistent and serious viral infections (211).

Some viral infections can directly result in immune suppression. Some strains of enterovirus are known to infect leucocytes (212). In particular some strains, such as coxsackievirus B3 and enterovirus 70, that have been implicated as causes of sepsis have shown this capacity (212). Infection of leucocytes in severe viral infections has been shown to result in an immunosuppressed state, as the death of infected leucocytes can result in a diminished ability to prime CD4 and CD8 cells and a reduced ability to control pathogens by phagocytosis. This greatly increases the susceptibility of the host to secondary infections (210). When compounded with other immune suppressive effects of severe infections, such as lymphocyte exhaustion, the ability to handle new infections can become drastically reduced (211). This phenomenon could explain the higher mortality observed in infections of relatively immunosuppressed patients, as well as the high level of mortality observed in sepsis after the initial inflammatory response has passed.

Serious infections that cause prolonged inflammation very often result in immune exhaustion. It was found in *in vivo* experiments that prolonged infections by a large variety of viruses can result in decreased differentiation of immature lymphocytes into CD8 T cells (213). Prolonged viral infections resulted in decreased expression of IL-7Rα, which in turn resulted in decreased numbers of circulating memory CD8 cells. It had also been observed that chronic infection with lymphocytic choriomeningitis virus (LCMV) resulted in poor CD8 T cell responses to cytokine stimulation and poor development of a memory CD8 response (214). A similar effect had been observed in a large number of mouse studies, suggesting that chronic viral infections could impair many aspects of CD8 effector T cell function (215–218).

Chronic viral infections have been found to have differential abilities to induce an immune suppressive state depending on the strain of virus. In a mouse model, high levels of T cell exhaustion have been induced by infections with LCMV, hepatitis B virus, hepatitis C virus, and HIV (217–221). Less pronounced immune exhaustion was produced in mouse CD8 cells by infection with Epstein-Barr virus (EBV), measles virus, and CMV (222–225). Why different chronic infections produce differing degrees of immune suppression is not well-understood, but could be due to novel immune evasion mechanisms, as in the case of HIV (220), the rate and degree of replication of a virus, its preferred replicative niche or its degree of interaction with PRRs. One well-characterised example of virally induced

immunosuppression can be found in measles virus (225). Measles virus has several ways in which it can interact with the immune system and suppress responses. One of these is by interaction with the CD150 receptor to increase the apoptosis of immune effector cells while also decreasing expression of IFN-γ (226). Infection with measles virus has also been shown to decrease the proliferation of lymphocytes for several weeks after the initial infection (227). One study found that measles virus infection may have immunosuppressive effects on the host immune system that can persist for 2–3 years (228). Although the mechanism by which this may occur was not characterised, it was hypothesised that measles virus employs a method of depleting memory B and T cells, resulting in a heightened susceptibility to infection by other pathogens (229). This process is multifactorial and not fully understood. One proposed mechanism is the inhibition of T cell replication by the measles virus proteins H and F_1-F_2 (230). It is possible that other viruses may utilise other immune evasion mechanisms that may result in immunosuppression.

Viral reactivation is a common feature of all forms of sepsis. As excessive inflammation gives way to immune exhaustion some latent infections can take advantage of this more forgiving environment, escape immunological control and replicate. Herpesviruses are most commonly associated with this phenomenon, owing to their ability to become latent for many years (231). Epidemiological studies have found CMV, EBV and HSV to be latent in around 50.4% (232), 66.5% (233), and 53.9% (234) of the non-paediatric population of the United States, respectively, with detection of viral RNA (indicating active replication) in cases of sepsis occurring in 24.2, 53.2, and 14.1% of cases, respectively (235).

Some reactivated viral infections are particularly associated with increased mortality during sepsis. While viral reactivation is associated with higher mortality during sepsis, the contribution of individual viruses to mortality is still controversial. CMV reactivation has been associated with the doubling of mortality rates, which is comparable to the increase in mortality rate associated with latent HIV infection during sepsis (236). This model has been questioned however, and it is possible that the increased mortality attributed to CMV reactivation could be due to the reactivation of multiple viruses simultaneously. One study found no single virus to be significantly associated with higher mortality upon reactivation in sepsis, but found the reactivation of multiple viruses at once to significantly associate with worsened outcome (237). Further research on the contributions of the reactivation of individual virus strains to mortality is required.

It has also been hypothesised that viral reactivation may contribute to a feedback loop within sepsis, wherein the reactivated viruses contribute even further to T cell exhaustion and immunosuppression, resulting in even greater susceptibility to viremia, compounded immune suppression (235) and elevated inflammation. This feedback loop would explain why a high degree of viral reactivation (of all viruses) correlates with increased mortality. It is still unclear whether elevated viral loads of herpesviruses following reactivation indeed impairs lymphocyte function or whether it is just a side effect of other kinds of immune suppression already taking place (238). It has been demonstrated that CMV infection can have an effect on the differentiation of immature lymphocytes into effector CD8 T cells; however, the overall effects this may have on the patient are not well-characterised (239, 240). The state of CD8 differentiation brought about by CMV infection is similar to the differentiation state of CD8 T cells in older adults. This suggests that CMV infection may contribute to immunosuppression, but more research is required to confirm if this effect has a significant effect on mortality during severe infections (240). Immunosuppressive effects have also been observed in EBV infection. EBV infection was found to be associated with reduced antibody responses to vaccines in Gambian infants (241). The mechanism of this is still not yet known. Interestingly CMV infection was not found to be associated with these lowered responses (241). In addition, there is evidence that these reactivated viral infections contribute to inflammation alongside any immunosuppressive effects, resulting in a heightened state of inflammation but without the capacity to resolve it. In particular, HSV and CMV have been associated with inflammatory responses upon reactivation (242, 243). Whether or not this phenomenon contributes significantly to mortality during sepsis is still controversial (235).

Understanding the role viral reactivation plays in the immunosuppressive phase and in the pathology of sepsis may provide avenues to treatment in the future. Possible approaches would be the application of antiviral medications specific to viruses that commonly experience reactivation, or drugs that could prevent immune exhaustion or ameliorate its effects (like inhibitors of negative co-stimulatory molecules discussed below). An improved understanding of the effects of viral reactivation is vital to expanding our understanding of sepsis and will contribute to better categorisation of illness and application of more appropriate treatments.

IS VIRAL SEPSIS DIFFERENT FROM BACTERIAL SEPSIS?

The diagnosis of viral sepsis can be useful to inform treatment in cases where antiviral medications are available and suitable; however, immunological data are scarce on viral sepsis. It cannot be said with any certainty if viral sepsis is meaningfully different from bacterial sepsis. Viral sepsis is only understood insofar as immune responses involved in severe viral infections are understood. The causes and character of sepsis can be highly heterogeneous (206). While knowledge of the causative pathogen provides with treatment options against that pathogen and against associated immune responses, sepsis and subtypes thereof are not characterised well enough for immune response-based therapies to proliferate and enter the mainstream. An important next step in the understanding of sepsis will be the characterisation and grouping of sepsis cases according to some criteria that may inform treatment, and the discovery of cheap and effective biomarkers which would allow these criteria to be defined.

Studies have been conducted which have aimed to develop methods to discriminate between viral and bacterial infections

(244–246). Some of these have analysed transcriptomics to identify gene signatures that can differentiate between viral and bacterial infections (244–246). Some genes identified in these studies include genes downstream of the IFN signalling pathways such as IFN-stimulated gene 15 (245) and IFN-α-inducible protein 27 (244) as well as cytokines such as IL-16 (245). The roles these genes play in viral infection is not yet known; however, that information is not required for their use as biomarkers, and as such they may have clinical utility regardless. The results of these studies may inform future research to identify biomarkers which can be used in a clinical setting to quickly differentiate between bacterial and viral infections. Applications of these methods to cases of sepsis may help us develop an understanding of how sepsis differs for differing aetiologies. Transcriptomic studies in sepsis have been performed before as discussed above (201, 202), but more focused studies aiming to understand the pathogen's role in the character of the disease will be essential to future sepsis research and providing an answer to the mysteries of viral sepsis. It may prove difficult to recruit sufficient sample sizes for a highly powered transcriptomics study in viral sepsis due to its underdiagnosis.

TREATMENTS FOR VIRAL INFECTIONS AND SEPSIS

Up to 42% of all cases of sepsis are culture negative, suggesting a possible non-bacterial cause of infection (21), if appropriate tests have been performed. Despite this, however, the preferred treatment of sepsis in all cases is the early administration of broad-spectrum antibiotics. The survival rate of patients presenting with septic shock decreases by an average of 7.6% for every hour that antimicrobials are not applied (247), with time to application of antimicrobial therapy being the single greatest indicator of outcome in the multivariate analysis performed in one study (247). However, the administration of antibiotics will not be effective in the case of viral sepsis and can be associated with adverse effects. Understanding a potential viral cause of the disease increases the possible treatment options, opening the possibility of using broad-spectrum antiviral medications, but also to treatments built on an understanding of both sepsis, and how the immune response to pathogens may contribute to it.

Prospective treatments for specific viruses implicated in sepsis are being developed. Pleconaril is an antiviral against enteroviral infection which inhibits viral attachment to the hosts cell receptors and prevents uncoating of the viral nucleic acids. There are data suggesting that the drug would be effective and safe in neonatal virally induced sepsis (248). The drug recently completed a small phase 2 clinical trial (248); however, it is no longer under development and is not available, even for compassionate use. The benefits of more effective antiviral medicines are clear. They could help both in cases of severe viral infections and sepsis while also providing treatment for more benign infections. It could prove useful to use such drugs alongside antibiotics in sepsis cases, to allow for the possibility of a viral cause, although this would also open the possibility of the development of viral resistance. There are many specific antiviral drugs that have been developed and gone through trials; however, none have yet been tested specifically for sepsis. Examples that may be beneficial in presentations of sepsis in certain situations include acyclovir, which has been proven effective in HSV infections (249), amantadine, rimantadine, oseltamivir, and zanamivir for influenza (250, 251), and more broad-spectrum antiviral drugs like ribavirin and favipiravir (250).

Antiviral medicines may also have a role in the treatment of viral reactivation, which may improve outcomes even in non-virally induced sepsis. Ganciclovir has been demonstrated to measurably decrease CMV reactivation in mice (248); however, human trials did not show any significant decrease in CMV-induced inflammatory cytokine levels (252). There have been numerous other drugs developed against CMV (253) which could be effective in preventing viral reactivation.

With the advent of research into personalised medicine, the idea of treating the host immune response in sepsis has become popular. By understanding the host response to pathogens and modifying it we may prevent serious infections that can result in sepsis or sepsis-like-illness. One common strategy of treating the host response is the use of immunomodulatory molecules to prevent harmful excessive inflammation in infections. Immunomodulation in sepsis aims to decrease the harmful effects of excessive inflammation by altering or counteracting the effects of inflammatory mediator molecules (254), such as TNF-α (255) or by using broad anti-inflammatory molecules, such as corticosteroids (256). Most trials for immunomodulatory drugs, however, have failed (257). This approach, has fallen out of favour in recent years as it either proves ineffective [e.g., dengue (258, 259)] or in some cases has the opposite of the desired effect and dampen the immune system in such a way as to allow the pathogen freedom to replicate and proliferate into an uncontrolled infection that causes greater harm [e.g., corticosteroids in influenza (260, 261)]. However, adjunctive corticosteroid therapy may be beneficial and can be considered in patients with varicella zoster virus encephalitis (262, 263) or HSV encephalitis (264).

In recent years, an immunostimulatory approach to immunotherapy against sepsis has become much more popular (265). This approach aims to promote rapid pathogen clearance, decreasing the chance for it to proliferate and cause a more severe infection (265). One proposed method of doing this is selective application of immunostimulatory cytokines such as IL-7 and granulocyte-macrophage colony-stimulating factor (GM-CSF) which some studies have shown to contribute to more effective viral clearance (266, 267). A recent study demonstrated that the majority of deaths which occur due to sepsis occur on the third day or later (203), after immunosuppression has taken hold, suggests an immunostimulatory approach may prove to be beneficial.

In order for the treatment of the immune response to work as a meaningful way to decrease overall mortality we must better understand how the immune response to infections contributes to the development of sepsis. To this end, the development of biomarkers that could determine the likelihood of an infection becoming harmful due to a lack of ability to clear an infection,

or indeed whether the initial immune response will be excessive and harmful, would make these relatively brute force treatments much more effective by timing and targeting their application. An understanding of the excessive inflammatory response to pathogens will allow us to better categorise sepsis states, and group them according to treatability. Some strides forward have been made in this field. A 2016 study (201) identified two distinct host response signatures to sepsis by unsupervised hierarchical clustering, one of which significantly associated with higher 14-day mortality. While this study only analysed bacterial sepsis, the methodology would be extremely useful in expanding our understanding of viral sepsis. The identified signature was characterised by relative immunosuppression as well as increased tolerance to endotoxins, T lymphocyte exhaustion and metabolic dysfunction. It was hypothesised that these individuals experienced more severe disease due to an inability to control infections, resulting in increased pathogen replication. These individuals would also be more susceptible to secondary infection. This study, however, only examined sepsis in adult patients with community acquired pneumonia, which is not necessarily informative of paediatric populations with immature immune systems, other immunocompromised groups or viral infections. A similar study focusing primarily on viral sepsis would be invaluable to the understanding of viral sepsis and would open numerous new avenues for prospective treatments. To be effective in a clinical setting this approach will require the development of rapid transcriptomic analysis methods to be clinically useful in a case of a patient acutely presenting with sepsis. Another study aiming to identify a predictive genomic signature had similar results (202), grouping cases of sepsis using a hierarchical clustering method into two distinct subgroups, one of which correlated with a higher rate of mortality, and was characterised by a more immunosuppressed phenotype. Genes involved in the function of lymphocytes were suppressed, despite the relatively high lymphocyte counts in patients in this group. This study had similar limitations to the other study mentioned above.

This knowledge could potentially be used to inform treatment and the development of immunotherapeutics. It was suggested that patients presenting with this immunosuppressed gene expression signature may benefit from drugs that modulate aspects of the immune response, for example treatment with various cytokines (such as IFN-γ, IL-7, or IL-15) or blockade of receptors that can induce cell death in T lymphocytes (201), such as programmed death 1 (PD-1).

The PD-1 ligand is a promising target for the treatment of viral sepsis. PD-1 has been implicated in the development of the immunosuppressive phase of sepsis by inducing the apoptosis of effector T cells. Continued elevation of PD-1 expression in septic patients has been found to correlate highly with patient mortality (238). PD-1 has been implicated in the pathogenesis of highly pathogenic influenza infections. In more severe Influenza infections PD-1 was expressed at a higher level, while blocking it led to increased CD8+ numbers and reduced viral titres *in vivo* (268). Blocking of PD-1 may allow for vastly improved clearance of serious viral infections, preventing the patient from becoming septic. PD-1 blockade treatment may also be useful in the immunosuppressive phase of sepsis by maintaining the competence of the immune system to clear secondary infections and thus decreasing overall mortality (269–271).

One common feature of sepsis that has emerged as a promising target for treatments is the dysregulation of endothelial barriers (272). The endothelial barrier is a continuous layer coating the vascular system which separates the fluid from the tissue compartments. The barrier is important in maintaining bodily homeostasis, regulating the passage of gases, liquids, proteins, cells and micro-organisms, among other things from the blood into the tissues. This dysregulation has been found to be central to the pathology of sepsis (273). In states of septic shock the tight junctions between cells become disrupted due to platelets and neutrophils adhering to the endothelial wall, the release of inflammatory and toxic mediators by these cells, and an increased expression of binding molecules like selectins and integrins, which allow leucocytes to bind to the endothelial layer, and then migrate through it (274). The process of dysregulation also compounds any damage to the endothelial barrier by increasing the level of inflammatory mediators at its surface. This leads to fluid leaking into interstitial tissues, and the recruitment of macrophages and other inflammatory cells to tissues they cannot normally access resulting in tissue damage (272). While these responses generally allow immune effector cells to reach sites of localised infection or damage, during the dysregulation and move away from homeostasis that occurs in sepsis, they can allow for considerable damage to be done to tissues.

This phenomenon has been found to also occur in serious viral infections, and also plays a major role in the pathology of viral sepsis in these cases (275). Viruses such as hantavirus, dengue viruses, and HSV have presentations that suggest a role for the endothelial barrier in the pathogenesis of serious disease (276). There is a lack of data on the relevance of endothelial leaking in most other viral infections; however, there is evidence that it is involved in the pathogenesis of avian influenza A (H5N1) virus, which produces a cytokine storm effect (277), and is known to increase vascular permeability and immune cell infiltration into the tissues.

Therefore, endothelial barrier dysfunction presents an attractive target for the treatment of severe viral infections and sepsis. There are several drugs which are known to help preserve endothelial integrity which may be of use in treating severe viral infections or sepsis. These include common medications such as statins and angiotensin receptor blockers (278), which have proven benefits in both sepsis, influenza and other critical illnesses. They are thought to work by maintaining or restoring endothelial barrier integrity (278) and could be promising treatments of viral infections known to disrupt endothelial integrity. These drugs have had some success in treating severe viral infections such as Ebola and influenza (279–281) suggesting their use in viral sepsis is a promising area for future research. One trial aimed to control the severity of sepsis using the drug "atorvastatin." The trial, while aborted due to subpar recruitment, demonstrated interesting results. It was calculated that assuming the drug would bring about a 15% reduction in cases of progression to sepsis, 414 patients would be required

to achieve statistical significance. While only a quarter of this number were recruited, an 83% decrease in progression to sepsis (from all causes) was observed, far exceeding expectations (282). However, due to the insufficient sample size, the result was insignificant (282). With the addition of more data these results would suggest that statins may be a particularly promising route for further research particularly in cases of viruses known to interfere with endothelial barrier function.

CONCLUSIONS

Viral sepsis is a continually underdiagnosed and heterogeneous form of sepsis that can be caused by a wide variety of viruses. The most common of these pathogens are HSV, enteroviruses, HPeVs, influenza, and dengue viruses. Some populations are at a much higher risk of viral sepsis than others for many reasons. The populations at the highest risk are young children, pregnant women, older adults, and immunosuppressed individuals. This heightened risk and severity is due to relative immunosuppression present in these populations. The viruses that most often cause viral sepsis tend to have the capacity to evade killing by the immune system while still inducing powerful inflammatory responses, often characterised by high levels of TNF-α and IL-6 expression alongside low IFN-γ expression that can damage the host. The prolonged inflammation that can be brought on by these infections can then result in an immunosuppressed state, further reducing the body's capacity to clear infections, and drastically increasing the risk of death from the original viral infection, a newly acquired infection or a reactivated infection. Understanding the viral cause of sepsis and the immune responses to common viral infections could lead to improved treatment of sepsis by use of specific antiviral medications. In the future it may be possible to apply immunotherapies built around the understanding of the specifics of viral infections to either aid in viral clearance or reduce harm from viral infections. Sepsis from differing causes seems to differ little in its clinical presentation; however, use of modern transcriptomic methods is demonstrating that there are meaningful differences in immune responses that may be used to distinguish between viral and bacterial sepsis, which may aid in the development of future immunomodulatory drugs.

AUTHOR CONTRIBUTIONS

AP, G-LL, and JM conceived the topic and scope of this review. G-LL and JM drafted the first version of the manuscript. G-LL compiled the table. JM designed the figure. AP and SD provided critical revision of the manuscript. All authors made significant editorial contributions, read and approved the submitted version of the article.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Immune Functional Assays, From Custom to Standardized Tests for Precision Medicine

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The immune response is a dynamic system that maintains the integrity of the body, and more specifically fight against infections. However, an unbalanced host immune response is highlighted in many diseases. Exacerbated responses lead to autoimmune and allergic diseases, whereas, low or inefficient responses favor opportunistic infections and viral reactivations. Conflicting situations may also occur, such as in sepsis where inflammation and compensatory immunosuppression make it difficult to deploy the appropriate drug treatment. Until the current day, assessing the immune profile of patients remains a challenge. This is especially due to the inter-individual variability—a key feature of the immune system—which hinders precise diagnosis, prognosis, and therapeutic stratification. Our incapacity to practically interpret the host response may contribute to a high morbidity and mortality, such as the annual 6 million worldwide deaths in sepsis alone. Therefore, there is a high and increasing demand to assess patient immune function in routine clinical practice, currently met by Immune Functional Assays. Immune Functional Assays (IFA) hold a plethora of potentials that include the precise diagnosis of infections, as well as prediction of secondary and latent infections. Current available products are devoted to indirect pathogen detection such as Mycobacteria tuberculosis interferon gamma release assays (IGRA). In addition, identifying the status and the underlying factors of immune dysfunction (e.g., in septic patients) may guide immune targeted therapies. Tools to monitor and stratify the immune status are currently being studied but they still have many limitations such as technical standardization, biomarkers relevance, systematic interpretation and need to be simplified, in order to set the boundaries of "healthy," "ill," and "critically ill" responses. Thus, the design of new tools that give a comprehensive insight into the immune functionality, at the bedside, and in a timely manner represents a leap toward immunoprofiling of patients.

Keywords: immune functional assay, host response, immune monitoring, IGRA, stimulation, critically-ill patients, immunoprofiling, sepsis

INTRODUCTION

The immune system plays a key role in protecting our body from internal and external threats, contributing to the maintenance of homeostasis. This explains why it is involved is many diseases, being the lead cause, or contributing to their pathophysiology. Excessive or insufficient responses, inborn or acquired, may therefore lead to chronic inflammatory diseases, allergy, or immune deficiencies and increased infectious risk (1). In sepsis, defined as dysregulated host response to infection, the immune system also plays a key role. Our current understanding underline that both pro- and anti-inflammatory responses are involved in a complex and dynamic process, which may lead to organ failures and secondary infections, both contributing to a high morbi-mortality (2). The ability to closely monitor the immune status is thus a critical unmet medical need, which may help stratify patients for personalized care.

However, monitoring the immune system is complex. First, assessing such system relies on a precise knowledge of its components and functions. Innate and adaptive arms of the immune system are both composed of several cell types and humoral components, that act together to maintain immune homeostasis. Counting cells, measuring soluble or cell surface biomarkers, are several options to routinely assess the system (1). However, as many tasks of the immune systems are performed through complex interactions between its components, these routine assays also have limitations and may miss key alterations. Such functional assessment is better performed through Immune Functional Assays (IFA) (3).

Immune Functional Assays are assays that record a response to a given stimulation. Various assays have been developed to better describe or understand the immune system, as well as to monitor diseases in which the immune system is involved. These assays, their advantages and drawbacks, with a special emphasis on their use in sepsis, are the focus of the present review. The development and use of IFA came along with the study of the immune system. Since the times of Edward Jenner, the conception of IFA started when he injected pathogen extracts subcutaneously to assess the humoral response to immunization against smallpox (4). Similarly, Koch and Mantoux noticed that the subcutaneous injection of tuberculin lead to a strong skin reaction in patients with active tuberculosis (TB), and invented the first IFA to diagnose infections (5). Later on, immunologists developed several IFA to assess the immune system and decrypt primary immune deficiencies (PID). Tests such as lymphocyte proliferation or complement assays are still used routinely in the first steps of PID diagnosis (6).

Immune Functional Assays to Diagnose and Manage Infections

In tuberculosis, *Mycobacterium tuberculosis* cannot be cultured by classical microbiological techniques so IFA are used to detect a recent contact with the pathogen, and help in the diagnosis of an infection where the causal agent is difficult to isolate and cultivate. Tuberculosis is among the top 10 causes of death worldwide (7). Active TB accounts for 5–10% of the cases and is suspected when the symptoms manifest such as a severe cough that lasts 3 weeks or longer, pain in the chest and coughing up sputum or blood. Active TB is well managed with antibiotics when detected in an early stage. However, latent tuberculosis infection (LTBI, which presents 90–95% of the cases) lurking in the host is asymptomatic and has a high probability of being activated in a hampered immune system. The ability to detect latent TB is therefore critical in situations where patient's management implies iatrogenic immune-suppression such as chemotherapy, transplantation, or chronic inflammatory diseases (8).

Classical tuberculin skin test (TST) has been the only practical mean to diagnose TB over the last century. TST measures the T-lymphocyte response to the intradermal injection of purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Mtb). Positivity to this test is possible from 4 to 12 weeks after the infection and results are obtained in 48 to 72 h after carrying out the test. This test has many limitations as it needs an *in vivo* intradermal injection by trained staff, requires two visits to obtain the results, and its interpretation remains subjective, making assessment difficult. Moreover, its low specificity and sensitivity makes this test barely reliable for active and latent TB diagnosis (8, 9).

In the last few years, T-cells blood based assays have been developed to offer new and more precise diagnostic tools. Interferon-Gamma Release Assays (IGRAs) measure a person's immune reactivity to Mtb (10). IGRA tests have revolutionized the detection of TB as being the first standardized and accurate test currently commercialized. The progress of genomic analysis in mycobacterium including Mtb allowed to find Mtb-specific antigens located into the RD-1 region, early secreted antigenic target (ESAT-6) and culture filtrate protein (CFP-10), which induce strong interferon-gamma (IFN-γ) release from sensitized T cells, signaling an ongoing infection. Since ESAT-6 and CFP-10 are absent from all Bacillus Calmette–Guérin (BCG) substrains (RD-1 region is deleted) and most of non-tuberculous mycobacterium (NTB), these diagnostic tests are not confounded with BCG vaccination and infection with the majority of NTB (11).

To conduct IGRA test, fresh blood samples are mixed with specific Mtb-antigens and controls, and a specific response is detected through the quantification of IFN-γ release. Currently, there are two FDA-approved commercially available tests: QuantiFERON-TB Gold (QFT) (Qiagen) and T-SPOT.TB (Oxford Immunotec). QFT test uses a peptide cocktail targeting Mtb proteins to stimulate cells in heparinized whole blood. Detection of IFN-γ by enzyme-linked immunosorbent assay (ELISA) is used to identify *in vitro* responses to these Mtb-associated peptides. T-SPOT.TB is a peripheral blood mononuclear cell (PBMC)-based assay which quantifies the number of IFN-γ secreting lymphocytes by ELISpot technique (**Figure 1**). The advantages of both tests over TST are that they only require a single patient visit to conduct the test, results can be available within 48–72 h. Excellent specificity has been described for latent TB [against controls, (90–91%)], without false-positive in BCG-vaccinated subjects, and a limited number of false-positive due to Non-Tuberculous mycobacterium/Mycobacterium other than Tuberculosis

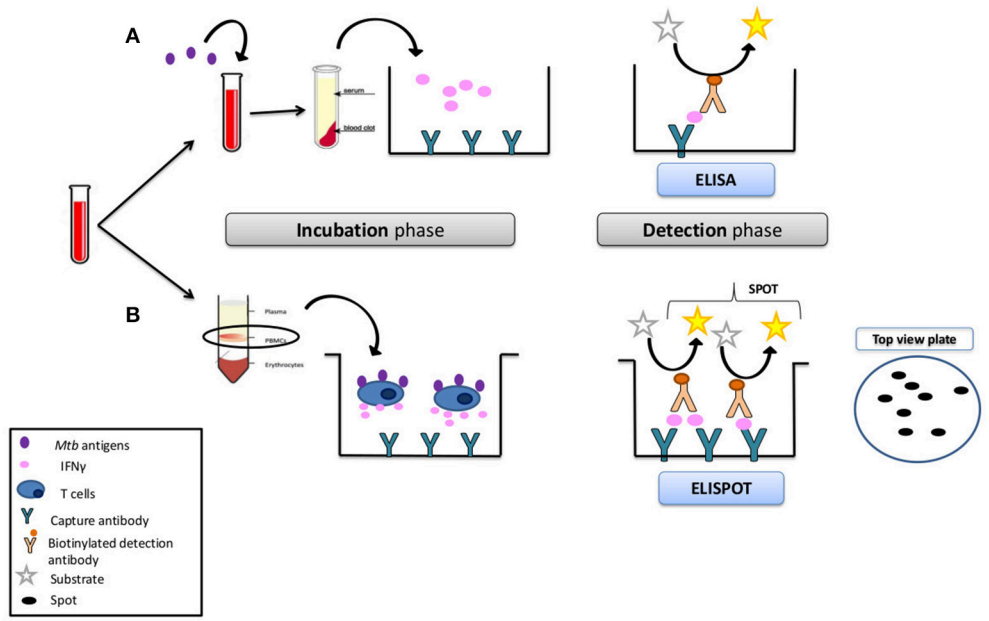

FIGURE 1 | Interferon-gamma-based *in vitro* assays (IGRA). **(A)** Mycobacterium tuberculosis IFN-γ-release assay. In the ELISA method (QuantiFERON®-TB Gold In-Tube Test; Quest Diagnostics, USA), whole blood is stimulated with *M. tuberculosis* antigens, and the amount of IFN-γ secreted into the supernatant is quantified by ELISA. **(B)** In the ELISPOT method (T-SPOT.TB; Oxford Immunotec, UK), PBMCs are prepared by density gradient centrifugation (Ficoll method). A defined number of cells is then stimulated with *M. tuberculosis* antigens for 24 h on plates coated with anti-IFN-γ antibodies. Antigen-responsive cells secrete IFN-γ, which binds to these antibodies. After removal of the cells, antigens are detected by a second labeled anti-IFN-γ antibody. The number of spots on the plate corresponds to the number of IFN-γ+ cells in the sample.

(NTM/MOTT). IGRA test can help determine the full efficacy of BCG vaccine, which can have key implications for its use in current immunization programs as well as in the future development of new improved tuberculosis vaccines (12). Among the disadvantages of TB IGRA is the poor sensitivity to latent TB, poor reproducibility, high number of indeterminate results, high cost, and the inability to discriminate between latent and active TB.

Identification and treatment (i.e., preventive therapy or prophylaxis) of LTBI can substantially reduce the risk of active disease development (by as much as 60%), and is an effective TB control strategy (13). Promising work on new antigens such as mycobacterial Heparin-Binding Haemagglutinin Adhesin (HBHA) antigen might help to improve the ability of IGRA to discriminate between latent and active TB, and identify populations that have dormant TB with a high reactivation potential (14).

Immune functional assays might also be interesting in the management of other infectious diseases. **Table 1** lists various pathogens for which IGRA tests have been considered as an option. Human cytomegalovirus (CMV) and Chagas disease are two other examples illustrating how IGRA IFA could help in patient management. In organ transplantation, the risk of CMV reactivation in patients is high and can be well managed with the administration of prophylactic treatment while viral reactivation is easily monitored through specific PCR assays. However, the ability to precisely define the right timing to stop such prophylaxis is pending. Immune Functional Assay could help there by demonstrating patients' recovery and the ability to control CMV replication and fight infection. The main immune response against CMV is cell-mediated, with specific $CD8^+$ T cells that produce IFN-γ against CMV. These cells are critical to eliminate viremia from blood. Similarly to TB, IFA tools were developed to quantify IFN-γ and assess the CMV-cell mediated immunity. (e.g., T-Track® CMV kit ELISPOT or QuantiFERON-CMV®). These assays are able to predict risk of developing CMV infection after prophylaxis and can aid in the decision to initiate, delay or discontinue antiviral therapy (15, 16).

Immune Functional Assays for Immune Monitoring

Since IFA directly measures *ex-vivo* the capacity of a cell population to respond to an immune challenge, functional testing theoretically represents the best way to monitor immune functions. Although widely used in the research setting, only a few IFA are available routinely in the clinical practice. Most developments have been made along the study of primary immune deficiencies, allergy, and transplantation. However, the rise of immunotherapies in cancer, and the potential applications in sepsis have given these assays a new momentum.

Primary Immune Deficiencies (PID) encompass at least 300 single gene inborn errors, associated to a wide range of phenotypes as diverse as increased risk of infection or malignancies, allergy, or inflammatory/auto-immune diseases (17). The study and characterization of PID has been instrumental in understanding how the immune system works.

TABLE 1 | Immune functional assay potentials in identification of latent and/or active infections, monitoring of therapy or vaccination success, and risk stratification for high risk groups.

Disease	Aim of the functional test	Target to be explored	Action based of the test
Tuberculosis	Detection of latent infection	Immune memory	Administer prophylaxis
CMV, Epstein-Barr Virus (EBV)	Prognosis of viral reactivation in organ transplant (Risk stratification)	Specific immune competence	Continue or discontinue prophylaxis
Chagas	Monitoring the efficacy of treatment	Immune system activity	Stop treatment
Lyme disease (*Borrelia*)	Diagnosis/ monitoring therapy	Specific immune competence	Adapt therapy accordingly
HIV	Prediction of development of active infection	Specific immune competence	Administer prophylaxis

These studies and the precise diagnosis of PID rely on various IFA that allow the precise characterization of immune defects. Indeed, the clinical and immunological heterogeneity in PID makes diagnosis challenging, while an early and accurate diagnosis facilitates prompt management (18).

Lymphocyte proliferation (also known as lymphocyte transformation test, LTT) is routinely used in clinical immunology labs to assess lymphocyte function. The evaluation of lymphocyte proliferative response is routinely performed by the measurement of tritiated thymidine uptake after stimulation with mitogens or recall antigens (19, 20). For example, children with unusual infections or unusually severe course of infection, that fail to thrive from early infancy (intractable diarrhea, severe eczema), or with recurrent infections with the same type of pathogen should be explored through a protocol that comprise LTT (20). Lymphocyte proliferation assays are also of particular interest when cell counts are normal, such as in Functional T Cell immunodeficiencies (21). However, even if alternatives exist to avoid the use of radioactivity (22), such tests remain cumbersome and difficult to implement.

Chronic Granulomatous disease (CGD) is a relatively rare PID with an incidence of ∼1 in 200,000–250,000 individuals characterized by genetic defects in the oxidative burst pathway (NADPH oxidase complexes) that is linked with phagocytosis in myeloid cells, such as neutrophils. Clinically, CGD is characterized by recurrent or persistent bacterial and fungal infections in addition to granuloma formation. Flow cytometric analysis to evaluate NADPH oxidase activity (oxidative burst) are performed using dihydrorhodamine (DHR) 1, 2, 3 as a fluorescent marker of hydrogen peroxide generation before and after stimulation of neutrophils with phorbol myristate acetate (PMA). This is a relatively rapid and highly sensitive assay that allows the use of whole blood without purification of neutrophils, and tends to replace nitroblue tetrazolium test or chemoluminescence tests (23).

The second field where IFA are routinely used in clinical practice is allergy. A typical exacerbated response triggered by food and/or environmental factors is observed in allergy. The gold standard in the field is based on skin prick testing to confirm sensitization in IgE-mediated allergy (24). The recommended method of prick testing includes the appropriate use of specific allergen extracts, positive and negative controls, interpretation of the test after 15–20 min of application, finally a positive result is defined as a wheal of $\geq$3 mm diameter (25). These tests measure sensitization not the clinical allergy which can be influenced by other factors, interpretation can be liable to over- or under-diagnosis. When there is a confrontation with a doubtful response or limitations to carry out these tests on subjects, the basophil activation test (BAT) is performed. BAT is a flow cytometry based *in vitro* assay that evaluates the expression of activation markers on the surface of basophils after being stimulated with the allergen (26).

The precise monitoring of immunosuppression is also key to ensure long-term viability of solid organ allografts without increasing risk of infection. Monitoring of this dual risks of rejection and infection through immune functional assays could help assess the immune function of the transplant recipient and individualize the immunosuppressive therapy.

Currently, after solid organ transplant or hematopoietic cell transplantation (HCT), levels of immunosuppression are determined by assessing clinical toxicity (e.g., leukopenia, renal failure) and by therapeutic drug monitoring (TDM) when available. However, drug levels are a poor surrogate of the immune status, and may vary a lot among individuals. The main value of TDM is the avoidance of toxic levels. There are currently two IFA available to assess the immune function in transplant patients.

ImmunKnow (Cylex, Inc., Columbia, MD, USA) has been developed to assess the risk of infection and prediction of organ transplant rejection (15). The assay measures intracellular ATP produced by purified $CD4^+$ T lymphocytes after *in vitro* whole blood incubation with phytohaemagglutinin (PHA). The samples are incubated for 15-18 h (with or without PHA) and the production of ATP after stimulation is compared to the basal ATP level. Several studies have reported correlation between lower levels of ATP and a higher risk of infection, while increased production of ATP seems to be associated with rejection. The latter could be used as a tool to determine the threshold of immunosuppression and as an indicator of increased risk of infection or rejection (3), such thresholds are hard to determine due to heterogeneity in the studies with various settings and designs.

Pleximmune™ assesses the activity of T-cytotoxic memory cells through the expression of an inflammatory/activation marker: CD154. The expression of CD154 on patient's cells is compared to the basal level of expression on third party cells, an increased ratio being in favor of an acute rejection. This is not an IFA *per se*, as no stimulation step is performed, but rather a surrogate marker of the activity of T-cytotoxic memory cells (27).

In Hematopoietic Stem Cell Transplants (HSCT), recipients exhibit a profound immunosuppression with an increased risk of infection with most pathogens, followed by a gradual recovery. Infections are the most frequent complications after HSCT and have therefore a huge impact on recipient's outcome. Many of these infections are vaccine-preventable infections but no

clear vaccination schedule has been established so far and vaccination paradoxically occurs after the highest risk period. In such case, lymphocyte count is not a good indicator as it does not correlate to the vaccine response. IFA may help evaluate the reconstitution of T-lymphocyte pool and immune function recovery after chemo-therapy induced aplasia. Indeed, being able to determine the earliest time for immune responsiveness could dramatically change the prognosis of these patients. The vaccine response in HSCT patients can be measured using conjugate pneumococcal or varicella zoster virus (VZV) vaccine as mitogens (28, 29). In CEREDIH (France) which belongs to the European network RITA (Rare Immunodeficiencies auto-inflammatory and autoimmune diseases network), patients are evaluated for the number of lymphocytes in blood circulation and if those reach a threshold, an *ex vivo* test with PHA is performed. A positive response, i.e., proliferation is observed, allows the patient to be vaccinated. One month after the 3rd booster dose, immune cells are again tested in *ex vivo* condition, this time against recall antigens. A positive response indicates that T cells have recovered and are fully functional.

IFA could be a potential asset in management of sepsis which accounts for 31.5 million cases per year with 6 million deaths and 3 million suffering from frequent hospital re-admission post-sepsis, and long-term morbidity (30, 31). The current definition of sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection (2). Although infection is the initial trigger of the response, the dysregulated immune response remains even after the successful treatment of the infection (32). Sepsis patients develop an early hyper-inflammatory response where a cytokine storm is associated to early death and organ dysfunctions. Simultaneously, an anti-inflammatory response tries to compensate causing the patient to plunge in an immunosuppressive phase, thus increasing susceptibility to secondary infections and viral reactivation (33). These responses are very dynamic and vary from one patient to another. The choice of the right treatment can be daunting as the immune status remains hard to predict. IFA could provide useful information on the septic patients' immune status. Being able to assess the immune status at a given point during sepsis time course could be instrumental in reducing morbi-mortality associated to sepsis. What makes IFA superior is the ability to monitor the dynamics of sepsis and possibly stratify patients rather than the static readout in available traditional tests.

The complexity of the sepsis phenotypes are observed not only at the whole-organism level (disease course) but also at the molecular level. It remains unclear which mechanisms drive sepsis-associated pathology and which are secondary disturbances. This is why it is important to understand the biochemical and immunological profile of every patient to help in the dissection of every septic setting (32). A plausible approach is risk stratification to monitor this disease-population. High-risk patients may benefit from earlier clinical interventions, whereas low-risk patients may recover without unnecessary intervention (34).

Researchers have done extensive studies to understand the immune alterations that occur during sepsis providing insights on the valuable biomarkers that can be employed in immunoprofilling of sepsis patients. A range of pro-inflammatory cyto- and chemokines are secreted, and relevant genes are upregulated as an alert state to recruit immune cells, complement, and coagulation systems, endothelial and epithelial cell responses.

Some alterations are observed on the markers expressed on the cell surface such as the decrease of HLA-DR on monocytes, increase of CD64 on neutrophils upon activation, modulation of PD-1 on lymphocytes and other cell markers that can be measured by FACS to help guide patients' management (35). Many of the released proteins can be used as markers to identify the early onset of sepsis and to stratify patients at risk of organ failure caused by the overwhelming inflammatory host-response. Such markers include IL-6, IL-8, procalcitonin (PCT), C-reactive protein (CRP), pentraxin-3 (PTX3), and many others (36, 37).

Nonetheless, in efforts to understand sepsis pathophysiology, it was hypothesized by Boomer et al. that a profound immunosuppression can occur upon sepsis onset and can persist even after the acute hyper-inflammatory phase (38). They conducted a study on the spleen and lungs of septic patients declared clinically dead where they identified changes in the cytokine profile compared to non-septic controls after stimulation with a mitogen. It was observed that both the pro- and anti-inflammatory cytokines production of TNF, IL-6, IL-10, and IFN-γ were impaired at 5 h post-stimulation. Moreover, T-cells expressed higher PD-1, TIM-3, and LAG-3, and a lower expression of CD127 and CD62L, all identified as exhaustion-related markers. The ability to reach levels similar to controls after 22 h of culture in some patients suggest that these alterations might be reversible, and immune recovery possible (38).

The current markers that address the immunocompromised state are the lymphocyte count and human leukocyte antigen-D related (HLA-DR) expression on monocytes measured by flow cytometry (39). Reduced monocyte HLA-DR was found associated to acquisition of nosocomial infections (40), as were elevated regulatory T cells and diminished neutrophil CD88 expression (41). These markers were assessed as stratification tools for immune therapies such as the effect of GM-CSF or rIL-7 on sepsis-induced immunosuppression restoration (42, 43).

A potential gold standard assay to diagnose the immunosuppression is the *ex vivo* stimulation of patients' PBMC with lipopolysaccharide (LPS), measuring TNF-α release with ELISA technique (44). Immunosuppressed patients tend to secrete less TNF-α than healthy subjects, reminiscent of the endotoxin tolerance model, and confirms their immune dysfunction. Limitations reside in the lack of current standardization of such assay, the inter-subject variability in the response to LPS (45), and in the within-individual compartmentalization of tolerance to endotoxin (46).

Neutrophils, key effector cells in clearance of bacteria and fungi infections, can also be assessed by IFA. Indeed, one of the sepsis hallmark is the acquired neutrophil dysfunction that is common during critical illness (47). Phagocytosis, apoptosis,

chemotaxis, and oxidative burst are among the neutrophil's defense weapons that are impaired in sepsis setting. Phagocytosis assays (48) are used to assess neutrophils' capacity to clear pathogens; from recognition, engulfment, to intracellular killing. Cells are exposed to *ex vivo* zymosan particles (derived from Saccharomyces cerevisiae cell wall) and incubated for 30–60 min. Phagocytic capacity is determined with light microscopy by the percentage of neutrophils having ingested 2 or more particles (49). Headway to standardize these assays, BD Bioscience-Europe launched PhagotestTM (CE/IVD) which allows the quantitative determination of granulocytes and monocyte phagocytosis in heparinized whole blood. It works with fluorescein (FITC)-labeled opsonized *E. coli* bacteria and determination is performed with flow cytometry. pH sensitive probes also exist in the market (pHrodo® dye) to evaluate neutrophils phagocytosis directly on whole blood, and therefore avoid the variability introduced by sample preparation. Morton et al. tested P4 peptide activity to evaluate severe sepsis patient neutrophils' capacity to engulf and kill bacteria after *ex vivo* stimulation. The increased neutrophil functions observed after incubation with P4 peptide could be determinant in the potential use of augmented passive immunotherapy for patients with severe infection (50). Phagocytosis of neutrophils may be conserved in some sepsis patient while other neutrophil functions may be affected, making these assays insufficient to determine neutrophil impairment. Patients can have adequate phagocytic activity with severely reduced oxidative burst activity, for that, PhagoBurst (BurstTest) from Allele Biotechne, is intended to investigate the altered oxidative burst but also to evaluate the effects of drugs. Cells are incubated with stimuli to promote oxidative burst, and intracellular fluorescence is measured to characterize leukocyte burst activity. Such assays could be of value to stratify septic patients for therapies targeting the innate system such as GM-CSF, and follow-up of the patient's response (51).

Extensive studies were done to monitor the real time changes of the cytokine profile after *ex vivo* stimulation in ICU patients. Antonakas *et al.* compared survivors and non-survivors groups at several time points: 24 h within the first organ dysfunction followed by day 3, 7, and 10. On day 3, PBMCs were stimulated with LPS and defective levels of TNF-α were detected that persisted till day 10. Day 3 recorded the lowest levels in all three cytokines TNF, IL6, and IL8, and they remained low until day 7 compared to the prior time points, this profile was characteristic of the non-survivors' profile (52). A step toward standardization was demonstrated by Monneret *et al.* showing that monocyte anergy could be identified in septic patients by quantifying intra-cellular TNF with flow cytometry following a simple no-wash, no-centrifuge workflow (53).

On the genomic and transcriptomic levels many teams have focused their efforts on identifying signatures that are able to identify immunosuppressed patients at high risk and discover prognosis markers such as the sepsis response signature (SRS1 and SRS2) (54), and the Molecular Diagnosis and Risk Stratification (MARS) consortium (55). In addition, novel bioinformatics approaches, such as a meta-analysis of several studies, showed that it is feasible to develop a prognostic model with good performances (56).

Transcriptome signatures hold great promise to address the complexity of the immune system within a single test, to determine the patient's immune status. Assessing such transcriptional response after *ex-vivo* stimulation may overcome the observed heterogeneity of sepsis cohorts, and provide a better assessment of the immune status on top of several confounders such as inter-individual variability or temporal effects. This approach is now possible with the recent advances in "omics" and multiplexing technologies.

Current Challenges and Future Perspectives

Factors Having an Impact on Immune Response

In physiological context, different parameters impact the human immune system. Several studies have highlighted different factors causing inter-individual variability which consequently has an impact on the evaluation of the immune function. Intrinsic factors like age, sex, co-morbidities, heterogeneity of blood composition, and genetic—and even epigenetic—factors are responsible for the physiological variations and differences observed in response to a pathogen challenge among healthy individuals (57–59).

Age represents one of the contributing factors to variability, in particular extreme ages. Two periods of life represented by neonates (especially preterm neonates) and the elderly are often marked by impairments in the immune system. The development of immature immune system in newborns and the deterioration of immune function in the elderly contribute to higher risk of infections observed in these populations. The term immunosenescence has been coined to describe the progressive deterioration of immune system with aging, notably characterized by a decrease of the immune memory. Other flawed mechanisms include, an inverse CD4/CD8 ratio, loss of naïve T cells, increase in the numbers of well-differentiated T cells and alteration of natural killer cells are all hallmarks of immunosenescence (60). However, functional rather than anatomical impairment is the probable underlying cause of immune alteration which can be accompanied by a defective production of inflammatory mediators (61).

The decrease in immune memory related to immunosenescence highlights the key role of this cellular repertoire against infections. Vaccination is one way to induce this immune memory to confer protection against pathogens. It is well known that vaccines act on the adaptive arm of the immunity essentially through the production of antibodies which targets specific pathogens. Recently, new approaches of vaccination target the innate arm of the immunity to protect against various pathogens and clear infections. Arts et al. studied the epigenetic changes, specifically the "reprogramming" of monocytes post-BCG vaccination. Monocytes stimulation with BCG led to functional changes in the innate immunity especially in the pro-inflammatory cytokine profiles. The investigators observed that this intervention led to the clearing of unrelated viral infections such as yellow fever virus (62). BCG vaccination

can generate innate immune memory, also known as "trained immunity" that can help prevent certain respiratory infections and neonatal sepsis. To evaluate the efficacy and success of vaccination, recall antigens are used to elicit an *ex vivo* response in order to evaluate whether the induced protection is based on the principle of prior exposure (63). Consequently, the immune response observed after *ex vivo* stimulation in an IFA is largely impacted by innate and adaptive immune memory, contributing to the inter-individual variability.

Besides protection obtained through preventive interventions, diverse studies have highlighted the importance and role of genetic factors in the safeguard against infections. A recent study by Piasecka et al. attempted to delineate the inter-individual variability by exploring the effect of host intrinsic factors such as genes, gender and age on the transcriptional responses and immune cells proportions of healthy volunteers. A thousand donors' blood stratified by age and sex was tested before and after the immune activation with different microbial stimulus that included bacterial, fungal, and viral. The transcriptional response of 560 immune-related genes was quantified as well as the measurement of eight major immune cell types. Finally the investigators measured the contribution of genetic factors to the immune gene expression variation by mapping the expression quantitative trait loci (eQTLs) for associations between genome-wide SNPs and 560 expression traits. The study concluded that the effect of age and gender was moderate but widespread across numerous immune genes and was not relevant to the immune cell composition. Meanwhile, genetic variations had a stronger effect on the regulation of immune genes although they affected only a limited number of gene set, with some genetic variants elucidated their regulatory effect only upon stimulation (64).

Finally, in the recent years light was shed on the important role of the microbiota to alter and modulate the immune response. A conception was developed implicating that symbiosis between immune system and the microbiota can establish a threshold of activation and regulation to maintain homeostasis (65). The disruption of this "alliance" caused by injury or antibiotics was associated to several disorders such as autoimmune diseases, allergy, and even cancer (66). New research observed that critically ill patients, in particular sepsis patients, had a significant shift in the gut microbiota populations marked by the disappearance of bacteria genera that are essential in the host metabolic activity and anti-inflammatory function, which might explain the diversity encountered in the immune status of these patients (67).

Diversity conferred by those intrinsic factors are responsible for the inter-individual differences observed in the immune response and accounts for almost 20% of the variability in the immune response (64). Knowing this inter-individual variability observed in the healthy physiological context, the definition of "Healthy Immune System" has to be well established in order to measure the immune function in a pathological condition.

This concept has been indirectly addressed in the design of IFA in order to be able to interpret patients' results (**Table 2**). For example, Neuvonen et al. tested the recommended antigens by the WHO for delayed hypersensitivity skin test in a large cohort of healthy population thus setting references for assessing the immune-competence in patients (73). In like manner, Pottumarthy et al. used healthy responses to evaluate the potentials of replacing the traditional skin test with tuberculin gamma interferon assay (74). While, Ulrichs et al. tried to grade the use of ESAT-6 as specific antigen in the development of the IGRA test comparing stimulations of healthy and patients with tuberculosis (75). The Milieu Interieur consortium took the initiative of setting preliminary reference ranges of a healthy immune response and its natural heterogeneity. They challenged healthy blood with different types of stimulus, from Toll-like receptors (TLR) agonists to complex whole microbes, to evaluate and decorticate the response of the immune cells. Healthy population from European ancestry was selected to reduce the

TABLE 2 | Immune Functional Assays applications in different pathologies.

Disease area	IFA	Assessment	Evaluation	Reference
Allergy	Skin test	Diagnosis of food allergy	Wheal diameter on skin	Gupta, (24)
	Basophil Activation test	Diagnosis of food allergy	Markers for basophile granulocytes identification	Hoffmann, (26)
	Histamine Release test	Diagnosis of drug allergy	Histamine release	Dona, (68)
Primary Immunodeficiency Disorder (PID)	Oxidative burst	Diagnosis	NADPH oxidase activity	Abrahams, (23)
	Lymphocyte transformation test	Diagnosis	Proliferation	Moylett EH, (69)
Tuberculosis	IGRA	Diagnosis	IFN-γ release	Van Pinxteren, (70)
Immunosuppression	Endotoxin test	Diagnosis anergy	TNF-α secretion	Cavaillon, (44)
Lymphoma	TLR agonist stimulation on PBMC	Response to therapy	Cytokine profile evaluation	Dietsch (71)
Autoimmunity	IGRA	Biotherapy	IFN-γ release	Mir Viladrich, et al., (72)
Organ Transplant	Immuknow	Outcome prediction	Intracellular ATP production	Lindermann, (3)
Vaccination	Recall antigens for lymphocyte proliferation	Immune function evaluation	Lymphocyte proliferation	Disteler, (28), Hoshina, (29)

inter-individual variability among the donors. Decreasing inter-individual variability readout is a relevant and key aspect to consider when designing an IFA, especially for the interpretation of the results. The efforts of the group highlighted the separation of different immune arms based on induced inflammatory signatures, which can contribute to the monitoring of immune function and the possibility of quantifying dysregulations (76). Setting reference is indispensable for the evaluation of the "in-range" and "out-range," as a base to map and identify disorders accordingly.

Need of Standardization and Precision Medicine

Taking into consideration the challenge to interpret results due to inter-individual diversity, technical variability should be kept minimum to avoid complexity of interpretation. IFA is composed of three main parts; the biological sample, the stimulant, and the cellular response, where the aim is to minimize technical variability to increase reproducibility.

Currently, the wider matrix used in IFA are PBMCs which despite technical advances in immunophenotyping, still relies on 50-year-old artisanal skills for the separation of mononuclear cells from whole blood using the Ficoll method (77). Besides, this technique has many limitations as being time-consuming and causing non-specific cell activation and cell death, thus reducing the quality of the sample. Moreover, sample manipulation increases the risk of contamination and introduces technical variability linked to the investigator. It is challenging to standardize protocols within and across laboratories. Some techniques such as intracellular cytokine staining, isolation of specific cells and *ex vivo* stimulations are accompanied with technical complexity and hard access to the testing platform. A primordial requirement of an IFA test is to preserve cell composition and interactions among cell populations and soluble factors should not be disturbed. Hence, whole blood is a more practical solution since it contains the same mix of cells and factors that reflect the inner environment of the subject with minimal handling.

The selection of stimuli to challenge immune cells is of relevant importance to promote a reiterative response at every use. LPS, a specific TLR-4 ligand, is the most widely used compound to stimulate whole blood, PBMCs and most of the isolated cell-population. LPS is purified from gram negative bacteria, nonetheless the difference in bacterial source elicits a distinct response and has different effects on the cells (78). Method of preparation and purity level (79) of LPS adds a higher degree of variability, making its reproducibility difficult from one study to another and the elicited response is hardly comparable. Arens and team illustrated this point when they stimulated *ex vivo* whole blood from controls and sepsis patients with LPS and no statistical difference was observed. Although it is commonly used across laboratories, LPS is not always a good-group discriminator, even between healthy and sick subjects (80). Moreover, the stimulation time is a critical factor in IFA design, since a short boost has an influence on the early and acute gene responders, while a longer period of incubation will favor the stimulation of a long-term response; influencing the arm of the immunity that comes into play.

Unbiased-immune response evaluation after *ex vivo* stimulation is a key part in the development of an IFA. It is widely accepted that only one marker is not enough to diagnose a clinical condition, predict an outcome, or assess a treatment. A combination of biomarkers is favored to obtain a holistic view of the patients' immune status to drive therapeutic decisions. The emergence of -omics studies have led to the

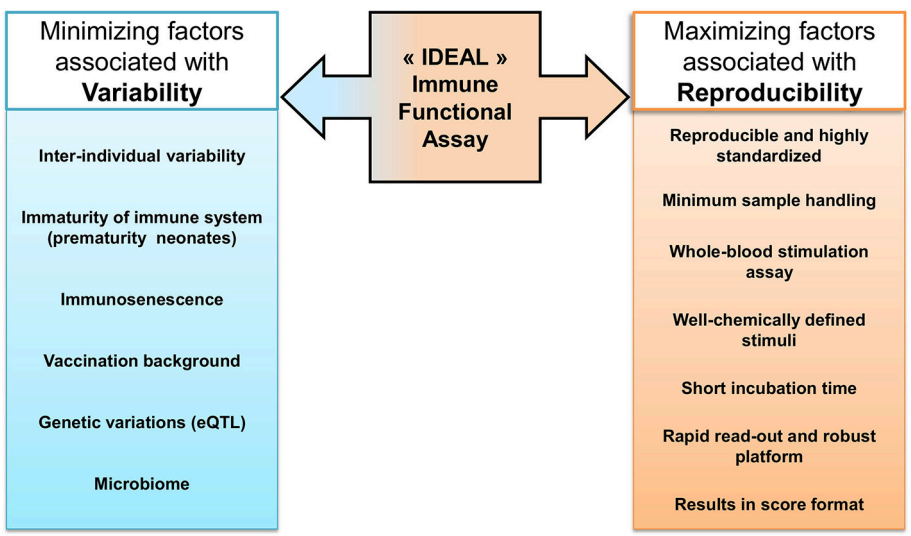

FIGURE 2 | "Ideal" Immune Function Assay. Many intrinsic factors contribute to the increased variability among subjects, such as genetic factors or vaccination record. The ideal IFA should be minimally impacted by those factors that create diversity in the physiological context. Taking into consideration the challenge to interpret results due to inter-individual diversity, technical factors, as minimal sample handling or robust platforms, can be optimized to allow reproducibility between tests and decrease the bias accumulated during the manipulation workflow.

development of advanced technologies and ready-to-use devices. Multiplexing in proteomics and transcriptomics allow the analysis of a high number of targeted analytes at the same time. Although caution must be taken with automated algorithms, readout and interpretation must be customized according to the clinical context. As one size does not fit all, in case of sepsis the interpretation must take into account intrinsic factors like SNPs or the natural diversity of patients, external factors such as pathogen (including the site of infection, load, and virulence) which in turn might alter the readout due to the pleiotropic nature of the immune response. A criteria was recently proposed by Shanker-Hari et al. to employ biomarkers and genetic signatures in precision medicine to address response and host heterogeneity. The team suggested that the patient endotypes should be consistent that is, when a pattern is observed among a population, it shall remain the same and persevere against bootstrap testing. The second aspect is to stratify the patients into subgroups, and each subgroup should be biologically and clinically plausible based on the systematic collection of a relevant and known biological basis. Finally, the patient groups should be realistic and are feasible to be clinically managed (81). This approach is already being applied in many research, where sepsis patients are classified in different endotypes according to their genomic information (55).

Until the current day, there is no molecular host biomarker panel available as a point of care for physicians to take an informed decision of a precise intervention based on the diagnosis of immune function or the ability to monitor the changes in the status of sepsis patients. Recent studies such as the INFECT study underline the gain in performances to predict patient outcome using three immune markers together rather than each one solely. Morris et al. showed that the use of neutrophil CD88, HLA-DR, and T_{regs} percentage in standardized flow cytometry was able to predict the occurrence of secondary infection in critically ill patients. Furthermore, the team was able to propose a cut-off to identify immune dysfunction from day 3 to 9 of ICU admission (82). Such study and signature discovery studies emphasize the importance of using a multiplex-based panel of markers. Current ongoing studies such as the REALISM study might shed the light on the impact of using immune functional assays in combination with immunosuppression biomarkers to stratify critically ill patients (83).

To finally put all the pieces together, an array of biomarkers is needed to tackle the heterogeneity of endotypes and identifying different host responses. Eventually, such biomarkers could help personalize treatment based on where a patient resides in the spectrum of inflammation, or whether specific organs are failing (84). In order to benefit from immunotherapy for instance, patients need to be stratified using a multiplexing technology of a biomarker-based panel to characterize the immune status of the patients. Therefore, a "bundle" of biomarkers combined with IFA could provide a robust tool to help achieve the desired stratification of high-risk patients such as the immunosuppressed profile, to map the altered pathways, and achieve a tailored management.

Research efforts are now directed toward precision medicine using several biomarkers to identify susceptible endotypes and predict the outcome of disease in order to intervene accurately. IFA paves the way to anticipate treatment responders from non-responders and functional measurement of the immune cells. Current immune function assays used in clinical routine for different applications have some limitations that hamper standardized reproducibility, however they are still used by default as being the better option. To develop an "ideal IFA," as illustrated in **Figure 2.**, the first requirement is to be highly standardized, reproducible across labs, demand a minimum of sample handling and require less technical skills. To accomplish this aim, the stimulant has to be consistent and a chemically well-defined molecule used worldwide with the same properties. Besides, the stimulation time has to be shortened as much as possible to be used at the bedside. For the evaluation of the response, the choice of the read-out and the technical platform is critical to obtain quick and accurate upshots. Ideally, the results should be processed within the day to get back to the patient in the least time. Results have to be obtained or smoothly transformed into a special format that can be promptly interpreted by clinicians, to be used in their evaluation and decision-making. Indeed, an acceptable result has to be able to map a patient's disorder into a specific category of treatment and/or management care. The future objective for IFA development is the procurement of the result in a "score" format. This resulting score has to reflect the accuracy and sensitivity of the test but above all can be easily interpreted by clinicians to guide in the decision making. Laboratory assay, preclinical development and clinical relevance are key steps to translate as a point of care. Interaction among multidisciplinary staff and opening channels of discussion are indispensable to improve the rigor of diagnostic performance to achieve precision in patient management. Although, the heterogeneity among subjects will always exist and will remain a challenge for the classification of endotype to be evaluated by an IFA test.

AUTHOR CONTRIBUTIONS

Conception and structuring of the manuscript were done by JT, ST-A, and FM. Drafting the manuscript was done by CA-V, DT, and JT. Final revision and editing was done by all authors.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Polymicrobial Sepsis Chronic Immunoparalysis Is Defined by Diminished Ag-Specific T Cell-Dependent B Cell Responses

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Immunosuppression is one hallmark of sepsis, decreasing the host response to the primary septic pathogens and/or secondary nosocomial infections. CD4 T cells and B cells are among the array of immune cells that experience reductions in number and function during sepsis. "Help" from follicular helper (Tfh) CD4 T cells to B cells is needed for productive and protective humoral immunity, but there is a paucity of data defining the effect of sepsis on a primary CD4 T cell-dependent B cell response. Using the cecal ligation and puncture (CLP) mouse model of sepsis induction, we observed reduced antibody production in mice challenged with influenza A virus or TNP-KLH in alum early (2 days) and late (30 days) after CLP surgery compared to mice subjected to sham surgery. To better understand how these CD4 T cell-dependent B cell responses were altered by a septic event, we immunized mice with a Complete Freund's Adjuvant emulsion containing the MHC II-restricted peptide 2W1S$_{56-68}$ coupled to the fluorochrome phycoerythrin (PE). Immunization with 2W1S-PE/CFA results in T cell-dependent B cell activation, giving us the ability to track defined populations of antigen-specific CD4 T cells and B cells responding to the same immunogen in the same mouse. Compared to sham mice, differentiation and class switching in PE-specific B cells were blunted in mice subjected to CLP surgery. Similarly, mice subjected to CLP had reduced expansion of 2W1S-specific T cells and Tfh differentiation after immunization. Our data suggest CLP-induced sepsis impacts humoral immunity by affecting the number and function of both antigen-specific B cells and CD4 Tfh cells, further defining the period of chronic immunoparalysis after sepsis induction.

Keywords: sepsis, B cells, immune suppression, antibody, CD4 T cells

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INTRODUCTION

Vaccination or infection is one of the most effective ways to generate immunity to microbes. Efficacious vaccinations and natural infection elicit antibody (Ab) production by B cells and their progeny, providing a first line of defense against subsequent microbial invasion. B cells recognize a wide variety of antigens (Ag), including proteins, lipids, polysaccharides, nucleic acids, and chemicals that bind to surface IgM or IgD (1). While serving as a major means of protection against extracellular pathogens and the various toxins they produce, Ab are also a vital means of defense against intracellular pathogens (including viruses) because of their ability to neutralize the pathogen before they can enter a cell, preventing the spread of infection (2, 3). Ab responses can be classified as "T cell-dependent or –independent," based on the use of CD4 T cell help (4). B cell responses to protein Ag in the absence of CD4 T cell help are weak, producing Ab with low affinity. In contrast, B cell responses generated with the help of CD4 T cells produce high affinity, class-switched Ab.

There has been considerable interest in recent years in $CXCR5^+PD\text{-}1^+Bcl6^+$ follicular helper CD4 T (Tfh) cells—the specialized CD4 T cell subset that provides help to B cells—and understanding the role they play in facilitating the proliferation and function of primary and memory B cells (5, 6). When Tfh cells detect B cells presenting their cognate Ag, they upregulate CD154 expression and secrete a number of cytokines to promote B cell proliferation and differentiation into plasma cells (7, 8). During the early Ab response plasma cells secrete Ab and some degree of isotype switching occurs. A few of the activated B cells return to the follicle, accompanied by Tfh cells, where they proliferate and form a germinal center (GC) in response to the Tfh cell-derived signals. The proliferating GC B cells undergo immunoglobulin (Ig) heavy chain isotype switching, somatic hypermutation of Ab gene variable regions, and affinity maturation. Repeated exposure to their cognate Ag promotes the B cells to produce the highest affinity and most efficacious Ab for neutralization of microbes and their toxic products and differentiate into long-lived plasma cells and memory B cells (9–11).

The importance of both the humoral and cellular arms of the adaptive immune system for overall health is dramatically illustrated by individuals with immune system defects being highly susceptible to serious and often life-threatening infections. States of immune deficiency can be congenital (e.g., impaired T and/or B cell development) or acquired (e.g., HIV infection, iatrogenic (post-organ transplant) immune suppression, or surgery/trauma). The combination of quantitative and qualitative impairments to multiple compartments of the immune system that develop in the wake of a septic event lead to an acquired immune deficiency (12). Sepsis, currently defined as life-threatening organ dysfunction resulting from the dysregulated host response to infection (13), is responsible for thousands of deaths annually (14). As the host recovers from the initial septic event, the immune system becomes hyporesponsive, resulting in a long-lasting immunosuppressive state. Advances in critical care and life support medicine have greatly improved survival rates of patients in the initial hyperinflammatory phase of sepsis, such that the acute cytokine storm is responsible for only ∼30% of the sepsis-related mortality. Today the majority of sepsis-related deaths occur after the patient has recovered from the initial hyperinflammatory phase, with many patient deaths occurring weeks and months later (15, 16). Reduced numbers of immune cells in septic patients contribute to the decreased responses to new and secondary infections (17, 18). While the characteristic sepsis-induced lymphopenia is transient, the prolonged immune suppression that develops after a septic event and remains even once lymphocyte numbers normalize is now considered a leading cause of prolonged susceptibility to secondary pathogens normally handled by the immune system in healthy individuals (19).

Studies in human septic patients show both CD4 T cells and B cells are reduced during the hyperinflammatory phase of sepsis (20), but there is limited data detailing the long-term impact of sepsis on these cells within the context of a CD4 T cell-dependent B cell response. We have taken advantage of using peptide:MHC I or II tetramers to track the number and function of endogenous Ag-specific CD8 or CD4 T cell populations (21–23) to investigate how specific subsets of the T cell compartment are quantitatively and qualitatively affected in the mouse model of cecal ligation and puncture (CLP)-induced polymicrobial sepsis (24). Similar approaches can identify endogenous Ag-specific B cells, such as B cells specific for the commonly used fluorochrome phycoerythrin (PE) (25–27). The objective of this study was to define the mechanism(s) responsible for the impairment of primary CD4 T cell-dependent B cell responses in the septic host using the CLP model followed by immunization with an Ag containing defined CD4 T cell and B cell epitopes. Our data suggest CLP-induced sepsis impacts humoral immunity by affecting the number and function of both Ag-specific B cells and CD4 Tfh cells.

MATERIALS AND METHODS

Mice

8 week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and maintained in AALAC-approved animal facilities at the University of Minnesota and University of Iowa at the appropriate biosafety level. Experimental procedures were approved by the University of Minnesota and University of Iowa Institutional Animal Care and Use Committees and performed following the Office of Laboratory Animal Welfare guidelines and PHS Policy on Human Cancer and Use of Laboratory Animals.

Cecal Ligation and Puncture (CLP)

Sepsis was induced by CLP (24). Briefly, mice were anesthetized using isoflurane (2.5% gas via inhalation) or Ketamine/xylazine (87.5 and 12.5 mg/kg, respectively, i.p.). The abdomen was shaved and disinfected with 5% povidone-iodine antiseptic. Bupivicaine (6 mg/kg s.c.) was then administered at the site where a midline incision was made. The distal third of the cecum was ligated with 4-0 silk suture and punctured once with a 25-g needle to extrude a small amount of cecal content. The cecum was returned to the abdomen, the peritoneum was closed via continuous suture, and the skin was sealed using surgical glue (Vetbond; 3M, St. Paul,

MN). Meloxicam (2 mg/kg) in 1 ml saline was administered at the conclusion of surgery and the following 3 days for post-operative analgesia and fluid resuscitation. Mice were monitored daily for weight loss and pain for at least 5 days post-surgery. To control for non-specific changes from the surgery, sham mice underwent the same laparotomy procedure excluding ligation and puncture.

Immunizations

On days 2 or 30 after sham or CLP surgery, B6 mice were immunized with the following reagents: (1) influenza A virus (A/PR/8; 10^5 PFU in 100 μl PBS i.p.; obtained from Dr. Ryan Langlois, University of Minnesota); (2) 2,4,6 trinitrophenyl-conjugated keyhole limpet hemocyanin [TNP-KLH; 50 μg i.p. (Biosearch Technologies, Novato, CA)] precipitated in alum (100 μg) or mixed with CpG containing oligonucleotide 1826 (10 μg; TCCATGA*CG*TTCCTGA*CG*TT), followed 3 weeks later by a second immunization; or (3) 2W1S:PE conjugates [i.p. injection of 0.6 μg 2W1S peptide (EAWGALANWAVDSA; GenScript, Piscataway, NJ) conjugated to 2.4 μg PE (ProZyme; Hayward, CA) emulsified in Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St. Louis, MO)] (28). The 2W1S:PE conjugate was formed by combining biotinylated 2W1S peptide with streptavidin-PE at a 4:1 ratio.

Enrichment and Analysis of Ag-Specific B Cells and CD4 T Cells and B Cells

To quantify the number of PE-specific B cells and 2W1S-specific CD4 T cells in mice following sham or CLP surgery, an enrichment protocol was used (25–27, 29). Briefly, spleens and peripheral LN (axillary, brachial, cervical, inguinal, and mesenteric) were harvested for each mouse analyzed. Pooled LN [in 1 ml of FACS buffer (PBS containing 0.1% NaN_3 and 2% FBS)] were mashed on a nylon mesh into a single-cell suspension. The spleen from the same mouse was then added, along with 1 ml of RPMI-1640 medium containing Collagenase P (0.2 mg/ml final), Dispase (0.8 mg/ml final), and DNase I (01 mg/ml final). A single-cell suspension from these pooled lymphoid tissues was then generated using a GentleMACS dissociator (Miltenyi Biotech). This suspension was incubated in a 37°C water bath for 20 min, and then run on the GentleMACS a second time. Ten (10) ml of ice cold FACS buffer containing 5 mM EDTA was added to the dissociator tubes, which were inverted several times to wash the top of the tubes before decanting into new 50 ml conical tubes. The dissociator tubes were rinsed with an additional 5 ml of ice cold FACS buffer, which was then decanted into the corresponding 50 ml conical tubes. The cells were pelleted by centrifugation, and then resuspended in 400 μl FACS buffer containing 5 mM EDTA and anti-CD16/32 mAb (clone 93, 1:100 dilution; BioLegend) to block Fc receptors. In some cases, the single-cell suspension was divided to permit separate enrichments for the B cells and CD4 T cells from the same sample.

B Cell Enrichment

Cells were incubated with PE (1 μg; Prozyme, Hayward, CA) for 30 min on ice. After washing with 10 ml cold FACS buffer with 5 mM EDTA, the cells were then incubated with 25 μl anti-PE-conjugated magnetic microbeads (Miltenyi Biotec) for 30 min on ice. The cells were washed, resuspended in 3 ml FACS buffer, and then passed over a magnetized LS column to enrich for the PE-specific cells. The column was washed twice with 3 ml of FACS buffer, and the bound cells were eluted from the column by pushing 5 ml of buffer with a plunger.

CD4 T Cell Enrichment

I-A^b-specific tetramers containing 2W1S (EAWGALANWAVDSA) were used to identify and enrich Ag-specific CD4 T cells (29–31). Briefly, biotinylated I-A^b molecules containing the 2W1S peptide covalently linked to the I-A^b β chain were produced in *Drosophila melanogaster* S2 cell along with the I-A^b α chain (29). The monomers were purified, and then made into tetramers with streptavidin-allophycocyanin (SA-APC; Prozyme). Tetramers (10 nM final concentration) were then added to single-cell suspensions in 300 μl tetramer staining buffer (PBS containing 5% FBS, 2 mM EDTA, and 50 μ? Dasatinib, 1:50 normal mouse serum, and 1:100 anti-CD16/32 mAb). The cells were incubated in the dark at room temperature for 1 h, followed by a wash in 10 ml ice cold FACS Buffer. The tetramer-stained cells were then resuspended in 300 μl FACS Buffer, mixed with 25 μl of anti-APC mAb-conjugated magnetic microbeads (StemCell Technologies), and incubated in the dark on ice for 30 min. The cells were washed, resuspended in 3 ml cold FACS Buffer, and passed through an EasySep Magnet (StemCell Technologies) to yield an enriched tetramer positive population.

The resulting enriched fractions were stained with a cocktail of fluorochrome-labeled mAb (see below). Cell numbers for each sample were determined using AccuCheck Counting Beads (Invitrogen). Samples were then analyzed using an LSR II flow cytometer (BD) and FlowJo software (TreeStar Inc., Ashland, OR). The percentage of PE^+ or 2W1S:I-A^{b+} events was multiplied by the total number of cells in the enriched fraction to calculate the total number of PE-specific B cells or 2W1S:I-A^b-specific CD4 T cells, respectively.

Flow Cytometry

To assess the expression of cell surface proteins, cells were incubated with fluorochrome-conjugated mAb at 4°C for 30 min. The cells were then washed with FACS buffer. For some experiments, the cells were then fixed with PBS containing 2% paraformaldeyhe. In procedures requiring intracellular staining, cells were permeabilized following surface staining using the transcription factor staining kit (eBioscience), stained for 1 h at 4°C with a second set of fluorochrome-conjugated mAb, and suspended in FACS buffer for acquisition. The fluorochrome-conjugated mAb used in surface and intracellular staining were as follows: *B cell panel*—FITC IgA, PerCP-eF710 IgM, AF594 IgG3, AF647 IgG2b, AF700 CD38, APC-eF780 "dump" (CD90.2, CD11c, F4-80, and GR1), BV510 IgE, BV605 IgG1, BV711 IgG2A, BV786 IgD, AF350 IgG (H+L), BUV395 B220; *T cell surface panel* –PE-Cy7 PD-1, AlexaFluor® (AF) 700 CD44, APC-eFluor® (eF) 780 "dump" (CD11b, CD11c, and B220), Brilliant Violet™ (BV) 421 CXCR5, BV650 CD8a, and Brilliant Ultraviolet™ (BUV) 395

CD4; and *T cell ICS panel*—AF488 Bcl6, BV605 Tbet, AF700 CD44, APC-eF780 "dump" (CD11b, CD11c, and B220), and BUV395 CD4.

Assessment of Ab Production After TNP-KLH or Influenza a Virus Immunization

Mice immunized with TNP-KLH were bled 7 days after the boost to collect serum to measure TNP-specific Ab levels. Mice challenged with influenza A virus were bled after 28 days to collect serum to measure anti-IAV Ab titers. Mice were anesthetized and blood was collected retro-orbitally. Blood samples were clotted and separated serum was stored at −80°C until use in enzyme-linked immunosorbent assay (ELISA) to determine the presence of Ag-specific Ab.

TNP-specific Ab were determined as follows: 96-well ELISA plates (Immulon 2, Thermo, Milford, MA) were coated with goat anti-mouse IgM (10 μg/ml; Southern Biotech, Birmingham, AL), goat anti-mouse IgG1 (5 μg/ml; Southern Biotech), or goat anti-mouse IgG2b (5 μg/ml; Southern Biotech) in 0.05 M Tris-HCl buffer (pH 9.5) overnight at 4°C. Coated plates were blocked with 5% w/v dry milk in phosphate buffered saline (PBS). Control anti-TNP mAb (for standard curves) or serum samples appropriately diluted in 5% dry milk-PBS were added, and similarly incubated. After washing, 2μg/ml TNP-human gamma globulin-biotin diluted in 5% dry milk-PBS was added to each well, and the plates further incubated. Alkaline phosphatase streptavidin (3 μg/ml; Zymed, San Francisco, CA) diluted in 5% dry milk-PBS was added after washing. Substrate (2 mg/ml; Sigma Chemical Co., St. Louis, MO) diluted in substrate buffer [50 mM Na_2CO_3 and 1 mM $MgCl_2$ $6H_2O$ in H_2O (pH 9.8)] was added to each well, and absorbance measured at a dual wavelength of 405 and 540 nm using a Microplate Autoreader EL311 (Bio-Tek Instruments, Winooski, VT). All washes between steps were performed with a 0.9% NaCl, 0.05% Tween-20 buffer (pH 7.0) and all incubation steps were done at 37°C in 5% CO_2. Ab concentrations were determined from standard curves using DeltaSOFT software (Bio-Tek Instruments). Control mAb used for standard curves were 49.2 (mouse IgG2b anti-TNP mAb; Pharmingen, San Diego, CA), 4G2F8 (mouse IgM anti-TNP mAb), and 1B7 (mouse IgG1 anti-TNP mAb. 4G2F8 and 1B7 were affinity purified by passage of hybridoma culture supernatants over TNP-bovine gamma globulin-Sepharose 6B followed by elution with TNP-glycine (Sigma Chemical Co.).

Influenza-specific Ab were determined as follows: 96-well ELISA plates were coated with purified A/PR/8 Influenza A virus (50 μl/well of 2 mg/ml PBS virus) overnight at 4°C. Coated plates were blocked for 1 h at room temperature with 5% normal goat or donkey serum in PBS, followed by incubation with diluted serum samples from IAV-challenged mice overnight at 4°C. After washing, plates were incubated with either an alkaline phosphatase-conjugated goat anti-mouse Ig (Southern Biotech) or donkey anti-mouse IgG (Jackson ImmunoResearch). Substrate was added and absorbance was measured as described above.

Statistical Analyses

Data shown are presented as mean values ± SEM. GraphPad Prism 7 was used for statistical analysis, where statistical significance was determined using two-tailed Student *t*-test (for 2 individual groups, if unequal variance Mann-Whitney U test was used) or group-wise, one-way ANOVA analyses followed by multiple-testing correction using the Holm-Sidak method, with $\alpha = 0.05$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

RESULTS

Sepsis Induces a Transient Reduction in B Cells and CD4 T Cells

Patients surviving a septic event often have suppressed immune function, as data showing reduced immune cell function in critically ill sepsis patients date back over 40 years (32). While some data suggested a phenotypic switch in CD4 T cells from Th1 to Th2 (33), other data indicated that the reduced cellular activity was more likely due to a global dysfunction (34). This idea is reinforced by decreased expression of Tbet, GATA3, and RORγt, the transcription factors regulating Th1, Th2, and Th17 phenotypes, respectively, in CD4 T cells from septic patients (35). More recently, post-mortem assessment of T cells from patients who died from severe sepsis showed almost no production of IFNγ, TNFα, IL-6, and IL-10 after anti-CD3/CD28 mAb stimulation compared to samples from non-septic, control patients (36)—further supporting the idea that sepsis affects general T cell function. Indirect evidence of defective CD4 T cell function has come from other studies describing altered humoral responses after sepsis, specifically in terms of Ag-specific immunity (e.g., T cell-dependent Ab responses) (37, 38). With this clinical information in mind, we wanted to further investigate how sepsis affects the generation of a primary CD4 T cell-dependent B cell response using the CLP mouse model of polymicrobial sepsis. The severity of the CLP we performed was marked by a significant, but transient, loss of weight that was recovered by 7 days after surgery (**Supplemental Figure 1A**), as well as the rapid production of IL-1β, IL-6, IFNγ, and TNF detectable in the serum during the first 24 h after surgery (**Supplemental Figure 1B**). Both of these parameters are consistent with previous reports (39–43). In addition, we see a mortality rate of ∼25% in the group of mice receiving CLP surgery (**Supplemental Figure 1C**), which is consistent with clinical rates (44).

We initially wanted to define the numerical changes that occur within the total B cell and CD4 T cell compartments—the cells that participate in CD4 T cell-dependent B cell responses—following a septic event. B cells and CD4 T cells present in the blood and secondary lymphoid organs were enumerated by flow cytometry early (day 2) and late (day 30) after sham or CLP surgery (**Figure 1A**). B cell numbers in the blood, spleen, and inguinal lymph nodes (iLN) were significantly reduced 2 days after sepsis induction, decreasing 11-fold, 2-fold, and 6-fold, respectively, before recovering to sham levels by day 30 (**Figures 1B,C**). Interestingly, there was no reduction in B cells isolated from mesenteric lymph nodes (mLN) on day 2 and there was a slight (but insignificant) increase in number on day 30. Similar trends were observed with CD4 T cells—transient numerical reductions in the blood (5-fold), spleens (2-fold),

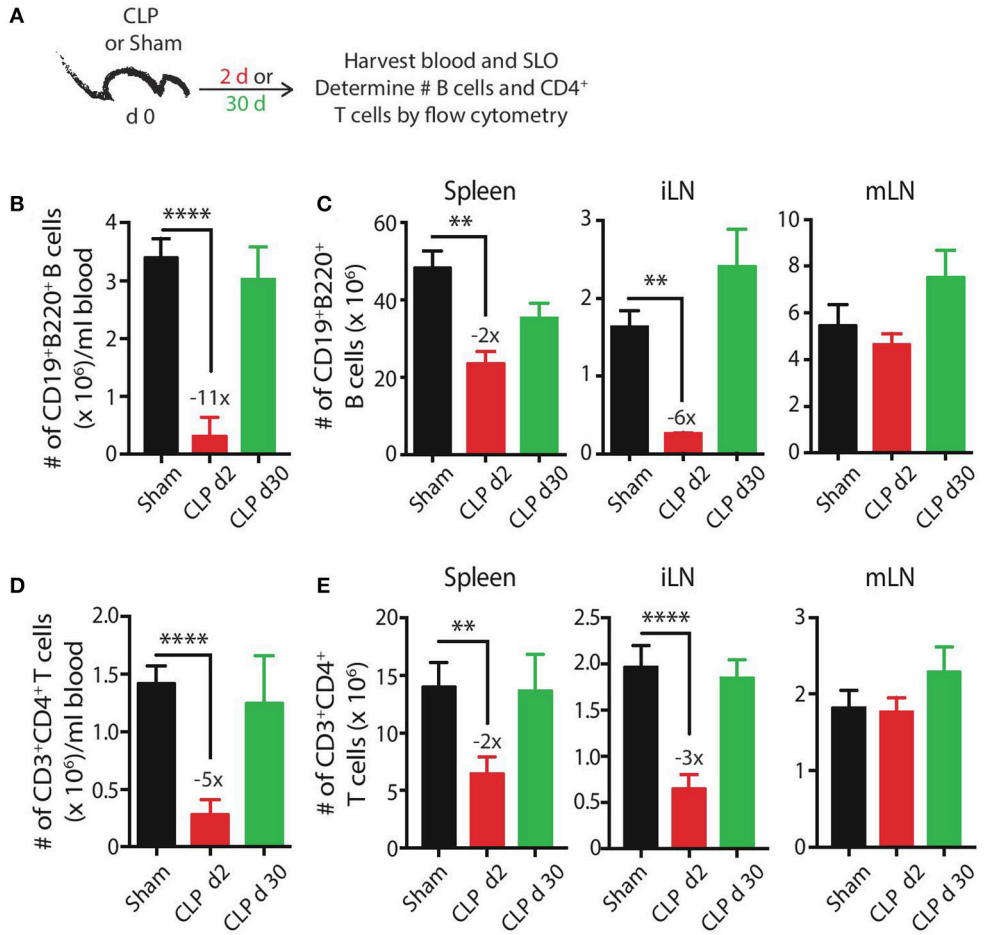

FIGURE 1 | CLP induces transient reductions in the number of B cells and CD4 T cells**. (A)** Experimental design—blood and secondary lymphoid organs (SLO) including spleens, inguinal lymph nodes (iLN), and mesenteric lymph nodes (mLN) were harvested 2 and 30 days after sham or CLP surgery. The number of **(B,C)** $CD19^+$ $B220^+$ B cells or **(D,E)** $CD3^+$ $CD4^+$ T cells in blood (per ml), spleen, iLN, and mLN was determined by flow cytometry. $n = 5–7$ mice/group. $^{**}p < 0.01$, $^{****}p < 0.001$. Numbers above bars indicate the average fold change in number compared to sham mice. Data are representative of at least 3 independent experiments.

and iLN (3-fold) but no change in the mLN (**Figures 1D,E**). The mLN drain the gut mucosa and are located within the site of the initial polymicrobial septic insult, suggesting the proximity to the intraperitoneal inflammation may either prevent the sepsis-induced death of lymphocytes seen in the periphery and/or recruit cells from periphery through the production of inflammatory cues. However, migration of cells to mLN cannot fully account for the diminished cellularity observed in other tissues. We have previously shown dendritic cells follow the same pattern of numerical reduction, with losses in the blood, spleen, and iLN, and no change in the mLN (45), indicating cells of the lymphoid and myeloid lineages are similarly maintained numerically in the anatomical locations where the nidus of the septic event is found. Data examining the B cell and CD4 T cell compartments at the "total population" level suggest the immune system has returned to its pre-sepsis state by day 30 in terms of B cell and CD4 T cell numbers.

Sepsis-Induced Effects on Ag-Specific B Cells and CD4 T Cell

The results presented in **Figure 1** suggest the re-establishment of a "normal" immune system within 30 days after CLP-induced sepsis. However, previous work from our group revealed distinct differences in the ability of individual Ag-specific CD4 T cell populations to numerically and functionally recover after sepsis (22). We hypothesized that similar differences may occur for an individual Ag-specific B cell population within the total B cell compartment, prompting us to employ a system where we could directly monitor defined populations of Ag-specific B cells and CD4 T cells within the total B cell and CD4 T cell compartments to more rigorously study the effect of sepsis on CD4 T cell-dependent B cell responses. Specifically, we used enrichment protocols to identify B cells specific for the fluorochrome phycoerythrin (PE) (25–27) and CD4 T cells specific for the 2W1S variant of peptide 52-68 from the I-E α-chain (29, 46).

As a first step in this analysis, we determined how sepsis affected the number and phenotype of PE-specific B cells (**Figure 2A**). We were able to clearly detect and quantify the PE-specific B cells, as well as the $B220^{hi}$IgG $[H+L]^{int}CD38^{+}GL7^{-}$ naïve/memory B cells, $B220^{hi}$IgG $[H+L]^{int}CD38^{-}GL7^{+}$ germinal center (GC) B cells, and $B220^{lo}$IgG $[H+L]^{hi}CD38^{-}GL7^{-}$ plasma cells within the PE-specific B cell population (**Figure 2B**). Similar to the transient loss and recovery within the total B cell compartment (**Figures 1B,C**), there was a significant reduction (5.2-fold less compared to sham mice) in number of PE-specific B cells 2 days after CLP surgery that recovered by day 30 (**Figure 2C**). The number of PE-specific naïve/memory B cells decreased ∼4-fold 2 days after CLP surgery, but did not fully recover by day 30 to the number found in mice that underwent sham surgery (**Figure 2D**). Interestingly, while there were ∼9- and 1.5-fold reductions in number of PE-specific GC B cells and plasma cells, respectively, 2 days after CLP surgery, this was followed by a ∼3-fold increase in both of these subsets by day 30 after CLP surgery (**Figures 2E,F**). In addition to determining the number of these PE-specific B cell subsets, we also evaluated how the septic event affected the Ab isotypes they produced. As expected, there were numerical reductions in several populations 2 days after CLP surgery, including $IgM^{+}IgD^{-}$ naïve/memory, $IgG1^{+}$ and IgE^{+} GC B cells, and IgA^{+} plasma cells (**Figures 2G–I**). It is also important to note several populations were increased by day 30 after CLP, namely the IgG2b-producing PE-specific naïve/memory B cells, GC B cells, and plasma cells 30 days after CLP compared to sham mice (**Figures 2G–I**). IgG1- and IgG2c-producing GC B cells also increased in number by day 30 after CLP surgery. These data show sepsis can induce a variety of small numerical changes within the B cell compartment when examined at the Ag-specific level.

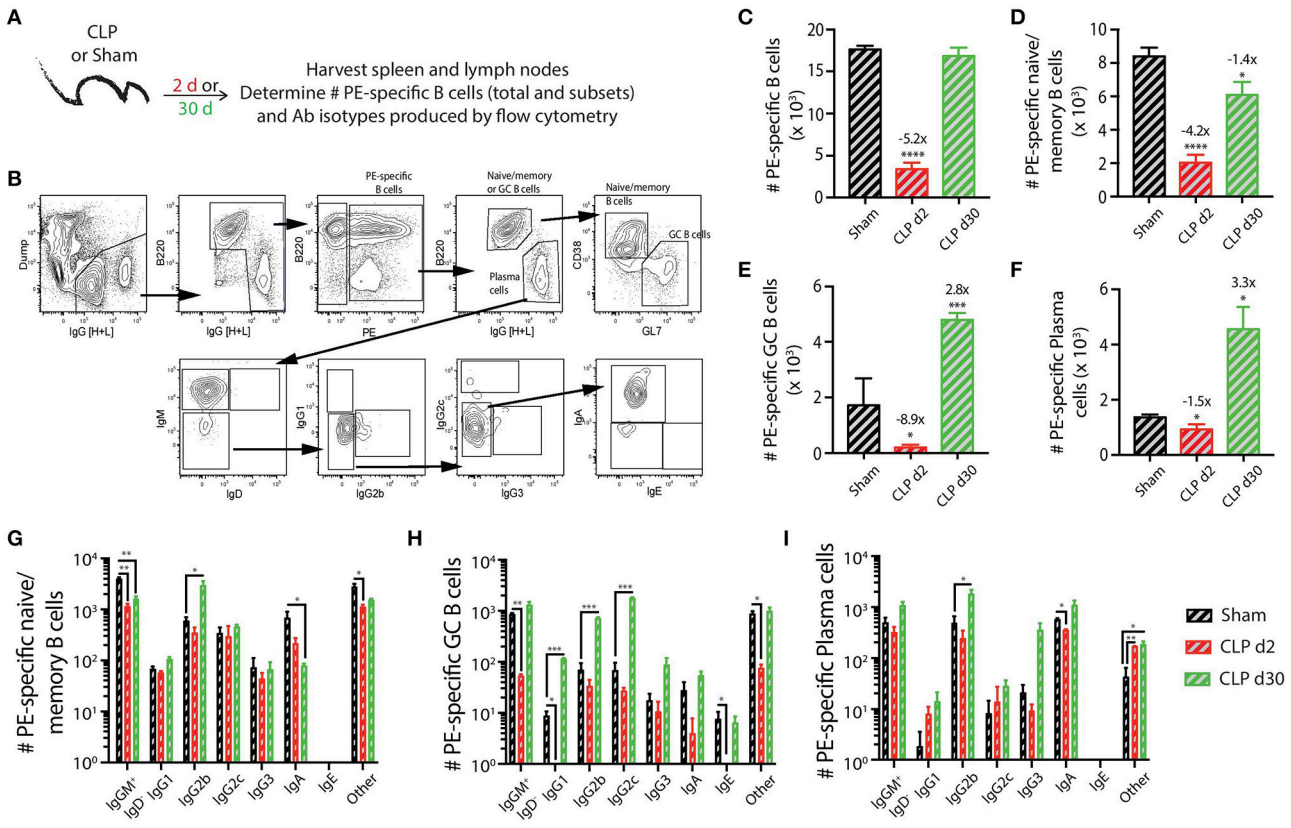

FIGURE 2 | Numerical changes in PE-specific B cells after sepsis. **(A)** Experimental design—Spleens and peripheral lymph nodes (axillary, brachial, cervical, inguinal, and mesenteric) were harvested from mice on day 2 and 30 post-sham or CLP surgery and combined. **(B)** Gating scheme used to identify PE-specific B cells, as well as the $B220^{hi}$IgG $[H+L]^{lo}$ $CD38^{+}GL7^{-}$ naïve/memory B cells, $B220^{hi}$IgG $[H+L]^{lo}$ $CD38^{-}GL7^{+}$ germinal center (GC) B cells, and $B220^{lo}$IgG $[H+L]^{hi}CD38^{-}GL7^{-}$ plasma cells. This representative scheme also shows the gating used to determine the extent of class switching within the plasma cell population, but similar gating was used on the naïve/memory and GC B cell populations. Representative flow plots are from a mouse that underwent sham surgery. The number of **(C)** total PE-specific B cells and PE-specific **(D)** naïve/memory B cells, defined as $B220^{hi}$ IgG $[H+L]^{lo}$ $CD38^{+}GL7^{-}$, **(E)** germinal center (GC) B cells, defined as $B220^{hi}$ IgG $[H+L]^{lo}$ $CD38^{-}$ $GL7^{+}$, and **(F)** plasma cells, defined as $B220^{lo}$ IgG $[H+L]^{hi}$ $CD38^{-}$ $GL7^{-}$, were determined. Numbers above bars indicate the average fold change in number compared to sham mice. **(G–I)** The naïve/memory B cell, GC B cell, and plasma cell subsets from the unimmunized mice were additionally subdivided based on the Ab isotype being produced. $n = 5$–7 mice/group. Statistical comparisons were made between sham mice and either CLP d2 or d30 mice, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.001$. Data are representative of at least 3 independent experiments.

In contrast to the PE-specific B cells and consistent with our previous data (22), we did not observe the same numerical recovery of 2W1S:I-A^b-specific T cells after CLP surgery that was seen for the total CD4 T cell population. In fact, the number of 2W1S:I-A^b-specific CD4 T cells was reduced ~3-fold at day 2 post-CLP, which was maintained at day 30 (**Figures 3A–C**). Our analysis of the 2W1S:I-A^b-specific T cells also included an assessment of CD4 T cell lineage-specific master regulators Tbet (Th1) and Bcl6 (Tfh) (47, 48). A small number of the 2W1S:I-A^b-specific CD4 T cells expressed Tbet by day 30 after CLP surgery (**Figure 3D**), but we were unable to detect any 2W1S:I-A^b-specific CD4 T cells expressing Bcl6 or PD-1 and CXCR5, indicators of Tfh differentiation, after CLP. Thus, the data in **Figures 2**, **3** show how sepsis affects the number of PE-specific B cells and 2W1S:I-A^b-specific CD4 T cells.

Prolonged Impairment in Primary CD4 T Cell-Dependent B Cell Responses Is Associated With Reduced Germinal Center T Follicular Helper (Tfh) Cell Differentiation

We next determined how sepsis affected the ability of the PE-specific B cells and 2W1S:I-A^b-specific CD4 T cells to respond after immunization with a CFA emulsion containing the MHC

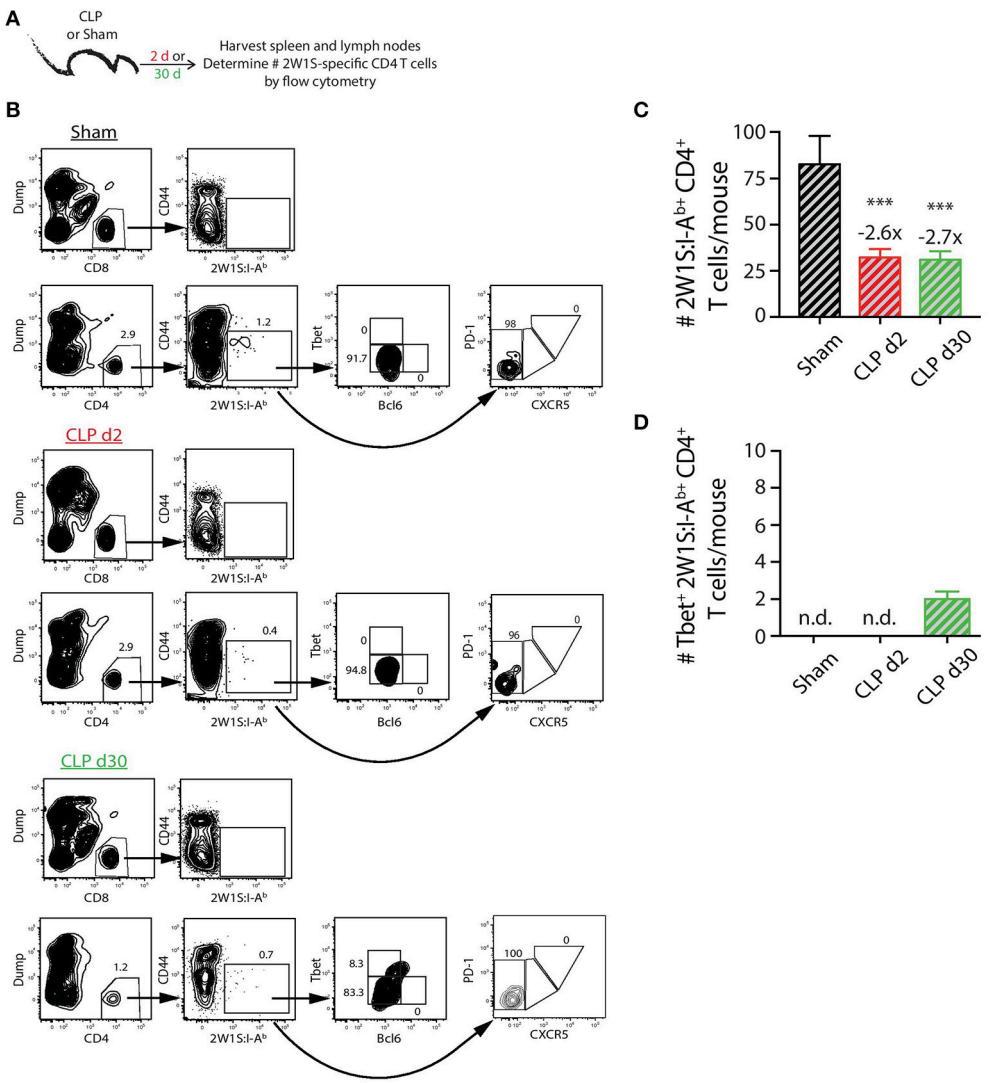

FIGURE 3 | Numerical changes in 2W1S-specific CD4 T cells after sepsis. **(A)** Experimental design—Spleens and peripheral lymph nodes (axillary, brachial, cervical, inguinal, and mesenteric) were harvested from mice on day 2 and 30 post-sham or CLP surgery and combined. **(B)** Gating scheme used to identify 2W1S:I-A^b-specific CD4 T cells (CD8 T cells were used as the internal negative control for tetramer binding), as well as the 2W1S:I-A^b-specific CD4 T cells expressing Tbet, Bcl-6, or PD-1 and CXCR5. The frequency of cells within the gated populations is indicated. The number of **(C)** total and **(D)** Tbet$^+$ 2W1S:I-A^b-specific CD4 T cells was determined. n = 9–10 mice/group in **(B,C)**. Statistical comparisons were made between sham mice and either CLP d2 or d30 mice, where ***$p < 0.005$. Numbers above bars indicate the average fold change in number compared to sham-treated mice.

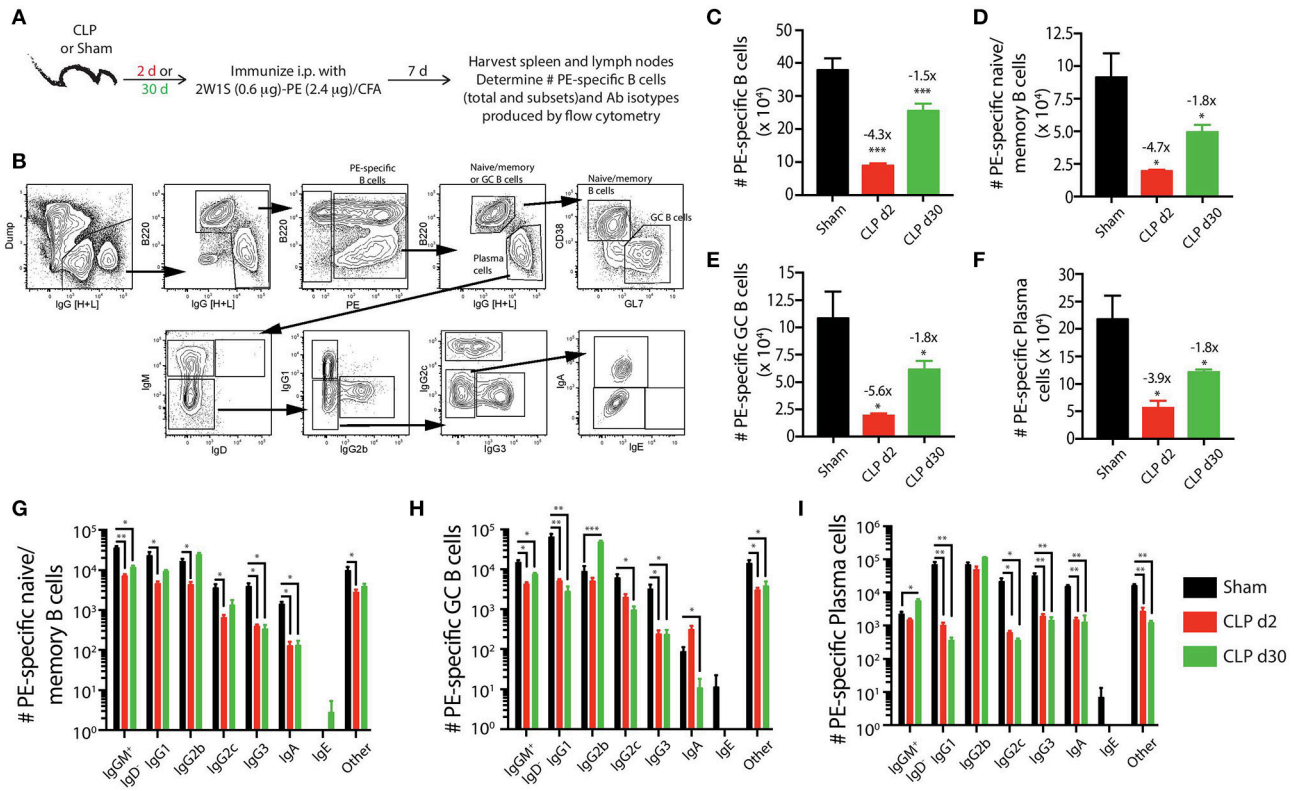

FIGURE 4 | Sepsis hinders B cell differentiation and class-switching during a CD4 T cell-dependent B cell response. **(A)** Experimental design—mice underwent sham or CLP surgery and were then immunized on day 2 or 30 after surgery with 2W1S-PE/CFA i.p. Spleens and peripheral lymph nodes were harvested 7 days after immunization and combined. **(B)** Gating scheme used to identify PE-specific B cells, as well as the $B220^{hi}$IgG [H+L]^{lo} $CD38^{+}GL7^{-}$ naïve/memory B cells, $B220^{hi}$IgG [H+L]^{lo} $CD38^{-}GL7^{+}$ germinal center (GC) B cells, and $B220^{lo}$IgG [H+L]$^{hi}CD38^{-}GL7^{-}$ plasma cells. This representative scheme also shows the gating used to determine the extent of class switching within the plasma cell population, but similar gating was used on the naïve/memory and GC B cell populations. Representative flow plots are from a mouse immunized 2 days after sham surgery. The number of **(C)** total PE-specific B cells and PE-specific **(D)** naïve/memory B cells, **(E)** GC B cells, and **(F)** plasma cells were determined. Numbers above bars indicate the average fold change in number compared to sham-treated mice. **(G–I)** The naïve/memory B cell, GC B cell, and plasma cell subsets from the immunized mice were additionally subdivided based on the Ab isotype being produced. $n = 5$–7 mice/group. Statistical comparisons were made between sham mice and either CLP d2 or d30 mice, where $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$. Data are representative of at least 3 independent experiments.

II-restricted $2W1S_{56-68}$ peptide coupled to PE (28) (**Figure 4A**). The 2W1S-PE immunogen is internalized by the B cell receptor of PE-specific B cells, which then present 2W1S in MHC II complexes to 2W1S-specific CD4 T cells who provide the necessary help to generate a robust B cell response. This immunization method allowed us to simultaneously track the numerical and phenotypic changes among PE-specific B cells and 2W1S:I-A^{b}-specific CD4 T cells in the same mouse during a CD4 T cell-dependent B cell response.

Using the identical Ab panels and gating schemes that were used above (**Figure 4B**) to identify PE-specific B cells in mice that had only experienced sham or CLP surgery, we found the immunization-induced expansion for each PE-specific B cell population was greatest in the sham-treated mice (**Figures 4C–F**). While the number of total PE-specific B cells and each subset had recovered or expanded by day 30 after CLP surgery, the response of these populations to immunization with 2W1S-PE/CFA was significantly reduced in mice subjected to CLP surgery compared to sham mice. We noted 4–6-fold fewer PE-specific naïve/memory, GC, and plasma cells in mice immunized 2 days after CLP surgery compared to sham mice, and this numerical reduction (∼2-fold less) was maintained in the CLP mice immunized 30 days after surgery. Thus, the PE-specific B cell compartment showed long-lasting functional impairment in terms of cellular proliferative capacity, extending well-past the resolution of the septic event. Sepsis also affected the degree of class switching after immunization with 2W1S-PE/CFA for each subset of PE-specific B cells. In general, there were reductions in the number of multiple isotypes produced by the PE-specific B cell subsets (**Figures 4G–I**). This was most evident in the PE-specific plasma cells, as there were significantly fewer cells producing IgG1, IgG2c, IgG3, and IgA after immunization either on day 2 or 30 after CLP surgery (**Figure 4I**).

The extent of Tfh cell differentiation by the 2W1S:I-A^{b}-specific CD4 T cells after 2W1S-PE/CFA immunization (**Figure 5A**) was also impaired by sepsis. There were ∼4-fold

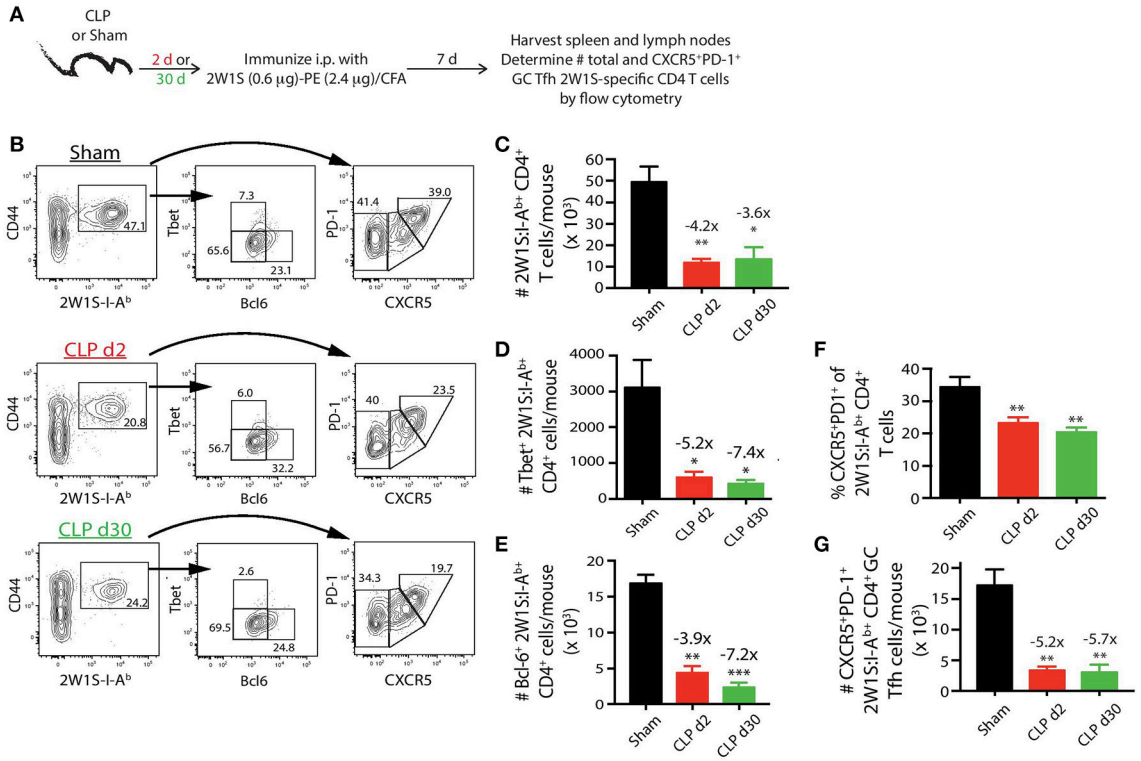

FIGURE 5 | Sepsis restricts the ability of 2W1S:I-A^b-specific CD4 T cells to differentiate into Tfh during a CD4 T cell-dependent B cell response. **(A)** Experimental design—mice underwent sham or CLP surgery and were then immunized on day 2 or 30 after surgery with 2W1S-PE/CFA i.p. Spleens and peripheral lymph nodes were harvested 7 days after immunization and combined. **(B)** Gating scheme used to identify 2W1S:I-A^b-specific CD4 T cells (CD8 T cells were used as the internal negative control for tetramer binding), as well as the 2W1S:I-A^b-specific CD4 T cells expressing Tbet, Bcl-6, or PD-1 and CXCR5. The frequency of cells within the gated populations is indicated. The number of **(C)** total, **(D)** Tbet$^+$, **(E)** Bcl-6$^+$ 2W1S:I-A^b-specific CD4 T cells, as well as **(F)** frequency, and **(G)** number of 2W1S:I-A^b-specific CD4 T cells expressing CXCR5 and PD-1, was determined. $n = 5$–7 mice/group. Statistical comparisons were made between sham-treated mice and either CLP d2- or d30-treated mice, where $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$. Data are representative of at least 3 independent experiments.

fewer total 2W1S:I-A^b-specific CD4 T cells in the mice subjected to CLP surgery, regardless of when the immunization occurred (day 2 or 30 after surgery), compared to sham mice (**Figures 5B,C**). Moreover, there was a significant reduction in the frequency and number (∼5-fold less than sham-treated mice) of 2W1S:I-A^b-specific CD4 T cells that differentiated into GC Tfh cells, based on expression of Bcl-6 (**Figure 5E**) or CXCR5 and PD-1 (**Figures 5F,G**). The data in **Figures 2–5** reveal the profound quantitative and qualitative effects of sepsis on both Ag-specific B cells and CD4 T cells, especially after immunization with a model Ag designed to elicit a CD4 T cell-dependent B cell response, that were not apparent when evaluating the bulk B cell and CD4 T cell compartments.

To bolster our findings above, we wanted to determine the impact of sepsis on Ab production during a primary CD4 T cell-dependent B cell response to a pathogen challenge commonly used to probe B cell and/or T cell response and a second model Ag classically used to evaluate the fitness of the humoral arm of adaptive immunity. B6 mice were challenged with live influenza A virus (IAV) on day 2 or 30 after sham or CLP surgery, and serum was collected 28 days later to measure the amount of anti-IAV Ab produced (**Figure 6A**). We noted marked reductions in the amount of total Ab and IgG specific for IAV in the serum of CLP-treated mice challenged on days 2 or 30 after CLP surgery (**Figures 6B,C**). Similar results were seen after immunization with TNP-KLH, a common Ag used to test various aspect of humoral immunity (**Figure 7**). Interestingly, the sepsis-induced deficiency Ab production was most pronounced and sustained when the TNP-KLH was administered with the Th2-polarizing adjuvant alum (49, 50) (**Figure 7B**). There remained a significant reduction in anti-TNP IgM, IgG_1, and IgG_2 even when the mice were first immunized 30 days after CLP surgery, and boosted 21 days later. By comparison, CLP-treated mice demonstrated a significant reduction in anti-TNP IgM and IgG_2 when immunized with TNP-KLH mixed with Th1-polarizing adjuvant CpG (51) only on day 2, but these reductions were not maintained when immunization occurred 30 days after surgery (**Figure 7C**). The differences in response depending on the adjuvant used are intriguing, but it is important to note that our primary goal of these experiments was not to directly compare "Th1" vs. "Th2" priming conditions. Rather, we reasoned the comparison between control (sham) to CLP mice after the same duration post-surgery was more critical. Sham surgery likely causes some low-level abdominal inflammation,

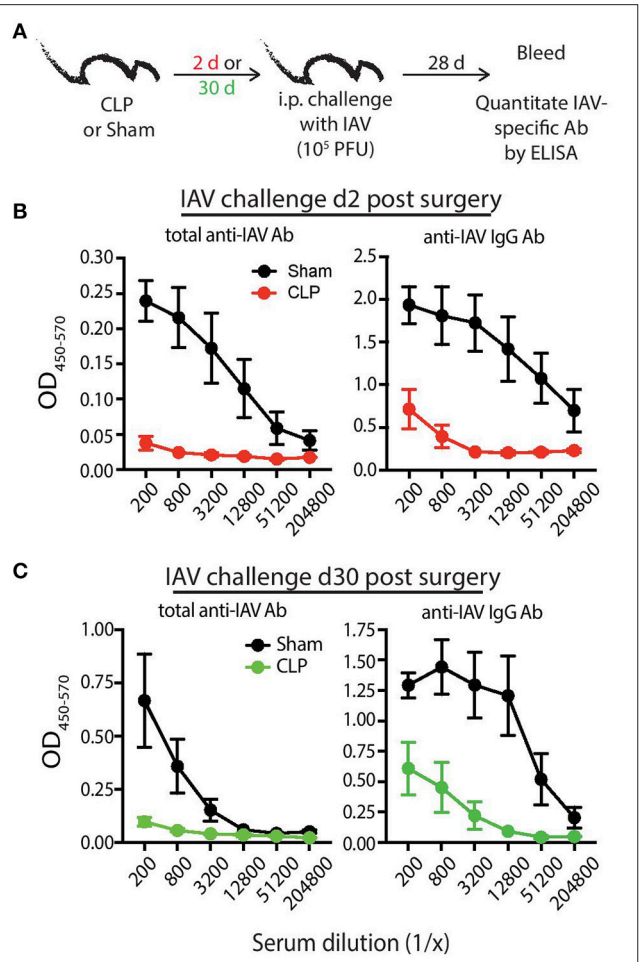

FIGURE 6 | Septic mice have reduced primary Ab response following influenza A virus (IAV) challenge. **(A)** Experimental design—B6 mice were challenged with live IAV (A/PR/8; 10^5 PFU in 100 μl PBS i.p.) 2 or 30 days after sham or CLP surgery. Serum was collected 28 days after immunization. **(B)** Absorbance of total anti-IAV Ab (left) and IgG specific for IAV (right) from the indicated dilutions of serum from mice challenged 2 days after surgery. **(C)** Absorbance of total anti-IAV Ab (left) and IgG specific for IAV (right) from the indicated dilutions of serum from mice challenged 30 days after surgery. *n* = 4–6 mice/group. Data are representative of at least 2 independent experimental replicates.

as the abdomen/peritoneum is surgically opened and the cecum exposed and put back into the mouse (without any needle puncture) followed by closure. While the mice received surgery at the same time, the peritoneum is going to be much different 2 days after this surgical event compared with 30 days where healing has taken place. Together, these results highlight the compromised ability to produce Ab during a primary B cell response following a septic event.

DISCUSSION

Defects in humoral immunity are associated with increased susceptibility to infection (52). Reduced immune function after septic injury is well-documented in the clinic, and a number of mechanisms have been posited to explain sepsis-induced immune suppression (12). Clinical data show acute reduction in both CD4 T cells and B cells in sepsis patients (20), as well as IgM levels in the circulation (53). The reduction of these components of the adaptive immune system contribute to the increased risk of nosocomial bacterial infections and viral reactivation, and poor chances for a favorable outcome (36, 54). While there is a reasonable understanding of the numerical changes in total B cells and CD4 T cells within several days after sepsis onset, there is a paucity of information describing the qualitative long-term impact of sepsis on these cells within the context of a primary CD4 T cell-dependent B cell response. The reduced Ab response following antigenic challenge in mice that experienced CLP-induced sepsis we have described here is consistent with the recent data by Mohr et al. (37). Further, the data we have presented importantly extend our understanding of what happens to the cellular components of the adaptive immune system following a septic event that affect the generation of a primary CD4 T cell-dependent B cells response. Our results show that following sepsis, mice subjected to CLP surgery have long-term reductions in B cell differentiation and class switching after vaccination or infection, ultimately resulting in suboptimal Ab production. The data reported here also suggest the reduced Ab production in mice challenged with Ag early during the septic event or late after sepsis resolution is due in part to insufficient help from Tfh cells, leading to inadequate B cell differentiation and class switching.

Work reported in a number of publications have examined basic numerical and functional changes of various immune cell subsets after sepsis (almost) exclusively at the total population level. In the present study we have evaluated endogenous Ag-specific CD4 T cell and B cell populations within the context of a CD4 T cell-dependent B cell response following a septic event. Tracking Ag-specific T cells and B cells permits the most rigorous and sensitive functional analysis of these cells during the response to vaccination or infection by allowing us to identify changes at the Ag-specific level that may not be resolvable when examining the total populations (23). Additionally, we were able to evaluate the sepsis-induced numerical and phenotypic changes in endogenous Ag-specific CD4 T cells and B cells (2W1S:I-A^b- and PE-specific, respectively) responding to the same foreign Ag (2W1S peptide covalently coupled to PE in CFA) (28). As expected, the 2W1S:I-A^b-specific CD4 T cells expand and differentiate into Tfh cells as well as other defined CD4 T cell subsets, such as $Tbet^+$ "Th1" CD4 T cells (**Figure 5D**), in sham mice after 2W1S-PE/CFA vaccination. PE-specific B cells can then interact with the 2W1S:I-A^b-specific Tfh cells within the GC. There is robust expansion, differentiation, and class switching seen in the PE-specific B cells in this setting. Our data suggest all of these factors are affected by sepsis, even once the host has recovered from the acute hyperinflammatory response and transient lymphopenia characteristic of a septic event.

While there were a number of results that were expected, our analyses also revealed some unexpected findings. For example, the total number of PE-specific B cells was not different in the sham and day 30 CLP groups (see **Figure 2C**). There was just a redistribution among the different subsets. CLP-induced

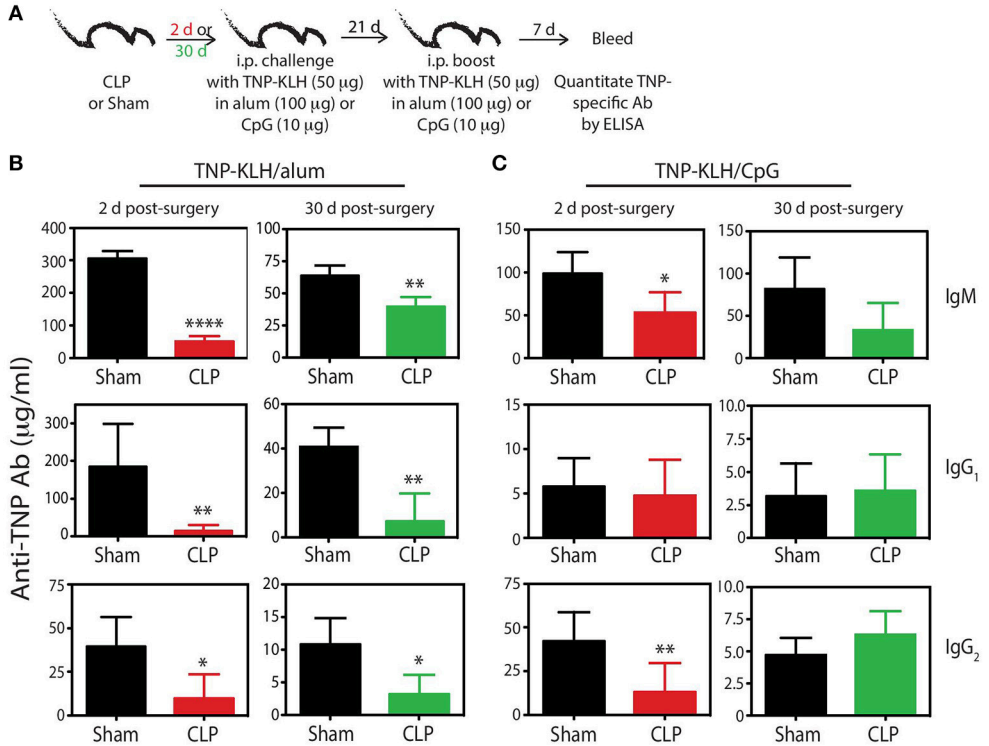

FIGURE 7 | Generation of anti-TNP mAb is reduced in septic mice**. (A)** Experimental design—B6 mice were immunized i.p. with 50 μg TNP-KLH in alum or CpG 2 or 30 days after sham or CLP surgery. A second immunization was administered 21 days later. Blood was collected for serum 7 days after the second immunization. Serum concentrations (quantified via ELISA) of anti-TNP Ab from **(B)** TNP-KLH/alum- and **(C)** TNP-KLH/CpG-immunized mice 2 and 30 days after sham and CLP surgeries. $n = 4$–6 mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.001$. Data are representative of at least 2 independent experiments.

polymicrobial sepsis induces a highly inflammatory event within the peritoneum, making it possible that this inflammation can drive a small number of the PE-specific cells to differentiate into GC B cells and plasma cells. The small but significant numerical increase in PE-specific GC B cells and plasma cells 30 days after CLP, prior to 2W1S-PE/CFA immunization (**Figures 2E,F**) was surprising, and was in contrast to the reduction seen in total 2W1S:I-A^b-specific CD4 T cells (**Figure 3C**). While 2W1S:I-A^b-specific CD4 T cells recognize a defined peptide sequence presented by MHC II, PE is a 250 kD multi-subunit protein originally isolated from red algae with multiple epitopes available for Ab recognition (55, 56). Thus, while being PE-specific, the B cells we detect using the enrichment protocol are likely polyclonal in composition, and bind to different epitopes within the PE protein. One possible explanation for the increase in PE-specific GC B cells and plasma cells 30 days after CLP is that some of the PE-specific B cells cross-react with antigenic epitopes expressed by the numerous gut commensal microbes released during the CLP surgery that establish the polymicrobial peritonitis. Such cross reactivity among B cells has been observed previously; for example, gut commensal bacteria can prime for the production of antibodies specific for HIV-1 envelope gp41 (57). Similarly, CD4 T cell cross-reactivity with gut commensal bacteria can drive responses to self Ag (glucose-6-phosphate isomerase) and foreign microbes that ultimately has an impact on host health (58–61). Future studies are needed to investigate this interesting possibility of cross-reactivity. Despite the increases seen in the PE-specific B cell populations after CLP, sepsis dramatically reduced the ability of these cells to respond to their cognate Ag following 2W1S-PE/CFA immunization. 2W1S:I-A^b-specific CD4 T cells have reduced proliferative capacity and cytokine production after sepsis, as well has developing changes within the TCR Vβ repertoire (22). In addition, numerical and functional deficits occur among dendritic cells following sepsis (45), suggesting the potential contribution for both T cell-intrinsic and -extrinsic factors to reduced function.

Another interesting finding was the skewing of the Ab response in the mice subjected to CLP surgery to IgG2b after immunization (and even prior to immunization). The presence of TGF-β, which has been reported to be elevated after sepsis (62), promotes switching to IgG2b and IgA (63). While class switching to IgG2b was elevated across the board, IgA was slightly elevated only in GC B cells and plasma B cells in unimmunized mice. There is clear redundancy in regard to the ability of certain cytokines to drive IgG2 (as well as other) isotype switching in murine B cells. IFNγ and type I IFN can promote switching to IgG2, as exemplified by the presence of normal levels of induced IgG2b in mice unable to express the type II TGF-β receptor on their B cells (64). Interestingly, it has also been reported that the absence of T cell help and presence of LPS favors switching

to IgG2b (63). Thus, given these redundancies and variety of cytokines produced during a septic event, we hesitate to suggest TGF-β (or any other single cytokine) is solely responsible for the IgG2b skewing after CLP.

While it is difficult to know how long the effects of a septic event will have on the function of the immune system, sepsis survivors have decreased 5-year survival compared to "control" patients (16, 65, 66). Perturbations, such as sepsis, that result in long-term impairments to the immune system have the potential to severely diminish vaccination efficacy. Typically, adults are recommended to receive a number of vaccinations to seasonal (e.g., influenza) and non-seasonal (e.g., pneumococcus and varicella zoster virus) pathogens to develop and maintain adequate protection from infection (67). These vaccinations work best when the host immune system is optimally functional. However, in a patient with a history of sepsis, these vaccinations may provide little-to-no protection, leaving these patients with an increased risk of secondary infection.

AUTHOR CONTRIBUTIONS

FS, SC, JK, KP, CD, DD, TK, LT, KM, JC-P, and TG performed experiments and analyzed data. TW, VB, and TG provided input on the research design, and FS, SC, JK, CD, DD, TW, VB, TG wrote and edited the manuscript. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02532/full#supplementary-material

Supplemental Figure 1 | Morbidity, cytokine production, and mortality following CLP surgery. B6 mice underwent sham or CLP surgery. **(A)** Body weight was measured before and the 7 days after surgery. Weight loss for each was determined based on their starting weight. **(B)** Serum samples were collected at the indicated time points after sham or CLP surgery. The amount of IL-1β, IL-6, IFNγ, and TNF in samples was determined by bioplex. $n = 11$ sham and 38 CLP mice for **(A,C)**; $n = 5$ mice/time point/group in **(B)** $^{*}p < 0.05$; $^{**}p < 0.01$, $^{****}p < 0.001$ for SPF—CLP vs. cohoused—CLP at the indicated time points.

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PRL2 Controls Phagocyte Bactericidal Activity by Sensing and Regulating ROS

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Although it is well-recognized that inflammation enhances leukocyte bactericidal activity, the underlying mechanisms are not clear. Here we report that PRL2 is sensitive to oxidative stress at inflamed sites. Reduced PRL2 in phagocytes causes increased respiratory burst activity and enhances phagocyte bactericidal activity. PRL2 (Phosphatase Regenerating Liver 2) is highly expressed in resting immune cells, but is markedly downregulated by inflammation. *in vitro* experiments showed that PRL2 was sensitive to hydrogen peroxide (H_2O_2), a common damage signal at inflamed sites. In response to infection, PRL2 knockout (KO) phagocytes were hyper activated, produced more reactive oxygen species (ROS) and exhibited enhanced bactericidal activity. Mice with PRL2 deficiency in the myeloid cell compartment were resistant to lethal listeria infection and cleared the bacteria more rapidly and effectively. Moreover, *in vitro* experiments demonstrated that PRL2 binds to GTPase Rac and regulates ROS production. Rac GTPases were more active in PRL2 (KO) phagocytes than in wild type cells after bacterium infection. Our findings indicate that PRL2 senses ROS at inflamed sites and regulates ROS production in phagocytes. This positive feedback mechanism promotes bactericidal activity of phagocytes and may play an important role in innate anti-bacterial immunity.

Keywords: PRL2, oxidative burst, bactericidal activity, Rac GTPase, neutrophil, macrophage

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INTRODUCTION

Phagocytes are used by our immune system to remove and destroy pathogens (1, 2). To defend against pathogens, these specialized phagocytic immune cells use a process called "respiratory burst," releasing reactive oxygen species (ROS) to degrade internalized microbes (3, 4). The key producer of ROS in phagocytes is the NADPH-oxidase complex which is made up of 5 phagocytic oxidase units (Nox2, p22phox, p40phox, p47phox, and p67phox) and a Rac GTPase (5). Under normal circumstances the NADPH complex is latent but it is activated to assemble during infection. In order to destroy microbes efficiently and avoid self-harm the activation of NADPH and the generation of ROS are precisely regulated (6). It has been shown that phagocytes in inflamed tissues produce ROS more quickly and generate more ROS than cells in normal tissues (7). However, the precise mechanisms by which ROS generation is controlled are still not fully elucidated.

The PRL (Phosphatase of Regenerating Liver) family of phosphatases are coded for by the protein tyrosine phosphatase IVA *(PTP4A)* gene and have 3 members, PRL1, PRL2, and PRL3 (8). PRLs have been validated as biomarkers and therapeutic targets in cancer and studies have shown that individual PRLs, especially PRL3, are expressed at high levels in variety of cancer cells and tissues (9, 10) and that overexpression of PRLs promote cell proliferation, migration, invasion, tumor growth and metastasis (11). Among the PRL family, PRL2 is the least studied member. While studies have reported that PRL2 is overexpressed in pancreatic, breast and lung cancer samples and its level is associated with tumor progression, there is still a lack of information available regarding the function of PRL2 (12). The expression of PRL2 is the most abundant of the three PRLs and PRL2 mRNA is almost ubiquitously expressed at high levels in normal adult human immune tissues, suggesting that PRL2 may have a specific role in immune function (13). Here we report that PRL2 is highly expressed in murine innate immune cells, acting as a ROS sensor and regulator. In innate phagocytes, PRL2 rapidly responds to the oxidative stress at the inflamed site by down regulating its own expression, leading to enhanced respiratory burst. This positive feedback mechanism promotes bactericidal activity of phagocytes and may play an important role in innate anti-bacterial immunity.

MATERIALS AND METHODS

Ethics Statement

The conducts and procedures involving animal experiments were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine (project number 2012008, A-2016–028). It is according to the Regulations for the Administration of Affairs Concerning Experimental Animals (approved by the State Council of the People's Republic of China) and the Guide for the Care and Use of Laboratory Animals (Department of Laboratory Science, Shanghai Jiao Tong University School of Medicine, laboratory animal usage license number SYXK 2013–0050, certificated by Shanghai Committee of Science and Technology).

Mice

Wide-type C57BL/6 mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Mice in which the *Ptp4a2* exon 4 are flanked by LoxP sites (*Ptp4a2*$^{fl/fl}$mice, 129S$_6$/S$_v$E$_v$ background) were generated by Shanghai Model Organism Center. C57BL/6 mice that carry a *Ptp4a2*$^{fl/fl}$ gene were generated by backcrossing *Ptp4a2*$^{fl/fl}$ mice to C57BL/6 mice for 10 generations. LysM$^{WT/cre}$ B6 background transgenic mice were kindly provided by Feng Qian's Lab in School of Life Science, Fudan University. Ptp4a2$^{fl/fl}$ B6 mice were crossed with LysM$^{WT/cre}$ mice. Ptp4a2$^{fl/fl}$LysM^{Cre+} mice (CKO) and their wild-type (WT) littermates (Ptp4a2$^{fl/fl}$LysM^{Cre-} mice) are the offsprings of the Ptp4a2$^{fl/fl}$ B6 mice and the LysM$^{WT/cre}$ B6 mice. Mice were housed in the Shanghai Jiaotong University School of Medicine Animal Care Facilities under specific pathogen-free conditions.

Protein Extraction and Immunoblotting

The thymus, bone marrow, spleen and lymph nodes were harvested from mice and passed through a cell strainer to generate single cell suspensions. The blood was collected from mice and the red blood cells were removed using ACK lysis buffer. Cells were used for protein extraction after being washed twice in phosphate-buffered saline (PBS). Whole cell lysates were prepared by resuspending cells in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 100 μM Na_3VO_4, 5 mM EDTA, 1 mM PMSF) supplemented with 1x complete protease inhibitor cocktail (Roche), followed by determination of protein concentration by BCA assay (Pierce). Equal quantities of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted using specific antibodies [anti-PRL2 antibody (Millipore), anti-GAPDH antibody (Sigma), anti-Rac1/2/3, anti-β-actin, anti-iNOS and anti-Arginase1 antibodies (CST), anti-Myc antibody (Invitrogen)] and HPR conjugated anti-mouse or anti-rabbit IgG (CST). The membrane was developed using Pierce SuperSignal reagent (Pierce) and detected by ImageQuant LAS 4000 mini (GE).

Primary Neutrophil and Macrophage Isolation and Generation

Morphologically mature neutrophils were purified from murine bone marrow by Percoll gradient centrifugation, as previously described (14). Briefly, bone marrow cells were harvested from mice using neutrophil isolation buffer (1× HBSS without Ca^{2+} and Mg^{2+} containing 0.25% BSA). After RBC lysis, cells were layered on a 3-step Percoll gradient (81%, 62%, 55%), centrifuged at 1,200 g for 30 min at room temperature and the cells at the 81%:62% interface were collected and washed. To obtain neutrophils from an inflamed site, 2% (w/v) casein in PBS (2 mL for each) was injected i.p., in mice. Four hours later the peritoneal exudate was collected and neutrophils were isolated and purified as above. After purification, neutrophil viability was assessed as >95% by trypan blue staining. Purity was typically >80% as assessed by flow cytometry based on the forward and side scatter and high Gr1 staining.

Macrophages were isolated from the peritoneal cavity of naïve mice or from mice that had been injected with 2 ml of 4% thioglycollate i.p. for 72 h. Peritoneal cavity cells were collected using ice cold PBS, washed, suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin (D10), and plated in petri dishes. Non-adherent cells were removed 2 h later and the adherent macrophages were used for further experiment. The purity of macrophages was >95%, as determined by flow cytometry analysis using F4/80 and CD11b markers.

To generate bone marrow-derived macrophages (BMDMs), bone marrow cells were harvested from mice and cultured in D10 with 50 ng/ml M-CSF for 7 days. Macrophages were treated and collected using 5 mM EDTA in cold PBS, centrifuged and resuspended in D10, followed by seeding and resting for 24 h

before functional assays. BMDMs were >95% $CD11b^+$ and $F4/80^+$ as determined by flow cytometry.

Cell Culture and Transfection

The murine Raw 264.7 (ATCC), COS7 (ATCC) and HEK293T cell lines (ATCC) were cultured in DMEM containing 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin (D10). Transfections were performed using Attractene transfection reagent according to manufacturer's instruction (QIAGEN).

Plasmid Constructs

Mouse PRL2-pRK5 plasmid was generated by Dr. YH Chen' Lab (University of Pennsylvania). Full length PRL2 was generated from the cDNA clone by PCR and subcloned into pRK5 with Myc tag at the N-terminal. Myc-Rac1, Myc-Rac2, and HRasG12V plasmids were obtained from Dr. YH Chen's lab and were as described previously (15). All constructs and mutations were confirmed by DNA sequencing.

In vivo L. Monocytogenes Infection Model

Wild-type *Listeria monocytogenes* (10403s) were provided by Dr. H. Shen (University of Pennsylvania) and grown at 37°C in Brain-Heart-Infusion medium (Becton Dickinson). Mid-log-phase bacteria were used for the experiments. $Ptp4a2^{fl/fl}$LysMCre$^+$ mice and WT controls were infected i.v., with 3.75×10^5 bacteria in 150 μl PBS. Mice were observed daily post-infection. For measurements of the bacterial burden, liver and spleens were homogenized 24 h after inoculation in 0.1% Triton in PBS, before plating serial dilutions of the homogenate on Brain-Heart-Infusion agar plates. The colonies were counted 24 h later. Serum alanine aminotransferase (ALT) was measured by Beckman-Coulter chemistry analyzer AU5800.

Phagocytosis and Killing Assays

Phagocytosis and killing assays were performed in 12-well plates. A total of 1×10^6 BMDMs were seeded in each well and cultured overnight in D10. Mid-log-phase *L. monocytogenes* or *E. coli* (DH5α) transformed with pRK5 plasmid, or fluorescently labeled 2 μm beads, were added to BMDMs at different multiplicity of infection (MOI). Centrifugation was performed at 500 g for 2 min to synchronize binding and internalization. For the phagocytosis assay following a 30 min incubation at 37°C the plates were rapidly washed with ice cold PBS twice. The cells were digested with 5 mM EDTA-PBS followed by 2% paraformaldehyde for fixing, and analyzed by flow cytometry. Cells incubated with bacteria were washed twice with PBS and lysed with 0.1% (v/v) Triton X-100 in PBS.

For the killing assay, 20 min post infection was considered to be the starting point of killing progress and the media was changed to fresh media containing 50 ng/mL gentamycin at this point. After 2 h the cells were washed and lysed with 0.1% (v/v) Triton X-100 in PBS. To determine the number of remaining intracellular bacteria, serial dilutions of the samples were plated on LB agar plates with ampicillin (*E. coli* transformed with PRK5 plasmid) or BHI agar plates with streptomycin (*L. monocytogenes*). The colonies were counted 24 h later.

Detection of ROS

Neutrophil ROS production was measured by a luminol-dependent chemiluminescence assay. 3×10^5 cells were plated in a 96-well luminometer plate (Coster) and prewarmed for 5 min. Pre-warmed fMLP (5 μM, Sigma-Aldrich) or Zymosan (100 μg/ml, Sigma-Aldrich) were added together with luminol (100 μM, Sigma-Aldrich) and HRP (20 U/ml, Sigma-Aldrich) and measurements started immediately. Chemiluminescence was measured at 2.5 min intervals for 30–60 min with a luminometer (BioTek Synergy HT microplate reader).

ROS production from BMDMs and Raw cells was detected using 2′,7′-dichlorofluorescein diacetate (DCFDA Sigma-Aldrich). Cells were incubated with the fluorogenic probe DCFDA for 30 min at 37°C in 5% CO_2 and ROS was determined using a microplate reader.

PAK Pull-Down

In order to measure Rac activity in mouse macrophages or transfected 293T cells, cells were washed in PBS and lysed in PBD lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl2, 0.2 M NaCl, 0.5% NP-40, and 1x protease inhibitors cocktail) (Roche). The lysate was incubated with 20 μg of PAK-GST protein beads (Cytoskeleton) for 30 min at 4°C, washed and then subjected to Western blot.

Immunofluorescence and Confocal Assay

COS7 cells were grown on chamber slides (Lab-Tec) for 24 h. Myc-PRL2 with Rac1-EGFP or Rac2-EGFP were co-transfected to COS7 cells. After 24 h of transfection, cells were washed twice with pre-heated PBS and fixed with 2% paraformaldehyde in PBS for 10 min at 37°C, followed by treatment with 0.1% saponin/0.3% BSA for 15 min, and blocking using 3% BSA for 45 min. The cells were then stained with anti-Myc Alexa 647 antibody (ebioscience) for 1 h, washed and mounted in ProLong Gold anti-fade reagent with DAPI (Molecular probes). Fluorescence was captured by a laser confocal microscope (Leica TCS SP8) at 63X magnification.

Co-immunoprecipitation

To determine the interaction between PRL2 and Rac1/2, 293T cells were transfected with Myc-PRL2 or Myc-Rac1/Myc-Rac2, respectively. Cell lysates were prepared 24 h after transfection using lysis buffer (50 mM HEPEs, 150 mM NaCl, 1 mM EDTA, pH 7.0, 0.1% ICEPAL) supplemented with 1× complete protease inhibitors mixture (Roche). Immunoprecipitation was performed using Dynabeads protein G (Invitrogen). In brief, 1.5 mg Protein G Dynabeads were coated with 5 μg anti-Myc antibody (Invitrogen) or Ig control for 1 h at room temperature with rotation. After removing unbound antibody, the bead-antibody complex was incubated with cell lysate overnight at 4°C with rotation. The captured Dynabead/Ab/Ag complex was washed 4 times with PBS and boiled in 2 × Laemmli buffer. The eluted proteins were subjected to 12% SDS-PAGE for Western blotting.

Statistical Analyses

Statistical differences in phagocytosis, bacterial killing and DCFH assay were analyzed by unpaired Student's *t*-test. Statistical differences in survival rate were analyzed by Log-rank (Mantel-Cox) test and area under curve (AUC) was analyzed using GraphPad Prism software.

RESULTS

Innate Immune Cells From Inflammatory Sites Show Less PRL2 Expression

To understand the functional role of PRL2 in the host immune system we analyzed the expression of PRL2 in cells from different immune tissues. Cells were prepared from central and peripheral immune tissues of normal mice and PRL2 expression levels were analyzed by western blot. Similarly to the expression profile in human tissues, PRL2 was widely expressed in mouse immune tissues and readily detected in thymus, bone marrow, spleen, lymph nodes and blood (**Figure 1A**).

We next analyzed PRL2 expression under normal and inflammatory conditions, using resting naïve cells or inflammatory peritoneal cells isolated after casein or thioglycollate (TG)-induced peritonitis. As shown in **Figure 1B**, neutrophils isolated from the inflammatory site showed significantly less PRL2 expression compared with resting neutrophils from naïve mouse bone marrow. A similar reduction in PRL2 levels was seen when analyzing thioglycollate—elicited peritoneal macrophages compared to naïve resident peritoneal macrophages (**Figure 1B**).

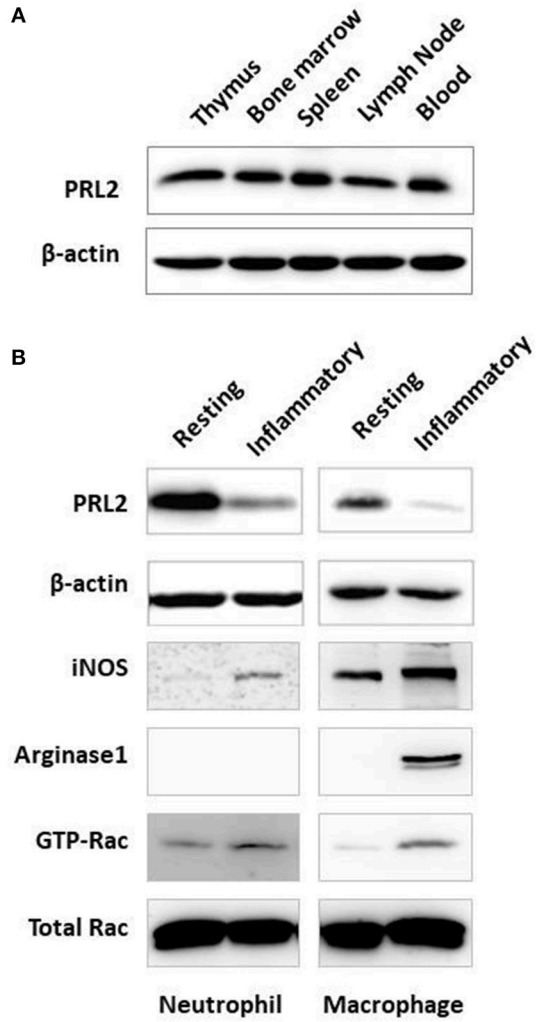

FIGURE 1 | Expression of PRL2 in murine immune tissues and cells. **(A)** Lysates of mouse immune tissues were subjected to SDS-PAGE followed by immunoblot analysis using the indicated antibodies. **(B)** Neutrophils were purified from naïve mouse bone marrow (resting) or abdominal cavity exudate (inflammatory) as described in Material and Methods. Resting and inflammatory macrophages were collected from naïve mice or from the peritoneal cavity after thioglycollate-elicited peritonitis, respectively. Cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using the indicated antibodies. Data are representative of two or three independent experiments.

PRL2 Is Susceptible to Oxidative Stress

Reactive oxygen species (ROS) are key signaling molecules in the process of inflammation (16) while PRL proteins belong to protein tyrosine phosphatases (PTPs) which are commonly susceptible to oxidative stress (17). To investigate if the reduced levels of PRL2 observed in inflammatory leukocytes was associated with oxidative stress, we examined PRL2 protein levels in murine bone marrow-derived macrophages (BMDMs) before and after treatment with hydrogen peroxide (H_2O_2). As shown in **Figure 2A**, upon treatment with H_2O_2 the protein levels of PRL2 were significantly reduced after 30 min, and the effects of H_2O_2 on primary BMDMs were dose and time-dependent. Similar effects were observed on primary neutrophils isolated from murine bone marrow where a significant decrease in the levels of PRL2 was observed after 15 min, with almost complete loss of PRL2 protein expression after 20 min (**Figure 2B**). The above results suggest that PRL2 responds to ROS rapidly and may be involved in immediate innate immune responses.

PRL2 Myeloid Cell Specific-Deficient Mice Are Resistant to Lethal Listeria Infection

To further investigate the role of PRL2 in innate immunity, we generated PRL2 myeloid cell conditional knockout (CKO) mice. Targeting of *Ptp4a2* was achieved by introduction of LoxP sites flanking exon 4 of the *Ptp4a2* gene (**Figure 3A**). The resulting $Ptp4a2^{fl/fl}$ B6 mice were crossed with $LysM^{WT/cre}$ mice to generate $Ptp4a2^{fl/fl}LysM^{Cre+}$ mice where PRL2 is deleted in the myeloid-cell lineage (**Figure 3B**). The $Ptp4a2^{fl/fl}LysM^{Cre+}$ CKO mice were born and developed normally. Adult CKO mice had similar body size, and blood, spleen and bone marrow cellularity to that of their wild-type (WT) littermates ($Ptp4a2^{fl/fl}$ $LysM^{Cre-}$ mice) (**Supplementary Figure 1**). In order to analyse the myeloid cell development we generated bone marrow derived macrophages (BMDMs) from CKO and WT mice, and analyzed them by flow cytometry. No significant differences were observed between BMDMs with or without PRL2.

To investigate the role of PRL2 in the innate immune response we infected CKO and WT mice with a lethal dose of *L.monocytogenes*. The majority of WT mice succumbed within

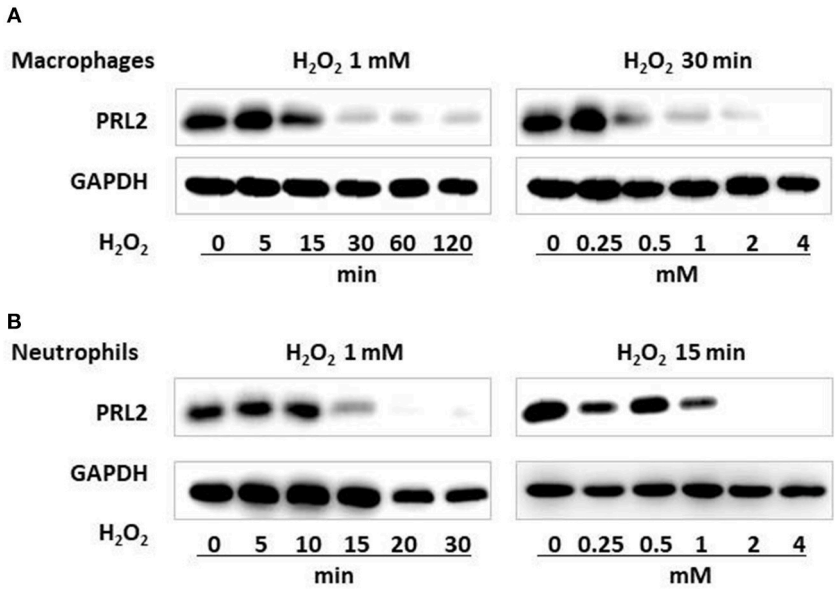

FIGURE 2 | PRL2 is susceptible to oxidative stress. **(A)** Wild-type bone marrow-derived macrophages were treated with 1 mM H_2O_2 for the indicated times, or with the indicated concentrations of H_2O_2 for 30 min. Cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using the indicated antibodies. **(B)** Mouse bone marrow neutrophils were treated with 1 mM H_2O_2 for the indicated times, or with the indicated concentrations of H_2O_2 for 15 min. Cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using the indicated antibodies. Data are representative of three independent experiments.

5 days of infection, whereas the majority of the CKO mice survived (**Figure 3C**). The serum alanine transaminase (ALT) is an indicator of hepatic injury. After Listeria infection, the serum ALT levels were higher in WT than in CKO mice (**Figure 3D**). Mortality of WT mice was also associated with high titers of bacteria in liver and spleen while the listeria titers in the CKO mice was significantly lower at 24 h post infection (**Figures 3E,F**). Taken together, these results demonstrate that PRL2 act as an inhibitor of innate immunity to bacteria.

PRL2 Negatively Regulates Bactericidal Activity of Phagocytes

Phagocytes play a major role in innate immune responses against bacteria through phagocytosis and killing of microbes. We analyzed the role of PRL2 in phagocytosis by incubating $PRL2^{-/-}$ and WT macrophages with fluorescently labeled beads, followed by measurement of fluorescent-positive cells by flow cytometry. As can be seen in **Figure 4A**, there was no difference in phagocytic ability between $PRL2^{-/-}$ cells and WT cells. Similarly, when the cells were incubated with *L.monocytogenes* or *E.coli* at a ratio of 1:10 for 30 min there was no difference in the number of bacteria taken up by the cells (**Figure 4B**). We next measured bactericidal activity of phagocytes. After 2 h of bacterial infection, PRL2-deficient macrophages killed *L. monocytogenes* and *E. coli* more efficiently than WT cells (**Figure 4C**). Killing of bacteria occurs very quickly in neutrophils, no *E.coli* survived in WT or PRL2 deficient neutrophils. As an intracellular bacterium, some *L. monocytogenes* can survive in WT and $PRL2^{-/-}$ neutrophils. More *L. moncytogenes* survived in WT cells than in $PRL2^{-/-}$ cells (**Figure 4D**). To further confirm the effect of PRL2 on bacterial killing, we overexpressed PRL2 in the murine RAW 264.7 macrophage cell line. Overexpression of PRL2 significantly increased bacterial survival in macrophages (**Figure 4E**). Taken together, the above results suggest that PRL2 negatively regulates bactericidal activity in phagocytes.

PRL2 Inhibits Oxidative Burst in Bacterial Infection

The bacterial killing ability of phagocytes is highly related to oxidative burst capacity. In order to evaluate the role of PRL2 on oxidative burst we stimulated cells with pathogen components and measured ROS production using a horseradish peroxidase (HRP) enhanced chemiluminescence (CL) system. $PRL2^{-/-}$ and WT neutrophils were stimulated with the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) or Zymosan A from *Saccharomyces cerevisiae*, two well-known stimuli of NADPH-oxidase activation. In response to fMLP stimulation, neutrophils quickly increased ROS generation. The peak values of chemiluminescence were observed within 5 min of stimulation, with $PRL2^{-/-}$ neutrophils producing significantly more ROS than WT cells. When neutrophils were stimulated with Zymosan the peak values appeared around 30 min after stimulation and $PRL2^{-/-}$ neutrophils again generated more ROS than WT cells (**Figure 5A**).

ROS production in macrophages was detected by incubating cells with H_2-DCFDA and measuring the intracellular levels of ROS by using a fluorescence microplate reader. As shown in **Figure 5B**, incubating macrophages with either *L. monocytogenes* or *E. coli* induced a strong ROS response,

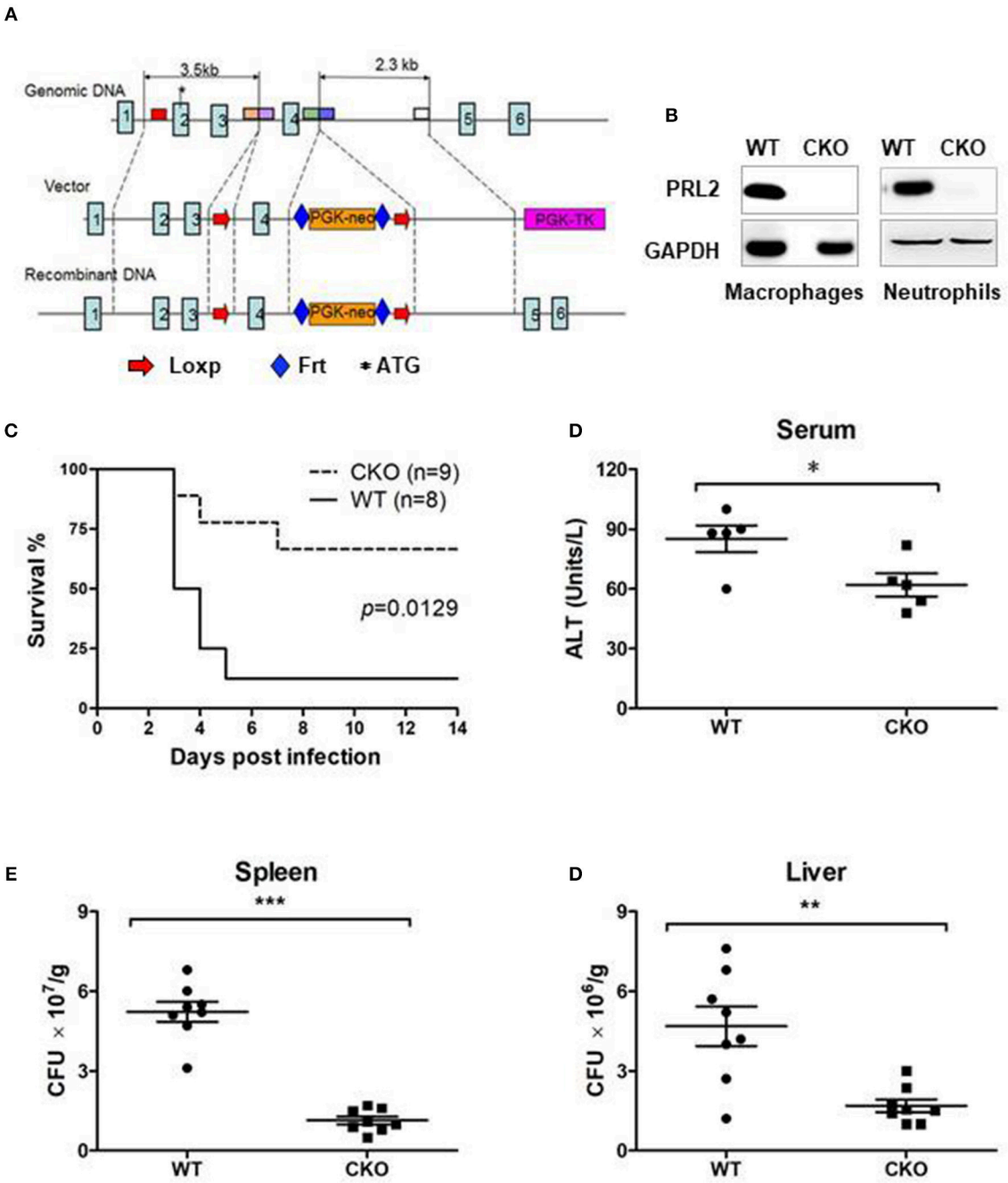

FIGURE 3 | PRL2 myeloid cell-deficient mice are resistant to lethal listeria infection**. (A)** The strategy of Ptp4a2$^{fl/fl}$ mice. LoxP sites were inserted at both ends of exon 4 of the *Ptp4a2* gene. **(B)** Peritoneal macrophages and bone marrow neutrophils were isolated from PRL2 myeloid cell conditional knockout mice (CKO) and their wild-type littermates (WT). Cell lysates were subjected to SDS-PAGE followed by immunoblot analysis using the indicated antibodies. **(C)** WT ($n = 8$) and PRL2 CKO mice ($n = 9$) were infected with 3.75×10^5 CFUs of *L. monocytogenes* i.v and the survival monitored. **(D)** Serum ALT levels were measured 24 h post infection. **(E,F)** Liver and spleen CFUs were determined 24 h post infection by colony-forming unit assay. Data are pooled from 2 independent experiments. Error bars represent the SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

and PRL2$^{-/-}$ macrophages producing significantly more ROS than WT cells. On the other hand, ectopic overexpression of PRL2 significantly inhibited ROS production in macrophages (**Figure 5C**).

In phagocytes, oxygen-dependent killing can be mediated by ROS or nitric oxide (NO). NO is produced by inducible NO synthase (iNOS) which require the cofactor NADPH (18). We measured iNOS expression and NO production in WT

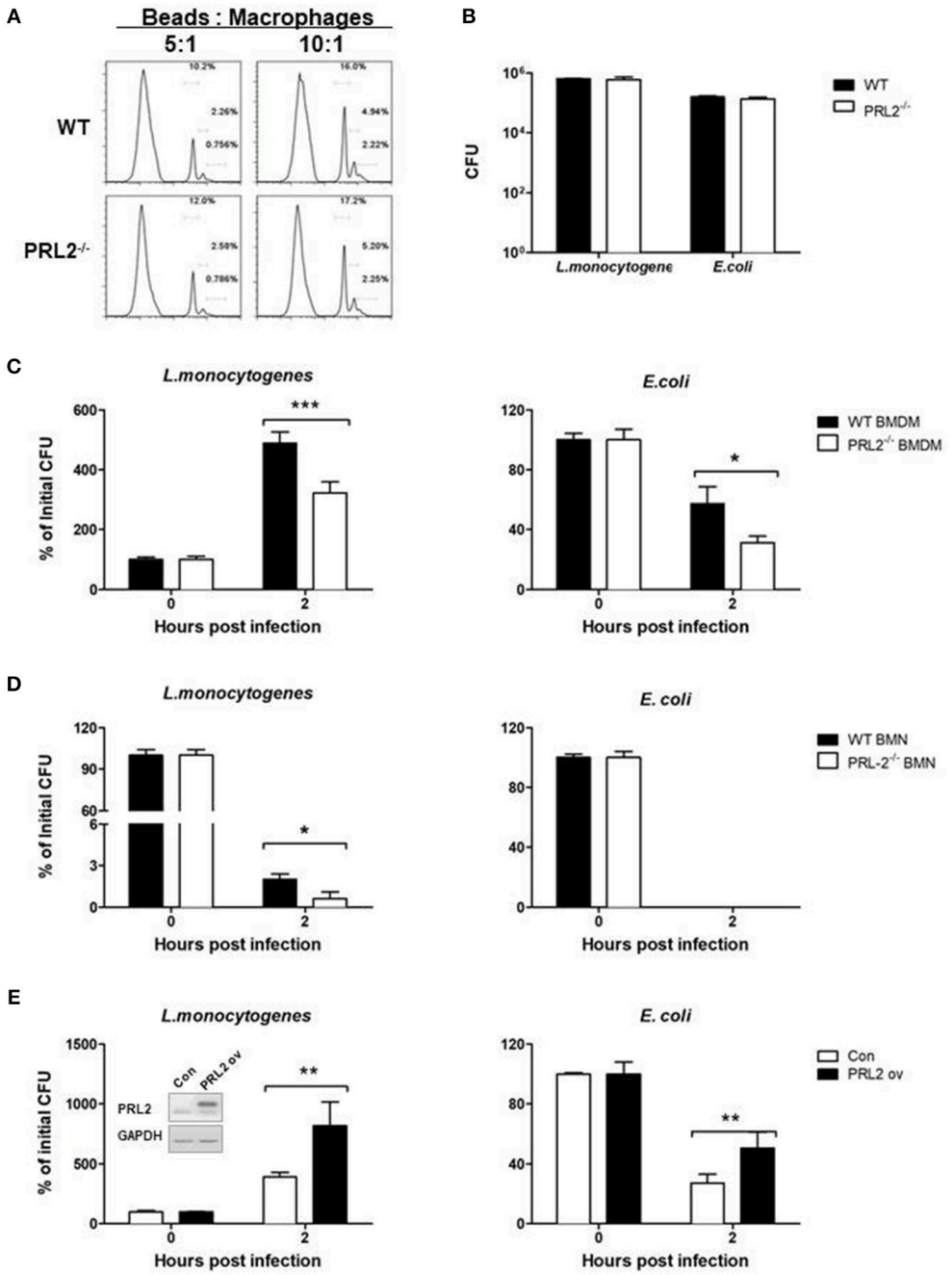

FIGURE 4 | PRL2 negatively regulates bactericidal activity of phagocytes. **(A)** Bone marrow-derived macrophages (BMDMs) from PRL2 CKO mice and wild-type littermates were incubated with fluorescently labeled 2 μm beads at a ratio of 5:1 or 10:1 (Beads: cells) for 30 min, The phagocytosis function of BMDMs was evaluated by flow cytometer assay. **(B)** BMDMs from PRL2 CKO mice and wild-type littermates were incubated with *Listeria monocytogenes* (*L. monocytogenes*) or *Escherichia coli* (*E. coli*) at MOI 10 (bacteria:cell) for phagocytosis analysis. Surviving intracellular bacteria were determined by colony-forming unit assay. **(C,D)** BMDMs and bone marrow neutrophils (BMNs) from WT or CKO mice were infected with *L. monocytogenes* or *E. coli* at a ratio of 1:1 for 20 min. After washing cells were incubated for additional 2 h. Bacterial numbers in the cells were determined by colony-forming unit assay. **(E)** Raw 264.7 cells were transfected with PRL2, or a control plasmid, and incubated with *L. monocytogenes* or *E. coli* at a ratio of 1:1 for 20 min. After washing cells were incubated for additional 2 h. The intracellular bacteria were determined by colony-forming unit assay. Data are pooled from three to four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

and PRL2 deficient macrophages. In response to bacterial component stimulation, PRL2 deficient macrophages produced more NO than WT cells, while the expression of iNOS was similar (**Supplementary Figure 2**). The above results suggested the activity of NADPH might be important in PRL2 associated bacterial killing.

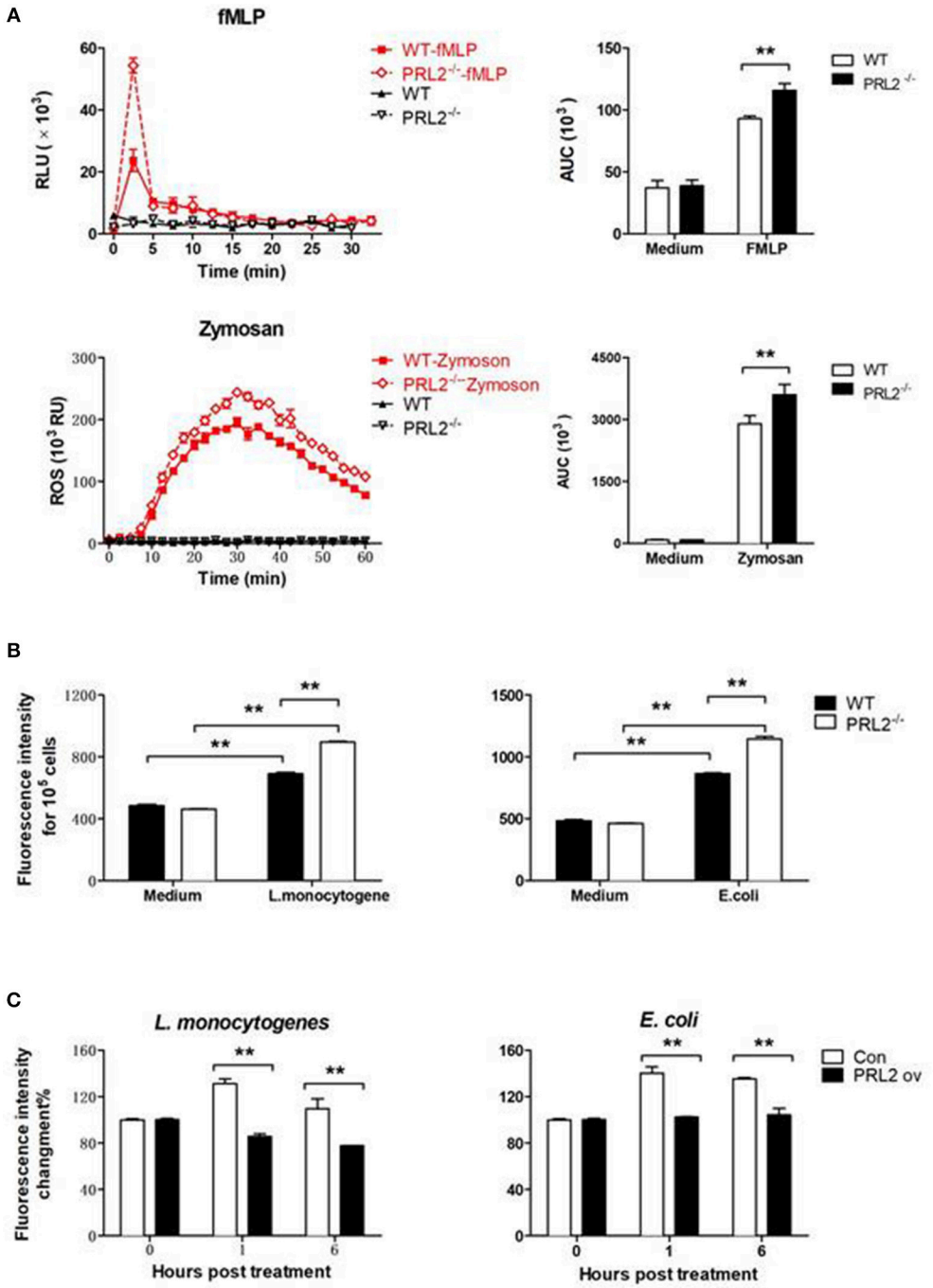

FIGURE 5 | PRL2 controls oxidative burst. **(A)** Neutrophils collected from PRL2 CKO mice and wild-type littermates were stimulated with 5 μM fMlP or 100 μg/ml Zymosan and ROS production was measured by chemiluminiscence assay as described in section Materials and Methods. Left, ROS kinetic plots of a representative experiment. Right, averaged area under cure (AUC) from 3 independent experiments. **(B)** WT and PRL2$^{-/-}$ BMDMs were treated with bacteria as indicated in graph for 30 min and ROS production was measured by fluorescent staining. **(C)** Raw264.7 cells were transfected with PRK5 or Myc-PRL2 PRK5 for 24 h. Cells were treated with *L. monocytogenes* or *E. coli* at MOI = 10 (bacteria vs. cells) for 30 min. ROS production was measured by fluorescent staining assay as above. Results shown are means ± SEM and are representative of more than three independent experiments. $^{**}p < 0.01$.

PRL2 Binds to Rac GTPase and Regulates its Activation

In antibacterial immune responses ROS are mainly generated by the NADPH-oxidase complex which is made up of 5 phagocytic oxidase units and a Rac GTPase. It has been reported that Rac GTPase is involved in the PRL signaling pathway (11), so we asked whether PRL2 could regulate Rac activation in innate immune responses as well. To address this question, we

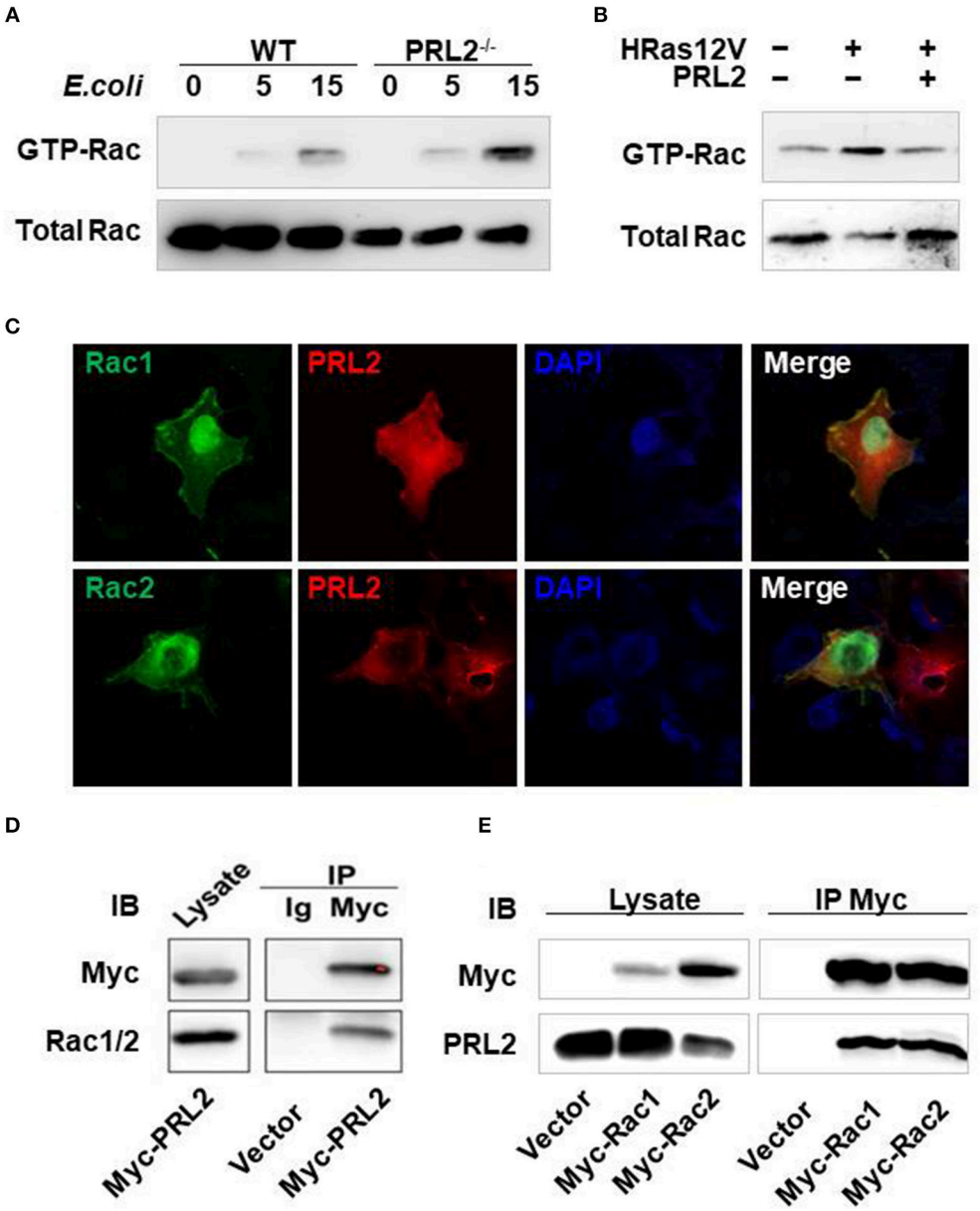

FIGURE 6 | PRL2 binds to, and regulates the activity of, Rac GTPase. **(A)** WT or PRL2$^{-/-}$ BMDMs were treated with *E.coli* (MOI = 10, bacteria vs. cell) for the indicated times. Cell lysates were subjected to pull-down using PAK GST beads. Lysate from pull-down and total lysate were subjected to SDS-PAGE followed by immunoblot assay with anti-Rac antibody. The experiments were repeated 2 times with similar results. **(B)** 293T cell was transfected with HRas12V or HRas12V and PRL2 for 8 hours. Active and total Rac was detected as above. The experiments were repeated 2 times with similar results. **(C)** COS7 cells on chamber slides were co-transfected with Rac1-EGFP, Rac2-EGFP and Myc-PRL2 and stained with anti-Myc Alexa 647 antibody (red). Images are representative of two independent experiments. **(D)** Lysate of 293T cells transiently transfected with Myc–PRL2 pRK5 constructs were immunoprecipitated with anti-Myc beads or anti-IgG beads. Immunoprecipitates and total cell lysate were subjected to SDS-PAGE for western blot. **(E)** 293T cells were transfected with pRK5 vector or expression plasmids for Myc-tagged Rac1 or Rac2. Cell lysates were collected for co-immunoprecipitation with anti-Myc beads 24 h post transfection. Immunoprecipitates and total cell lysates were treated as above. The experiments in panel d and e were repeated at least three times with similar results.

took 2 complementary approaches. First, we tested the effect of PRL2 deficiency on Rac activation in macrophages using a PAK pulldown assay. When cells were stimulated with *E. coli*, activated Rac GTPase was strongly induced in PRL2$^{-/-}$ macrophages (**Figure 6A**). Second, we tested the effect of PRL2 in 293T cells that did, or did not, express HRas12V. Rac serves as an essential downstream component of the signaling pathway by which oncogenic RAS induces cell transformation (19) and HRas12V is a mutation which replaces the amino acid glycine with the amino acid valine at position 12. This altered HRAS

protein is permanently active within the cell and causes Rac activation (15). As shown in **Figure 6B**, we found that PRL2 transfection inhibited HRas-induced Rac activation.

There are two members of Rac GTPases, Rac1 and Rac2. To further investigate the relationship between PRL2 and Rac we co-overexpressed Rac1/Rac2 and PRL2 in COS7 cells and measured their subcellular localization using immunofluorescence. Both Rac and PRL2 are membrane proteins. As expected, Rac1/Rac2 was enriched at the plasma membrane where it co-localized with PRL2. Co-localization of PRL2 and Rac was also observed in the cytosol. Overexpressed Rac1/Rac2 was also enriched in nucleus while PRL2 was distributed mainly at the nuclear envelope (**Figure 6C**). Since PRL2 and Rac were co-localized both in the cell membrane and the cytosol, we tested whether PRL2 could bind to Rac using a co-immunoprecipitation (Co-IP) assay. First, myc-PRL2 was expressed in 293T cells and upon blotting using an anti-Rac1/2 antibody a strong PRL2 signal was detected in the precipitates (**Figure 6D**), indicating that PRL2 interacted with endogenous Rac protein. Second, we expressed Myc-tagged Rac1 or Rac2 in 293T cells and detected endogenous PRL2 protein. We found that PRL2 interacted with both Rac1 and Rac2 (**Figure 6E**).

DISCUSSION

PRL proteins represent a group of protein tyrosine phosphatases that has been implicated in the development and metastasis of various types of cancer, however, little is known about their function in immune system. Here we report that PRL2 plays an important role in the innate immune response by sensing ROS and regulating ROS production. PRL2 is highly expressed across all tissues and organ systems (13). Orthology data show that PRL2 is highly conserved in mammalians and contain homologs in protozoa, worms, insects and vertebrates suggesting it may have a critical function. ROS represent an evolutionary ancient part of the innate immune response for fighting invading microbes (20). They are a highly reactive group of oxygen-containing molecules which act as important signaling messengers to regulate various biological and physiological processes, including certain immune response mechanisms (7). In this study, we revealed a relationship between mammalian PRL2 and ROS. We propose that PRL2 is a controller of respiratory burst. High levels of PRLs in resting leukocytes maintain redox homeostasis under normal conditions, while under inflammatory conditions reduced levels of PRL2 promotes oxidative burst in order to damage invading pathogens.

Data from PRL2 genomic knockout (KO)mice has shown that deletion of PRL2 leads to retarded growth both at birth and adult stage. PRL2 genomic KO mice are 20% smaller compared to their wild-type littermates throughout their adulthood, although their bone marrow cellularity is normal when normalized to total body weight (21). PRL2 was found to be a repressor of PTEN and required for a number of development processes (placenta formation, spermatogenesis and stem cell self-renewal) (21–23). In this study, we have focused on the role of PRL2 in innate immunity. We generated PRL2 myeloid cell-specific conditional knockout mice (CKO) and found that they display normal body weight as well as normal blood, spleen and bone marrow cellularity. Both *in vitro* data, using cells, and *in vivo* data, using *L.monocytogenes* infection, suggest PRL2 is involved in the innate immune response rather than in innate immune cell development. At the molecular level, we found the colocation of PRL2 and Rac on cell membrane and in cytosol. Co-IP data suggested PRL2 bind with Rac1 and Rac2. The most important source of ROS in phagocytes is NADPH-oxidase which is made up of 5 phagocytic oxidase units and a Rac GTPase (24). In the resting state, the oxidase units and Rac are separated; upon cell stimulation they assemble to form the active enzyme. This may explain our results where PRL2-deficient phagocytes only show differences after stimulation and not in a resting state.

PRLs share the CX5R active site, P-loop, and WDP loop motifs typical of PTPs, while the presence a CAAX prenylation motif next to a polybasic region make them a unique subfamily of PTPs (25). PTPs commonly possess a unique cysteine (cys) residue that is highly sensitive to oxidation by ROS (17) and it has been reported that the oxidation of PRL1 and 3 induces both intramolecular and intermolecular disulfide bond formation and that the biological function of PRLs can be regulated by oxidation (26). It is well established that ROS contributes to both physiological and pathological conditions via its involvement in redox signaling and oxidative stress (7). Here, we focus on the role of PRL2 in inflammation. Neutrophils and macrophages from inflamed sites expressed less PRL2 protein than cells from normal tissues. We found that the reduced levels of PRL2 were associated with high ROS levels in the tissue environment. In response to H_2O_2 treatment the PRL2 protein expression levels in neutrophils and macrophages were diminished within 15∼30 min suggesting that the stability of the PRL2 protein may be altered under conditions of oxidative stress. It has been reported that the cys residue at the active site and CAAX terminal of PRL are both sensitive to oxidation and that the complete oxidation of full-length PRL leads to protein precipitation (27, 28). This may contribute to the instability of PRL2 under oxidative stress.

ROS are used by the immune system as weapons against pathogens; however ROS may also cause tissue damage. Precisely regulating the intensity and timing of ROS production is critical for the host antibacterial immune response. Our findings may reveal a basic ROS regulation signal in animals and the identification of PRL2 functions in innate immunity may be useful in providing novel insights into the mechanisms of ROS generation and regulation, and might eventually lead to the development of more effective therapies against infectious diseases or for the control of immunopathogenic responses.

AUTHOR CONTRIBUTIONS

CY and ZW wrote the paper. ZW, CY ,and CW designed the experiments. YF, CW, XD, JW, LT, and JP performed and analyzed the data. WZ, YW, XG, and GC contributed reagents. GC and XG read the paper, and ZW oversaw the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02609/full#supplementary-material

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Sepsis Induces a Long-Lasting State of Trained Immunity in Bone Marrow Monocytes

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Innate immune memory describes the functional reprogramming of innate immune cells after pathogen contact, leading to either a boosted (trained immunity) or a diminished (immune tolerance) response to a secondary stimulus. Immune tolerance or "sepsis-induced immunosuppression" is a typical hallmark of patients after sepsis survival, characterized by hypo-responsiveness of the host's immune system. This condition renders the host vulnerable for a persisting infection or the occurrence of secondary, often opportunistic infections, along with an increased mortality rate. The mechanisms involved in the maintenance of this long-lasting condition are not examined yet. Polymicrobial abdominal sepsis was induced in 12 week old male C57BL/6 mice by cecal ligation and puncture. Mice were euthanized 3 months after insult. Immune cell composition of the spleen and whole blood, as well as stem and progenitor cells of the bone marrow, were assessed by flow cytometry. Whole blood and bone marrow monocytes were stimulated with LPS and supernatant levels of TNF and IL-6 detected by ELISA. Furthermore, naïve bone marrow monocytes were analyzed for metabolic (Seahorse technology) and transcriptomic (RNA sequencing) changes. Flow cytometric analysis revealed an increase of inflammatory monocytes and regulatory T cells in the spleen, whereby immune composition of whole blood kept unchanged. Granulocyte-monocyte progenitor cells are increased in sepsis survivors. Systemic cytokine response was unchanged after LPS challenge. In contrast, cytokine response of post-septic naïve bone marrow monocytes was increased. Metabolic analysis revealed enhanced glycolytic activity, whereas mitochondrial indices were not affected. In addition, RNA sequencing analysis of global gene expression in monocytes revealed a sustained signature of 367 differentially expressed genes. We here demonstrate that sepsis via functional reprogramming of naïve bone marrow monocytes induces a cellular state of trained immunity, which might be counteracted depending on the compartmental localization of the cell. These findings shed new light on the complex aftermath of sepsis and open up a new pathophysiological framework in need for further research.

Keywords: SIRS, CARS, immunosuppression, metabolism, immune memory

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INTRODUCTION

The immune system is historically divided into an innate and an adaptive branch. Adaptive immune responses, involving lymphoid immune cells, are slow, but specific for certain pathogens. Cells of the adaptive immune system are able to build up an organism-level immune memory as a prerequisite to respond faster to a second encounter (1). In contrast, innate immune responses representing the first line of host defense against invading pathogens are mediated by cells of the myeloid lineage with a fast kinetic and pathogen-unspecific recognition of conserved patterns (2). Further, they were classically proposed to lack memory function (3). In the last decades, this concept was counteracted. It has been shown that an activation of innate immune cells is believed to leave "immunological scars" on the cellular level, leading to either an boosted (training) or a diminished (tolerance) response to a secondary stimulus. This phenomenon of cellular adaption was termed "innate immune memory" (4–6).

Mechanisms involved in the regulation of this long-term innate immune memory is regulated are still under consideration. Recent studies demonstrate the involvement of metabolic reprogramming in this process (7, 8). For example, as a central mechanistic prerequisite of trained immunity, the shift from oxidative phosphorylation toward aerobic glycolysis has been proposed (Warburg effect) (9, 10). Furthermore, not only glucose metabolism but also such pathways as glutaminolysis or cholesterol synthesis were shown to play critical roles in the induction of a trained state (11, 12). Underlying these metabolic changes, epigenetic reprogramming is considered to be the main regulator of trained immunity (13). Concerning the short half-life of innate immune cells, especially monocytes in the circulation, a reprogramming of hematopoietic progenitor cells in the bone marrow was shown to be involved in the long-term maintenance of those effects (13–15).

Sepsis is a life-threatening syndrome, triggered by an infection which initiates a strong systemic release of inflammatory mediators. This cytokine storm during the acute phase of sepsis can lead to hypotension, cardiovascular dysfunction, tissue damage, and multiple organ failure. Simultaneously, an output of anti-inflammatory cytokines occurs to restrict the damage of the inflammatory reaction (16, 17). In the late phase of sepsis, the latter reaction overwhelms, counteracts host's initial response and can lead to a systemic state of immune tolerance. This so-called sepsis-induced immunosuppression can persist for years, rendering patients susceptible to persistent and secondary nosocomial infections, associated with an increased mortality rate (16–19).

Since there is growing evidence that innate immune memory plays a crucial role in the maintenance of post-septic immunosuppression (20), we used a polymicrobial animal model of sepsis to shed light on this point. We here prove against our expectations that sepsis induces a trained immunity phenotype in naïve bone marrow monocytes months after the initial insult, whereas the systemic response remained unaltered.

MATERIALS AND METHODS

Mice

All animal procedures were conducted in accordance with the German Animal Welfare Act law and were approved by the regional council Karlsruhe (reference number G-132/15).

Twelve week old male C57BL/6 mice were purchased from Janvier Laboratories (Le Genest Saint Isle, France). All animals were housed in a 12 h light/dark cycle at 22°C, receiving food and water *ad libitum*. Mice were acclimatized for 7 days before conduct of any experimental procedure.

Polymicrobial Sepsis Model

For the investigation of post-septic immunological consequences a cecal ligation and puncture (CLP) mouse model was used as described before (21). Briefly, mice were anesthetized by an intraperitoneal injection of 100 mg/kg ketamine (Ketanest®S, Pfizer Pharma, Berlin, Germany) and 20 mg/kg xylazine (Xylavet, CP-Pharma, Burgdorf, Germany). After median laparotomy, cecum was mobilized, ligated (5 mm).and punctured once with a 23 G needle (BD Microlance™ 3, BD Medical, Heidelberg, Germany). Fecal content was gently extruded and the cecum afterwards relocated. Mice were supplemented with 400 µL 0.9% sodium chloride (B. Braun, Melsungen, Germany), given directly in the abdominal cavity and abdomen was closed thereafter with a double suture. Control animals received a sham surgery without cecal ligation and puncture. For pain relieve, mice were treated with 0.05 mg/kg bodyweight buprenorphine (Temgesic, RB Pharmaceuticals, Slough, UK) every 8 h for 2 days after surgery. In total, 15 animals received a CLP and 9 animals a sham surgery. All animals were subsequently housed till euthanasia 12 weeks after intervention (**Figure 1A**).

Cell Isolation

Mice were euthanized via cardiac puncture. Whole blood was collected in heparinized (Heparin-Natrium-25000-Ratiopharm, Ulm, Germany) syringes (BD Plastipak, BD Medical, Heidelberg, Germany).

Spleen was dissected and weighed. For dissociation of splenic tissue, the spleen was passed through a 70 µm cell strainer (Greiner BioOne, Frickenhausen, Germany). Cells were centrifuged and resuspended in ACK lysis buffer [0.15 M NH_4Cl (Riedel-de-Haen, Seelze, Germany), 1 mM $KHCO_3$ (Merck, Darmstadt, Germany), 0.1 mM Na_2EDTA (Life Technologies, Darmstadt, Germany)] for red blood cell lysis. After centrifugation, pellet was resupended in PBS and cells counted.

Bone marrow was extracted from femurae and tibiae of CLP and control mice as follows. Bones were prepared by removing all soft tissue without fracturing, followed by a decapping of the bone on both sides. Subsequently, bone marrow was flushed out with pre-warmed RPMI 1640 media (Life Technologies, Darmstadt, Germany) supplemented with 10% Ultra-low endotoxin FCS (Cell concepts, Umkirch, Germany). 10^7 cells were set aside for flow cytometric analysis.

Naïve bone marrow monocytes were isolated by negative selection using magnetic bead-based MACS technology

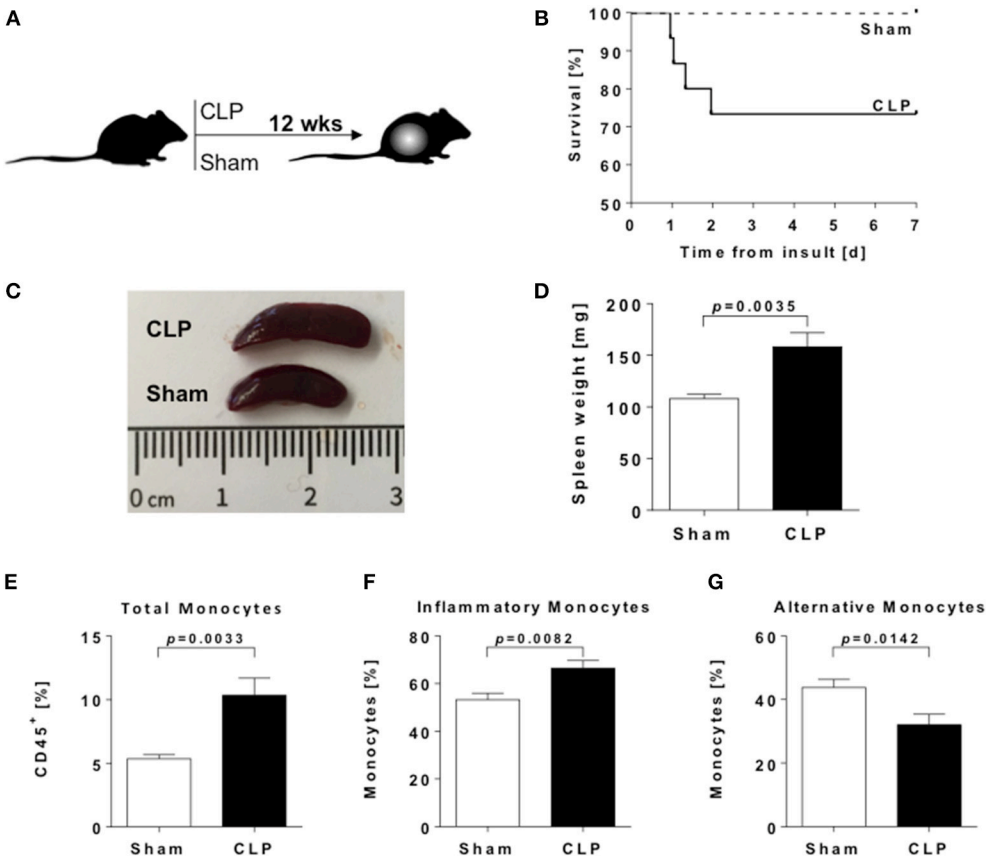

FIGURE 1 | Splenomegaly and enhanced frequencies of splenic myeloid cells in sepsis-survivors. **(A)** Experimental design of the study. **(B)** Survival rate of male C57BL/6 mice after CLP ($n = 15$; solid line) or sham ($n = 9$; dashed line) procedure. **(C)** Representative spleens and **(D)** spleen weights from CLP (black bar) and sham (open bar) animals twelve weeks postoperative ($n = 9$ per condition). Spleens were dissected and immune cell populations measured by flow cytometry. Frequencies of **(E)** total monocytes ($CD11b^+$ $F4/80^-$), **(F)** inflammatory ($CD11b^+$ $F4/80^-$ $Ly6C^+$), and **(G)** alternative monocytes ($CD11b^+$ $F4/80^-$ $Ly6C^-$). Data are represented as mean ± SEM, $n = 8$ per condition.

(Monocyte Isolation Kit (BM), Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, bone marrow cells were incubated with FcR Blocking Reagent and Monocyte Biotin-Antibody Cocktail. After washing, cells were incubated with Anti-Biotin MicroBeads and separation was facilitated on an AutoMACS Pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany) via depletion of labeled non-monocyte cells. Quality control was performed by flow cytometry.

Flow Cytometry

Whole blood (50 µl) was incubated with CD45 Pacific Blue (BioLegend, San Diego, USA), CD11b FITC (BioLegend, San Diego, USA), F4/80 PE (BioLegend, San Diego, USA), and Ly6C PerCP-Cy5.5 (BD Biosciences, Heidelberg, Germany) for identification of myeloid cells and with CD45 APC-Cy7 (BioLegend, San Diego, USA), CD4 PerCP-Cy5.5 (BioLegend, San Diego, USA), CD25 PE (BioLegend, San Diego, USA), and CD127 APC (BioLegend, San Diego, USA) for identification of regulatory T cells. Incubation was done for 30 min at 4°C in the dark. Subsequently, erythrocytes were lysed by adding FACS lysing solution (BD Biosciences, Heidelberg, Germany) and cells analyzed on a FACSverse flow cytometer (BD Biosciences, Heidelberg, Germany).

Splenic cells (10^6 cells per tube) were stained with CD45 APC (BioLegend, San Diego, USA), CD4 PerCP/Cy5.5 (BD Biosciences, Heidelberg, Germany), CD25 PE (BioLegend, San Diego, USA), CD127 APC (BioLegend, San Diego, USA), CD3e FITC (BD Biosciences, Heidelberg, Germany), CD8a PE/Cy7 (BioLegend, San Diego, USA) and CD19 eFluor 450 (Thermo Fisher Scientific, Dreieich, Germany) for regulatory T cells, CD45 eFluor450 (Thermo Fisher Scientific, Dreieich, Germany), CD11b FITC (BD Biosciences, Heidelberg, Germany), F4/80 PE (BioLegend, San Diego, USA), Ly6C PerCP/Cy5.5 (BioLegend, San Diego, USA), and CD11c (BioLegend, San Diego, USA) for myeloid cells. FMO control for Ly6C was used for proper gate placement.

For the analysis of hematopoietic progenitor cell composition in bone marrow, isolated cells were stained with Lineage Pacific Blue (Biolegend, San Diego, USA), Sca-1 PE/Cy7 (Biolegend, San Diego, USA), CD117 (c-Kit) APC (Biolegend, San Diego, USA), CD34 FITC (Miltenyi Biotec, Bergisch Gladbach, Germany),

CD16/32 PE (Miltenyi Biotec, Bergisch Gladbach, Germany), CD48 APC/Cy7(Biolegend, San Diego, USA), and CD150 PerCP/Cy5.5 (Biolegend, San Diego, USA). FMO control tubes for CD34, CD16/32 and CD150 were included for proper gate adjustment. Cells were fixed with 4% PFA and detection was performed on a FACSverse flow cytometer (BD Biosciences, Heidelberg, Germany).

Quality control was performed via flow cytometry. Therefore, bone marrow monocytes ($1{\cdot}10^5$ cells) were centrifuged and stained with CD11b PE antibody (BioLegend, San Diego, USA) and Ly6C FITC antibody (BioLegend, San Diego, USA).

Ex vivo Stimulation and Cytokine Analysis

Heparinized whole blood from left-ventricular cardiac puncture was transferred into a 96-well plate (Sarstedt, Nümbrecht, Germany) and diluted 1:1 with RPMI 1640 Media (Life Technolgies, Darmstadt, Germany) supplemented with 10% Ultra-low endotoxin FCS (Cell Concepts, Umkirch, Germany). Stimulation was performed with 200 ng/mL LPS (O111:B4, Ultrapure, Invivogen, Toulouse, France) or solvent as control.

Isolated monocytes were counted using a Scepter™ 2.0 cell counter (Merck, Darmstadt, Germany). 2×10^5 cells were seeded into a 96-well plate (Sarstedt, Nuembrecht, Germany) with RPMI 1640 Media (Life Technologies, Darmstadt, Germany) supplemented with 10% Ultra-low endotoxin FCS (Cell Concepts, Umkirch, Germany). After 1 h rest, cells were stimulated as described above.

After incubation for 24 h (37°C, 5% CO_2), the supernatants from whole blood as well as monocytes were collected and TNF-α and IL-6 concentrations were determined using Mouse TNF-α and IL-6 DuoSet ELISA (R&D Systems, Minneapolis, USA), respectively, according to manufacturer's instruction.

Extracellular Flux Analysis

Mitochondrial function (oxidative phosphorylation) and glycolytic rate of naïve bone marrow monocytes were assessed using a modified variant of the XFp Cell Mito Stress Kit and a Seahorse XFp Analyzer (both Agilent Technologies, Waldbronn, Germany). In brief, monocytes were washed with Seahorse XF Base Medium (Agilent Technologies, Waldbronn, Germany) supplemented with 10 mM glucose (Sigma Aldrich, Taufkirchen, Germany), 5 mM HEPES (Agilent Technologies, Waldbronn, Germany), 2 mM glutamine (Life Technologies, Darmstadt, Germany) and 1 mM pyruvate (Life Technologies, Darmstadt, Germany), and seeded in Cell-Tak (Corning, Kaiserslautern, Germany) coated 8-well Seahorse plates (1.5×10^5 cells per well). All experiments were performed with two technical replicates. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured consecutively three times under basal conditions and after sequential injection of Oligomycin A (1 μM), FCCP (2 μM), Rotenone/Antimycin A (0.5 μM; all three reagents included in XFp Cell Mito Stress Kit, Agilent Technologies, Waldbronn, Germany) and finally 2-Deoxyglucose (50 mM) (Sigma Aldrich, Taufkirchen, Germany). Evaluation and calculation of mitochondrial and glycolytic indices was done using Wave software (Agilent Technologies, Waldbronn, Germany).

RNA-Seq

RNA from naïve bone marrow monocytes was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions after lysis with RLT Buffer containing 1% β-mercaptoethanol (Roth, Karlsruhe, Germany) and homogenization using QIAshredder (Qiagen, Hilden, Germany). Concentration and purity was determined with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany) and integrity of RNA was assessed using the RNA 6000 Nano Kit and a Bioanalyzer (both Agilent Technologies, Waldbronn, Germany). RNA-seq including sample preparation, library preparation, and Illumina sequencing was performed by GATC Biotech AG (Konstanz, Germany). Raw sequencing data of individual samples is publicly available from the NCBI BioProject repository (https://www.ncbi.nlm.nih.gov/bioproject/) under the BioProject ID PRJNA488339.

RNA-Seq Reads Processing, Mapping

Initial quality control using FastQC (22) was performed for the available RNA-seq datasets. Subsequent processing included filtering with SortMeRNA (23) to remove contaminants of ribosomal RNA as well as trimming of short or low quality reads and TruSeq adapter sequences by Trimmomatic (24) software.

For main processing, the remaining reads were mapped to *Mus musculus* release M17 (GRCm38.p6) reference genome available from the GENCODE project (https://www.gencodegenes.org) using STAR (24) alignment software. Comprehensive gene annotation on the primary assembly (chromosomes and scaffolds) was chosen as superset of the main annotation. Unambiguously mapped and unique reads were kept. SAMtools (25) was used to convert the resulting sequence alignment maps to sorted binary alignment format (BAM) for downstream analysis.

Differential Expression, Gene-Ontology Term Analyses

Feature counting was performed using HTSeq (26) for all replicates against the respective GRCm38.p6 gene transfer file. Since all samples were represented by two technical replicates, count data was initially merged per sample to conserve existing counts while maintaining independence of the available biological replicates. DESeq2 (27) was used for differential expression analysis of count data. The differentially expressed genes as identified by DESeq2 were filtered to results with absolute linear fold change values above 1.5 and p-values below 0.02.

Over-represented GO-terms were identified by use of Genomatix Genome Analyzer (Genomatix, Munich, Germany) separately for both up- and down-regulated gene sets.

Heatmap Generation, Principle Component Analysis

For heatmap visualization, library-size normalized count data of filtered differentially expressed genes were selected. To generate an informative heatmap, normalized count values per

gene were standardized to z-scores. The final heatmap was clustered per gene as well as per experimental sample based on Ward's hierarchical agglomerative clustering method (Euclidean distance measure; Ward2 criterion).

Principle component analysis was performed on the standardized library-size normalized count data for differentially expressed genes.

Statistics

Statistical data analysis was carried out in GraphPad Prism (V6.0 for Windows, GraphPad Software, La Jolla, USA). For group comparisons, unpaired *t*-test (two-tailed) was performed. Bar-graph data is represented as mean ± SEM. Survival rate is represented as Kaplan-Meier curve. For all analysis, statistical significance was assumed at $p < 0.05$.

RESULTS

Sepsis Induces Sustained Alterations of Spleen Morphology and Cell Composition

To investigate the long-term consequences of sepsis on innate immunity, we used the polymicrobial CLP mouse model. Overall mortality rate of the CLP group was 28% (4/15 animals), while no animal of the control group (Sham) died (**Figure 1B**). Enhanced loss of body weight and a higher clinical score in the CLP group indicates the successful induction of sepsis (**Supplementary Figures 1A,B**).

Twelve weeks after CLP or Sham surgery, we found markedly enlarged spleens with higher weights in post-septic animals (**Figures 1C,D**). Frequencies of monocyte populations were analyzed by flow cytometry (**Supplementary Figure 1C**). We found a significant increase of frequencies in total $CD11b^+$ $F4/80^-$ splenic monocytes after sepsis (**Figure 1E**). We further distinguished monocytes due to their functional properties: $Ly6C^+$ inflammatory monocytes (**Figure 1F**) were significantly increased, whereas $Ly6C^-$ alternative monocytes (**Figure 1G**) were significantly decreased in the post-septic spleen. Frequencies of splenic $CD11b^+$ $F4/80^+$ macrophages were enhanced as well (**Supplementary Figure 1D**).

Further evaluation of $CD4^+$ $CD25^+$ $CD127^{dim}$ regulatory T cell (T_{reg}) frequencies (**Supplementary Figure 1E**) revealed an increase of these immunosuppressive cells (**Supplementary Figure 1F**) as well.

The Systemic Immune Response Is Not Altered in Post-septic Mice

Next we asked, if such a condition can be found in the blood as well. We performed flow cytometric analysis of monocyte as well as T_{reg} frequencies in whole blood (**Supplementary Figures 2A,B**). We found no significant change in either total monocyte frequency or the subgroups of inflammatory or alternative monocytes in survivors of sepsis (**Figures 2A–C**). Nevertheless, contrasting to the results in spleen, $Ly6C^+$ inflammatory monocytes (**Figure 2B**) rather showed a decreasing trend, whereas $Ly6C^-$ alternative monocytes (**Figure 2C**) were slightly increased in whole blood. T_{Regs} were also marginally increased after CLP (**Supplementary Figure 2C**), in line with the results of the spleen. We evaluated the overall responsivity of the blood cells upon LPS stimulation and found no differences in the TNF-α and IL-6 output between CLP and sham mice (**Figures 2D,E**).

Together, these results show that there is no long-term impact of sepsis on the systemic immune response in mice 12 weeks after insult.

Sepsis Induces a Sustained Shift Toward an Increased Myeloid Hematopoiesis

Bone marrow is largely constituted of hematopoietic stem cells and thereby the source of newly generated immune cells for the steady replenishment of circulating cells as well for as the recruitment to tissues during e.g., infection. Evidence is mounting that inflammatory processes impact monocyte development and alter cellular functions already on this level (28). We characterized hematopoietic and progenitor cells from whole bone marrow of post-sepsis and sham mice by flow cytometry (**Supplementary Figure 3**).

Twelve weeks after insult, no differences in frequencies of long-term hematopoietic stem cells (LT-HSCs) (**Figure 3A**) were observed, while frequencies of short-term hematopoietic stem cells (ST-HSCs) were slightly decreased (**Figure 3B**). Other progenitor cells populations, including multipotent and common myeloid progenitors (MPP/CMP) as well as megakaryocyte-erythrocyte progenitors (MEPs) (**Figure 3E**).did only show subtle differences between the groups (**Figures 3C–E**). In contrast, late-stage granulocyte-monocyte progenitors (GMP) are significantly enriched (**Figure 3F**), indicating a sustained shift to myelopoiesis.

Naïve Bone Marrow Monocytes of Post-septic Animals Exhibit a Trained Immunity Phenotype

To evaluate if sepsis alters the function of naïve monocytes in a long-term manner, we stimulated MACS-sorted bone marrow monocytes with LPS and measured the production of TNF and IL-6. Compared to animals without experienced sepsis, supernatant concentrations of both cytokines were significantly elevated after stimulation (**Figures 4A,B**), resembling a typical hallmark of trained immunity.

An additional characteristic of trained immune cells is the shift of cellular metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis (7, 28). To proof, whether bone marrow monocytes are "trained" by sepsis, we conducted measurements of mitochondrial and glycolytic function by Seahorse. Basal glycolysis was significantly enhanced, while compensatory glycolysis was not elevated in CLP animals (**Figures 4C,D**). Also, indices of mitochondrial function were not altered (**Supplementary Figure 4**).

In summary, our finding of enhanced cytokine response as well as increased glycolysis is indicative for a trained state of naïve bone marrow monocytes in the post-septic mice.

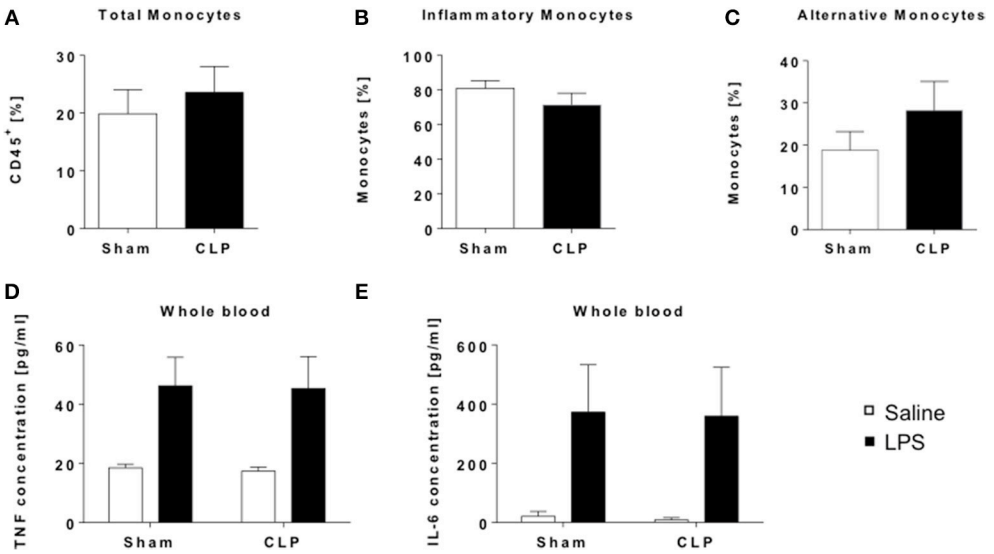

FIGURE 2 | Whole blood analysis revealed no systemic alterations in the immune appearance of CLP mice. Immune cell composition of whole blood from CLP (black bar) and sham mice (open bar) was determined by flow cytometry. Frequencies of **(A)** total monocytes (CD11b$^+$ F4/80$^-$), **(B)** inflammatory (CD11b$^+$ F4/80$^-$ Ly6C$^+$), and **(C)** alternative monocytes (CD11b$^+$ F4/80$^-$ Ly6C). For functionality assessment whole blood was stimulated with either LPS (black bar) or saline (open bar) for 24 h and cytokine levels in supernatants detected by ELISA: **(D)** TNF, and **(E)** IL-6. Data are represented as mean ± SEM, $n = 9$ per condition for FACS and $n = 5$ per condition for stimulation experiments.

RNA-Seq Analysis Reveals Long-Term Transcriptomic Alterations in Naïve Bone Marrow Monocytes

Transcriptional alterations were described to be involved in trained immunity (20). To elucidate a mechanistic background for our functional findings, we performed RNA-seq analysis of naïve bone marrow monocytes. We found a total of 367 differentially expressed genes in post-septic monocytes compared to control, from which 73 were up- and 294 down-regulated (linear expression changes >1.5-fold; $p < 0.02$) (full list available as **Supplementary File 1**). Principal component analysis of those 367 genes revealed a distinct separation of the animals according to their prior exposure (**Figure 5A**). Additionally, genes were analyzed for overrepresentation of biological functions. Upregulated genes were involved in processes like "cell migration" or "response to cytokines" (**Figures 5B,C**), whereas down-regulated genes were involved in "positive regulation of cilium assembly" or "carbohydrate derivative metabolic process" (**Figures 5B,D**).

Altogether our findings indicate an induction of trained immunity in naïve bone marrow monocytes as a long-term consequence of sepsis. Metabolic, as well as transcriptomic alterations, are involved in establishing this state.

DISCUSSION

In our study, we used an animal model of polymicrobial sepsis to investigate the consequences of a survived sepsis on innate immune cells, especially monocytes, in a clinically relevant timeframe.

The first finding was a persisting splenomegaly in animals after sepsis. Splenomegaly is typically associated with the expansion of immune cell populations and therefore an important feature of acute and chronic infections. We found increased frequencies of monocytes in post-septic spleens, with enhanced inflammatory and decreased alternative monocytes. Using a similar model, Valdes-Ferrer and colleagues found splenomegaly as well as increased frequencies of splenic monocyte populations within 10 days after CLP, but decreasing over (29). Contrasting to our findings, 12 weeks post-CLP both values returned back to baseline in their study. The opposing outcomes might be explained by the use of different mouse strains: Valdes-Ferrer used Th2-biased Balb/c mice, whereas we used Th1-biased C67BL/6 mice (30, 31).

Regulatory T cells are associated with immunosuppression and involved in the maintenance of self-tolerance (32). Our study shows enhanced frequencies of regulatory T cells in spleen and blood of animals 12 weeks after sepsis. Elevation of splenic regulatory T cells with higher repressive functionality in the post-septic environment has been described before in mice 15 days after CLP (33). This study further demonstrated that the expansion was caused by a dendritic cell mediated conversion of CD4$^+$ CD25$^-$ T cells to CD4$^+$ CD25$^+$ regulatory T cells. The co-occurrence of both inflammatory as well as immunosuppressive conditions hints toward a compartmentalized and cell-mediated counterbalance of innate immunity within the spleen.

Bone marrow is the central source of immune cells in the developed organism and is capable to respond to changing demands of the organism, e.g., during infection or injuries. Sepsis has been described to exhibit a suppressive influence on hematopoiesis, resulting in myelosuppression (34, 35).

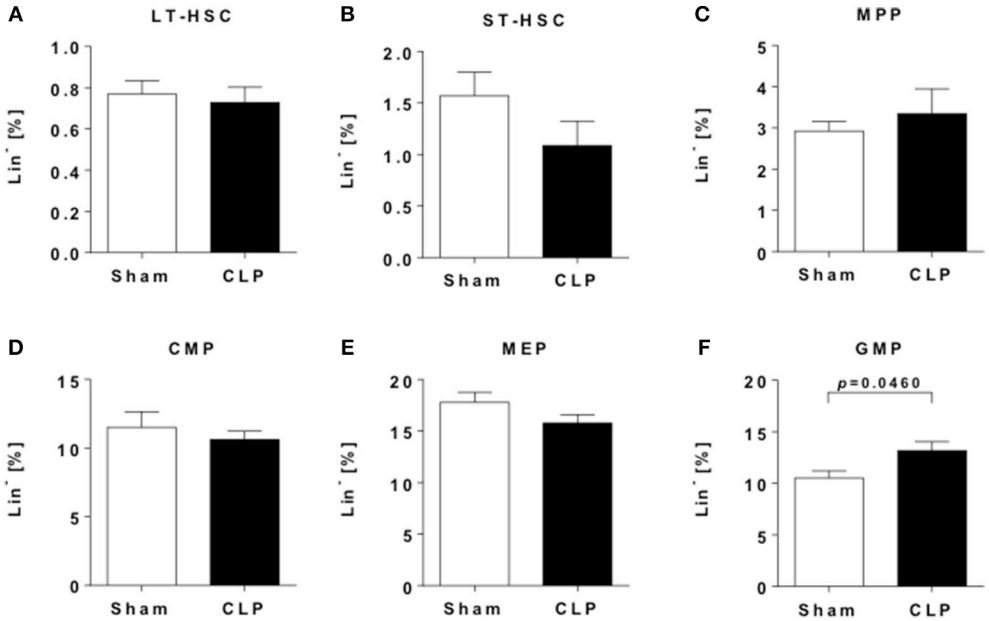

FIGURE 3 | Sepsis induces an increase in myeloid progenitor cells. Hematopoietic progenitor and stem cells (HPSCs) from the bone marrow of CLP (black bar) and sham (open bar) mice were evaluated by flow cytometry. Frequencies of **(A)** LT-HSC (LSK CD48$^-$ CD150$^+$), **(B)** ST-HSC (LSK CD48$^-$ CD150$^-$), **(C)** MPP (LSK CD48$^+$ CD150$^-$), **(D)** CMP (LS$^-$K CD16/32^{int} CD34$^+$), **(E)** MEP (LS$^-$K CD16/32$^-$ CD34$^-$), and **(F)** GMP (LS$^-$K CD16/32$^+$ CD34$^+$). Data are represented as mean ± SEM, $n = 9$ per condition.

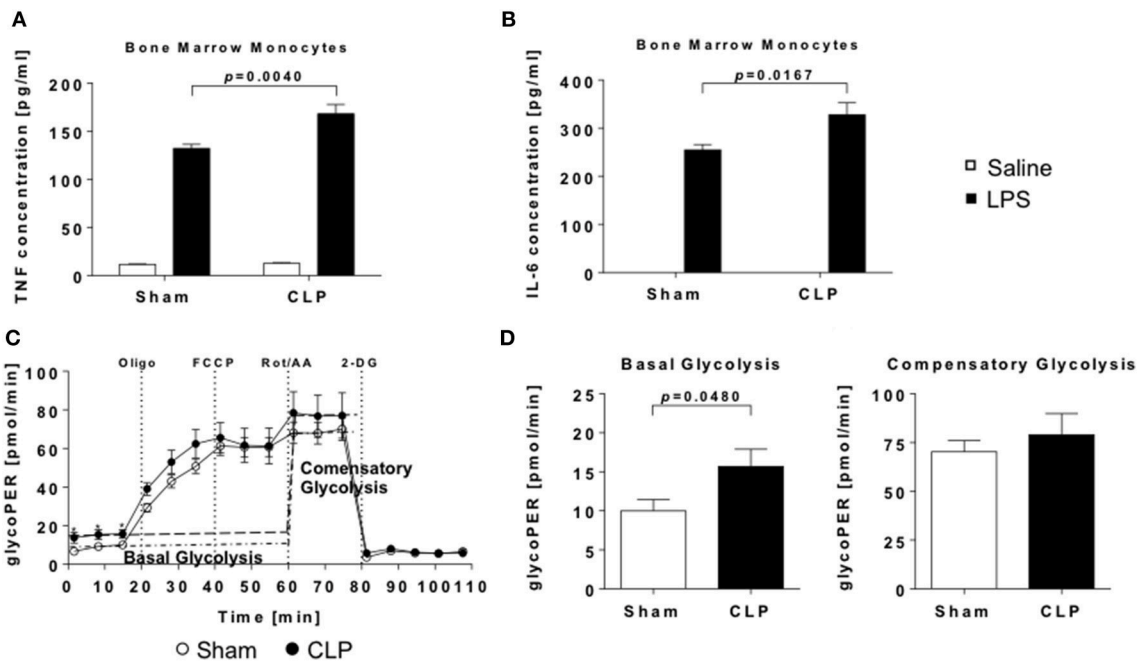

FIGURE 4 | Enhanced inflammatory response to LPS and increased glycolytic activity of post-septic naïve bone-marrow monocytes. Monocytes were stimulated with LPS or saline for 24 h and levels of **(A)** TNF and **(B)** IL-6. in supernatants detected by ELISA. Metabolic activity of unstimulated monocytes was measured using Seahorse technology. **(C)** Cumulative glycolytic proton efflux rate and **(D)** evaluation of basal and compensatory glycolysis of bone marrow monocytes from CLP (black points/bars) or sham (open points/bars) mice. Data are represented as mean ± SEM, $n = 9$ per condition.

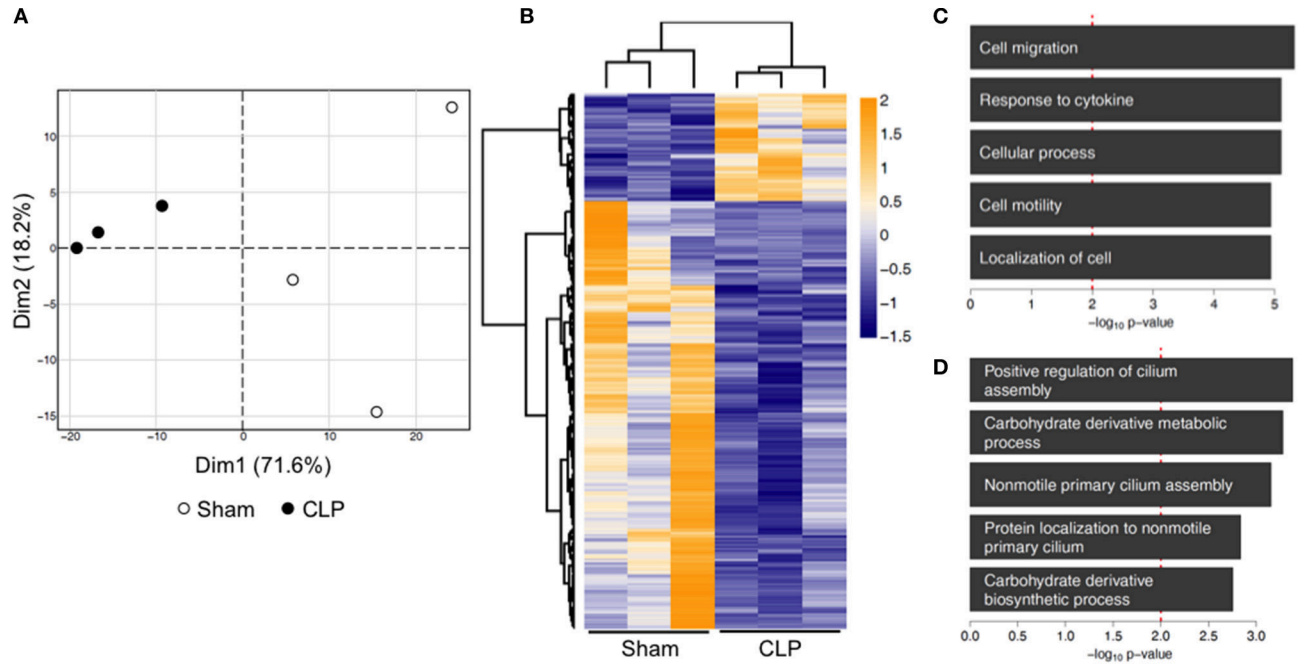

FIGURE 5 | Transcriptional reprogramming of naïve bone-marrow monocytes 12 weeks post-CLP or sham. RNA of bone-marrow monocytes was isolated and whole genome transcriptome analysis performed by RNA-seq. **(A)** Principal component analysis of 367 genes identified as differentially expressed (linear change in expression of >1.5-fold; $p < 0.02$). **(B)** The heat map is representing standardized counts of differentially expressed genes in post-septic bone marrow monocytes. **(C)** Gene ontology term analysis, showing the top five upregulated and **(D)** downregulated biological processes. $n = 3$ per condition.

Opposing to these results, we found a significant increase of GMPs in post-septic animals, indicating a rather enhanced myelopoiesis. Moreover, we found slightly increased MPP populations, whereas ST-HSCs are marginally decreased. The controversial results compared to literature could be explained by different time points of assessment: both, the studies of Rodriguez et al. and Zhang et al. investigated HSPCs as early as 24 h after septic insult, a time point where early compensatory mechanism might still be present to counteract initial responses. Our investigation was performed long after insult, proving a sustained shift from myelosuppression to enhanced myelopoiesis, possibly as an adaptive mechanism after the clearance of the acute inflammatory tone of the system.

Our results are consistent with (36), who recently demonstrated enhanced myelopoiesis along with an increase of MPP populations as well as decreased ST-HSCs in animals after stimulation with β-glucan, the archetypical model of "trained immunity." This effect diminished 28 days after stimulation, but GMPs remained significantly increased, similar to our results. These effects associated with trained immunity, induced by the stimulation with the *Candida albicans* cell wall component β-glucan can also be triggered by western diet, most probably due to a change in the microbiota composition in the gut (37). Considering our polymicrobal sepsis model, trained immunity could be induced by gastrointestinal-derived fungi, which enter circulation together with other bacterial pathogens via the extruded feces.

Since trained monocytes are characterized by an enhanced pro-inflammatory cytokine release after a second stimulus, we isolated bone marrow monocytes and analyzed their responsivity. We found an enhanced response after LPS stimulation, indicated by significantly increased level of TNF-α as well as IL-6 in the supernatant. Those functional results point toward a trained immune state after sepsis on the level of the bone marrow, similar to what has been described before for β-glucan stimulation (38, 39).

Another hallmark of trained immune cells is enhanced glycolysis, often described in combination with a reduction of oxidative phosphorylation and termed "aerobic glycolysis" (40, 41). We found indeed an increased glycolysis in monocytes of CLP animals, but could not determine any changes in oxygen consumption. A possible explanation for that phenomenon is, that monocytes investigated in our study were unstimulated, whereas innate immune cells described in metabolic studies of trained immunity were regularly analyzed after LPS re-stimulation. This second stimulus might be necessary for the full shift to aerobic glycolysis. Nevertheless, unstimulated cells seem to be already primed toward an enhanced glycolysis.

As described by several publications, transcriptional upregulation of genes involved in glycolysis as well as genes involved in mTOR (mammalian target of rapamycin) signaling, a key regulator of glucose metabolism, are responsible for the enhanced glycolytic activity (7, 11, 12, 36). To investigate, if post-septic bone-marrow monocytes show similar alterations, we performed whole-genome sequencing by RNA-seq. We found

several genes linked to metabolic processes to be differentially expressed. For example, the gene encoding Ribulosephosphat-3-epimerase (RPE) was downregulated in monocytes of post-septic animals. RPE is involved in the regulation of the non-oxidative branch of the pentose phosphate pathway (PPP) and catalyzes the back reaction from PPP to glycolysis. Arts et al. (11) showed by NMR experiments with ^{13}C-labeled glucose that the non-oxidative branch of PPP is inactive in trained immune cells. That seems to be induced by reduced RPE, driving PPP output toward purine metabolism.

Increased purine metabolism was described in trained immune cells to fulfill the increased nucleotide demand evoked by enriched transcription (8, 11). As described above, PPP output is skewed toward purine synthesis due to RPE downregulation. However, the gene *Nme7*, whose product catalyzes the transfer of phosphate from nucleoside triphosphates to nucleoside diphosphates and vice versa, is downregulated in post-septic monocytes. Also, we found genes involved in purine catabolism to be differentially expressed. For example, the enzymes Guanine deaminase (GDA) and Xanthine dehydrogenase (XDH) were upregulated. Both enzymes are involved in the degradation from guanine or hypoxanthine to xanthine and further to urate. Since we analyzed unstimulated cells, there is no acute need for enhanced purine synthesis, and therefore the overproduction resulting from the restriction of the non-oxidative PPP branch seems to be compensated by these gene expression changes. Further work is necessary to examine if the cells switch from purine catabolism to anabolism upon further immunological stimulation.

Another important metabolic pathway for the induction of trained immunity is glutaminolysis. Thereby, glutamine is converted via glutamate to α-ketoglutarate, an anaplerotic substrate of the tricarboxylic acid cycle (TCA). Enhanced glutaminolysis in trained immune cells replenishes the TCA cycle and increases amounts of the TCA metabolites succinate, fumarate, or malate. Fumarate itself was described to induce trained immunity by modulating epigenetic changes at gene promotors and therefore modulates the transcription of proinflammatory cytokines (11).

We did not find transcriptional changes directly correlated with glutaminolysis. Nevertheless, Histidine deaminase (HAL), an enzyme involved in the degradation from histidine to glutamate via urocanic acid, is upregulated in post-septic monocytes (42), representing a source of glutamate after a second pathogen challenge.

Immune regulatory genes involved in cytokine signaling are upregulated in monocytes of post-septic mice. One of those genes is myeloid differentiation protein 88 (MyD88). MyD88 is a crucial adapter protein involved in downstream signaling of several Toll-like receptors. Activation of MyD88 leads to the activation of NF-kappa-B and therefore to the induction of pro-inflammatory gene-transcription (43, 44). Another up-regulated adaptor, Janus kinase 3 (Jak3) is part of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and regulates cytokine-mediated cellular responses, e.g., downstream of the IL-6 receptor. JAK/STAT signaling is also involved in various developmental and homeostatic processes, including hematopoiesis and immune cell development (45–47). Upregulation of those genes can foster pro-inflammatory cytokine outflow after stimulation, leading to the increased TNF and IL-6 output as observed in our experiments.

Epigenetic reprogramming is also involved in the establishment and maintenance of trained immunity, but was not analyzed in our study (7, 13, 48). Therefore, this point is open for further investigations. Compared to other groups and earlier studies, we solely analyzed the molecular changes occurring in unstimulated monocytes. An evaluation of metabolic, transcriptional, and epigenetic alterations in post-septic naïve bone marrow monocytes after a second stimulus like LPS remains to be elucidated, especially considering a selective "training" for certain pathogens or stimuli. Also, the impact of the disease severity evoked during the model needs to be clarified, as our study made use of a "mild" CLP approach with only 25% lethality. Higher severity can be associated with a higher bacterial burden and might implicate divergent long-term consequences on immunity.

Several animal studies approached the concept of "post-septic immunosuppression" using double-hit models and combined an initial CLP (or pneumonia) with a second encounter by bacterial (e.g., *P. aeriugunosa* or *S. pneumoniae*) or viral (respiratory syncytial virus) pathogens. Most of those studies analyzed the susceptibility to a second stimulus only a few hours or days after the insult (49–52). Only Nascimento and colleagues demonstrated an enhanced vulnerability to opportunistic infections up to 30 days (50). Importantly, several of those studies are focused on the lung, thereby inducing secondary pneumonia and demonstrating the organ-specific induction of immune tolerance via e.g., TGF-β and accumulation of regulatory T cells (49–52). Especially, Roquilly et al. demonstrated in their pneumonia/pneumonia double-hit model the induction of local immune tolerance in bone marrow derived dendritic cells and macrophages after lung migration (52).

Our findings of a trained phenotype might seem contradictory on first sight, but actually, they complement the current state of knowledge regarding the long-term temporal dimension and indicate the inherent complexity of systemic diseases. Our study is the first approaching immune function 3 months after sepsis, resembling a clinically relevant post-ICU timeframe. One might hypothesize that after an early state of tolerance, as extensively proven before, the body overcompensates such state by acquiring a trained phenotype with increased myelopoiesis and cellular alterations, resembling potentially favorable long-term adaption of innate immunity. Considering the complex host response during sepsis with extensive crosstalk of organs, the possibility of a combined spatiotemporal impact, depending on both the site of initial infection as well as the time of observation is likely. Moreover, each model itself has several dimensions to be taken into account: from the age and strain of the mice, the characteristics, and quantity of the infectious agent, up to the evoked disease severity. In the future, mixed second hit models (abdomen/lung, lung/soft tissue, etc.) will be of tremendous importance to assess which (distant) organs and body compartments might have adapted and if this is detrimental

or protective for the host. As mentioned before, organs like the lung itself adapt and generate an immunosuppressive milieu, which further shapes the invading cells function. Comparably, we did not observe alterations of the blood regarding an evoked cytokine secretion. The reasons for this surprising finding are elusive. However, upon release from the bone marrow, monocytes enter their short life cycle in blood, changing from inflammatory monocytes to alternative monocytes within a day before subsequently leaving circulation. We observed a slight increase in the frequency of alternative monocytes in the blood, hinting for a faster conversion rate or extended halftime of alternative monocytes until extravasation. Vice versa, inflammatory monocytes decrease, and this might blur the "trained" response observed in isolated monocytes of the bone marrow. Alternatively, persisting humoral factors like, e.g., metabolites might contribute to this peripheral phenomenon. Importantly, the question of adaption does not only extend solely to immune function, but rather to the fundamental functions of each organ or tissue.

In conclusion, the present study proves for the first time to our knowledge that sepsis induces a state of trained immunity both on a cellular level in naïve monocytes as well as in the hematopoietic niche of the bone marrow. These results refine the current hypothesis of a persisting global post-septic immunosuppression on the cellular level and therefore underline the requirement for further investigation of the immune system in sepsis survivors to understand the complex adaptions present.

AUTHOR CONTRIBUTIONS

KB and FU planned and performed the animal experiments. KB, JS, IS, and DS conducted the analysis. KB, DS, MW, and FU performed data interpretation and statistics. KB, FU, and MW wrote the manuscript. All authors critically revised and drafted the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02685/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Protection Against Invasive Infections in Children Caused by Encapsulated Bacteria

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The encapsulated bacteria *Streptococcus pneumoniae*, *Neisseria meningitis*, *Haemophilus influenzae*, and *Streptococcus agalactiae* (Group B Streptococcus) have been responsible for the majority of severe infections in children for decades, specifically bacteremia and meningitis. Isolates which cause invasive disease are usually surrounded by a polysaccharide capsule, which is a major virulence factor and the key antigen in protective protein-polysaccharide conjugate vaccines. Protection against these bacteria is largely mediated via polysaccharide-specific antibody and complement, although the contribution of these and other components, and the precise mechanisms, vary between species and include opsonophagocytosis and complement-dependent bacteriolysis. Further studies are required to more precisely elucidate mechanisms of protection against non-type b *H. influenzae* and Group B Streptococcus.

Keywords: sepsis, bacteremia, meningitis, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, Group B Streptococcus, *Streptococcus agalactiae*

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INTRODUCTION

Encapsulated bacteria have been responsible for the majority of bacteremia and meningitis in children for many decades (1–3). In children aged >3 months, *Streptococcus pneumoniae* (pneumococcus), *Neisseria meningitidis* (meningococcus), and *Haemophilus influenzae* have been the predominant pathogens, with *H. influenzae* being a less significant problem since the introduction of vaccines against type b organisms. Disease is most common in young children <5 years of age and older adults aged >65 years. In neonates and young infants, Group B Streptococcus (GBS) is the major cause of bacteremia and meningitis (1). These organisms have the shared characteristic of being surrounded by a polysaccharide capsule, which is a key virulence factor because it helps the bacteria evade complement deposition and subsequent phagocytosis and killing. These polysaccharides have also been the basis for successful vaccines against all except GBS, because immune responses against the polysaccharide capsule are the primary mechanism of protection for the human host. Each species can be encapsulated by polysaccharides of different biochemical compositions, which has been used for categorization into capsular groups or serotypes.

Protection against these organisms is highly dependent on circulating serum antibody because of the rapid development of disease following infection, which can result in death within hours (**Figure 1**). While vaccination and/or infection with these organisms does result in development of B cell memory, at least 2–7 days is required following pathogen exposure for a detectable response to occur—which is too slow to mediate protection (4–7). B cell immunity

is important to protection against encapsulated bacteria, with common themes across responses to all of the polysaccharide capsules discussed further below. The majority of B cell responses are T-dependent, but responses to polysaccharides are T-independent. Cross-linking of the surface-expressed B cell receptor results in differentiation of polysaccharide-specific B cells into plasma cells, without generation of memory B cells and thus depletes the naïve B cell pool from which future memory cells must be derived (8, 9). As a result, polysaccharide vaccines are generally poorly immunogenic in young children (under 2 years of age), there is no memory generated and no anamnestic response on future exposure to pathogen or booster vaccine doses (10–12). The development of conjugation chemistry, whereby polysaccharide antigens are linked to carrier proteins, resulted in vaccine antigens which recruited T cell help and thus generation of polysaccharide-specific memory B cells even in young children, and which can provide rapid responses upon administration of future vaccine doses (9). Complement and the spleen also play a critical role in protection against encapsulated bacteria. The spleen has a central role in protection against infection by encapsulated bacteria, via phagocytosis and production of opsonins and components of the complement pathway (**Figure 1**). Asplenic or hyposplenic individuals (e.g., post-splenectomy, sickle cell disease) are therefore rendered at much higher risk of life-threatening infection, reflected by the increased rates of infection by *S. pneumoniae* in particular—but also other encapsulated bacteria such as *H. influenzae* and *N. meningitidis* (13–19). Such individuals are therefore recommended to receive vaccination against all of these three pathogens, even when not in a high risk age group, in addition to long-term antibiotic prophylaxis to prevent infection (19).

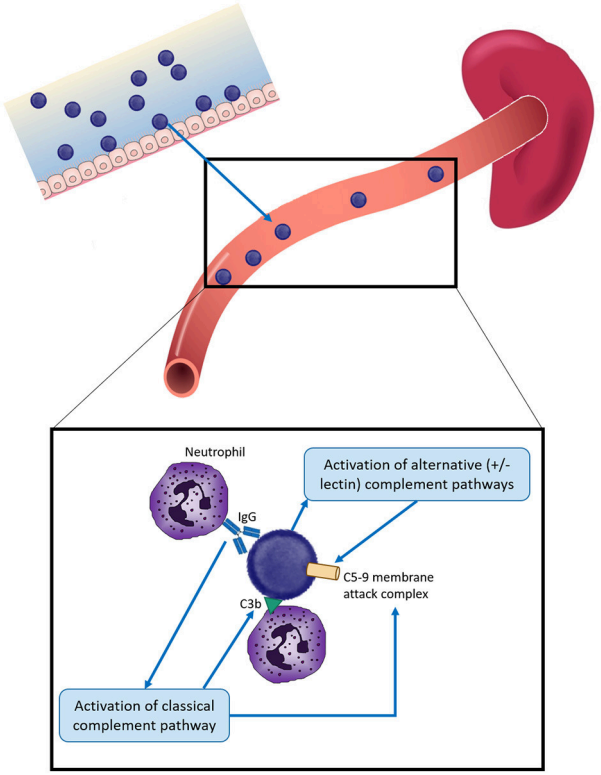

FIGURE 1 | Overview of protection against infection by encapsulated bacteria in children. Encapsulated bacteria initially infect the mucosal surface—nasopharynx for *S. pneumoniae*, *N. meningitidis,* and *H. influenzae* and the gastrointestinal or vaginal tracts for Group B Streptococcus (GBS). Occasionally bacteria enter the bloodstream to cause severe infection. Protection against invasive infection includes: direct activation of the alternative and/or lectin complement pathways, resulting in insertion of the C5-9 membrane attack complex into the bacterial membrane and bacterial death; binding by specific antibody and activation of the classical complement pathway, resulting in bacteriolysis by the C5-9 membrane attack complex and/or complement C3b deposition. Both antibody and C3b can result in opsonophagocytosis, most commonly by neutrophils. The spleen plays a key role by facilitating phagocytosis and producing components of the complement cascade. Different mechanisms have different relative importance depending on the pathogen, as described in the text.

STREPTOCOCCUS PNEUMONIAE

The Role of Antibody, Including Opsonophagocytosis

The uptake and killing of pneumococci by phagocytic cells, opsonophagocytosis (OP), is thought to be the predominant mechanism of bacterial killing. OP can be mediated by antigen-specific antibody or complement bound to the bacterial surface. OP against pneumococci is largely mediated via neutrophils (**Figure 1**). Following opsonization by antibody or complement component C3b on the bacterial surface, bacteria are phagocytosed by neutrophils and killed via serine proteases contained within neutrophil granules (20). Antibody coats the bacterial surface, following which the Fc portion binds to neutrophil receptors, initiating bacterial uptake.

In clinical trials of pneumococcal conjugate vaccines (PCVs), the protective antibody threshold was estimated to be 0.35 mg/mL polysaccharide-specific IgG (based on data from 3 clinical trials) and this has been used as the basis for licensure of these vaccines (21–24). A more recent analysis suggested that the actual amount of antibody required for protection varies depending on serotype, between 0.14 and 2.83 mg/mL for the 13 serotypes in the 13-valent PCV (PCV13) (25). In children immunized with the 7-valent PCV (PCV7) at 2, 4, 6, and 12 months of age, antibody levels increased following the 12-month booster, declined slightly by age 24 months and then did not decline much further to age 60 months (26). Antibody levels against serotypes which were frequently seen in carriage (and therefore presumed to be highly circulating) were higher, suggesting that ongoing exposure to *S. pneumoniae* was important in maintaining antibody levels. As well as increasing the total level of antibody, a booster vaccine dose at 12 months of age results in affinity maturation, resulting in higher avidity antibodies. This has been reported with booster doses of both conjugate and polysaccharide vaccines following priming doses with conjugate vaccine in infants (26–28).

The predominant anti-polysaccharide antibody in unvaccinated adults is IgG2 (29). This in part explains the high susceptibility of young children to pneumococcal disease, since

IgG2 production in the first 2 years of life is low (30). Although IgG2 provides some immunity against pneumococcal infection it is less efficient than other IgG subclasses in facilitating OP. IgG1 is up to 1,000-fold more efficient in inducing pneumococcal killing compared with IgG2 because IgG1 is a much more effective activator of the complement cascade (31–33). The ability of IgG2 to mediate protection against pneumococci is also impaired by its dependence on a polymorphic Fc receptor, Fcγ-IIA receptor. A single nucleotide polymorphism results in either a histidine (Fcγ-IIA-H131) or arginine (Fcγ-IIA-R131) residue at amino acid 131, with Fcγ-IIA-H131 having a higher affinity for IgG2 compared with Fcγ-IIA-R131 (34, 35). Fcγ-IIA-R131 is present in 30–50% of Caucasian populations (36). Adults who are homozygous for Fcγ-IIA-R131 are more likely to develop severe pneumococcal disease, and this form of the Fc receptor is also more commonly found in those with bacteremic pneumococcal pneumonia compared with healthy individuals and patients with non-bacteremic pneumonia (37–39). Most studies of IgG2-mediated OP have used post-vaccination serum (40, 41), and it is possible that natural immunity also relies on antibody against other bacterial components. In serum from unvaccinated individuals the classical complement pathway has a greater influence on OP than antibody level, and was more reliant on natural IgM, suggesting that IgG may be less important in natural immunity (42). This may also explain why rates of IPD are high in adults aged over 65 years despite high levels of anti-capsular IgG (43).

Natural IgM is encoded by germ-line genes which have not undergone somatic hypermutation. The B-cell repertoire of natural IgM is limited, of low affinity and does not adapt as a consequence of antigen-specific interactions (44). Such antibodies are cross-reactive and recognize antigens common to several pathogens, such as phosphorycholine found in the cell wall of the pneumococcus. Mice depleted natural IgM-producing B cells are highly susceptible to pneumococcal infection (45).

The Role of Complement

The importance of complement is highlighted by increased susceptibility to pneumococcal infections in individuals with complement pathway defects (46). In humans, activation of the classical complement pathway predominates in protection against pneumococcal infections, with some contribution from the alternative pathway. This has been confirmed by *in vitro* opsonization studies of pneumococci using serum from patients with complement C2 deficiency or depleted of C1q or factor B (42). Similar data have been obtained in mouse studies (47). The importance of the mannose-binding lectin (MBL) pathway differs between mice and humans. In mice the role of the MBL pathway is thought to be negligible whilst in humans mutations in the *MBL* gene are found in higher frequencies in individuals with IPD in comparison to healthy individuals (48).

In vitro anti-polysaccharide antibody alone can facilitate OP for some serotypes, but the addition of complement markedly increases phagocytosis (49, 50). This suggests that antibody-mediated killing occurs through both activation of the complement pathway as well as direct initiation of OP (42). The relative contribution of these two pathways in IgG-mediated protection is not clear. In a mouse model antibody-mediated protection was not reduced when antibody-dependent OP was blocked, whereas complement depletion resulted in bacteremia. These data suggest that IgG-mediated protection is predominantly through complement activation (51). In human sera it has been demonstrated that complement activation may be more important for the protection via IgG1 compared IgG2, in experiments where complement is depleted by heat inactivation (52).

In addition to its role in OP, complement C3b additionally stimulates B cells to increase antibody production via CD21 (53). The complement protein C5b enhances vascular permeability and chemotaxin release that guides neutrophils to the site of infection. The membrane attack complex (consisting of terminal complement components C5-9) is a major endpoint of the classical complement pathway, but does not play a significant role in protection against *S. pneumoniae*, unlike *N. meningitidis* (see below). The reasons for this are unclear, but may be related to the different structure of the Gram-positive *S. pneumoniae* where the cell wall is the predominant outer structure, compared with the Gram-negative *N. meningitidis* where the outer membrane surrounds a smaller cell wall.

Non-polysaccharide Directed Immunity

There is some evidence that antibodies against pneumococcal proteins may mediate some protection against pneumococcal infection. Firstly, IgG targeting different pneumococcal proteins, such as pneumolysin, are reduced in older compared with younger adults. Secondly, anti-pneumolysin IgG from patients with pneumonia confer protection against IPD in mice in passive protection studies (54). Finally, the age-related decline in rates of IPD after the age of 2 years is uniform across all serotypes, not only those that are more likely to be found causing carriage, suggesting non-polysaccharide-mediated protection (55).

NEISSERIA MENINGITIDIS

The Role of Antibody, Including Bacteriolysis

Disease incidence and activity of complement-dependent serum bactericidal antibody (SBA) show an inverse correlation, with the level of SBA being highest at birth and among adults, and lowest in children aged between 6 months and 2 years when the highest incidence of disease occurs (56, 57). Such antibodies occur naturally following asymptomatic carriage of both pathogenic and non-pathogenic Neisseria, such as *Neisseria lactamica*, and other antigenically related Gram negative bacteria. Polysaccharide-protein conjugate vaccines are available for capsular groups A, C, W, and Y; however only protein vaccines are available for group B because the polysialic acid capsule resembles human neuronal cell adhesion molecule and is therefore not immunogenic as a vaccine antigen (58). For the meningococcal capsular group C conjugate vaccine, an SBA titer of $\geq$8 correlated strongly with postlicensure

vaccine effectiveness (59). Following immunization with the capsular group C conjugate vaccine, protection after infant immunization does not persist into the second year of life (60), whereas immunization at age 12 months results in 1–2 years of protection (61), immunization at 1–9 years of age provides 2–5 years of protection, and the most durable responses resulting in ≥5 years of protection occur in children vaccinated when 10 years or older (62–65). For capsular group B disease, the proportions of capsular group B vaccine recipients with ≥4-fold rises in SBA following vaccination or SBA titers ≥4 have been correlated with clinical effectiveness in studies of outer membrane vesicle vaccines (66–68). The predicted effectiveness of a group B protein vaccine in the UK based on SBA (69) was supported by an observational study after vaccine introduction (70), further supporting these thresholds for protection. These cutoffs are, therefore, currently used for regulatory approval of new meningococcal vaccines. Previous studies have reported variable correlation between total antibody and SBA, highlighting the importance of antibody function for protection against meningococcal disease (67, 71–80). Hence, total antibody level is not considered relevant when considering protection against meningococcal disease. The strong association between disease risk and genetic variation in human complement factor H (81), further supports the importance of complement-mediated protection against disease.

The Role of Complement

Complement is a key factor in protection against meningococcal disease. The risk of developing meningococcal disease in individuals with primary immunodeficiency who have reduced or absent levels of properdin, factor D, or terminal complement components is up to 1,000-fold higher compared with the rest of the healthy population. Disease risk is also higher in individuals with nephrotic syndrome, systemic lupus erythematosus, hepatic failure, and other diseases which are associated with acquired decreases in levels of complement components, and in patients treated with eculizumab, a monoclonal antibody against complement protein C5. In individuals with complement deficiencies, disease tends to occur during late childhood and adolescence, concordant with higher rates of nasopharyngeal carriage. In addition, infections may be recurrent, which is extremely rare in otherwise healthy individuals. Meningococcal disease cases in those with complement defects are frequently reported as being less severe than in complement-sufficient persons (82), with the exception of properdin deficiency and occasionally in those with late complement component deficiency, perhaps because these cases are often caused by unusual capsular groups. In one study, one-third of individuals with meningococcal disease caused by capsular groups X, Y, and W had a complement deficiency, and group B disease (common in resource-rich countries) has only occasionally been described in series of complement-deficient individuals with IMD. Extensive complement activation and bacteriolysis are protective against early infection, but likely contribute to the pathogenesis of severe disease following bacterial invasion.

Other Mechanisms of Protection

There is evidence that mechanisms other than complement-dependent bactericidal antibodies may be important in determining protection against meningococcal disease. Firstly, the relationship between incidence of disease and prevalence of SBA have not been observed in studies in the UK and Canada (83, 84), After the first 2 years of life, disease incidence declined through childhood, but this was surprisingly not associated with an increase in bactericidal antibodies. In the UK study, the second, smaller peak of disease occurring in adolescents and young adults coincided with a paradoxical increase in the proportion of individuals with a protective SBA titer of ≥1:4 and adults had a low risk of disease despite a much lower prevalence of protective bactericidal antibodies. Secondly, in a large study in Iceland SBA titres underestimated vaccine efficacy (85). Thirdly, disease in individuals with complement deficiency has a different age distribution, is often less severe, and often involves unusual capsular groups (82).

Alternative important factors in protection include OP (86) and antibody avidity (77), but there are no data linking these mechanisms with either vaccine efficacy or effectiveness, as has been found with SBA. Protection in the absence of SBA activity is probably conferred by OP (87). This is observed in rats who are deficient in complement factor C6, where opsonization can occur, but bacteriolysis cannot. Both SBA and OP activity occur in animals and humans after immunization with protein and lipopolysaccharide (LPS), but there are few data to suggest that OP without SBA can prevent meningococcal disease or improve outcomes. The importance of antibody avidity and the ability of vaccines to stimulate avidity maturation has been demonstrated for MenC (87). SBA did not correlate well with IgG titres after vaccination with an OMV vaccine, possibly because only high avidity antibodies were bactericidal (88).

The risk ratio for siblings of individuals with invasive disease due to *N. meningitidis* is similar to that for other diseases where susceptibility shows polygenic inheritance. Multiple host genetic factors have been identified which influence either susceptibility to or severity of disease. The molecules implicated involved polymorphisms in genes expressed at epithelial surfaces, the complement cascade, pattern recognition receptors, clotting factors, or inflammatory mediators. Deficiencies in the complement pathways are consistently associated with an increased risk of meningococcal disease, with specific polymorphisms in MBL, and factor H found to be associated with disease susceptibility. A genome-wide association study of 7,522 individuals in Europe identified single-nucleotide polymorphisms within genes encoding complement factor H *(CFH)* and CFH-related protein 3 (*CFHR3*), which were associated with host susceptibility to meningococcal disease (81). Complement-mediated bacteriolysis is known to be extremely important in protection against meningococcal disease, giving these associations biologic plausibility. In particular, factor H attaches to various binding proteins expressed on the bacterial surface, downregulating complement activation and allowing the organism to evade host responses.

HAEMOPHILUS INFLUENZAE

Haemophilus influenzae type b

In concurrence with the other encapsulated bacteria, the most important mediator of host defense against *H. influenzae* type b (Hib; the best studied serotype) is antibody directed against the type b capsular polysaccharide polyribosylribitol phosphate (PRP). Anti-PRP antibody is acquired in an age-related fashion and mediates bacterial killing, in part via OP. The importance of anti-polysaccharide antibodies comes from studies where administration of immunoglobulin enriched for these antibodies protected against disease, in both humans and rats (89, 90). Further studies in rats demonstrated that both IgG1 and IgG2 had equivalent functional activities, suggesting multiple effector mechanisms (91). Antibodies against non-polysaccharide antigens, such as OMPs, have also been demonstrated to be protective in the rat model (92). Both the classic and alternative complement pathways are important in defense against *H. influenzae* type b. Protection against Hib appears to correlate with the concentration of anti-PRP antibody, with a serum antibody concentration of 0.15–1.0 μg/mL considered protective against invasive infection (93). Unimmunized infants and young children aged between 6 months and 5 years usually lack this level of anti-PRP antibody and are therefore susceptible to invasive Hib disease (94). Following immunization in infancy (<12 months of age), antibody levels fall by 18 months of age, below protective levels (95). After a booster dose in the 2nd year of life, however, high antibody levels persist for up to 8–10 years (95–97). Unlike the rise and subsequent decline in antibody levels seen after booster doses of vaccine, antibody avidity appears to increase more consistently over time following vaccination. After vaccination with Hib conjugate vaccine at age 2, 3, and 4 months of age, antibody avidity increases over time even while antibody levels fall (98). After a booster dose, both avidity and levels increased in this study, although lower avidity was evident in children with low antibody levels after initial priming doses, suggesting high avidity may be a marker of better priming. In addition, lower antibody avidity has been reported in vaccine failures (99), highlighting the importance of antibody function as well as concentration, although the level of antibody produced post-vaccination remains the sole basis for vaccine licensure.

Non-type b *H. influenzae*

Much less is known about immunity to other *H. influenzae* serotypes or to non-typable isolates, which lack a polysaccharide capsule. Non-typable bacteria are very uncommon in invasive disease, and usually cause mucosal infections such as otitis media and sinusitis. For these strains, evidence suggests that antibodies directed against OMPs are bactericidal and protect against experimental challenge (100, 101). Disease caused by *H. influenzae* type a (Hia) has been most commonly reported in indigenous populations of North America, the reasons for which are unclear (102). In a recent study in Canada, SBA activity in Aboriginal adults was higher than in non-Aboriginal adults, suggesting higher rates of exposure and that exposure results in production of SBA (103). In this study, the SBA activity appeared to be mediated by IgM rather than IgG. This is akin to what has been described for *S. pneumoniae*, but contrasts with SBA against *N. meningitidis*, which is mediated by IgG. The precise role of this IgM-mediated SBA in protection against disease is not clear and further studies are awaited. Development of a protein-polysaccharide conjugate vaccine against Hia is ongoing, so determination of mechanisms of protection against Hia remains an important area of research (104, 105).

GROUP B STREPTOCOCCUS

There is currently no licensed vaccine for protection against GBS and one of the reasons for this is the mechanism of protection is less definite then for the other encapsulated bacteria described. An added difficulty is that any studies of GBS immunity need to involve the mother-infant dyad.

An association between anti group B-polysaccharide antibody levels and invasive GBS disease in newborns was first described in 1976 (106). In most studies, low levels of anti-polysaccharide antibodies occurred in women who had neonates with GBS disease, compared with women with unaffected infants. There is a high correlation of antibody levels between the mothers and infants, indicating the importance of transplacental antibody transfer in neonatal immunity to GBS. In a recent meta-analysis the odds ratios of having an antibody level <2 μg/ml were 6.6 (95% CI: 2.1–20.6) and 2.4 (95% CI: 1.2–4.7) among those with types III and Ia GBS disease, respectively, compared to those without GBS disease (107). For capsule types Ia and III, one study suggested a threshold of 1 μg/ml as a correlate for protection (108), but much higher thresholds have been identified in other studies using different case-control designs (109, 110) and different methods, making direct comparisons difficult (111). Levels of polysaccharide-specific antibodies correlate with *in vitro* killing activity and *in vivo* protection (108), although several studies have suggested that some laboratory antibody detection methods may underestimate protection (112–114). In one study OP activity of anti-GBS polysaccharide IgG declined significantly from a 4-week post-immunization peak, but substantial functional activity (>1-log reduction in GBS cfu/mL), was preserved at 18–24 months post-immunization for each GBS type assessed (112). Animal challenge models (mostly in mouse, but also rat and rabbit) have reported that GBS killing is mediated by antibody- and complement-dependent OP via neutrophils.

Significant gaps remaining in understanding protection against invasive GBS disease. Interpretation of studies done to date is confounded by variation in methodologies and lack of standardized reference ranges for serotype-specific antibody levels. Prospective studies in diverse settings are needed to establish thresholds of protection for the most common serotypes IgG capsular antibodies are unlikely to be the only determinant of protection, so measures of functional antibodies are also required. Such work could facilitate the licensure pathway of a GBS vaccine without the need for large-scale efficacy trials in pregnant women (115).

CONCLUDING REMARKS

Antigen-specific antibody directed at the capsular polysaccharide clearly has the central role in protection against invasive infection by encapsulated bacteria, in both children and adults. Antibody-dependent mechanisms mediating this protection differ between organisms, with OP predominant in protection against *S. pneumoniae* and SBA against *N. meningitidis*. The precise mechanism is less-well defined for *H. influenzae* and GBS, and further information for these pathogens will aid development of vaccines against *H. influenzae* type a and GBS. Data from studies to date highlight that even in these cases no single mechanism is exclusively responsible for protection. The role of complement is also critical in disease protection, highlighted by the increased rates of severe infection against encapsulated bacteria in individuals with complement deficiencies. Protection against colonization and mucosal infections from these same pathogens is less well-understood and further studies are required, to inform development of vaccines which are better able to prevent these infection, for example pneumococcal pneumonia in older adults and vaccines based on non-polysaccharide antigens. Increased understanding of protection against non-b serotypes of *H. influenzae* and GBS would also be useful in development of new vaccines against these infections.

AUTHOR CONTRIBUTIONS

MS is the sole author and was responsible for conceiving the idea for the manuscript and writing the manuscript.

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Resveratrol-Mediated Attenuation of *Staphylococcus aureus* Enterotoxin B-Induced Acute Liver Injury Is Associated With Regulation of microRNA and Induction of Myeloid-Derived Suppressor Cells

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Resveratrol (RES) is a polyphenolic compound found abundantly in plant products including red grapes, peanuts, and mulberries. Because of potent anti-inflammatory properties of RES, we investigated whether RES can protect from Staphylococcal enterotoxin B (SEB)-induced acute liver injury in mice. SEB is a potent super antigen that induces robust inflammation and releases inflammatory cytokines that can be fatal. We observed that SEB caused acute liver injury in mice with increases in enzyme aspartate transaminase (AST) levels, and massive infiltration of immune cells into the liver. Treatment with RES (100 mg/kg body weight) attenuated SEB-induced acute liver injury, as indicated by decreased AST levels and cellular infiltration in the liver. Interestingly, RES treatment increased the number of myeloid derived suppressor cells (MDSCs) in the liver. RES treatment led to alterations in the microRNA (miR) profile in liver mononuclear cells (MNCs) of mice exposed to SEB, and pathway analysis indicated these miRs targeted many inflammatory pathways. Of these, we identified miR-185, which was down-regulated by RES, to specifically target Colony Stimulating Factor (CSF1) using transfection studies. Moreover, the levels of CSF1 were significantly increased in RES-treated SEB mice. Because CSF1 is critical in MDSC induction, our studies suggest that RES may induce MDSCs by down-regulating miR-185 leading to increase the expression of CSF1. The data presented demonstrate for the first time that RES can effectively attenuates SEB-induced acute liver injury and that this may result from its action on miRs and induction of MDSCs.

Keywords: resveratrol, *Staphylococcus aureus* enterotoxin B, acute liver injury, microRNA, myeloid derived suppressor cells

INTRODUCTION

Resveratrol (RES: *trans*-3,5,4′-trihydroxystilbene) is a non-flavonoid polyphenolic compound found abundantly in a large number of plant products including red grapes, red wine, peanut, mulberries and the like (Jang et al., 1997; Jang and Surh, 2001). RES is produced as a part of a plant's defense system against fungal infection and is a member of the class of plant antibiotic compounds (Soleas et al., 1997a,b). Most importantly, RES was an essential component of Ko-jo-kon, an oriental medicine, used to treat diseases of the blood vessels, heart (Soleas et al., 1997a,b), and liver (Soleas et al., 1997a,b). In recent years, RES has been the focus of many studies, including our own laboratory, for its pharmacological and beneficial properties on a wide range of diseases, including cardiovascular, autoimmune, neurological, and hepatic (Baur and Sinclair, 2006; Singh et al., 2007a, 2011). We have previously shown the beneficial effects of RES on autoimmune and inflammatory diseases (Singh et al., 2007a, 2010, 2011; Singh U.P. et al., 2012). In addition, RES also has antibacterial activity. Ma et al. (2018) recently described the potential antibacterial activity of RES against foodborne pathogens, its mechanisms of action, and its possible applications in food packing and processing. We have also demonstrated the protective effect of RES against Staphylococcal enterotoxin B-induced lung injury (Rieder et al., 2012; Alghetaa et al., 2018). In another study, Yang and Lim showed that RES ameliorates hepatic metaflammation, accompanied by alterations in NLRP3 inflammasome (Yang and Lim, 2014). Due to the wide-ranging beneficial effects of RES, it has recently been introduced as a nutritional supplement in the market (Aschemann-Witzel and Grunert, 2015) and presently, it is being used extensively, not only in United States but also worldwide.

Staphylococcal enterotoxin B (SEB) is a well-known super antigen that is highly toxic. SEB bypasses the normal antigen processing by antigen-presenting cells (APCs). It interacts outside of the peptide-binding groove of major histocompatibility complex class II (MHC II) molecule, with certain external Vβ domains located on T cell receptor (TCR) to activate such T cells and cause cytokine storm (Marrack and Kappler, 1990; Baker and Acharya, 2004). While normal antigens activate approximately 0.1% of host T cells, super antigens, on the other hand, can activate up to 30% of host T cells (Rieder et al., 2011). Such massive T cell activation leads to uncontrolled proinflammatory cytokine release, termed as a cytokine storm, including the release of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukins IL-1, IL-2, IL-6, IL-8, and IL-12 (Miethke et al., 1992; Pfeffer et al., 1993; Krakauer et al., 2010). Despite improvements in healthcare, *Staphylococcus aureus* (*S. aureus*) exposure still results in 20–30% mortality in the developed world, partly because of the ability of this bacterium to acquire antibiotic resistance (Chambers and Deleo, 2009). *S. aureus* secretes major virulence factor that causes community acquired diseases and nosocomial infections (Dinges et al., 2000; Pinchuk et al., 2010). Also, SEB exposure in humans can cause severe food poisoning and sometimes, it can cause even fatal conditions including toxic shock syndrome (Henghold, 2004). SEB is an extremely stable compound in acidic environments (gastrointestinal tract) and is highly resistant to heat and proteolytic digestion (Ler et al., 2006). Because of such toxicity and ability to cause death, it has the potential to be used as a biological weapon, and to that end, Centers for Disease Control (CDC) has classified SEB as a category B priority agent (Madsen, 2001). Currently, there is no effective treatment to prevent SEB-mediated toxicity and thus, there is a dire need for a more effective treatment modality to control rapid T cell activation and cytokine storm induced by SEB.

Whether RES, which has potent anti-inflammatory properties, can effectively protect the liver from SEB-induced acute liver injury acute liver injury has not been previously studied. In this study, we demonstrate that RES protects mice from acute liver injury. Moreover, this protection was associated with altered expression of microRNA and induction of MDSCs. Specifically, we found that miR-130a and miR-185 directly target CSF1 [also known as macrophage colony stimulating factor (M-CSF)] gene in liver MNCs, which plays a critical role in the induction of MDSCs. The present study demonstrates that RES protects mice against SEB-induced acute liver injury possibly through regulation of microRNA to induce immunosuppressive MDSCs.

MATERIALS AND METHODS

Animals

C57Bl/6 female mice were obtained from Jackson laboratory. The Institutional Animal Care and Use Committee (IACUC) of University of South Carolina approved the protocol and use of mice. The mice were housed in a pathogen-free AALAC approved animal facility at University of South Carolina School of Medicine.

Chemicals and Reagents

The following chemicals were purchased and used: SEB (Toxin Technologies, Sarasota, FL, United States), RES and DMSO (Sigma-Aldrich, St. Louis, MO, United States), culture medium (RPMI 1640), Penicillin/Streptomycin, HEPES, L-glutamine, FBS, and PBS (Invitrogen Life Technologies, Carlsbad, CA, United States). Fluorophore-labeled anti-mouse CD3, CD4, CD8, CD44, NK1.1, CD11b, and Gr1 antibodies were purchased from eBioScience (Carlsbad, CA, United States). Bio-Plex kit for mouse cytokines was purchased from Bio-Rad (Bio-Rad, Hercules, CA, United States). Polymerase chain reaction (PCR) reagents, Epicentre's PCR premix F and Platinum *Taq* Polymerase, were purchased form Invitrogen Life Technologies (Carlsbad, CA, United States). miRNeasy kit, miScript cDNA synthesis kit, miScript primer assays kit, miScript SYBR Green PCR kit, miR-185-5P mimic, and miR-185-5p inhibitor, SsoAdvanced SYBR green supermix from Bio-Rad (Hercules, CA, United States) were purchased from QIAGEN (Qiagen, Inc., Valencia, CA, United States).

SEB-Induced Acute Liver Injury and RES Treatment

We tested the efficacy of RES in an *in vivo* mouse model of acute liver injury induced by SEB. To that end, SEB was injected intraperitonally (i.p.) into C57BL/6 mice at a dose of 40 μg in PBS, as described previously (Rieder et al., 2011; Rao et al., 2014). The mice were first sensitized by injecting (i.p.) D-galactosamine (Dgal; 20 mg) in PBS 30 min prior to SEB injection (Hegde et al., 2011). The mice were then treated with RES (100 mg/kg bw) suspended in water by oral gavage in a total volume of 100 μl, 2 h post-SEB injection and then daily until the completion of the experiment. Because SEB is a super antigen, it activates a large proportion of T cells and thus, liver damage is acute and liver enzymes are induced as early as 8 h after SEB, as shown by us previously (Busbee et al., 2015). It is for this reason that we injected RES, 2 h after SEB to test if RES can be used both to treat liver injury induced by SEB. Next, the mice were euthanized either on days 2–3 depending on the nature of experiments as detailed later on. Mice were monitored on daily basis for any signs of distress or any other health-associated problems. In most experiments, we used four groups of mice: Naïve (control), RES only, SEB+Vehicle, and SEB+RES. The experiments were repeated at least three times and each experimental groups consisted of five mice.

Analysis of Mouse Liver Post-SEB and RES Treatment

Livers were harvested on day 3 post-SEB injection and treatment with VEH or RES. Parts of the livers were stored in 10% formalin for histology and immunohistochemistry and the remaining parts of the livers were used for isolation of MNCs. To examine cell infiltration in liver, fixed liver tissues were embedded in paraffin, 5 μm sections were cut, and then the tissue sections were stained with Hematoxylin and Eosin (H&E). Liver-infiltrating MNCs were isolated from the remaining livers using Percoll density gradient as described earlier (Hegde et al., 2011). In brief, single cell suspensions of livers were prepared using a tissue homogenizer and then passed sterile nylon mesh (70 μM). Cell suspension was washed once with PBS and then the pellet was suspended in 33% Percoll (Sigma-Aldrich) diluted in sterile PBS. The cells in Percoll gradient were centrifuged at 2000 rpm for 15 min at 25°C. MNCs were washed twice with PBS and contaminating RBCs were lysed using RBC-lysis solution (Sigma-Aldrich). Total number of purified MNCs were counted in various treated groups. Purified MNCs were then used for RNA isolation or for staining the cells for various cell markers. For staining MNCs, the cells were first blocked using mouse Fc-block (anti-CD16/CD32) and then stained for various cell surface markers (CD3, CD4, CD8, CD11b, and Gr1) using fluorescently labeled mAb (10 μg/mL, in PBS containing 2% FBS). After washing, stained cells were analyzed in a flow cytometer (FC500, Beckman Coulter). Only live cells were counted by setting gates on forward and side scatters to exclude cell debris and dead cells.

Analysis of Aspartate Transaminase (AST) Post-SEB and RES Treatment

To assess the liver damage, liver enzyme, aspartate transaminase (AST) was measured by spectrophotometric method at 340 nm in sera collected at 16 h post-treatment. To this end, we used AST assay kit from Pointe Scientific (Canton, MI, United States) as described previously (Hegde et al., 2011; Busbee et al., 2015).

Characterization of Immune Cells in Spleen Post-SEB and RES Treatment

Spleens from mice exposed to SEB and treated with VEH or RES were harvested on day 3 and single cell suspensions were prepared. The splenic cells were then stained using anti-mouse anti-CD3, anti-CD4, anti-CD8, and anti-NK1.1 monoclonal antibodies (Biolegend, United States). The stained splenic cells were analyzed using flow cytometry (FC500; Beckman Coulter). We also stained splenic cells and liver MNCs with anti-mouse anti-CD11b and -Gr1 to identify myeloid-derived suppressor cells (MDSCs) and analyzed using flow cytometry.

Analysis of CSF1 in Serum

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the expression of CSF1 levels in the serum samples. To this end, serum was collected from mice 24 h post-SEB+VEH or SEB+RES treatments.

miR Expression Profiling and Identification of Dysregulated miRs

Total RNAs including microRNAs were isolated from liver MNCs harvested from mice on day 3 post-SEB injection and treatment with VEH or RES. The concentration and purity of the isolated RNAs were determined using a spectrophotometer, and the integrity of the RNA was verified by Agilent 2100 BioAnalyzer (Agilent Tech, Palo Alto, CA, United States). Profiling of miR expression from samples was performed using the Affymetrix GeneChip miRNA 3.0 array platform (Affymetrix, Santa Clara, CA, United States). This array, composed of 2023 miR mouse probes and using the FlashTag biotin HSR hybridization technique, was performed as previously described (Singh N.P. et al., 2012; Guan et al., 2013; Hegde et al., 2013). A heat map was generated by taking the log transformation of fluorescent intensities obtained from the hybridization. Ward's method was used to carry out hierarchical clustering and similarities were measured using half square Euclidean distance. Fold changes in miR expression were obtained from the array and miRs with > 1.5-fold change were considered for further analysis. Predicted miR targets, alignments, and mirSVR scores were determined by using online miR and miR database (mirwalk v3).

Real-Time PCR (RT-PCR) to Validate miRs Expression

To validate the expression of miRs (miR-130a and miR-185), quantitative RT-PCR was performed using miScript SYBR green PCR kit and mouse primers for miR-130a- (5′-CAGUGCAAU GUUAAAAGGGCAU; MS00001547) and miR-185-specific

(5′-UGGAGAGAAAGGCAGUU CCUGA; MS00001736) from Qiagen (Valencia, CA, United States) were used. SNORD96a (MS00033733) also from Qiagen (Valencia, CA, United States) was used as a positive control for miR expression. The expression level of miR-130a and miR-185 was normalized to SNORD96a levels. Fold change in expression was calculated using the $2^{-\Delta\Delta C_T}$ method. Similarly, quantitative real-time PCR (qRT-PCR) was performed to determine the expression of CSF1. We used SsoAdvanced SYBR green supermix from Bio-Rad (Hercules, CA, United States) and mouse CSF1-specific forward (5′-GACCCTCGAGTCAACA GAGC-3′) and reverse (5′-GAGGGGGAAAACTTTGCTTC-3′) primer pairs. Expression level of CSF1was normalized to 18S as described earlier (Singh et al., 2007a,b, 2011).

Transfection With miR-185 Mimic and Its Inhibitor

We chose miR185 to characterize its role in CSF1 regulation. As shown in **Figure 7A**, miR-185 has strong binding affinity with complementary sequences of 3′ UTR regions of CSF1 gene. To this end, splenic cells from naïve mice (C57BL/6) were first cultured in complete RPMI 1640 medium at 2×10^5 cells per well in a tissue culture plate and activated with SEB (1 μg/ml). The splenic T cells were transfected using HiPerfect Transfection Reagent kit from Qiagen and following the protocol of the company (Qiagen, Valencia, CA, United States). The cells were transfected with transfection reagents without miR (MOCK) or miR-185 mimic or anti-miR-185 or miR-185 mimic+anti-miR-185 (1:10). Because transfection of primary T cells is difficult, we used activated T cells which are easier to transfect as seen in our previous studies (Alghetaa et al., 2018) leading to transfection rate of > 80–85%. To that end, spleen cells from naïve mice were first activated with SEB for 12 h and then transfection was performed. Such activated cells were transfected with transfection reagents without miR (MOCK) or 20 nM of synthetic mmu-miR-185–5p mimic (5′-UGGAGAGAAAGGCAGUUCCUGA-3′, Cat No: MSY0000214) or anti-mmu-miR-185–5p (5′-UGGAGAGAAAGGCAGUUCCUGA-3′; Cat No: MIN0000214) or combination of miR-185 mimic and anti-miR-185 (1:10). Forty-Eight hours post-transfection, the cells were analyzed for transfection efficiency by flow cytomntry for GFP expression (Positive control). The cells were then treated with VEH or RES (20 μM/ml) for 24 h. The cells were collected and the expression of miR-185 and CSF1 was determined by performing quantitative Real-Time (qRT-PCR) as described above.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism software (San Diego, CA, United States). Each experimental group had at least five mice. The *in vitro* assays were performed in triplicate. All experiments were repeated at least three times. We used one-way ANOVA to calculate significance and Tukey's *post hoc* test to analyze differences between the groups, unless otherwise indicated. We used a p-value of < 0.05 to determine the statistical significance.

RESULTS

Effect of RES on SEB-Induced Acute Liver Injury in Mice

To examine the efficacy of RES on SEB-induced liver injury in mice, SEB was administered into mice (C57BL/6) as described in Section "Materials and Methods." Mice exposed to SEB were treated with VEH or RES (100 mg/kg body weight). Naïve mice without SEB treatment and mice treated with RES alone were also included as controls.

Histopathological analysis was performed on liver tissues post H&E staining. The liver tissues from naïve mice showed normal hepatic parenchyma with intact portal triads, hepatic plates of normal thickness, and normal-appearing hepatocytes (**Figure 1A,a,b**). Similarly, the liver tissue from mice treated with RES alone showed normal hepatic parenchyma with undamaged portal triads and normal-appearing hepatocytes (**Figure 1A,c,d**), demonstrating no histopathologic evidence of hepatic injury. The liver tissues from mice exposed to SEB+VEH showed marked hepatocyte necrosis (more pronounced in the centrilobular region) affecting approximately 60% of the hepatic parenchyma with relative sparing of the portal triads (**Figure 1A,e,f**). There were signs of inflammation with focal increase in MNCs (**Figure 1A,e,f**). In addition, there was an increase in steatosis, when compared to control mice (**Figure 1A,e,f**). Additionally, the hepatic parenchyma also demonstrated the evidence of hepatic injury with ballooning degeneration and single cell necrosis (**Figure 1B**). In contrast, in SEB+RES treated mice, the portal triads showed relatively minimal portal triaditis (**Figure 1A,g,h**). Also, there was less steatosis in the liver tissues of SEB+RES mice (**Figure 1A,g,h**). Although inflammation was observed in the liver SEB+RES mice, the level of inflammation was significantly reduced, when compared to liver tissues from mice treated with SEB+VEH (**Figure 1A,g,h**). The liver tissues from mice exposed to SEB+RES showed significant decrease in centrilobular necrosis affecting approximately 10% of the hepatic parenchyma when compared to ∼60% in SEB+VEH groups (**Figure 1B**). Next, we studied the total number of purified liver MNCs. There was no significant change between Naïve and RES groups (**Figure 1C**) but there were significantly higher number of MNCs in livers from SEB+VEH-treated mice (**Figure 1C**), when compared to VEH- or RES-treated groups. In contrast, there was significantly less number of MNCs in SEB+RES-treated groups, when compared to SEB+VEH group (**Figure 1C**).

Upon examination of liver enzyme aspartate transaminase (AST) levels in sera, we noted minimal AST levels (<100 IU/L) in both naïve and RES alone treated mice (**Figure 1D**). However, SEB+VEH-treated mice showed significant increase (more than 2000 IU/L) in AST levels (**Figure 1D**), while mice exposed to SEB+RES showed significant decrease (∼1000 IU/L) in AST levels (**Figure 1D**). These data together demonstrated significant attenuating effect of RES on SEB-induced acute liver injury.

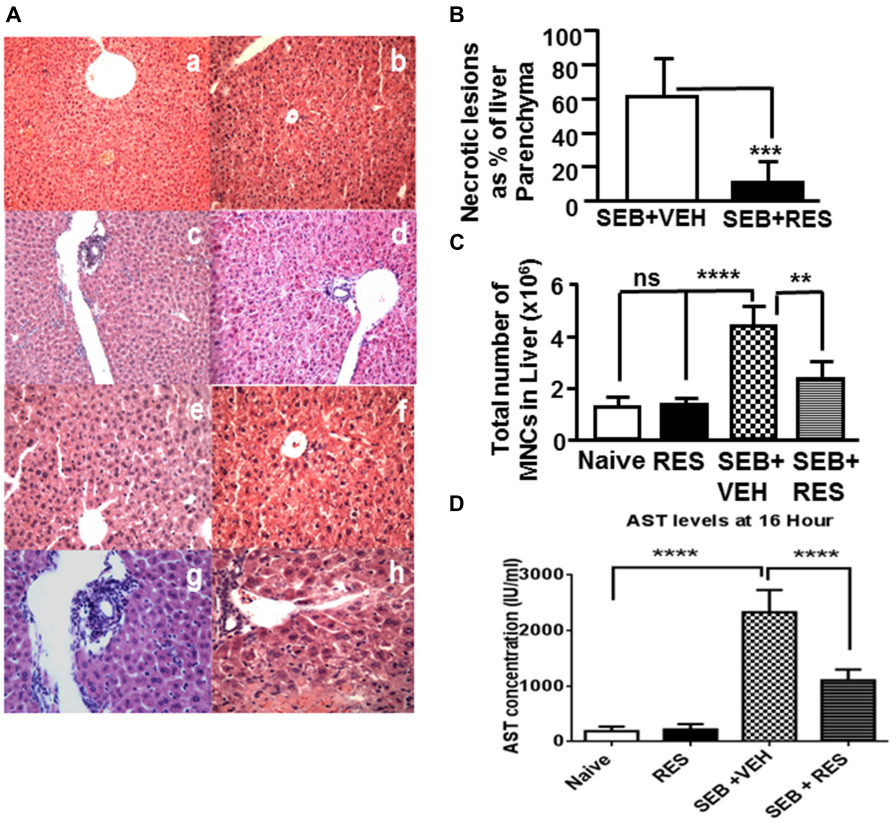

FIGURE 1 | Resveratrol (RES) attenuates SEB-induced liver damage. Mice were untreated (Naïve, Control), treated with RES (100 mg/kg bw) only, SEB+VEH or SEB+RES, as detailed in Section "Materials and Methods." Livers were harvested, sectioned and H&E staining was performed. **(A)** A representative of H&E stained livers from the four treatment groups; Naïve group (mice with no treatment: **a,b**), RES (mice treated with RES only: **c,d**), SEB+VEH (mice exposed to SEB and treated with VEH: **e,f**), and SEB+RES (mice exposed to SEB and treated with RES: **g,h**). **(B)** Depicts necrotic lesions in the liver of SEB+VEH **(e,f)** SEB+RES **(g,h)**. **(C)** Shows total number of MNCs in liver of various treated groups. **(D)** AST levels in sera. Data presented in **(B–D)** represent mean ± SEM from groups of five mice. Asterisks (*) in **(B–D)** indicate statistically significant ($p < 0.05$) difference between the groups.

RES Affects Immune Cell Populations of Both Spleen and Liver

Next, we examined the effect of RES on immune cells in the spleen as well as in the liver. There was a significant increase in total number of immune cells in spleen (**Figure 2A**) in mice exposed to SEB+VEH, when compared to mice that received either none (Naïve; control) or treated with RES alone. In contrast, there was a significant reduction in the number of immune cells in spleen (**Figure 2A**) of mice that were exposed to SEB+ RES, when compared to SEB+VEH group (**Figure 2A**).

Upon examination of the presence of various immune cells in the spleen and liver using various cell markers (CD3, CD4, CD8, and NK1.1), we noted no significant change in the total number of CD3+, CD4+, CD8+, and NK1.1+ cells in control and RES alone-treated groups of mice (**Figure 2B**). However, there was significant increase in total number of all the four cell populations in mice that were exposed to SEB+VEH, when compared to control or RES-treated mice (**Figure 2B**). Moreover, there was a significant decrease in cell populations of all the four cell types examined in mice exposed to SEB+RES, when compared to mice that received SEB+VEH (**Figure 2B**). These data demonstrated that SEB, being a super antigen induces proliferation of large numbers of T and NK1.1 cells while RES can suppress the induction of such cells.

RES Promotes Generation of Immunosuppressive MDSCs

Next, we analyzed for CD11b+/Gr1+ MDSCs cells by flow cytometry because previous studies from our laboratory have shown that RES induces MDSCs that are highly immunosuppressive (Singh U.P. et al., 2012). The data showed that there was no significant change in the percentage and absolute numbers of MDSCs in the spleens of naïve, RES only and SEB+VEH groups of mice tested. Interestingly, in SEB+RES group, there was a significant increase in the total numbers of MDSCs when compared to SEB+VEH treated group (**Figure 3A**). Upon analysis of MDSCs in liver MNCs, we noted significant increase in MDSCs in RES-treated group, when compared to Naïve control (**Figure 3B**). However, there was no significant change in MDSCs in SEB+VEH group, when compared to naïve control. Moreover, there was a significant increase in total number of MDSCs in liver of

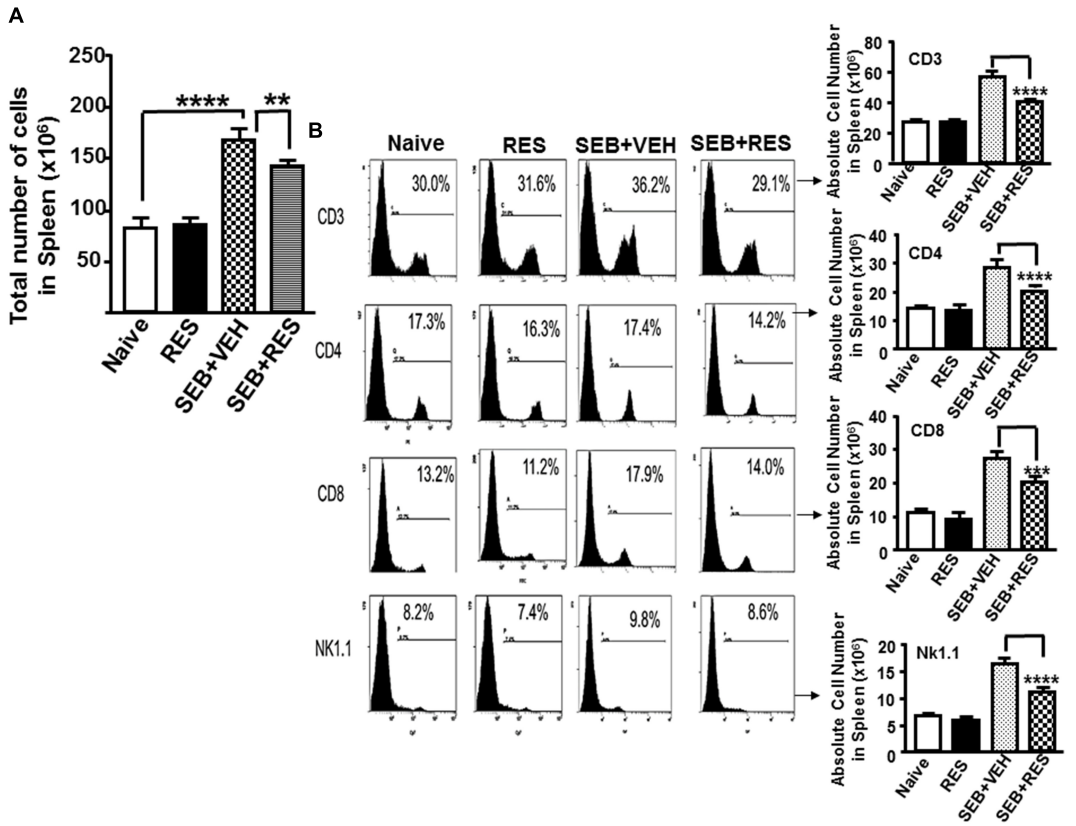

FIGURE 2 | Characterization of immune cells in mice with acute liver injury. Acute liver injury was induced as described in **Figure 1** legend. Spleen cells isolated from livers were further analyzed. **(A)** Shows total number of cells in spleen. **(B)** Spleen cells were stained with fluorophore-labeled anti-mouse anti- CD3, -CD4, -CD8, and -NK1.1 antibodies. The data are presented as percentage of various subsets of cells as well as total number of individual population of such cells in the spleen. Vertical bars represent mean ± SEM from groups of five mice. Asterisk (*) indicates statistically significant ($p < 0.05$) difference between groups.

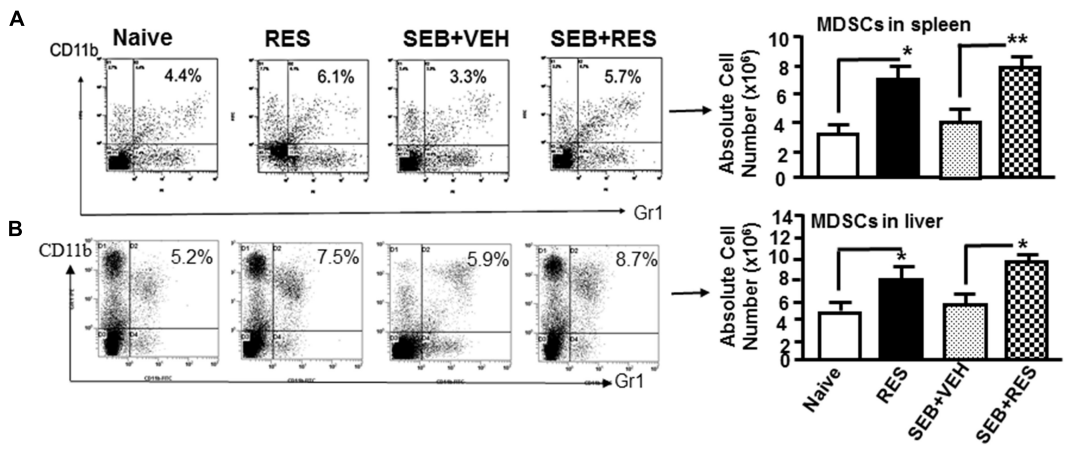

FIGURE 3 | Resveratrol induces MDSCs in the spleens and liver. Acute liver injury was induced as described in **Figure 1** legend. MDSCs were stained for the expression of Gr1 and CD11b. The percentage and total number of MDSCs in the spleen **(A)** and in the liver **(B)** is shown. Vertical bars in represent mean ± SEM using groups of five mice. Asterisk (*) indicates statistically significant ($p < 0.05$) differences between the groups.

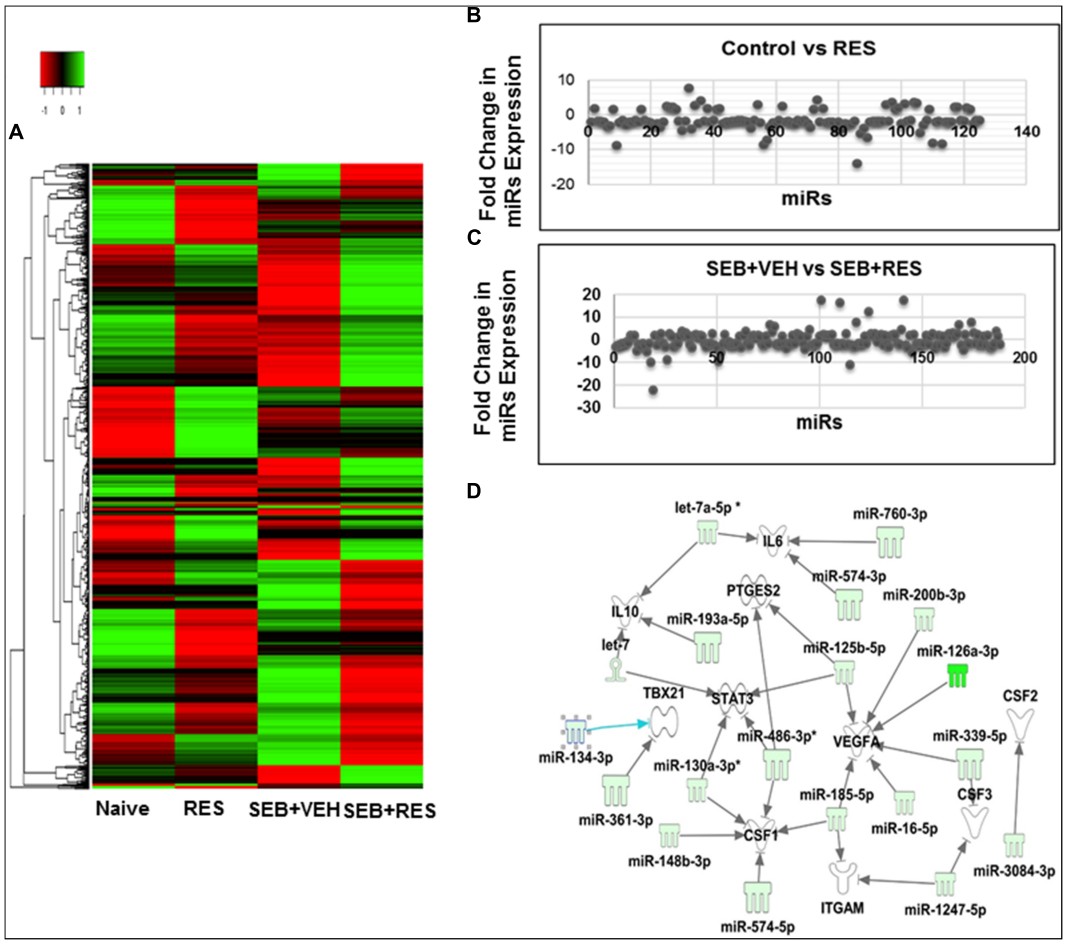

FIGURE 4 | Heat map of miR expression in liver MNCs in mice with acute liver injury and Pathway Analysis of dysregulated miRs in liver MNCs. Acute liver injury was induced as described in **Figure 1** legend. miR profile in liver MNCs was performed by using miR arrays on Affymetrix CGS300 System (Affymetrix). **(A)** Heat map depicting miR expression profile in liver MNCs of mice in the four groups. The expression pattern (green to red) represents the spectrum of downregulated to upregulated expression pattern of miRs, respectively. **(B)** Shows differential expression of miRs between Control and RES groups. **(C)** Shows differential expression of miRs between SEB+VEH and SEB+RES groups. **(D)** Dysregulated miRs in liver MNCs post-RES treatment in acute liver injury-induced mice were analyzed using Ingenuity pathway analysis (IPA) software online (Qiagen). There was a direct relationship of dysregulated miRs and several expected target genes including CSF gene families (CSF1, CSF2, CSF3), as well as VEGFA, PTGES2, STAT3, TBX21, and IL-10.

SEB+RES group when compared to SEB+VEH and Naïve groups (**Figure 3B**). These data suggested that RES might suppress SEB-mediated inflammation through induction of MDSCs.

RES Alters the miR Profile in Liver-Infiltrating MNCs

We next examined the miR profile in the liver-infiltrating MNCs cells of mice exposed to SEB and treated with VEH or RES. We performed cluster analysis of 2023 miRs (**Figure 4A**) using Ward's method and as defined by median absolute deviation in SEB+VEH and SEB+RES. We further measured the expression of miRs in the two groups using Half Square Euclidean Distance method. Ordering function of miRs was done based on input rank. Comparison of control vs. RES revealed 95 downregulated and 30 Upregulated miRs, while comparison of SEB+VEH vs. SEB+RES groups revealed 105 downregulated miRs and 74 upregulated miRs with a fold change of > 1.5 fold (**Figures 4B,C**).

RES-Regulated miRs Play Important Role in the Expression of CSF Family Genes

To understand the role of RES-mediated alterations in miRs in the regulation of genes that participate in anti-inflammatory responses, we analyzed some of the downregulated miRs using IPA software and the database of the company (Qiagen). These miRs were shown to target various genes including CSF1, CSF2, CSF3, VEGFA, PTGES2, STAT3, TBAX21 and IL-10 genes (**Figure 4D**). The CSF1 gene was particularly interesting because it has been shown to play a key role in the induction of MDSCs (Kumar et al., 2017), and we observed

significant induction of MDSCs following RES treatment (**Figures 3A,B**).

Validation of miR Expression by Real-Time PCR

Based on the analysis of miRs array data, we selected two downregulated miRs that targeted CSF1 (miR-130a and miR-185) (**Figure 5A**) to verify and validate their expression in liver MNCs harvested from naïve, RES, SEB+VEH, and SEB+RES groups of mice. We performed quantitative Real-Time PCR using cDNA generated from total RNAs including miRs isolated from MNCs of the four groups. Real-Time PCR data demonstrated significant downregulation in the expression of miR-130a and miR-185 in RES only treated mice, when compared to control mice (**Figure 5B**). Interestingly, SEB+VEH group showed robust upregulation of miR-130a and miR-185 when compared to the control group while SEB+RES group showed significant downregulation in the expression of these miRs (**Figure 5B**). Thus, the data obtained from Real-Time PCR validated the expression profile of miR-130a and miR-185 obtained from miR-array and demonstrated that SEB induces these miRs while RES decreases their expression.

RES Upregulated the Expression of CSF1 in Liver MNCs

Because CSF1 has been shown to play a key role in the induction of MDSCs (Kumar et al., 2017), and miR studies revealed down-regulation of miR-130a and miR-185 that target CSF1, we determined the expression of CSF1 in liver MNCs by quantitative Real-Time PCR. The data showed that there was a significant increase in the expression of CSF1 gene in RES only treated mice as well as SEB+RES groups when compared to control mice or those treated with SEB+VEH (**Figure 6A**). Additionally, we measured the levels of G-CSF in the sera of these mice and found that treatment with RES alone or SEB+RES significantly increased the G-CSF levels in the serum (**Figure 6B**). These data together demonstrated that RES significantly increases the expression of CSF1 in liver MNCs and G-CSF in the serum.

Analysis of miR-185-Associated CSF1 Expression

Because miR-185 showed strong binding affinity to CSF1 gene (mirwalk v3 and TargetScan), we investigated whether miR-185 plays a direct role in regulating the expression of CSF1 gene in mice. To that end, we transfected primary T cells with mature miR-185 mimic or anti-miR-185 or both, and cultured in the presence of SEB. Forty-eight hour post-transfection, the transfected T cells, were cultured in the presence of VEH or RES for 24 h. Next, we determined the expression of miR-185 and CSF1 by performing Real-Time PCR. T cells not transfected with miR-185 showed moderate miR-185 expression but RES significantly downregulated the expression of miR-185 (**Figure 7A**). In contrast, T cells transfected with miR-185 showed significantly higher expression of miR-185 but its expression was significantly downregulated in the presence of RES (**Figure 7A**).

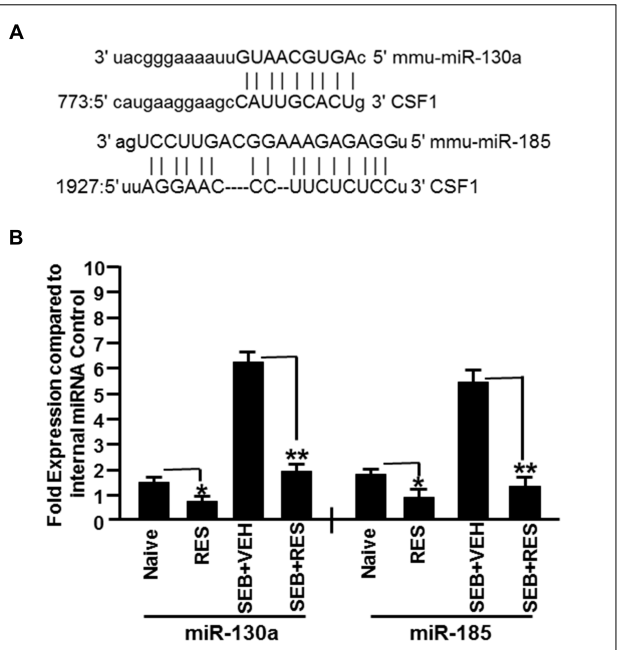

FIGURE 5 | Validation of expression profile of selected miRs in liver MNCs. We selected two miRs (miR-130a and miR-185) based on their ability to target CSF1 as suggested in figure for further validation. **(A)** Seed sequence and alignment of the miR-130a and miR-185 with 3′ UTR of CSF1 gene. **(B)** Using mouse miR-specific primers for mature miR-130a and miR-185, Real-Time PCR was performed to determine their expression in liver MNCs. SNORD96a was used as an internal control. Data presented for both miR-130a and miR-185 represent the mean ± SEM of three independent experiments **(B)**. Asterisk (*) indicates statistically significant ($p < 0.05$) difference between the groups compared.

T cells transfected with anti- miR-185, on the other hand, showed significantly downregulated miR-185 expression and its expression was further decreased in the presence of RES (**Figure 7A**). As suggested by the protocol of the manufacturer (Qiagen), T cells transfected with both miR-185 and anti-miR-185, showed significantly downregulated expression of miR-185 and RES further downregulated the expression of miR-185 (**Figure 7A**). These transfection studies confirmed that RES downregulates the expression of miR-185 in T cells.

We, next, examined the expression of CSF1 in T cells not transfected or transfected with miR-185 or anti-miR-185 or both, and treated with VEH or RES. As shown in **Figure 7B**, there was a moderate expression of CSF1 in VEH-treated untransfected T cells but CSF1 expression was significantly higher in T cells post-RES treatment (**Figure 7B**). However, CSF1 expression in T cells transfected with miR-185 significantly decreased, when compared to VEH-treated untransfected T cells (**Figure 7B**). Upon treatment with RES, there was further downregulation of CSF1 in T cells, when compared to T cells MOCK transfected and treated with VEH or RES (**Figure 7B**). T cells transfected with anti-miR-185 and treated with VEH, on the other hand, showed a significant increase in CSF1 expression, when compared to VEH-treated untransfected T cells (**Figure 7B**). Furthermore, RES treatment of anti-miR-185

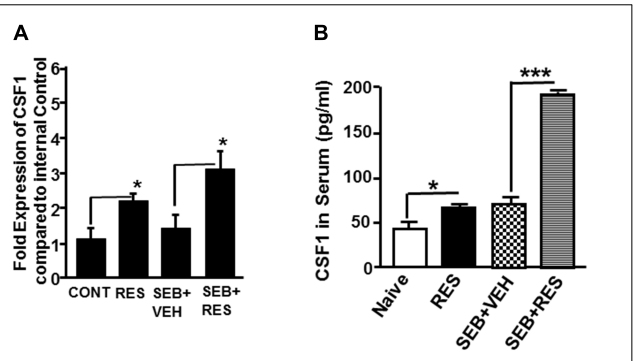

FIGURE 6 | Expression of CSF1 in liver MNCs and serum. Expression of CSF1 was measured in the four groups of mice that are described in **Figure 1** legend. **(A)** Expression of CSF1 gene in liver MNCs. Expression of mouse CSF1 gene in liver MNCs was determined by performing Real-Time quantitative PCR. Mouse CSF1-specific primers were used to perform Real-Time PCR on cDNAs generated from total RNAs isolated from liver MNCs of the four groups (naïve/control, RES, SEB+VEH, and SEB+RES). The internal control consisted of 18S. Data presented for CSF1 gene represent the mean ± SEM of three independent experiments and asterisk (*) indicates statistically significant ($p < 0.05$) difference between the groups. **(B)** Measurement of CSF1 levels in the sera of mice using ELISA. Vertical bars represent data with mean ± SEM of groups of five mice and asterisks (*) indicate statistically significant ($p < 0.05$) difference between groups compared.

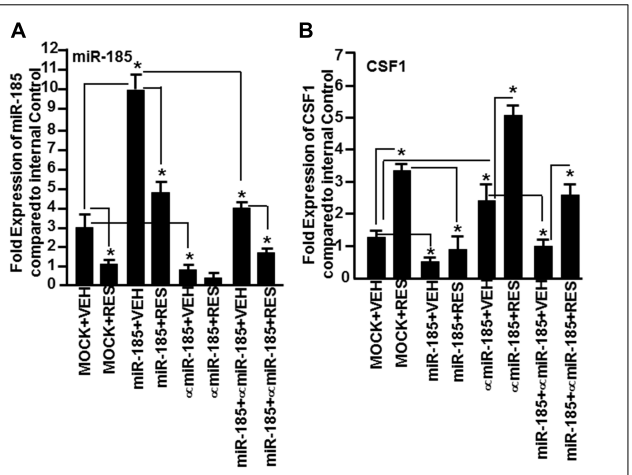

FIGURE 7 | Demonstration that miR-185 targets CSF1. Splenic T cells were transfected with MOCK (all transfection reagents without miR-185 mimic) or with mature miR-185 or anti-miR-185 or miR-185+anti-miR-185 (1:10 concentration). Forty-eight hours post-transfection, the cells were activated with SEB and treated with VEH or RES for 24 h. Expression of **(A)** miR-185 and CSF1 **(B)** genes was determined by performing Real-Time PCR. Data of Real-Time PCR are presented as fold change in expression relative to internal control (SNORD96a) for miR-185 or 18S for CSF1 gene. Data are depicted as mean ± SEM of three independent experiments and asterisks (*) indicate statistically significant ($p < 0.05$) difference between groups compared.

transfected T cells further significantly increased CSF1 expression (**Figure 7B**). When T cells were transfected with both miR-185 and anti-miR-185, CSF1 expression was downregulated, when compared to T cells transfected with anti-miR-185 (**Figure 7B**). Moreover, RES treatment significantly reversed the expression of CSF1 (**Figure 7B**). Taken together, these data suggested that RES decreases the expression of miR-185 which targets CSF1 thereby leading to induction of CSF1 expression.

DISCUSSION

Staphylococcus aureus is a ubiquitous Gram-positive bacteria that is found to colonize about one-third of the general population. It is an opportunistic pathogen that triggers infections through contaminated food. The enterotoxins such as SEB produced by these bacteria act as super antigens by way of activating a large proportion of T cells, triggering cytokine storm, acute toxic shock, multi-organ failure and mortality (McKallip et al., 2005; Krakauer et al., 2016). In the current study, we investigated whether RES, which is well-known for its potent anti-inflammatory property, can protect SEB-induced inflammation in liver and attenuate acute liver injury.

In the current study, we demonstrated that the administration of SEB caused an increase in AST levels as well as necrotic lesions in the liver. Additionally, the number of immune cells consisting of CD3+, CD4+ or CD8+ T cells, and NK1.1 cells, increased significantly in both the livers and spleens following SEB administration. RES treatment in SEB immunized mice caused an increase the population of MDSCs in the spleen as well as in the liver. Moreover, RES treatment led to reversal of all such inflammatory indicators and the livers were significantly protected from SEB-mediated injury. These data are consistent with our previous studies that RES is a potent anti-inflammatory agent that can protect mice from acute lung injury mediated by SEB (Rieder et al., 2012; Alghetaa et al., 2018), and extend these data by identifying the miR that regulates CSF1, which is involved in MDSC induction.

Myeloid derived suppressor cells are well-characterized by our lab and elsewhere as potent immunosuppressive cells (Gabrilovich et al., 2001; Hegde et al., 2008, 2013; Gabrilovich and Nagaraj, 2009; Singh U.P. et al., 2012). MDSCs have been well-studied and characterized in recent years in various tumor models as well as in cancer patients (Fujimura et al., 2010) for their immunosuppressive functions. In general, MDSC numbers increase significantly during cancer development, which in turn leads to suppression of anti-tumor immunity and enhanced tumor growth (Schmid and Varner, 2010). Hammerich and Tacke (2015) reported that the liver is a primary site of MDSCs *in vivo* and suggested that modulating the functionality of MDSCs might represent a promising therapeutic target for liver diseases (Hammerich and Tacke, 2015). MDSCs use several mechanisms to trigger immunosuppression, such as production of arginase I and inducible nitric oxide synthase (iNOS), leading to inhibition of T cell proliferation. However, more recently, it has been shown that MDSCs may also be induced at sites of inflammation and may prevent tissue injury by downregulating T cell responses (Bronte and Mocellin, 2009). An earlier

study demonstrated that repeated systemic administration of staphylococcal enterotoxin A (SEA) led to induction of tolerance via accumulation of MDSCs in the spleen, and this process was IFN-γ dependent (Cauley et al., 2000). We have also shown that RES attenuated chronic colitis in IL-10 knockout mice through MDSC induction (Singh U.P. et al., 2012). Also, RES was shown by our laboratory to induce immunosuppressive MDSCs in a lung-injury model (Rieder et al., 2012). In general, MDSCs get activated and expand in the presence of pro-inflammatory cytokines and other mediators such as G-CSF, CSF1 (M-CSF), GM-CSF, IL-1β, IL-12, and IFN-γ (Gabrilovich and Nagaraj, 2009). In the current study, we used miR pathway analysis to identify potential miRs that may trigger CSF1, a cytokine known to induce MDSCs. Interestingly, such studies led to identification of miR-130a and miR-185 that were found to target CSF1. These data also suggested that CSF1 induction by RES may play a critical role in MDSC induction inasmuch as previous studies showed that blocking CSF1 prevents the generation of MDSCs (Holmgaard et al., 2016).

Since the discovery of miRs, about a decade or so, these non-coding endogenous small RNAs have been shown to play a major role in the regulation of the immune responses (Pedersen and David, 2008), including autoimmunity and inflammation (Singh et al., 2013). Recent studies from our lab and elsewhere have shown that miRs play a critical role in promoting an anti-inflammatory state (Singh et al., 2013, 2014, 2016; Zhou et al., 2014; Guan et al., 2016). In the current study, therefore, we investigated if treatment with RES following SEB injection would alter the expression of miRs in the immune cells. The data demonstrated that RES treatment led to altered expression of a significant number of miRs in liver infiltrating MNCs of mice. Pathway analysis of miRs that are down-regulated by RES led to identification of two miRs (miR-130a and miR-185) that had strong binding affinity with complementary sequences of 3′ UTR regions of CSF1 gene. Because RES downregulated the expression of both miR-130a and miR-185, these data suggested the mechanisms through which RES may increase CSF1 expression. The role of miR-185 to regulate CSF1 expression was further confirmed by performing transfection experiments. There was significant downregulation of CSF1 expression in T cells that were transfected with miR-185 mimic while the expression of CSF1 was significantly upregulated in T cells that were transfected with anti-miR-185. These data also correlated with significant induction of CSF1 in liver MNCs and serum G-CSF concentrations in SEB+RES group when compared to SEB+VEH.

This is the first study that demonstrates the role of miR-185 in the regulation of CSF1. miR-130a and miR-185 have been shown to regulate other cytokines. For example, IL-10Rα has been shown to be directly regulated by miR-185 (Venza et al., 2015). Interestingly, miR-130a-3p was shown to target transforming growth factor-beta receptors (TGFBRs) 1 and 2. Thus, overexpression of miR-130a-3p in hepatic stellate cells (HSCs) inhibited their activation and proliferation, with the decreased expression of TGFBR1 and TGFBR2. TGF-β has been shown to control the generation of MDSCs (Lee et al., 2018). Thus, RES-mediated decrease in miR-130a may enhance TGF-β pathway to induce MDSCs. Woo et al. (2013) have shown that miR-130a regulates CSF1 mRNA decay in ovarian cancer cell demonstrating a role for miR-130a in CSF1 regulation.

In summary, the current study suggests that RES attenuates SEB-induced liver injury through modulation of miRs. Because SEB is a super antigen that drives cytokine storm, our studies suggest that RES is a potent anti-inflammatory agent that has the potential to be used as a therapeutic modality to treat acute inflammation triggered by bacterial enterotoxins. Our studies also suggest that RES may mediate its effects through alterations in the expression of miR-130a and miR-185, which target CSF1 expression and consequently trigger highly immunosuppressive MDSCs.

In addition to the immunosuppressive properties exerted by RES against SEB, a bacterial enterotoxin, as shown in the current study, RES has also been shown to exert antibacterial activity (Ma et al., 2018). Also, RES may act an antagonist, when used in combination of antimicrobial agents. Recently, Tosato et al. (2018) have shown that the antimicrobial capacity of levofloxacin or photodynamic therapy was significantly diminished when levofloxacin or methylene blue were co-administered together with RES, indicating that consumption of RES during antimicrobial treatment should be cautioned (Tosato et al., 2018). In another study, Liu et al. (2016) showed that RES antagonizes antimicrobial lethality and stimulates recovery of bacterial mutants. In the light of these reports, one should be cautious to use RES when antibiotic agents are being used to treat pathogenic bacteria. However, we also wish to add that our studies have focused on SEB which can also be used directly as a bioterrorism agent to cause toxicity and organ failure, and that in such instances, RES as well as miRNA identified may serve as a treatment modality.

AUTHOR CONTRIBUTIONS

SK, NS, PN, and MN conceptualized and designed this work. SK and NS were involved in sample processing and data collection, conducted the primary investigation and statistical analysis of the data, and wrote the manuscript. PN and MN provided resources, analysis tools, and responsible for funding acquisition. SK, NS, EZ, TC, SC, LH, PN, and MN reviewed and edited the manuscript. AA contributed to the design of one experiment and reviewed the manuscript. All authors approved the final manuscript.

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REVIEW
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Macrophage Activation-Like Syndrome: A Distinct Entity Leading to Early Death in Sepsis

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Hemophagocytic lymphohistocytosis (HLH) is characterized by fulminant cytokine storm leading to multiple organ dysfunction and high mortality. HLH is classified into familial (fHLH) and into secondary (sHLH). fHLH is rare and it is due to mutations of genes encoding for perforin or excretory granules of natural killer (NK) cells of CD8-lymphocytes. sHLH is also known as macrophage activation syndrome (MAS). Macrophage activation syndrome (MAS) in adults is poorly studied. Main features are fever, hepatosplenomegaly, hepatobiliary dysfunction (HBD), coagulopathy, cytopenia of two to three cell lineages, increased triglycerides and hemophagocytosis in the bone marrow. sHLH/MAS complicates hematologic malignancies, autoimmune disorders and infections mainly of viral origin. Pathogenesis is poorly understood and it is associated with increased activation of macrophages and NK cells. An autocrine loop of interleukin (IL)-1β over-secretion leads to cytokine storm of IL-6, IL-18, ferritin, and interferon-gamma; soluble CD163 is highly increased from macrophages. The true incidence of sHLH/MAS among patients with sepsis has only been studied in the cohort of the Hellenic Sepsis Study Group. Patients meeting the Sepsis-3 criteria and who had positive HSscore or co-presence of HBD and disseminated intravascular coagulation (DIC) were classified as patients with macrophage activation-like syndrome (MALS). The frequency of MALS ranged between 3 and 4% and it was an independent entity associated with early mortality after 10 days. Ferritin was proposed as a diagnostic and surrogate biomarker. Concentrations >4,420 ng/ml were associated with diagnosis of MALS with 97.1% specificity and 98% negative predictive value. Increased ferritin was also associated with increased IL-6, IL-18, IFNγ, and sCD163 and by decreased IL-10/TNFα ratio. A drop of ferritin by 15% the first 48 h was a surrogate finding of favorable outcome. There are 10 on-going trials in adults with sHLH; two for the development of biomarkers and eight for management. Only one of them is focusing in sepsis. The acronym of the trial is PROVIDE (ClinicalTrials.gov NCT03332225) and it is a double-blind randomized clinical trial aiming to deliver to patients with septic shock treatment targeting their precise immune state. Patients diagnosed with MALS are receiving randomized treatment with placebo or the IL-1β blocker anakinra.

Keywords: macrophage activation syndrome, sepsis, hemophagocytic lymphohistocytosis, ferritin, interleukins, interferon-gamma

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INTRODUCTION

The need for adjunctive therapies in sepsis is coming from the failure of antimicrobial treatment and source control to restrain the high lethality. This led to the concept that modulation of the overwhelming host response of an infection is the strategy that needs to be followed in order to improve outcomes. It is already more than 20 years since treatment strategies with drugs aiming to modulate the exaggerated host response to an infection have started to be studied in randomized clinical trials (RCTs). A large list of agents was studied the vast majority of which was targeting pro-inflammatory mediators like cytokines. The vast majority of these RCTs failed to meet the study primary endpoint that was, for most of these RCTs, mortality after 28 days. There are two suggested explanations for the failure of these RCTs: (a) the primary endpoint is not correct since sepsis is an infectious complication so the endpoint should address the criteria for infection resolution; (b) the timing of the strategy is not correct or partly correct e.g., inhibiting an exaggerated host response may be ineffective if given to a wrong time point when the host response has ceased to exist. This last hypothesis may mostly apply for patients who develop sepsis in the field of community-acquired infections where the time from start of infection until the development of sepsis-associated organ dysfunction varies greatly from one patient to the other. The failure of RCTs with inhibitors of pro-inflammatory mediators gave birth to the concept of sepsis-induced immunosuppression that has gained much of enthusiasm the last years. We elaborate on the hypothesis that patients with sepsis can be classified into three groups regarding the mechanism of organ dysfunction:

- Patients with dominant pro-inflammatory mechanism of organ dysfunction.
- Patients with dominant anti-inflammatory mechanism of organ dysfunction.
- Patients with co-existing pro-inflammatory and anti-inflammatory mechanisms of organ dysfunction that do not remain stable and fluctuate toward the one or the other direction over time.

If this hypothesis holds true, it can be postulated that treatment of patients with a dominant pro-inflammatory mechanism of organ dysfunction mandates inhibition of pro-inflammatory mediators. In this review, we try present for the first time all published evidence demonstrating that in adults with sepsis there is a small proportion of patients who develop and maintain sepsis responses through dominant pro-inflammatory mechanisms of organ dysfunction. We also aim to review and suggest specific biomarkers of the detection of patients who develop sepsis-associated organ dysfunction with dominant pro-inflammatory mechanism and to suggest therapies that act on this pro-inflammatory mechanism. Evidence coming from studies in children is used only when evidence coming from adults is missing.

HEMOPHAGOCYTIC LYMPHOHISTOCYTOSIS: CLASSIFICATION AND CURRENT CONCEPT

The situation of predominant pro-inflammatory sepsis in the adult is an expansion of our knowledge on the syndrome of hemophagocytic lymphohistiocytosis (HLH) described in children. There are two forms of HLH; the primary or familial HLH (fHLH) and the secondary HLH (sHLH). The sHLH is also known as macrophage activation syndrome (MAS). Familial HLH (fHLH) is caused by mutations affecting the cytolytic pathway of natural killer (NK) cells and CD8+ T lymphocytes. Normally, these cells recognize and kill infected cells. The majority of patients with fHLH carry mutations in *PRF1*, which encodes perforin, or in genes encoding proteins required for the docking and fusion of granules for excretory protein like *UNC13D* (encoding MUNC13–4), *STX11* (encoding syntaxin 11), and *STXBP2* (encoding syntaxin-binding protein 2). These mutations transform NK cells to become over-active and stimulate a fulminant cytokine storm leading to organ dysfunctions (1). Children are classified into HLH if they meet at least five of the eight criteria of the International Histiocyte Society (2004-HLH criteria) published in 2007: (a) fever, (b) splenomegaly, (c) cytopenia of at least two lineages; (d) fasting triglycerides $\geq$265 mg/dl and fibrinogen $\leq$150 mg/dl; (e) hemophagocytosis in the bone marrow; (f) low or absent NK-cell activity; (g) ferritin $\geq$500 ng/ml; and soluble CD25 $\geq$2,400 units/ml (2). These patients are further classified into fHLH or sHLH if they have or if they do not have positive molecular assay for one of the mutations listed above. There is large overlap between clinical signs of sHLH and of sepsis-associated organ dysfunction in children. Despite this overlap, the treatment strategy and associated prognosis are far different in children with sHLH than in children with sepsis. Management of sHLH mandates repeated cycles of chemotherapy whereas management of sepsis relies on the proper use of antimicrobials (3).

Macrophage Activation Syndrome in the Adults: Features, Classification Criteria, and Etiology

The classification criteria for sHLH or MAS were developed by the analysis of medical records of 312 patients by three experts. The experts classified patients as positive or negative for sHLH or undetermined through a consensus approach. The main clinical characteristics associated with sHLH entered multivariate logistic regression analysis and variables independently associated with sHLH were used to construct the HSscore. This score now contains nine variables. The score may range from 0 to 317 and values >169 provide the best cut-off for classification as they have sensitivity 93% and specificity 86% allowing correct classification of 90% of cases (4). The majority of analyzed cases developed sHLH as a complication of hematologic malignancy (57% of cases), infection (25% of cases), or both malignancies and infection (4% of cases).

TABLE 1 | Synopsis of observational case-series of adult patients with macrophage activation published the last 5 years.

References	No of patients	Main clinical and laboratory signs (n/total patients or %)	Mortality (n/total patients or %)
Barba et al. (5)	71 ICU admissions	Fever (92), hepatomegaly (44), splenomegaly (39), thrombocytopenia (45), leukopenia (10), ↑AST (71), hemophagocytosis in bone marrow (83)	38.1
Goemezano et al. (7)	17 with SLE and acute pancreatitis	Fever (94), hepatomegaly (47), splenomegaly (23), thrombocytopenia (65), leukopenia (82), ↑ triglycerides (87), ↓ fibrinogen (9), ↑AST (71)	23
Japtag et al. (8)	6	Fever (6/6), splenomegaly (6/6), pancytopenia (6/6), ↑ triglycerides (6/6), ↑ bilirubin (6/6), ↓ fibrinogen (6/6), ↑ALT/AST (6/6), hemophagocytosis in bone marrow (6/6)	2/6
Liu at al. (9)	32 with SLE	Fever (96.9), splenomegaly (56.3), anemia (87.5), thrombocytopenia (84.4), neutropenia (58.1), ↑ triglycerides (73), ↓ fibrinogen (38.7), ↑ALT (81.3), hemophagocytosis in bone marrow (100)	12.5
Schram et al. (6)	68	Fever (96), splenomegaly (73), anemia (94), thrombocytopenia (96), neutropenia (72), ↑ triglycerides (58.1), ↑ bilirubin (85), ↓ fibrinogen (62), ↑ALT (97), hemophagocytosis in bone marrow (89)	21
Schulert et al. (10)	16 with H1N1 influenza	Splenomegaly (6/16), hepatomegaly (14/16), thrombocytopenia (12/16), neutropenia (2/16), ↑ triglycerides (9/16), ↑AST (14/16), hemophagocytosis in bone marrow (13/16)	16/16

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ICU, intensive care unit; SLE, systemic lupus erythematosus; ↓, decreased; ↑, increased.

A total of 115 cases of patients hospitalized in Intensive Care Units (ICU) and undergoing bone marrow aspiration were retrospectively analyzed and classified using the HSscore; 71 cases were classified into confirmed sHLH. Malignancies and infection were the most common predisposing conditions complicated by HLH. The most common malignancy associated with sHLH was non-Hodgkin's lymphoma (21%) and the most common infections were those coming from Ebstein-Barr virus and from cytomegalovirus (18%) (5). These patients were admitted in the ICU with organ dysfunction mainly acute respiratory distress syndrome (ARDS, 35% of cases), circulatory shock (28% of cases) or multiple organ dysfunctions (MODS, 10% of cases). In another series of 68 analyzed cases, the most common predisposing conditions were hematologic malignancies (49% in total; of myeloid origin 13%; of B-lymphoid origin 19%; and of T-lymphoid origin 13%), and infections (33% total; viral 24% of cases) (6). The main clinical and laboratory features of the reported series of patients with sHLH published the last 5 years are provided in **Table 1**.

Pathogenesis

The mechanism of pathogenesis of sHLH/ MAS remains unclear. It seems that a trigger of persistent inflammation or antigen presentation caused by ineffective apoptosis of infected or activated or malignant cells leads, usually through continuous stimulation of Toll-like receptors (TLRs), to the activation and uncontrolled expansion of T lymphocytes, macrophages, and NK cells; this leads to marked hypercytokinemia. The reason for ineffective cytolytic killing of NK cells may rely on genetic defects in perforin-mediated cytotoxicity. Whole exome sequencing analysis has revealed a high frequency of SNP in genes causatively related to fHLH among patients with MAS developing in the field of systemic onset juvenile idiopathic arthritis (sJIA, 35.7% of cases) compared to sJIA controls (13.7% of cases; $p = 0.098$). A series of other variants involved in the trafficking and fusion of cytolytic granules of NK cells were also identified in this population. These findings can be an indication of a genetic overlap as well as a common pathogenesis pathway between sHLH/MAS and fHLH (11).

In a report of 16 patients with fatal infection by the H1N1 influenza virus, signs of hemophagocytosis were found in 13 patients. DNA sequencing for genes reported in patients with familial HLH was done. Carriage of rare variants associated with HLH was found in five patients. All five patients were carriers of mutations of the *LYST* gene; two of these five patients were also carrying mutation of the *PRF1* gene. Although the functional significance of these variants is unknown, it should be underscored that *PRF1* encodes for perforin that decreases NK cell cytotoxicity (10).

The main cytokines that play a major role in the pathogenesis of sHLH/MAS are interleukin (IL)-1β, IL-6, IL-18 and interferon-gamma (IFNγ) and one iron-binding protein, namely ferritin. Even though studies have not shown IL-1β levels to be consistently increased during sHLH (12), it could bear in mind that IL-1β is secreted locally and hence not measured. The implication of the role of IL-1β is coming indirectly both from the increase of circulating levels of the IL-1 receptor antagonist (IL-1ra) and by the therapeutic role of the IL-1β blocker anakinra in children with sHLH (13, 14).

In patients with MAS, free IL-18 concentrations significantly correlated with the clinical status and the biologic markers of MAS such as anemia ($p < 0.001$), hyper-triglyceridemia, and hyper-ferritinemia and also with markers of Th1 lymphocyte and macrophage activation, such as the elevated concentrations of IFNγ and of soluble IL-2 receptor and of the concentrations of the TNFα receptor (15).

Current knowledge on the pathogenesis of sHLH is coming from animal models in mice. These models suggest that there is no unique mechanism leading to sHLH. sHLH is commonly induced in mice after viral challenge using strains like the lymphocytic choriomeningitis virus (LCMV) and the murine cytomegalovirus (CMV). The induction of sHLH in

mice by LCMV mandates the challenge into *pfp*–/– mice with homogeneous deficiency of the perforin gene. These mice develop within 10 days full clinical signs of sHLH i.e., fever, cytopenia, hypofibrinogenemia, hypertriglyceridemia and splenomegaly. Excess levels of IFNγ mediated through the activation of CD8-lymphocytes is observed (16). However, it seems that the excess levels of IFNγ do not represent a real causal link between IFNγ and sHLH. In a mouse model of sHLH induced by CMV, viral infection of mice knocked-out for IFNγ led to more severe disease phenotype with more rapid progression into death and greater cytokine production so as to indicate that excess IFNγ is a reciprocal mechanism to prevent massive tissue destruction in sHLH. This model is also characterized by microthrombi in tissue vasculature consistent with disseminated intravascular coagulation (17). Although the use of LCMV and CMV as the triggering pathogens highlights the contribution of cells of the adaptive immune system in the pathogenesis of sHLH, sHLH may be induced in mice through serial challenge with the TLR9 agonist CpG. Single challenge with CpG cannot induce sHLH but this requires serial injections in consecutive days. CpG can be a powerful stimulant of sHLH even in *Rag2*$^{-/-}$ mice depleted by T- and B-lymphocytes. Two peaks of circulating IFNγ are observed; an early peak the first 24 h post LCMV infection and a second peak 7 days post LCMV infection. Both peaks are needed for the sHLH phenotype to be induced (18).

Girard-Guyonvarc'h and Weiss showed a causative link between free L-18 and MAS induction. Using two different mouse models of IL-18 over-activity [namely, mice over-expressing IL-18 and mice deficient in IL-18 binding protein (*Il18bp*–/–)], both research groups confirmed that high levels of free IL-18 unbound to IL-18BP increase the risk of developing MAS. Following an additional trigger through TLR9 activation both mouse models developed characteristic MAS manifestations, whereas wild-type mice did not (19, 20). Inhibition of IL-18 signaling using an antibody targeting the IL-18 receptor attenuated the severity of the MAS manifestations in *Il18bp*–/– mice. In patients suffering from auto-inflammatory disorders and hyper-ferritinemia a dramatic correlation of the risk for the development of MAS risk with the chronic (sometimes lifelong) elevation of free IL-18 was found (20). In a specific type of MAS associated with gain-of-function mutations in *NLRC4* that cause inflammasome hyperactivity, IL-18 was derived from the intestinal epithelium.

Increased ferritin is a common denominator of sHLH and of some cases of septic shock (21). It is not fully elucidated whether it is a bystander highly increased or a pro-inflammatory mediator *per se*. Mortality analysis was done in 405 adult patients with ultra-elevated ferritin >5,000 ng/ml. Overall mortality 30 days and 6 months after index serum ferritin measurement was 32 and 50%, respectively. For patients with serum ferritin between 5,000 and 10,000 ng/ml, mortality was 27% after 30 days and 49% after 6 months. For patients with serum ferritin between 10,000 and 20,000 ng/ml, mortality was 41% after 30 days and 50% after 6 months. For patients with serum ferritin between 20,000 and 40,000 ng/ml, mortality was 41% after 30 days and 52% after 6 months. For patients with serum ferritin >40,000 ng/ml, mortality was 52% after 30 days and 57% after 6 months. Sepsis was not identified as a cause of hyperferitinaemia among patients who were also suffering from malignancies (22).

Ferritin is an iron storage protein including heavy (H) and light (L) subunits. In a study evaluating bone marrow biopsies of patients with MAS, H-ferritin, IL-1β, TNFα, and IFNγ were significantly increased. Furthermore, an increased number of CD68+ /H-ferritin+ cells and an infiltrate of cells co-expressing H-ferritin and IL-12, suggesting an infiltrate of M1 macrophages, were found. H-ferritin levels and CD68+ /H-ferritin+ cells were correlated with hematological involvement of the disease, serum ferritin and C-reactive protein (23). Ferritin synthesis is up-regulated in response to hemoxygenase-1 activation to remove any iron that could exacerbate oxidative stress. Ferritin also acts as an antiapoptotic agent in ischemia-reperfusion injury. It may be the case that ferritin acts like a danger-associated molecular pattern (DAMP) ending with the stimulation of NF-κB and the over-production of IL-1β.

A schematic representation of the pathogenesis of sHLH/MAS in sepsis is shown in **Figure 1**.

Biomarkers

Apart from ferritin mentioned above, there is no consensus on which the best diagnostic biomarkers for the diagnosis of sHLH/MAS are. Soluble CD163 is the soluble counterpart of the CD163 receptor for the hemoglobin-haptoglobin complex on the cell membrane of M2 macrophages. Increases of sCD163 signify intense differentiation of M2 macrophages through the alternate pathway related to phagocytic activity. In a recent study, sCD163 was measured in the serum of 63 patients with sJIA. Concentrations were greater among patients with active sJIA associated with macrophage activation and they were decreased upon disease remission. Positive associations were found with circulating IL-18 and ferritin and with liver aminotransferases (24). Similar increases of sCD163 were reported for 34 patients with adult-onset Still'disease (AOSD) in whom sCD163 was elevated at similar levels as for 16 patients with sepsis. Immunohistochemistry of lymph nodes and of the tonsils revealed equal distribution of sCD163 in both B-rich and T-rich areas contrary to H-ferritin that was mainly expressed in B-rich areas (25).

Although not conducted in adults, a recent analysis supports concentrations of fibrinogen in plasma lower than 150 mg/dl as suggestive of the presence of sHLH/MAS. More precisely, 190 admissions of patients aged <21 years were retrospectively analyzed. Patients were split into those with hypofibrinogenemia (≤150 mg/dl, $n = 38$) and into those with normal of elevated fibrinogen (>150 mg/dl, $n = 154$) based on available fibrinogen measurements at the beginning of follow-up. A composite endpoint, namely, complicated course, was selected by the authors as the primary study endpoint. This was composed from the presence of at least one unfavorable outcome i.e., death after 28 days or ≥ two organ dysfunctions the first 7 days. This primary endpoint was met in 73.7% of patients with hypofibrinogenemia compared to 29.2% of comparators ($p < 0.0001$). Patients were also classified into sHLH/MAS based on the 2004-HLH criteria (2). The proportion of sHLH/MAS was 15.8% in the group of

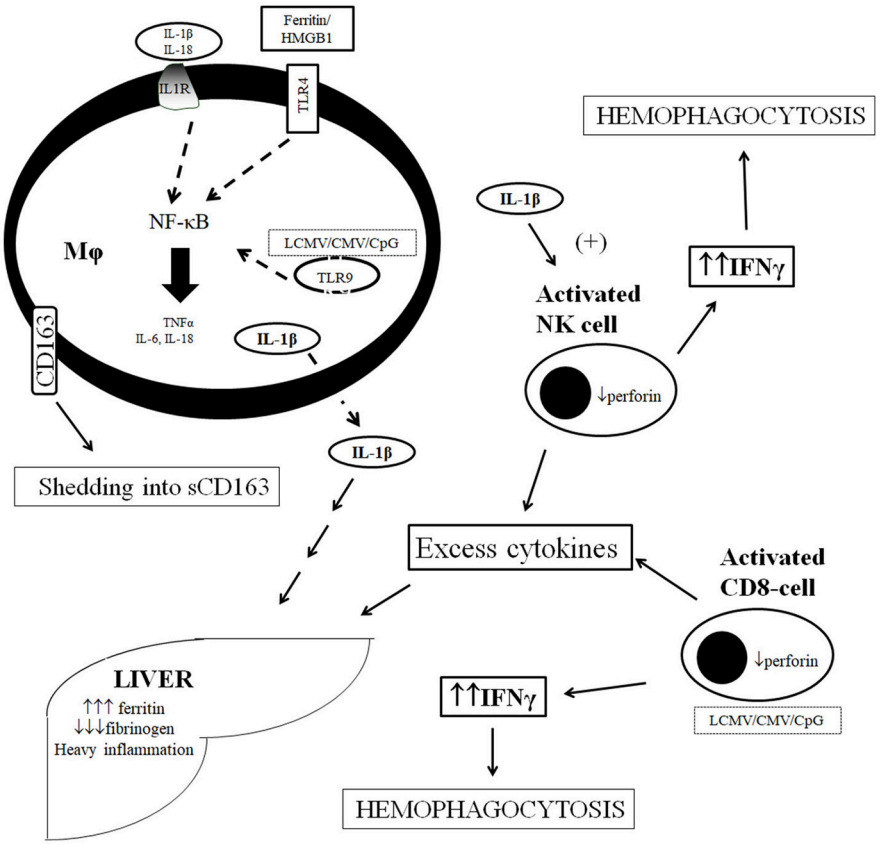

FIGURE 1 | Current concept of pathogenesis of macrophage activation syndrome in sepsis. Over-production of IL-1β is effected through the stimulation of TLRs by ferritin and HMGB1 and by the *per se* autocrine effect of IL-1β on IL-1R at the macrophage level. Stimulation leads to cytokine storm and shedding of CD163 from macrophage cell membrane. Over-produced cytokines stimulate further ferritin production by the liver and liver dysfunction whereas IL-1β leads to over-production of IFNγ by NK cells leading to hemophagocytosis. Excess cytokine production may results independently by distirbances in NK cell and CD-cell function leading to their over-activation. CMV, cytomegalovirus; CpG, bacterial oligonucleotides; IL, interleukin; IL-1R, receptor of IL-1; LCMV, lymphocytic choriomeningovirus; Mϕ, macrophage, NF-κB, nuclear factor kappa B; NK, natural killer; TLR, Toll-like receptor; TNFα, tumor necrosis factor-alpha.

hypofibrinogenemia whereas it was 1.3% among the comparators ($p < 0.0001$) (26).

In a recent retrospective study, positron emission tomography/computed tomography (PET/CT) with ^{18}F-fluorodeoxyglucose uptake was conducted in 34 patients classified with MAS using the HSscore. Fifteen patients with sepsis scoring negative for the HScore were studied as comparators. The spleen to liver maximal uptake value (SLR_{max}) was greater in MAS than sepsis. The best discriminator was for values >1.31 associated with odds ratio 8.175 for MAS. Patients with SLR_{max} >1.72 died earlier (27). Although these findings point the role of the spleen as a major lymphoid and macrophage pool for MAS, they also underscore the diagnostic and prognostic role of FDG-PET/CT.

MACROPHAGE ACTIVATION SYNDROME AND SEPSIS: THE BIRTH OF A HYPOTHESIS

As analyzed above among infections causing MAS, bacteria are the least common causes whereas viruses are the major causes. The dominant feature of MAS is the over-activation of tissue macrophages for the release of a storm of cytokines leading to rapidly progressing organ dysfunction where pancytopenia, tissue hemophagocytosis, hepatobiliary dysfunction (HBD), disseminated intravascular coagulation (DIC), and dysfunction of the central nervous system predominate. Macrophage activation syndrome (MAS) often leads to early death. The hallmark of pathogenesis is the over-production of IL-1β by tissues macrophages. IL-1β acts through an autocrine way on macrophages leading to a vicious cycle of further cytokine production and exaggerated inflammation.

Anakinra is the recombinant humanized form of IL-1 receptor antagonist that inhibits both IL-1β and IL-1α. In one phase 3 RCT conducted 25 years ago, anakinra was administered intravenously in 906 patients with severe sepsis. The study was prematurely stopped for futility (28). Twenty years after the conduct of this trial, clinical data of enrolled patients were retrospectively reviewed. Among enrolled patients, those presenting both with HBD and DIC were considered to have traits of MAS. A total of 43 patients were classified with MAS, 26 of whom were treated with anakinra and 17 with placebo; 28-day mortality was 35 and 65%, respectively and this difference was statistically significant

TABLE 2 | Suggested classification criteria for macrophage activation-like syndrome in sepsis used in the manuscript by Kyriazopoulou et al.

Sepsis (defined as total SOFA score ≥2 points for new admissions or as increase of total SOFA score ≥2 points for hospitalized patients)

+ either positive HSscore or both HBD and DIC

HSscore (more than 151 points are needed)	
	Points
• Infection by HIV or long term immunosuppressive treatment e.g., cyclosporine, glucocorticoids, azathioprine	18
• Core temperature	
<38.4°C	0
38.4–39.4°C	1
>39.5°C	2
• Organomegaly	
Hepatomegaly or splenomegaly	1
Hepatomegaly and splenomegaly	2
• Number of cytopenias	
1 lineage	0
2 lineages	24
3 lineages	34
• Ferritin (ng/ml)	
<2,000	0
2,000–6,000	35
>6,000	50
• Triglycerides (mmol/l)	
<1.5	0
1.5–4	44
>4	64
• Fibrinogen (mg/l)	
>2.5	0
≤2.5	30
• Serum aspartate aminotransferase (U/l)	
<30	0
≥30	19

HBD
Presence of at least 2 of the following:
• Serum bilirubin > 2.5 mg/dl
• Aspartate aminotransferase ≥2 × upper normal limit
• International normalized ratio (INR) >1.5

DIC score (more than 5 points are needed)	
	Points
• Platelet count (/mm^3)	
<100,000	1
<50,000	2
• D-dimers	
No increase	0
Moderate increase	2
Strong increase	3
• Prothrombin time	
<3 s	0
3–6 s	1
>6 s	2
• Fibrinogen (g/l)	
>1	0
<1	1

DIC, disseminated intravascular coagulation; HBD, hepatobiliary dysfunction; HIV, human immunodeficiency virus; HS, hemophagocytosis; SOFA, sequential organ failure assessment; <, less than; >, more than; ≤, less than or equal to; ≥, more than or equal to.

(p: 0.0006) (29). Although these results were inconclusive, they triggered the concept that in a small fraction of sepsis patients pro-inflammatory phenomena predominate and that treatment may be associated with better outcomes.

MACROPHAGE ACTIVATION-LIKE SYNDROME IN SEPSIS: DOES THIS EXIST?

In order to investigate if a situation like MAS exists in sepsis, we run an analysis of 5,121 patients registered in the prospective cohort of the Hellenic Sepsis Study Group using a test and validation approach. We set specific criteria of classification (presented in **Table 2**) and we called this syndrome macrophage activation-like syndrome (MALS) because routine bone marrow biopsy was not available for the patients. According to the criteria, patients with sepsis (as defined by the Sepsis-3 definition) and with HSscore more than 151 or who were presenting with both HBD and DIC were classified into MALS. 3.7% of the test cohort ($n = 3,417$ patients) and 4.3% of the validation cohort ($n = 1,704$) were classified into MALS. Macrophage activation-like syndrome (MALS) was an independent variable associated with early death after 10 days (odds ratio 1.86 in the test cohort; 2.81 in the validation cohort) (30).

We developed ferritin measurement of the first 24 h as a diagnostic biomarker of MALS. Ferritin has already been proposed by others to be the diagnostic hallmark of MAS. We selected a cut-off concentration of 4,420 ng/ml that was associated with 97.1% specificity and 98% negative predictive value for diagnosis. The reason of selection of this cut-off was the high diagnostic specificity that was necessary for the biomarker to be used for treatment guidance. Twenty-eight-day mortality for patients having ferritin more than 4,420 ng/ml was 66.7% in the test cohort and 66.0% in the validation cohort. This was 52.9% in a separate cohort of 109 Swedish patients with

severe sepsis/septic shock. Hyperferritinemia was accompanied by elevated serum concentrations of IL-18, IFNγ and of sCD163 and by decrease of the ratio IL-10/TNFα pointing toward the pro-inflammatory nature of these patients. Ferritin concentrations started to decrease within the first 48 h among survivors; more than 15% decrease was associated with 0.13 odds ratio for favorable outcome.

The above findings are compatible with the existence of a small proportion of 3–4% of sepsis patients who have MALS at sepsis onset. However, they do not provide evidence if MALS can develop later during the course of sepsis. Stimulation of pro-inflammatory innate responses can also come from DAMPs. One of these DAMPs is the non-histone nuclear protein high mobility group box-1 (HMGB1) that is released after cell destruction. HMGB1 can stimulate tissue macrophages. Serial measurements of circulating HMGB1 after sepsis onset allows classification of patients into those who present early peak of HMGB1 over the disease course (i.e., the first 48 h from sepsis onset) and into those who present late peak of HMGB1 over the disease course (i.e., after seven days from sepsis onset). Mortality after 28 days was 9.6 and 26.9%, respectively (*p*: 0.026). This late peak of HMGB-1 was accompanied by increased serum levels of ferritin and IFNγ implying that patients with late peak of HMGB1 were entering into a mechanism resembling MALS. Surprisingly, the late peak acted synergistically with the history of chronic comorbidities with a pro-inflammatory component, namely type 2 diabetes mellitus, chronic heart failure and chronic renal disease, and increased substantially the risk for 28-day mortality (31).

Serum levels of HMGB1 were measured in the sera of children with MAS developing as a complication of sJIA or of systemic lupus erythematosus (SLE). Samplings were done at MAS onset and repeated upon clinical improvement 2 and 8 weeks after start of etoposide treatment. HMGB1 was found increased and it was decreasing over-time (32). Similar time kinetics were also found for ferritin, IL-18 and IFNγ, that have already been described to be the combination of elevated cytokines that are characteristic of pro-inflammatory sepsis (30). This observation in children complements the observation in sepsis patients with late HMGB1 peaks and elevated ferritin and IFNγ accompanied by high mortality suggesting that HMGB1 can be a stimulator of MAS. What remains to be explained is how the late peak of HMGB1 acts synergistically with chronic comorbidities to prime unfavorable outcome. One explanation may come from the results of the CANTOS trial. In this RCT, survivors from a first myocardial infarct were randomized to blind treatment with placebo or with escalating doses of the IL-1β blocker canakinumab. Results showed substantial decrease of the risk of secondary cardiovascular death (33). This clinical benefit was achieved through IL-1β inhibition pointing toward tissue macrophage-derived elevated IL-1β production as a common denominator for situations with atherosclerosis like type 2 diabetes mellitus, chronic heart failure, and chronic renal disease. This sterile chronic pro-inflammation is definitively mediated by DAMPs. Since DAMPs like HMGB1 also participate in septic phenomena, it should not be surprising that a state of chronic stimulation of tissue macrophages synergizes with a sepsis state of peak in HMGB1.

The significance of elevated ferritin as a prognostic biomarker in sepsis, albeit not in adults, was recently published by Carcillo et al. (34). In a series of 100 consecutive admitted children with severe sepsis, a contingency Table was built using ferritin and CRP cutoffs of 1,980 ng/ml and 4.08 mg/dl, respectively. Patients with both markers above the cut-offs were classified with high mortality risk of 46.15%; those with one of the two biomarkers above the cut-offs as intermediate risk with mortality ranging between 0 and 4.65%; and those with both biomarkers below the cut-offs as low-risk with 0% mortality. High-risk defined by the two biomarkers was the only variable independently associated with 28-day mortality in the pediatric ICU (odds ratio = 9.58; $p = 0.019$; Z statistic = 2.35); whereas, bacterial infection ($p = 0.07$; Z statistic = 1.83), age ($p = 0.96$; Z statistic = 0.05), cancer diagnosis ($p = 0.45$; Z statistic = 0.76), PRISM score ($p = 0.88$ Z; statistic = 0.88), and maximum OFI ($p = 0.13$; *Z* statistic = 1.52) did not (34).

CURRENT TREATMENT MODALITIES AND THE NEED FOR LARGE-SCALE RANDOMIZED CLINICAL TRIALS

There is no gold-standard of treatment of sHLH/MAS. As a rule, treatment modalities like etoposide, glucocorticosteroids, anakinra, and intravenous immunoglobulins that are already in use in children (35) are also in use in adults. Available evidence is coming from case-series with limited number of patients. In a study of 25 critically ill patients with respiratory failure and/or hemodynamic instability following influenza A/H1N1 infection in Germany, nine patients were found to develop sHLH and consequently MODS (36). Of them, six were assigned to treatment; four patients with etoposide/ dexamethasone and two patients with steroids; only one patient survived. Although it is difficult to distinguish treatment failures from treatment delay or treatment harm, eight deaths occurred as a result of uncontrolled disease progress leading to MODS. After a retrospective survey in 19 adults with sHLH, Kumar et al. proposed that personalized decision making is warranted depending on the clinical presentation and course of disease. In their analysis, the majority of patients received anakinra, cyclosporine, intravenous immunoglobulins, and steroids. After excluding patients with hematologic malignancies, survival was 88% (37).

Although no data exist for adults, an open-label prospective study has been conducted in children with sHLH randomized to high-volume hemofiltration (HVHF) ($n = 17$) or not ($n = 16$) with a filter that was anticipated to remove pro-inflammatory mediators. Although the study was not powered for mortality, this was 29.4 and 56.3%, respectively, after 28 days; albeit this difference was not statistically significant. However, among patients receiving HVHF significant decrease over-time of ferritin, TNFα, and IL-6 was found; increase of NK cell activity was found as well (38).

The realm for the management of sHLH/MAS in adults mandates the results of one large-scale randomized clinical trial (RCT). **Table 3** summarizes the features of on-going clinical

TABLE 3 | Summary of on-going clinical trials for the diagnosis and management of secondary hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) in adults.

Registration at ClinicalTrials.gov	Design	Title	Intervention	Study population	Anticipated enrolment	Primary endpoint
NCT03510650	Prospective diagnostic	Diagnostic biomarkers for adult HLH in critically ill patients	Blood sampling for cytokine panel, EBV, and CMV viral loads, microRNAs	Adults with suspected or diagnosed HLH or sepsis	50 patients with sepsis and 50 patients with sHLH	Incidence of HLH in intensive care units based on HLH-2004 criteria
NCT03259230	Prospective, case-control	Diagnostic biomarkers for sHLH	Blood sampling	Adults and children with HLH at the context of malignancy	25 patients and 25 controls	Inflammatory markers and genetic variants in malignancy-associated HLH
NCT02631109	Single-arm, open-label	L-DEP regimen as a salvage therapy for refractory EBV-induced HLH	L-DEP + pegaspargase + (doxorubicin or etoposide + methylprednisolone)	>14 years with EBV-induced sHLH	120 patients	% treatment response
NCT02780583	RCT	Treatment of MAS with anakinra	• Placebo + methylprednisolone • Anakinra + methylprednisolone	• Children or adults with sJIA and two or more of: ↓platelets; ↑AST; ↓WBCs; ↓fibrinogen; or three or more of combined clinical / laboratory criteria (↓ platelets; ↑AST, ↓WBCs; ↓fibrinogen; central nervous system dysfunction; hemorrhages; hepatomegaly) • Children or adults without sJIA and ferritin >2,000 ng/ml and 3 out of the following: bicytopenia; ↑fasting triglycerides; splenomegaly; ↑ALT or AST; fever; ↓fibrinogen <1.5 g/L or INR >1.5 or D-dimers >500 ng/ml	40 patients	Number of acquired infections and deaths after 72 h
NCT03332225	Double-dummy RCT	A trial of validation and restoration of immune dysfunction in severe infections and sepsis	• Placebo • Anakinra or rhIFNγ	• Adults with septic shock due to lung infection or primary bacteremia or acute cholangitis • Ferritin >4,420 ng/ml (MALS) or low ferritin and CD14/HLA-DR<30%	278 patients	Mortality after 28 days
NCT02400463	Single-arm, open-label	Pilot study of ruxolitinib in secondary HLH	Ruxolitinib	Adults with at least 5 of the following): fever, splenomegaly, cytopenia, hypertriglyceridemia, or hypofibrinogenemia, tissue demonstration of hemophagocytosis, ↓ NK cell activity, ferritin ≥3,000 ng/ml, soluble IL-2 receptor >2,400 U/ml	10 patients	Survival after 2 months
NCT03533790	Single-arm, open-label	DEP-ruxolitinib regimen as a salvage therapy for refractory/relapsed HLH	DEP-Ru	Age 1–70 years old who meet HLH-2004 diagnostic criteria	80 patients	% treatment response
NCT02385110	Single-arm, open-label	Alemtuzumab or tocilizumab in combination with etoposide and dexamethasone for the treatment of adult patients with HLH	• Alemtuzumab + etoposide + dexamethasone • Etoposide + dexamethasone + tocilizumab	Adults with HLH-2004 criteria	40 patients	Response rate

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMV, cytomegalovirus; EBV, Ebstein-Barr virus; IL, interleukin; INR, international normalized ratio; NK, natural killer; RCT, randomized clinical trials; sJIA, systemic juvenile idiopathic arthritis; WBCs, white blood cells.

trials for the development of diagnostic biomarkers and for the management of sHLH/MAS in adults. The real flare coming from this Table is that our understanding of sHLH/MAS is still in its infancy since we are still in need of tools to recognize this syndrome whereas the majority of on-going clinical trials are open-label and single-arm. Only one of these trials is featuring in a population of patients with sepsis. PROVIDE (personalized randomized trial of validation and restoration of immune dysfunction in severe infections and sepsis; EudraCT 2017-002171-26; ClinicalTrials.gov identifier NCT03332225) is a RCT of personalized approach in sepsis. This is a double-blind, double-dummy phase 2 study in which enrolled patients are suffering from septic shock due to lung infection, primary bacteremia and acute cholangitis and laboratory signs of MALS or hypo-inflammation on two serial time measurements. Ferritin above 4,420 ng/ml is the diagnostic tool of MALS and CD14/HLA-DR <30% in the absence of high ferritin is the diagnostic tool of hypo-inflammation. The study is on-going in 14 study sites in Greece and the primary endpoint is 28-day mortality. Patients with MALS are randomized into treatment with placebo or anakinra.

CONCLUSIONS

The above analysis shows that we are still in a very early stage of our understanding of the frequency of sHLH/MAS in critically ill patients with sepsis. Current evidence suggests that about 3–4% of patients are in that state dominated by pro-inflammatory host responses. Our suggestion of using ferritin cut-offs >4,420 ng/ml does not guarantee the miss of great many of these patients but is just focusing on the use of a biomarker cut-off associated with great specificity for diagnosis. Future mandates the development of better diagnostic tools. Their development will favor the conduct of RCTs to tailor individualized needs.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ferritin Light Chain Confers Protection Against Sepsis-Induced Inflammation and Organ Injury

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Despite the prevalence and recognition of its detrimental impact, clinical complications of sepsis remain a major challenge. Here, we investigated the effects of myeloid ferritin heavy chain (FtH) in regulating the pathogenic sequelae of sepsis. We demonstrate that deletion of myeloid FtH leads to protection against lipopolysaccharide-induced endotoxemia and cecal ligation and puncture (CLP)-induced model of sepsis as evidenced by reduced cytokine levels, multi-organ dysfunction and mortality. We identified that such protection is predominantly mediated by the compensatory increase in circulating ferritin (ferritin light chain; FtL) in the absence of myeloid FtH. Our *in vitro* and *in vivo* studies indicate that prior exposure to ferritin light chain restrains an otherwise dysregulated response to infection. These findings are mediated by an inhibitory action of FtL on NF-κB activation, a key signaling pathway that is implicated in the pathogenesis of sepsis. We further identified that LPS mediated activation of MAPK pathways, specifically, JNK, and ERK were also reduced with FtL pre-treatment. Taken together, our findings elucidate a crucial immunomodulatory function for circulating ferritin that challenges the traditional view of this protein as a mere marker of body iron stores. Accordingly, these findings will stimulate investigations to the adaptive nature of this protein in diverse clinical settings.

Keywords: ferritin, inflammatory response, sepsis, NF-κB, cytokine, LPS, multi-organ injury, myeloid cells

INTRODUCTION

Sepsis is a severe, dynamic, and deranged immune response to an infectious insult (1, 2). The complexity of this clinical syndrome is perhaps best highlighted by its evolving definitions (3, 4). The most recent international consensus defines sepsis as life-threatening organ dysfunction resulting from a dysregulated host response to infection (4). The overall mortality rates of sepsis are alarmingly high and range from 10 to 50% of diagnosed patients with development of organ injury and septic shock as two major factors leading to higher mortality rates (5–7). Consequently,

the massive burden of morbidity, mortality, and healthcare expenditure associated with sepsis underscore the desperate need for gaining more insight into this devastating clinical syndrome and identifying novel targets for prevention and therapy. Discovery of penicillin by Sir Alexander Fleming was a major defining moment in human history and its struggle against infections. Nevertheless, despite vigorous investigations, overall improvement in our understanding of the molecular mechanisms involved in the pathogenesis of sepsis and numerous clinical trials, an "anti-sepsis" drug remains elusive (8, 9).

Ferritin is a spherical protein made of 24 subunits (10). These subunits are composed of heavy (FtH) and light (FtL) chains and their proportional contribution to the hollow spherical shell varies among tissues. Ferritin was traditionally considered to be a cytosolic protein with the mere function of iron storage. However, our understanding of this highly evolutionarily-preserved molecule has dramatically advanced in the past couple of decades. It is now clear that within the subcellular compartments, ferritin is present in the mitochondria and nucleus (11–13). Additionally, several functions other than iron storage including immunomodulatory attributes have been described [reviewed in (14, 15)]. Another interesting aspect of ferritin in the context of biological activity relates to serum ferritin. The precise source of serum ferritin was long debated. However, in an elegant study Cohen et al. revealed that serum ferritin is mainly a secreted protein, involving a non-classical lysosomal pathway (16). Furthermore, it was shown that FtL is the main form of circulatory protein, its iron content is low and more importantly, macrophages are the main source of serum ferritin (16). Serum ferritin level has been utilized by clinicians as part of a panel to assess iron stores. However, serum ferritin is also increased in response to infections, inflammation and malignancy and is generally accepted to be an acute phase reactant similar to C-reactive protein (17, 18). Despite this knowledge and our evolving understanding of the well-orchestrated pathways involved in cellular and systemic iron homeostasis, the precise role of serum ferritin and its impact on disease progression is yet to be conspicuously defined. While the connotation of iron's role in infection is extensively debated, lack of consistent evidence precludes us to comprehensively define the paradigm of such relationship and more importantly how serum ferritin may be involved in this context. Here, we asked whether myeloid cell specific FtH deletion modulates inflammation and organ injury in a commonly used and well-established model of poly-microbial sepsis, cecal ligation and puncture (CLP).

MATERIALS AND METHODS

Experimental Model and Subject Details

Male and female H-ferritin floxed mice ($FtH^{fl/fl}$) and myeloid-specific H-ferritin deletion mice ($FtH^{LysM-/-}$) (10–14 weeks of age), previously described (19, 20), were used in this study. $FtH^{fl/fl}$ mice were generated on a mixed background (C57BL/6 × 129/Sv) and were backcrossed to C57BL/6 for 10 generations. $LyzM^{Cre}$ mice were generated on a mixed background (Sv129 × C57BL/6 × CB.20) but were backcrossed to a C57BL/6 background for six generations before submission to Jackson Laboratories (Jackson Laboratories; stock 004781). Myeloid-specific FtH deletion mice on a C57BL/6 background were obtained by breeding $FtH^{fl/fl}$ and $LyzM^{Cre}$ mice. All mice were treated humanely and methods were approved by the Institutional Animal Care and Use Committee of UAB. Studies were performed in accordance with National Institutes of Health guidelines.

Cecal Ligation and Puncture-Induced Sepsis

Mice were anesthetized with isoflurane (1.5–2% isoflurane induction, 1–1.5% maintenance). Midline laparotomy incision (1–2 cm) was made under aseptic conditions to expose the cecum, and stool was milked to the tip and ligated 0.7 centimeter from the tip. Using a 21G needle, two punctures were made in the cecal wall and fecal material was expressed in to the peritoneal cavity. The cecum was placed back in the peritoneal cavity and musculature and skin were sutured. Sham animals underwent laparotomy and bowel manipulation without ligation or perforation. Mice were placed on a heating pad for recovery and provided unrestricted access to water and food. Mice were monitored closely for signs and symptoms of pain and distress (usually developed 6–12 h after surgery). Mice were sacrificed 8 and 24 h after surgery. Serum was collected for creatinine measurement by LC-MS/MS. Endotoxin-free recombinant FtL (1 mg per 25 g B.W.) was administered intravenously 24 h prior to CLP surgery.

Lipopolysaccharide (LPS)-Induced Sepsis

Mice were anesthetized with isoflurane 1.5–2% isoflurane induction, 1–1.5% maintenance and injected intraperitoneally with 8 mg/kg LPS (Invivogen) and were sacrificed 24 h after injection. Serum was collected for creatinine measurement by LC-MS/MS.

Radiotelemetric Blood Pressure Measurement

Blood pressure was recorded and analyzed via left carotid artery PA-C10 telemetry implant (DSI, Saint Paul, MN), as previously described (21, 22). Sepsis was induced by CLP after baseline measurements were performed and blood pressure was monitored. The device implantation and monitoring were performed by the UAB-UCSD O'Brien Center Core Facility.

Transcutaneous GFR Measurement

Transcutaneous GFR was measured in mice using fluorescein isothocyanate (FITC)-labeled sinistrin (MediBeacon), as previously described (23). GFR was monitored for 2 h, after which the monitors were removed and data were analyzed using elimination kinetics curve of sinistrin clearance, as previously described (24).

Measurement of Serum Cytokine Levels

Mouse serum cytokine levels were measured using mouse V-PLEX Pro-inflammatory Panel I Kit (Meso Scale Discovery), following manufacturers recommendations. Data was acquired using a MESO Sector S600 plate reader (Meso Scale Discovery).

Cytokine levels were represented as picograms or nanograms per milliliter.

Flow Cytometry

Mice were anesthetized with 1.5–2% isoflurane and blood was collected using cardiac puncture. Organs were perfused with cold PBS and spleen was harvested and weighed. Flow cytometry was performed as described previously (20, 25). Following antibodies (with their respective clone numbers) were obtained from eBioscience unless otherwise stated: 7-AAD, MHCII-FITC (M5/114.15.2), Gr-1-APC (1A8), Ly6C-eF450 (HK1.4), CD11b-SuperBright600 (M1/70), CD45.2-BV650 (104; BioLegend), F4/80-APC-eF780 (BM8), CD11c-BV785 (N418; BioLegend), NK1.1-PE (PK136), CD3e-APC (145-2C11), CD8a-eF450 (53-6.7), CD4-SuperBright600 (RM4-5), CD19-BV785 (6D5; BioLegend). AccuCheck beads (Life Technologies) were used to determine absolute numbers by normalizing to tissue mass. Data were collected with a Becton-Dickinson LSRII analyzer and analyzed using FlowJo software (Trecstar Software).

Peritoneal Colony Forming Unit (CFU) Measurement

Mice were anesthetized with 1.5–2% isoflurane and peritoneal lavage was sterilely collected in 0.9% sterile saline. Serial dilutions of the peritoneal lavage fluid were made and plated on Trypticase Soy Agar with Sheep Blood (Fisher Scientific). Agar plates were incubated at 37°C for 24 h. Single colonies were counted and data were represented as CFU per milliliter.

In vitro Phagocytosis

Bone marrow-derived macrophages (BMDMs) were changed to DMEM + 1% FBS media 24 h before being treated with LPS (100 ng/mL) or Apoferritin (0.1 mg/mL; Sigma). BMDMs were harvested and stained with CD11b-APC (M1/70; eBioscience), 7AAD (eBioscience). Cells were simultaneously incubated with MOI 10 pHrodo Red *Escherichia coli* BioParticles (ThermoFisher), as previously described with minor modifications (26). Cells were analyzed on a BD FACSCalibur and data was analyzed using FlowJo software.

Bacterial Propagation

Xen14 (bioluminescent *Escherichia coli* WS2572 parent strain, Perkin Elmer) was cultured overnight in an orbital shaker at 200 rotations per minute at 37°C in Luria Broth medium containing 30 μg/mL kanamycin (Fisher Scientific). Initial absorbance was determined at 600 nm and dilutions were prepared with antibiotic-free DMDM + 1% FBS for all experiments.

In vitro Bacterial Killing

Bone marrow-derived macrophages (BMDMs) were changed to DMEM + 1% FBS media 24 h before this assay. Absorbance of cultured Xen14 was determined at 600 nm and dilutions were prepared in antibiotic-antimycotic free cell culture media. BMDMs were treated with MOI 10 or 100 for 30 min. Bacterial media was aspirated and BMDMs were washed with PBS. DMEM + 1% FBS + 200 μg/mL gentamicin was added for 2 h and luminescence quantified on a Biotek Synergy HT plate reader. Data were expressed as arbitrary units (AU) of luminescence.

In vivo Bacterial Killing

Absorbance of cultured Xen14 was determined and dilutions were prepared in 0.9% sterile normal saline. The abdomen of the mice was shaved and injected intraperitoneally with 10^6 Xen14. Mice were immediately imaged using a Xenogen IVIS-50 Bioluminescence Reader, housed by the UAB Preclinical Imaging Shared Facility. Mice were imaged at 2, 4, 8, and 24 h after injection. Data were expressed as mean fluorescence index in AU.

Cecal Slurry Preparation

Cecal slurry was collected from male wild-type mice, as previously described (27). Equal volume of 30% glycerol in sterile PBS was added, then cecal slurry was aliquoted in to cryovials for storage at −80°C.

Purification of Recombinant Human FtL

Recombinant human FtL was generated as previously described (28). Briefly, FtL was expressed in *E. coli* using a pDS20pTrp vector. Gel purification was performed on a Sepharose 6B column, followed by a DEAE column. Endotoxin was removed using Pierce High Capacity Endotoxin Removal Spin Columns (ThermoFisher), per manufacturer protocol. Electrophoretic purity was determined (96% pure) and removal of endotoxin was confirmed using an E-Toxate kit (Sigma). Apoferritin derived from equine spleen was bought from Sigma (A3641).

Cell Culture

Bone marrow-derived macrophages (BMDMs) were isolated from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice, as previously described, with minor modifications (20). BMDMs were cultured for 6 days before experiments were performed. BMDMs were plated onto culture dishes and treated with L-ferritin, or Apoferritin (0.1 mg/ml) for 16 h. Media was removed, the cells were washed with PBS and treated with LPS (100 ng/mL) or cecal slurry (1 μL per one million cells) in DMEM + 1% FBS for the indicated times. Cells were then washed with PBS and collected for RNA or protein analysis.

Quantification of mRNA Expression

Gene expression analysis was performed, as previously described (29). Primers used to detect the specific genes are listed in **Table S4**. For the RNA sequencing studies, total RNA was isolated from blood via cardiac puncture using a Blood RNA isolation kit (ThermoFisher Scientific) and subsequently, globin mRNA was depleted using GLOBINclear kit (ThermoFisher Scientific). RNA was sequenced on NextSeq500 system and the library was prepared with the Agilent SureSelect Stranded mRNA kit.

Western Blot

All western blotting was performed as previously described (20). Membranes were blocked according to manufacturer's protocol (5% non-fat dry milk in TBST or 5% BSA in TBST) for 1 h and incubated with a mouse p-P65 (Santa Cruz, 1:1,000), rabbit total P65 (Cell Signaling, 1:2,000), mouse FtH (Santa Cruz, 1:1,000), mouse FtL (Santa Cruz, 1:1,000), or goat FtL

(Thermofisher or Santa Cruz, 1:1,000), followed by a peroxidase-conjugated goat anti-mouse or goat anti-rabbit or donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, 1:10,000). Horseradish peroxidase activity was detected using chemiluminescence (GE Healthcare) or KwikQuant detection system or LI-COR infrared Odyssey Imaging System. Membranes were stripped and reprobed with anti-GAPDH (Sigma-Aldrich, 1:10,000) or anti-β-actin (Sigma-Aldrich, 1:10,000) as a loading control. Densitometry analysis was performed using ImageStudio Lite and results were normalized to GAPDH or total P65 expression. Data were represented as fold change over controls.

Blood Collection, Serum Preparation, and Analysis

Mice were anesthetized with 1.5–2% isoflurane and blood was collected by intra-cardiac puncture. Serum was isolated from blood after 30-min room temperature incubation and centrifugation. Aspartate transaminase activity (BioAssay Systems), HMGB1 (LSBio), iron (Abcam), hepcidin (eLabScience), NGAL (ENZO Life Sciences), hemopexin (Alpha Diagnostic), haptoglobin (Alpha Diagnostic), and ferritin (Kamiya Biomed) were measured in the serum according to manufacturers' protocols.

HISTOLOGY

Organs were collected and fixed in 10% neutral buffered formalin for 16 h prior to paraffin embedding. Paraffin sections were cut in to 4 μm sections, deparaffinized, and rehydrated using CitriSolv and isopropanol. Tissues were stained with hematoxylin and eosin stain using standard protocol. All images were acquired using a Leica DMI 6000B microscope (Leica Microsystems) and Leica Application Suite V4.2 software.

RNA Sequencing Data Processing and Analysis

Raw sequencing (fastq) files were subjected to quality control analysis using FastQC (v0.11.5) [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/] and using Trim Galore (v0.4.1) [http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/], adapter sequences and poor quality reads were trimmed. Trimmed and cleaned sequence reads were aligned to mouse genome (GRCm38) using TopHat v2.1.0 (30) or STAR v2.5.3.a (31). The accepted BAM files from TopHat were sorted using samtools (Version: 1.3.1) (32) and reads aligning each annotated mouse gene were enumerated using HTSeq-count (33).

Differential expression analysis was performed using DESeq2 (34), following standard protocol [https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html]. Genes altered by absolute fold change of two or more with adjusted p-value < 0.01 were considered to be significantly differentially expressed [DEG]. DAVID (Database for Annotation, Visualization and Integrated Discovery) version 6.8 (35) was then used to conduct Gene Ontology (GO) and KEGG pathway enrichment analysis on DEG. Heatmap figures were generated in R 3.2.2 (https://cran.r-project.org/) using heatmap.2 function of gplots package [https://cran.r-project.org/web/packages/gplots/index.html].

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are represented as mean ± SEM. Unpaired 2-tailed t-test was used for comparisons between two groups. ANOVA and Tukey's multiple comparisons tests were used for comparisons between more than two groups. Survival significance was determined by Kaplan–Meier curve and log rank test. $P < 0.05$ were considered significant. All analysis was performed using GraphPad Prism 7.

DATA AVAILABILITY

The accession numbers for the RNA sequencing data reported in this paper are Gene Expression Omnibus (GEO): GSE114078.

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Subhashini Bolisetty (sbolisetty@uabmc.edu).

RESULTS

Myeloid FtH Deficiency Prevents Multi-organ Failure and Mortality in Experimental Sepsis

Following CLP, wild-type mice ($FtH^{fl/fl}$) succumbed to significant mortality within 72 h following surgery whereas mice deficient in myeloid FtH ($FtH^{LysM-/-}$) displayed significantly improved survival that was independent of gender (**Figures 1A,B**). Two important life-threatening attributes of sepsis are related to organ dysfunction and septic shock. Accordingly, we assessed the extent of organ injury following CLP and determined that myeloid FtH deficiency was associated with significantly improved preservation of renal function as evidenced by serum creatinine (**Figure 1C**) and transcutaneous glomerular filtration rate measurement (**Figure 1D**), lesser hepatic injury (AST, **Figure 1E**), and preserved circulatory status when compared to $FtH^{fl/fl}$ mice (mean arterial pressure, systolic and diastolic blood pressure, **Figures 1F–I**). Additionally, structural damage to the lung and liver was less prominent, as confirmed by histological analyses (**Figure S1**). These findings suggest that FtH deficiency in the myeloid cells is associated with defense against sepsis induced organ dysfunction.

Myeloid FtH Deficiency Dampens the Inflammatory Response in Sepsis

A dysregulated hyperinflammatory immune response is a hallmark of sepsis. In this context, we next assessed the levels

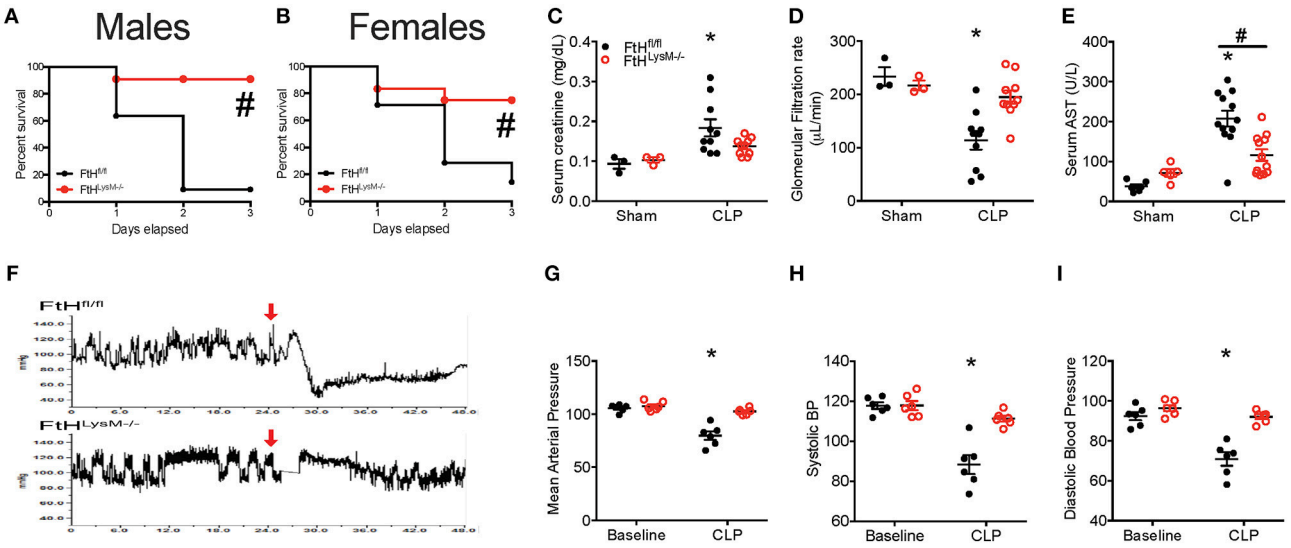

FIGURE 1 | Myeloid FtH deficiency prevents multi-organ failure and mortality in experimental sepsis. **(A,B)** Percent survival of mice deficient in myeloid-specific FtH ($FtH^{LysM-/-}$) and their floxed controls ($FtH^{fl/fl}$) after cecal ligation puncture (CLP) surgery. **(A)** Males, $n = 11$ per group. **(B)** Females, $n = 7$–12 per group. Data are from three independent experiments; $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$. **(C)** Serum creatinine levels were measured and expressed in milligrams per deciliter (mg/dL) as mean ± SEM 24 h after CLP or sham surgeries. Sham, $n = 3$ per group; CLP, $n = 10$ per group. $^{*}p < 0.05$ vs. sham. **(D)** Transcutaneous glomerular filtration rate (GFR) was measured using FITC-sinistrin 24 h after CLP or sham surgery and expressed in microliters per minute (μL/min) as mean ± SEM. Sham, $n = 3$ per group; CLP, $n = 10$ per group. $^{*}p < 0.05$ vs. sham. **(E)** Serum aspartate aminotransferase (AST) was measured 24 h after CLP surgery and expressed in units per liter (U/L) as mean ± SEM. Sham, $n = 5$–6 per group; CLP, $n = 12$ per group. $^{*}p < 0.05$ vs. sham **(F–I)**. **(F)** Blood pressure was measured using radiotelemetry. Baseline measurements were recorded 24 h prior to CLP. Mice were monitored for 48 h. **(G)** Mean arterial pressure, **(H)** systolic, and **(I)** diastolic blood pressure were recorded 24 h following CLP in $FtH^{LysM-/-}$ and $FtH^{fl/fl}$ controls and expressed as mean ± SEM. $n = 6$ per group; $^{*}p < 0.05$ vs. sham.

of cytokines in the sera of mice that underwent sham or CLP surgery. In accordance with organ dysfunction in the $FtH^{fl/fl}$ mice, 24 h after CLP surgery, there was a significant increase in the levels of cytokines that have been implicated in the pathogenesis of sepsis, including the pro-inflammatory cytokines, TNF-α, IFN-γ, IL-6, IL-12, CXCL1, IL-1β, and IL-2 (**Figure 2**). In contrast, $FtH^{LysM-/-}$ mice displayed a non-significant increase in the levels of these cytokines when compared to sham controls. This response was also associated with a lack of significant induction in the anti-inflammatory cytokines such as IL-4 and IL-10 and had no apparent difference in the expression of IL-5, a cytokine that is predominantly produced by non-myeloid cells such as mast cells and T cells.

To assess the cross-talk between parenchymal and immune cells, we next analyzed several well-established markers of inflammation. **Figure S2** depicts the expression profile of pro-inflammatory and anti-inflammatory cytokines and chemokines in the organs of myeloid FtH-deficient mice compared to their floxed wild-type controls that underwent sham or sepsis. Whether such regulation is contributed by the parenchymal cells or the resident myeloid cells within these organs is currently unknown. As expected, we found that splenic FtH induction following CLP was blunted in the $FtH^{LysM-/-}$ mice (**Figure S2**). However, we did not find any association of FtH expression and cytokine levels in the organs following CLP. Expression of Heme oxygenase-1 (HO-1), an anti-oxidant enzyme with potent anti-inflammatory properties, was not significantly different following CLP in the organs of $FtH^{fl/fl}$ compared to $FtH^{LysM-/-}$ mice, suggesting that the protective effects of myeloid FtH deletion were independent of HO-1 (**Figure S2**).

We next characterized the profile of immune cell populations in the blood, spleen and lung to determine whether the protective effect of myeloid FtH deficiency is secondary to its role in regulating inflammation (**Figures 3A,B** and **Figure S3A**). Corroborating previous reports, we demonstrate that sepsis results in marked leukopenia in mice, leading to reduced $CD45^{+}$ hematopoietic cells (**Figure 3C**) (36). More specifically, there was a significant reduction in the number of neutrophils in the blood and spleen following CLP in both $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice. Interestingly, there was no difference in monocytes/macrophages (MM) (**Figure 3C**) or inflammatory MM (as defined by expression of $Ly6C^{hi}$ and $CX3CR1^{+}CCR2^{+}$ macrophages) (**Figure S3B**). In support of previous reports, there is a significant reduction in peripheral as well as splenic T cells ($CD3^{+}$), and specifically a decrease in cytotoxic $CD8^{+}$ T cells following CLP in both the myeloid-ferritin knockout mice and controls (**Figure 3C**) (37–39). We also found a similar trend in the reduction of B cells following CLP (**Figure 3C**). In the spleen, we demonstrate a decrease in neutrophils and inflammatory macrophages ($Ly6C^{hi}$ and $CX3CR1^{+}CCR2^{+}$), but no difference in the total macrophage, dendritic cell or lymphoid cell populations (**Figure 3D** and **Figure S3C**). We

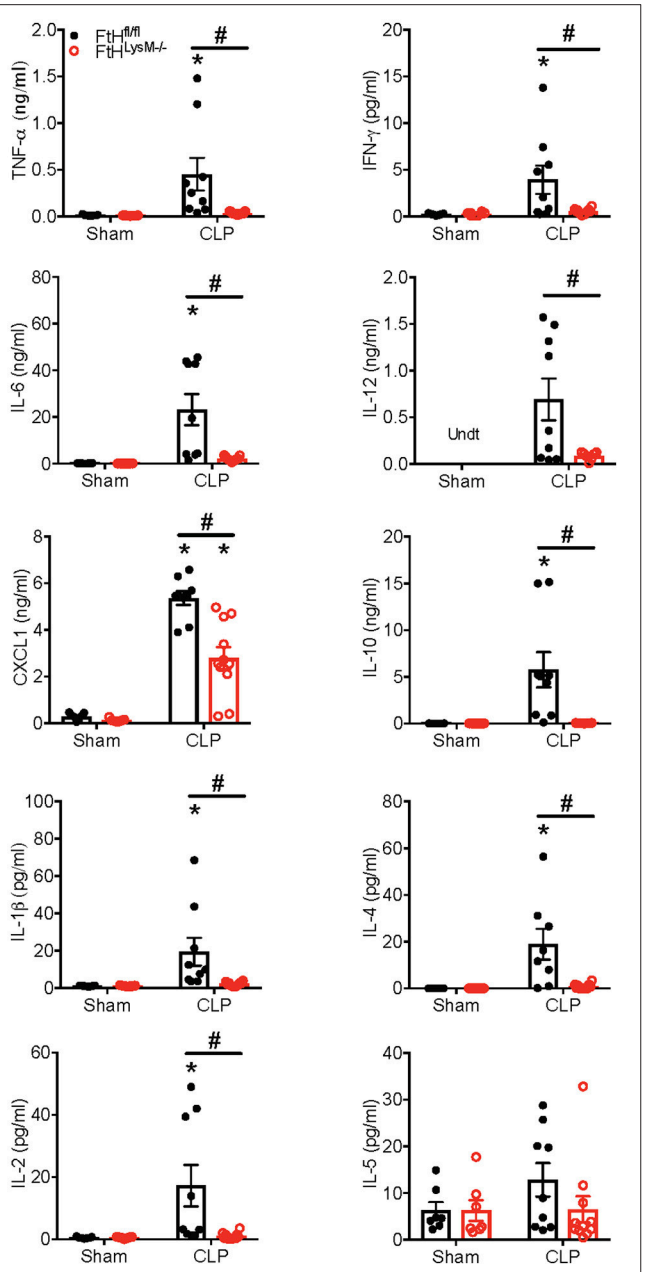

FIGURE 2 | Myeloid FtH deficiency dampens the inflammatory response during sepsis Serum levels of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-12, chemokine ligand 1 (CXCL1), IL-10, IL-1β, IL-4, IL-2, and IL-5 were measured 24 h following sham or CLP surgery. Data are expressed in nanograms or picograms per milliliter (ng/mL or pg/mL) as mean ± SEM. Sham, $n = 7$ per group; CLP, $n = 9$–11 per group. Data are from two independent experiments. $^{*}p < 0.05$ vs. sham, $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$.

further demonstrate that while there was a significant increase in the proportion of macrophages, there was no significant difference in the neutrophil or dendritic cell populations in lungs of both the transgenic mice following CLP compared to sham (**Figure S3D**). Taken together, there was no significant difference in the lymphoid and myeloid populations following sepsis in the $FtH^{fl/fl}$ vs. $FtH^{LysM-/-}$ mice.

FtH Expression Does Not Influence Phagocytosis or Bacterial Clearance

We next investigated whether the dampened cytokine response in the mice incompetent of FtH expression in the myeloid compartment was due to a difference in bacterial load or an inability of myeloid cells to detect, phagocytose, and clear bacteria. As evident from **Figure S4A**, there was no difference in the bacterial load in the peritoneal lavage of $FtH^{LysM-/-}$ vs. $FtH^{fl/fl}$ mice at 24 h following CLP. We also assessed the effect of myeloid FtH deficiency on bacterial clearance *in vivo*. Following intraperitoneal administration of chemiluminescent *E. coli* strain *Xen 14*, real-time IVIS imaging revealed no difference in bacterial clearance in $FtH^{LysM-/-}$ vs. $FtH^{fl/fl}$ mice (**Figure S4B**). These findings were further corroborated *in vitro* with infection of BMDMs with *Xen 14* (**Figure S4C**). Additionally, there was no significant difference in the phagocytic ability of myeloid FtH deficient BMDMs compared to wild-type BMDMs (**Figure S4D**). These results effectively rule out any attributable function of myeloid FtH in the context of bacterial recognition or phagocytosis by macrophages.

Myeloid FtH Deletion Diminishes Response to Lipopolysacharide

To further determine whether FtH expression regulates the response to lipopolysaccharide (LPS), a potent endotoxin that is implicated in the pathogenesis of gram-negative bacterial induced sepsis, we administered LPS to wild-type and myeloid FtH deficient mice. While $FtH^{fl/fl}$ mice exhibited a significant loss of kidney function, $FtH^{LysM-/-}$ mice were resistant to LPS-induced kidney injury (**Figure 4A**). Upon further investigation, we demonstrate that while LPS-induced IL-6 and IL-1β expression were significantly lower in $FtH^{LysM-/-}$ macrophages compared to $FtH^{fl/fl}$ macrophages, a non-significant trend was observed for TNF-α and NLRP3 mRNA expression (**Figures 4B–F**).

Absence of FtH Led to Blunted Activation of NF-κB Following LPS

We next addressed whether the protective effects of FtH deletion are related to the regulation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB; p65), a principal determinant of sepsis mediated injury (40). We demonstrate that LPS induced a time-dependent increase in the phosphorylation of p65 in $FtH^{fl/fl}$ macrophages, an effect that was remarkably blunted in the $FtH^{LysM-/-}$ cells (**Figure 4H**). We further evaluated macrophages for the expression of phosphorylated JNK and ERK, two key MAPK pathways that are activated following LPS stimulation. In corroboration with previous studies, absence of FtH led to an increase in LPS-induced phosphorylation of JNK (41) and ERK kinase (**Figures 4H,I**). Interestingly, we observed a bi-phasic activation of ERK following LPS stimulation, the latter activation is possibly driven by cytokines. As previously reported, lack of FtH in the macrophages from $FtH^{LysM-/-}$ mice was associated with a compensatory increase in the levels of FtL (**Figure 4G**) (20).

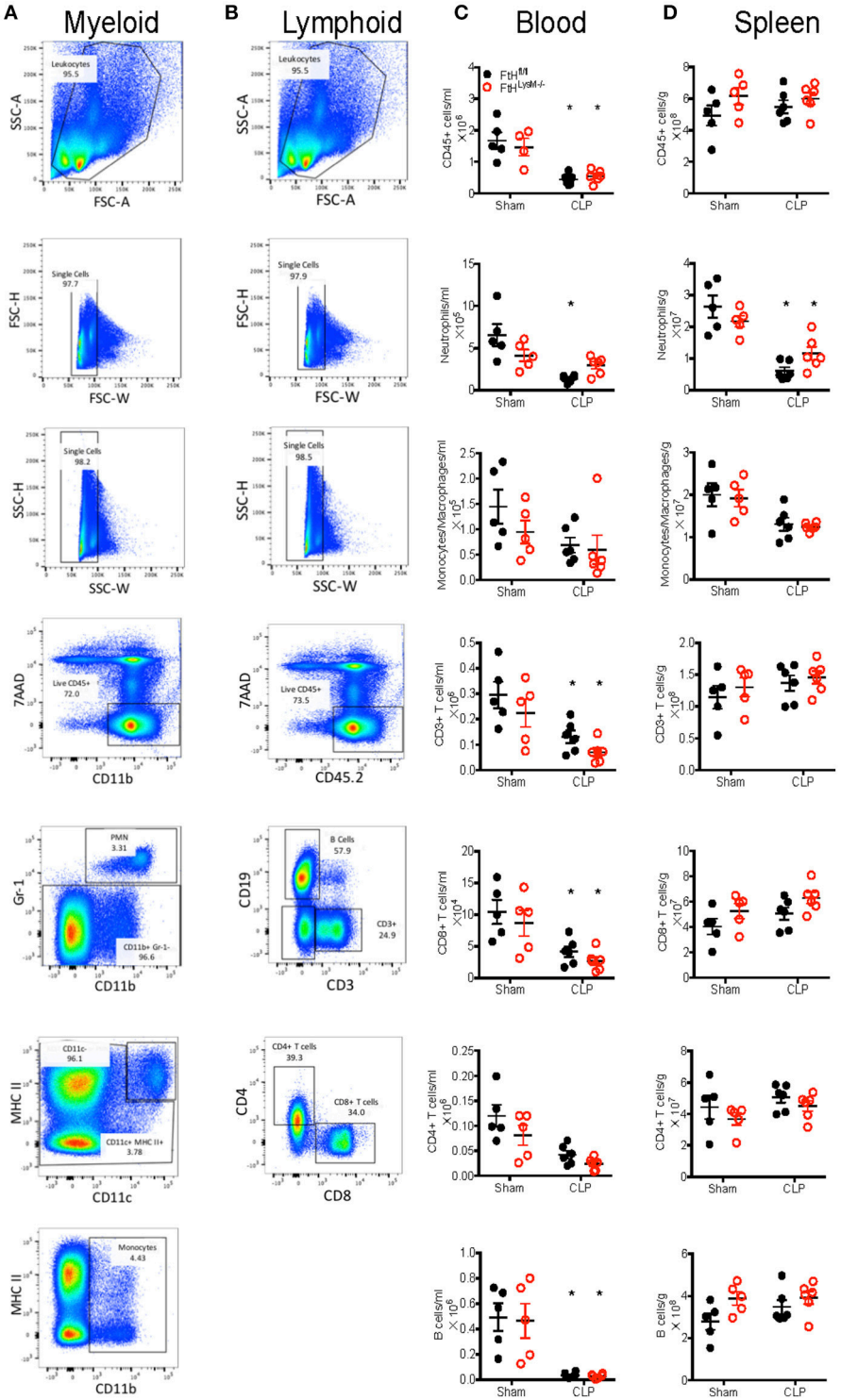

FIGURE 3 | Myeloid FtH deficiency dampens the inflammatory response without altering the proportions of immune cell populations. **(A,B)** Representative flow cytometry histograms of **(A)** myeloid and **(B)** lymphoid panels from $FtH^{fl/fl}$ sham control spleen demonstrating gating scheme and proportions of immune cell populations. **(C,D)** Quantification of number of cells in the **(C)** blood and **(D)** spleen 24 h after CLP. Data are from three independent experiments and are represented as number of cells per gram spleen (cells/g) or number of cells per ml blood as mean ± SEM. $n = 4–5$ per group. $^*p < 0.05$ vs. sham.

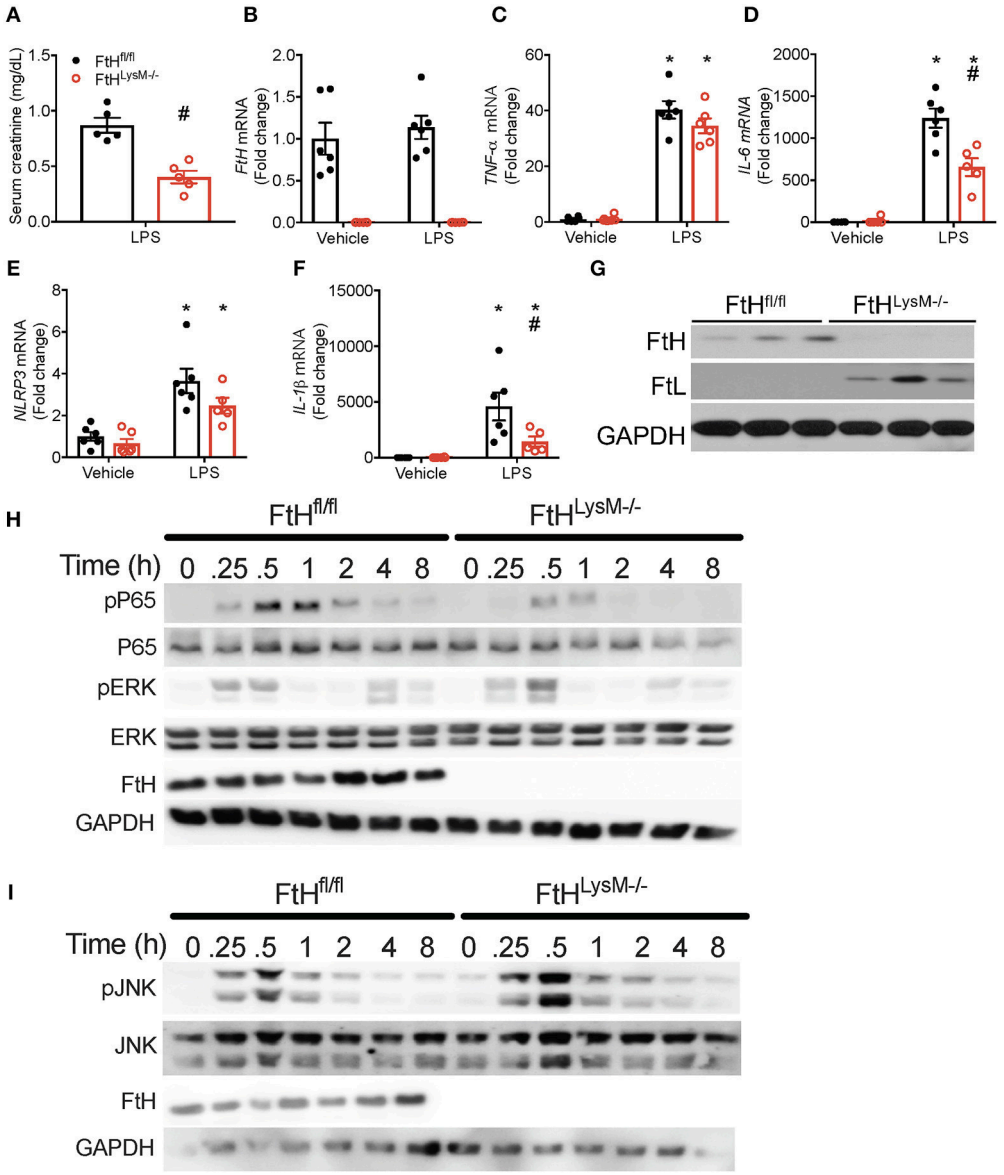

FIGURE 4 | Myeloid FtH deletion diminishes response to lipopolysaccharide (LPS). **(A)** Serum creatinine levels were measured and expressed in mg/dL as mean ± SEM 24 h after LPS (8 mg/kg) or vehicle (normal saline). $n = 5$ per group; $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$. **(B–G)** Real-time polymerase chain reaction (RT-PCR) analysis of mRNA expression of **(B)** *FtH*, **(C)** *TNF-α*, **(D)** *IL-6*, **(E)** *NACHT, LRR,* and *PYD domains-containing protein (NLRP3),* and **(F)** *IL-1β* in bone marrow-derived macrophages (BMDMs) of $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice that were treated with vehicle or LPS for 8 h. Data are analyzed and represented relative to GAPDH as mean ± SEM. $n = 5$–6 per group; $^{*}p < 0.05$ vs. vehicle, $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$. **(G)** Untreated BMDMs from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice were analyzed for expression of FtH and FtL. Membranes were stripped and reprobed for GAPDH expression to demonstrate equal loading. **(H,I)** BMDMs from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice were collected at 0, 0.25, 0.5, 1, 2, 4, and 8 h after LPS treatment and analyzed for expression of **(H)** phosphorylated p65 (pP65), total P65, phosphorylated extracellular signal-related kinase (pERK), FtH, and **(I)** phosphorylated c-Jun N-terminal kinase (pJNK), total JNK, and FtH. GAPDH was used as a loading control. Total ERK levels were examined in these lysates on a different membrane. Representative western blots from one out of three mice per genotype.

Serum Ferritin Is Significantly Higher in the Myeloid FtH Deficient Mice

At 24 h following CLP, there was no significant difference in the levels of high mobility group box 1 (HMGB1), a potential mediator of pathogenic events in sepsis (**Figure 5A**). HMGB1 is also implicated in leukocyte apoptosis and therefore may contribute to the significant decrease in leukocyte populations following CLP in both transgenic mice (**Figure 3C**). While serum iron levels were not different between sham-operated animals, CLP led to a significant reduction in serum iron levels in both the transgenic groups (**Figure 5B**). Additionally, serum iron levels were significantly higher in the $FtH^{LysM-/-}$ mice that underwent

CLP compared to $FtH^{fl/fl}$ mice. As expected, hepcidin, an anti-microbial peptide, and a master regulator of iron homeostasis, was significantly higher in both the $FtH^{LysM-/-}$ and $FtH^{fl/fl}$ mice after CLP when compared to their respective sham controls. However, hepcidin levels were significantly lower in $FtH^{LysM-/-}$ mice compared to $FtH^{fl/fl}$ mice that underwent CLP (**Figure 5C**), which may explain the higher serum iron levels in $FtH^{LysM-/-}$ mice following CLP. Furthermore, while neutrophil gelatinase-associated lipocalin (NGAL), another iron chelating protein with anti-bacterial properties, was significantly increased after CLP surgery, there was no significant difference attributed to myeloid FtH expression (**Figure 5D**). These results suggest that iron regulatory proteins, hepcidin and NGAL, do not account for the observed protective response in myeloid FtH deficient mice.

Hemolysis and resultant heme burden are implicated in the pathogenesis of sepsis (42). In fact, administration of hemopexin to quench free heme prevents sepsis injury in rodent models (42, 43). While we observed that hemopexin was significantly higher in mice that underwent CLP surgery, there was no influence of myeloid FtH on circulating hemopexin levels (**Figure 5E**). Haptoglobin, a protein that binds free hemoglobin is protective against sepsis pathogenesis and has been shown to decrease in the acute phase after sepsis (44, 45). We observed a significant reduction in serum haptogloboin levels 8 h after CLP, which was not different between $FtH^{fl/}$ and $FtH^{LysM-/-}$ mice (data not shown). However, we found significantly higher levels of haptoglobin at 24 h following CLP in the serum of $FtH^{LysM-/-}$ when compared to $FtH^{fl/fl}$ mice (**Figure 5F**).

We next investigated whether levels of serum ferritin, mainly comprised of ferritin light chain (FtL), were differentially regulated in the absence of myeloid FtH expression. Interestingly, we found that serum ferritin levels were significantly higher in the $FtH^{LysM-/-}$ mice compared to $FtH^{fl/fl}$ mice. Furthermore, we observed a significant increase in ferritin levels in mice that underwent CLP (irrespective of their myeloid FtH status) when compared to sham surgery (**Figure 5G**).

FtL Confers Protection Against Sepsis-Induced Hyperinflammation and Organ Dysfunction

To investigate whether increased serum ferritin levels offered resistance to sepsis, we administered recombinant FtL to $FtH^{fl/fl}$ mice, performed CLP surgery and measured the levels of inflammatory cytokines in the serum after 24 h (**Table S1**). We demonstrate a significant inhibitory effect on serum cytokine production in the presence of FtL following CLP surgery. Additionally, we also found that FtL administration led to significantly lesser hepatic injury as evidenced by AST (FtL CLP: 144 ± 9.4 vs. Vehicle CLP: 219 ± 16.8 U/L; $p < 0.001$). These findings suggest that FtL is associated with defense against sepsis-induced hyperinflammation and organ dysfunction.

FtL Mitigates LPS-Induced MAPK and NF-κB Activation in Macrophages

Pre-treatment of BMDMs with FtL led to significantly lower induction of expression of inflammatory markers (*IL-6, IL-1β, TNF-α, MCP-1*) and *inducible nitric oxide synthase* (*iNOS*; **Figure 6A**). Pre-treatment with apoferritin, a holosphere that contains equal proportions of FtH and FtL, yielded similar results (**Figure S5A**). Pre-treatment with FtL led to reduced activation of LPS-mediated phosphorylation of JNK and ERK (**Figure 6B**). Additionally, pre-treatment with apoferritin also led to reduced phosphorylation of JNK when compared to vehicle treated cells (**Figure 6D**). These JNK activation data contrast our findings in LPS treated $FtH^{LysM-/-}$ macrophages (**Figure 4I**), suggesting a cumulative effect of FtH and FtL expression on mitigating JNK activation. LPS treatment activates the NF-κB pathway (**Figure 4**), therefore, we determined whether FtL regulates NF-κB activation. Interestingly, we demonstrate that pre-treatment with FtL or apoferritin mitigates LPS-induced phosphorylation of p65 (**Figures 6C,D**). In addition, we found that LPS-mediated degradation of IκBα was markedly less pronounced in macrophages that were pre-treated with FtL or apoferritin (**Figures 6C,D**). Of note, we found that treatment with FtL led to an increase in expression of FtH expression. Also, phosphorylated p65 levels were significantly lower in the spleens of $FtH^{LysM-/-}$ mice compared to $FtH^{fl/fl}$ mice following CLP (**Figures S5C–E**). Taken together these data suggest that FtL diminishes NF-κB activation and downstream inflammatory response and confers resistance to sepsis.

To mimic the polymicrobial flora of our *in vivo* model, we prepared cecal slurry from $FtH^{fl/fl}$ mice as previously described (27). Our data demonstrated that pre-treatment with apoferritin led to an abated immune response in the presence of cecal slurry (**Figure S5B**). Specifically, induction of *IL-6, MCP-1, TNF-α, and IL-1β* was markedly lower in the presence of apoferritin. Corroborating our previous findings, we demonstrate that apoferritin treatment induced expression of *iNOS* (**Figure S5B**) (20).

Alteration of Gene Expression Profile of Blood Leukocytes Following Sepsis

We next performed transcriptomic analyses of blood leukocytes from $FtH^{LysM-/-}$ and $FtH^{fl/fl}$ mice following sham or CLP surgery. The heatmap shown in **Figure 7A** highlights the significant ($p < 0.01$) differential expression of transcripts following CLP in both the transgenic mice compared to sham controls. Corroborating our cytokine data, we found that CLP led to enrichment of biological processes that are associated with an immune response (**Tables S2**, **S3**). Interestingly, in comparison with $FtH^{fl/fl}$, CLP induced transcriptomic changes in the $FtH^{LysM-/-}$ mice demonstrated a clear pattern of dampened immune response. These include downregulation of genes associated with processes such as the inflammatory response, the response to LPS and cell proliferation (**Table S2**). Additionally, genes associated with processes such as oxidant detoxification and regulation of NF-κB signaling were upregulated in the myeloid FtH deficient mice (**Table S3**). Hierarchical clustering of

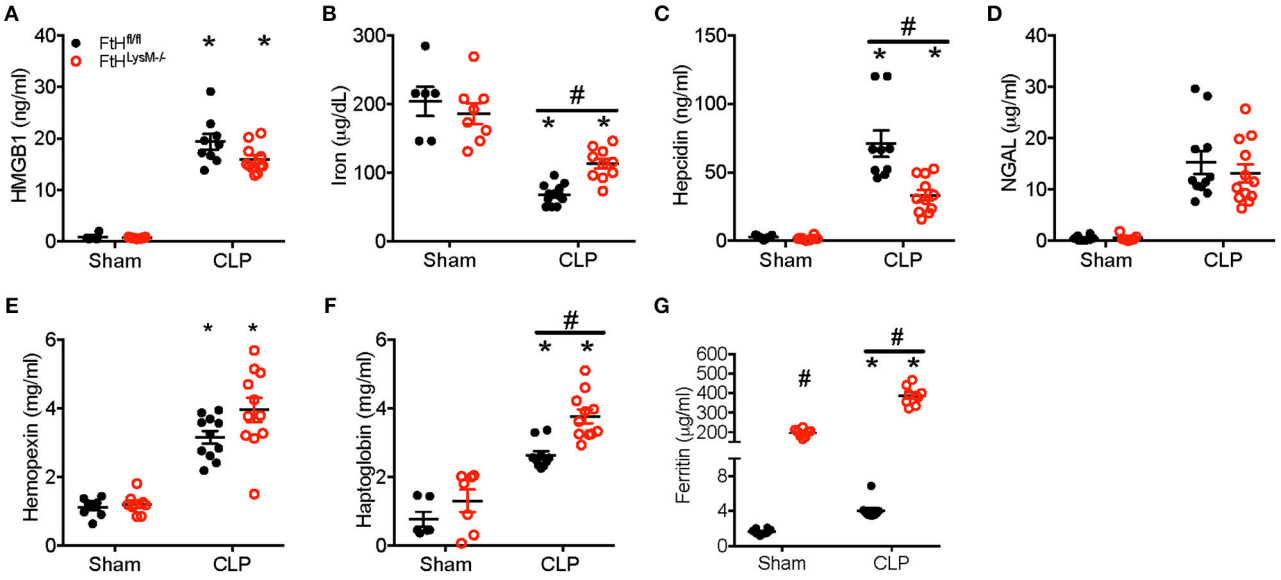

FIGURE 5 | Serum ferritin is significantly higher in myeloid FtH deficient mice. Serum from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice 24 h after CLP and sham surgeries was collected and analyzed for **(A)** high mobility group box-1 (HMGB-1; ng/ml), **(B)** iron (μg/dL), **(C)** hepcidin (ng/ml), **(D)** neutrophil gelatinase associated lipocalin (NGAL; μg/ml), **(E)** hemopexin (mg/ml), **(F)** haptoglobin (mg/ml), and **(G)** ferritin (μg/ml). Data are expressed as mean ± SEM. $n = 8$–10 per group. $^{*}p < 0.05$ vs. sham, $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$.

the NF-κB-dependent gene pathway identified several genes that were differentially regulated in the $FtH^{LysM-/-}$ mice (**Figure 7B**). Additionally, there was a clear difference in the expression levels of genes associated with the immune response between the $FtH^{LysM-/-}$ and $FtH^{fl/fl}$ mice that underwent CLP (**Figure 7C**).

DISCUSSION

In this study we investigated whether deletion of FtH in the myeloid cells impacts CLP-induced inflammation and organ injury. Our findings revealed that deletion of FtH was associated with significant disease protection against sepsis. This was evident by lower mortality, better preserved kidney function and blood pressure, lesser hepatic injury and serum cytokine levels, and decreased organ inflammation in myeloid FtH deficient mice. We found similar bacterial killing capabilities ruling out these processes as underlying mechanisms for the observed differences. Importantly, our results showed a significantly higher level of serum ferritin in both sham and CLP groups of $FtH^{LysM-/-}$ mice compared to their floxed counterparts. Since the macrophages are the main source of circulating ferritin (16), the higher serum ferritin was likely due to consequent compensatory overexpression of FtL in myeloid cells lacking FtH. Our *in vitro* and *in vivo* FtL administration studies indicate that prior exposure to FtL provides significant resistance to the septic process by mitigating overproduction of cytokines involved in the pathogenesis of sepsis. Our results indicate a major inhibitory action by ferritin on NF-κB activation and its downstream effects. RNA-Seq analyses of circulating leukocytes validate the paramount importance of serum ferritin in inhibition of the NF-κB pathway identifying novel functional properties of this protein.

The exact role of iron in the pathogenesis of sepsis and how iron regulatory proteins are involved in this process remains under investigation (46–49). In this context, little is known about the role of serum ferritin and its association with sepsis, particularly its pathogenesis and outcomes. Immediately following infection, a cascade of events shapes an environment known as state of hypoferremia that is intended to limit availability of iron to the infectious microbes (50, 51). This process is primarily driven by synthesis of hepcidin which in turn leads to endocytosis and degradation of ferroportin (52). Other pathways that are modulated by robust elevation of cytokines further solidify this strategy (53). The overall effect is decreased iron absorption and increased iron retention within reticuloendothelial cells. Serum ferritin (mainly FtL) is used to measure body iron stores in healthy individuals but its increased level in response to infection and inflammation limits its utility (17, 54). The perplexing evolutionary purpose of this increase in circulating iron poor ferritin during inflammatory processes is yet to be fully divulged.

To understand the adaptation to sepsis induced inflammation, we examined iron regulatory proteins that are beneficial during sepsis. Our findings do not support such protection to be mediated by hepcidin or NGAL, as neither of these proteins were significantly elevated in $FtH^{LysM-/-}$ mice compared to floxed controls. However, we found that serum ferritin was strikingly high in both groups of $FtH^{LysM-/-}$ mice undergoing sham or CLP surgeries. Further analysis revealed high levels of FtL expression in macrophages of $FtH^{LysM-/-}$ mice that underscores a compensatory mechanism to FtH gene deletion.

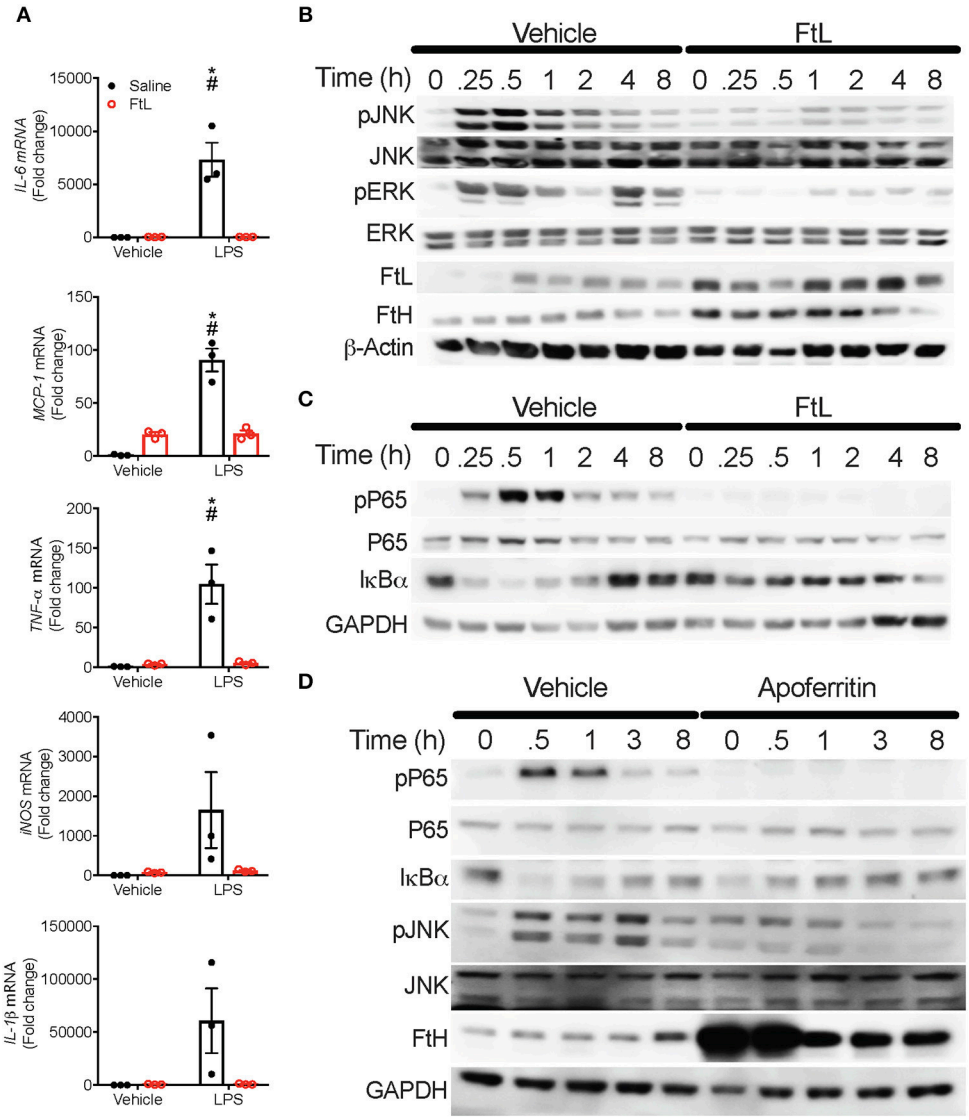

FIGURE 6 | Ferritin light chain (FtL) mitigates LPS-induced MAPK and NF-κB activation. **(A)** BMDMs from $FtH^{fl/fl}$ mice were pre-treated with FtL for 16 h followed by treatment with LPS or saline control. Cell lysates were collected 8 h later and analyzed for mRNA expression levels of *IL-6, monocyte chemoattractant protein-1 (MCP-1), TNF-α, inducible nitric oxide synthase (iNOS),* and *IL-1β,* Data are normalized to GAPDH and fold change relative to saline vehicle are expressed as mean ± SEM. $n = 3$ from three independent experiments. $^{*}p < 0.05$ vs. vehicle, $^{\#}p < 0.05$ vs. LPS+FtL. **(B,C)** $FtH^{fl/fl}$ BMDMs were pre-treated with FtL for 16 h, then washed and treated with LPS and collected at 0, 0.25, 0.5, 1, 2, 4, and 8 h. Cell lysates were analyzed for expression of **(B)** pJNK, total JNK, pERK, FtL, and FtH. β-actin was used as a loading control. Total ERK levels were examined in these lysates on a different membrane. **(C)** pP65, P65, and IκBα. GAPDH was used as a loading control. **(D)** $FtH^{fl/fl}$ BMDMs were pre-treated with Apoferritin (ApoF) for 16 h, then washed and treated with LPS and collected at 0, 0.5, 1, 3, and 8 h. Cell lysates were analyzed for expression of pP65, P65, IκBα, pJNK, total JNK, and FtH. GAPDH was used as a loading control. Representative western blot from one out of three mice per treatment group.

Since the major source of circulating ferritin is FtL (16), we postulated that this elevated serum ferritin may play a role in inflammation during sepsis. In support of this premise, we found a mitigated inflammatory response to LPS in macrophages with overexpression of FtL to compensate for FtH deletion. Moreover, when wild-type macrophages were treated with apoferritin or recombinant FtL (all devoid of iron) we found a similar pattern of resistance toward LPS or bacterial exposure that was evident by lesser induction of inflammatory markers, suggesting a less profound, and restrained response to infectious stimuli. As expected, we found a significant increase in intracellular FtL following administration of apoferritin and FtL. The cardinal pathway that mediates the response of inflammatory cells to infectious or injurious stimuli is the NF-κB pathway (40, 55). Our *in vitro* findings disclose a striking blockade of this pathway with preconditioning cells to various forms of ferritin. Also, a recent study demonstrated that transfection of macrophages with FtL led to reduced nuclear accumulation of p65 subunit after LPS

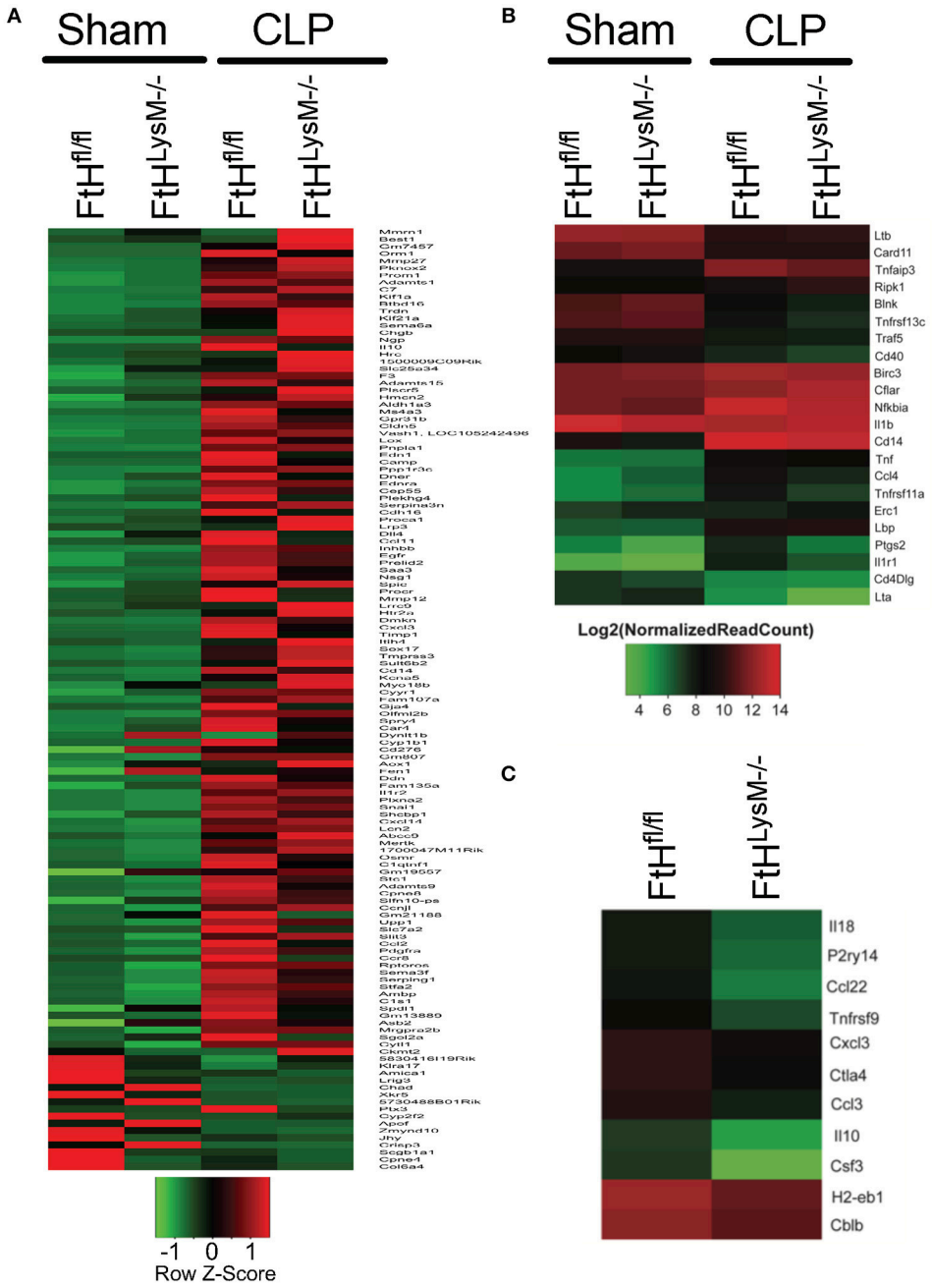

FIGURE 7 | Alteration of gene expression profile of blood leukocytes following sepsis. RNA sequencing was performed on blood leukocytes isolated from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice following CLP or sham surgery. **(A)** Hierarchical clustering of genes that are significantly altered ($p < 0.01$) between leukocytes from the transgenic mice at 8 h following surgery. Heatmap displays z-transformed expression values. $n = 3$ per group. **(B)** NF-κB signaling pathway genes differentially expressed in $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ CLP compared to $FtH^{fl/fl}$ sham. The differentially expressed genes were identified based on absolute fold change of two or more and adjusted p value < 0.01. $n = 3$ per group. **(C)** Genes associated with "immune response" biological process [GO: 0006955] down-regulated or differentially expressed in $FtH^{LysM-/-}$ CLP compared to $FtH^{fl/fl}$ CLP. The differentially expressed genes were identified based on absolute fold change of two or more and adjusted $p < 0.01$. $n = 3$ per group.

treatment (56). Given the importance of the spleen as a secondary lymphoid organ and its role in orchestrating adaptive immune responses, we validated the abrogation of the NF-κB pathway as a central regulator of the phenotypes observed in $FtH^{LysM-/-}$ mice. Moreover, taking advantage of the unbiased analysis of the transcriptome of circulating leukocytes by RNA-Seq, we further corroborate the unique signature of downregulation of the NF-κB pathway and genes associated with "immune response"

biological process in $FtH^{LysM-/-}$ mice following CLP induced sepsis.

Our understanding of iron metabolism and role of ferritin in various disease models is evolving. In a study published by Lipinski et al. in 1991, it was reported that tissue ferritin administration protects against *E. coli*-induced sepsis in mice (57). Furthermore, Weis et al. recently described how FtH mediated metabolic adaptation markedly improves disease tolerance to sepsis (58). It is important to note that these studies demonstrate how FtH induction plays a protective role during sepsis by inhibiting the iron-mediated oxidative inhibition of liver glucose-6-phosphatase and consequently sustain adequate gluconeogenesis. In contrast, our findings predominantly emphasize the significance of circulating FtL in modulating inflammatory cells toward a restrained response to infection.

It is recognized that a well-established anti-inflammatory cytokine, IL-10, is also robustly induced during inflammation to provide a counterbalance to an otherwise unchecked deleterious immune response. Similar pathways have been well-described in other physiological contexts such as the rapid induction of the anti-coagulation pathway during activation of the coagulation cascade. For the first time, our findings provide insight into the elusive nature and function of circulating ferritin. These findings suggest an important role for serum ferritin in regulating a controlled and measured response to infection in order to minimize a dysregulated and heightened injurious inflammatory process that is central to the detrimental outcomes of sepsis. These findings introduce a novel function for circulating FtL that establishes its role as an immunomodulatory cytokine. It must be noted that these results neither preclude utility of serum FtL as a marker of iron stores under physiological circumstances, nor do they rule out the association that has been described between high levels of circulating ferritin and worse outcomes in various inflammatory diseases (14). In contrast, our results suggest that similar to IL-10, the increment in the level of circulating ferritin should be regarded as an ongoing struggle to limit the body's inflammatory reaction toward injurious stimuli. Notably, elevated serum ferritin is a common finding in patients with end stage renal disease who require renal replacement therapy. Such elevated serum ferritin levels do not always mirror the iron status of these patients (17). Furthermore, a significant number of these patients dialyze via a permcath that distinctively predisposes them to bacteremia (59–61). Hence, future studies will need to investigate the rate of bacteremia/sepsis in these patients and their outcomes compared to patients with normal ferritin levels.

In summary, we provide novel findings that identify the NF-κB pathway as a main target of circulating ferritin to establish a restrained response to infection. These results will provide a novel platform for future studies to better understand the pathogenesis of sepsis and novel targets for potentially new strategies to challenge the significant burden of sepsis induced morbidity, mortality and substantial health care expenditure.

AUTHOR CONTRIBUTIONS

AZ and SB formulated the hypothesis, designed the study, performed most of the experiments, and wrote the manuscript. TH and RB performed and analyzed data from the flow cytometry experiments. SV and DC analyzed the RNA sequencing data and performed pathway analysis. WF performed radiotelemetry studies. LB performed the glomerular filtration measurements. LB, SB, and AZ performed bacterial clearance and phagocytosis experiments, all the remaining experiments were performed by AZ, KM, SE, and SB. PA and MP generated recombinant ferritin light chain protein. PA and JB provided scientific input. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00131/full#supplementary-material

Figure S1 | Myeloid FtH deficiency prevents multi-organ failure and mortality in experimental sepsis. Hematoxylin and eosin (HandE) staining of liver and lung tissue from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice 24 h following CLP. Structural damage to the liver is evidenced by steatosis, necrosis, and ballooning degeneration of hepatocytes. Histologic examination of the lung demonstrates interstitial edema and infiltration of immune cells. Scale bar = 100 μm.

Figure S2 | Myeloid FtH deficiency dampens the inflammatory response following sepsis. Spleen, liver, lung, and kidney tissues were analyzed for gene expression levels of *IL-6, IL-10, MCP-1, TNF-α, iNOS, HO-1,* and *FtH* in $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice 24 h after CLP. Data are expressed as fold change relative to GAPDH as mean ± SEM. Sham, $n = 4$ per group; CLP, $n = 8$ per group. $^{*}p < 0.05$ vs. sham, $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$.

Figure S3 | Myeloid FtH deficiency dampens the inflammatory response without altering the proportions of immune cell populations. **(A)** Representative flow cytometry histograms of monocyte/macrophage populations from $FtH^{fl/fl}$ sham control demonstrating gating scheme and proportions of immune cell populations. **(B–D)** Quantification of number of cells in the **(B)** blood, **(C)** spleen, and **(D)** lung 24 h after CLP. Data are represented as number of cells per gram kidney (cells/g) as mean ± SEM. $n = 4$–5 per group. $^{*}p < 0.05$ vs. sham.

Figure S4 | FtH expression does not influence phagocytosis or bacterial clearance. **(A)** Colony forming units (CFU) in $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice

peritoneal fluid 24 h after CLP. Data are expressed in CFU/mL as mean ± SEM. $n = 9$–12 per group. **(B)** *In vivo* bacterial clearance was assessed by IVIS bioluminescence. Mice were infected with *E. coli* Xen14 (10^6) intraperitoneally and imaged. Data are expressed in photons per second (photons/sec) as mean ± SEM. $n = 7$–8 per group; $^*p < 0.05$ vs. 0H. **(C)** *In vitro* bacterial killing was measured in mouse bone marrow-derived macrophages infected with *E. coli* Xen14 (MOI 10 and 100). Data are expressed as mean ± SEM in A.U. $n = 6$ per group. **(D)** *In vitro* phagocytic activity was assessed using pHrodo Red *E. coli* BioParticles using flow cytometry. Median fluorescence intensity is expressed as arbitrary units (A.U.) ± SEM. $n = 3$ per group.

Figure S5 | FtL confers protection against sepsis by mitigating NF-κB activation. **(A)** $FtH^{fl/fl}$ BMDMs were treated with LPS with or without pre-treatment with apoferritin or vehicle control for 16 h. Cells were collected after 8 h and analyzed for expression of *FtH, IL-6, TNF-α, NLRP3,* and *IL-1β mRNA.* Data are normalized to GAPDH and fold change relative to controls are expressed as mean ± SEM. $n = 6$ per group. $^*p < 0.05$ vs. vehicle, $^{\#}p < 0.05$ vs. LPS+Apoferritin. **(B)** $FtH^{fl/fl}$ BMDMs were treated with cecal slurry (C.S.) after pretreatment with apoferritin or saline control. Cells were collected after 8 h and analyzed for expression of *MCP-1, IL-6, TNF-α, IL-1β,* and *iNOS.* Data are normalized to GAPDH and fold change relative to controls are expressed as mean ± SEM. $n = 5$ per group. **(C–E)** Spleens from $FtH^{fl/fl}$ and $FtH^{LysM-/-}$ mice 24 h after CLP were analyzed for expression of pP65, P65, and FtH. GAPDH was used as a loading control. **(D)** pP65 was normalized to P65 and **(E)** FtH was normalized to GAPDH and densitometric values were expressed as A.U. $n = 7$ per group. $^{\#}p < 0.05$ vs. $FtH^{fl/fl}$.

Table S1 | Serum cytokine profile of $FtH^{fl/fl}$ + FtL compared to $FtH^{fl/fl}$.

Table S2 | Downregulated genes in $FtH^{LysM-/-}$ compared to $FtH^{fl/fl}$ during sepsis.

Table S3 | Upregulated genes in $FtH^{LysM-/-}$ compared to $FtH^{fl/fl}$ during sepsis.

Table S4 | Primers for Real-time PCR analysis.

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Myeloid-Derived Suppressor Cells in Sepsis

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Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells characterized by their immunosuppressive functions. MDSCs expand during chronic and acute inflammatory conditions, the best described being cancer. Recent studies uncovered an important role of MDSCs in the pathogenesis of infectious diseases along with sepsis. Here we discuss the mechanisms underlying the expansion and immunosuppressive functions of MDSCs, and the results of preclinical and clinical studies linking MDSCs to sepsis pathogenesis. Strikingly, all clinical studies to date suggest that high proportions of blood MDSCs are associated with clinical worsening, the incidence of nosocomial infections and/or mortality. Hence, MDSCs are attractive biomarkers and therapeutic targets for sepsis, especially because these cells are barely detectable in healthy subjects. Blocking MDSC-mediated immunosuppression and trafficking or depleting MDSCs might all improve sepsis outcome. While some key aspects of MDSCs biology need in depth investigations, exploring these avenues may participate to pave the way toward the implementation of personalized medicine and precision immunotherapy for patients suffering from sepsis.

Keywords: sepsis, infectious disease, innate immunity, myeloid-derived suppressor cells, biomarker, immunosuppression, inflammation, personalized medicine

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INTRODUCTION

Sepsis is one of the leading causes of preventable death. Sepsis is defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (1). The mortality rate of sepsis accounts for five-to-six million deaths of ∼30 million cases per year worldwide. Sepsis incidence is rising due to the aging of the population, the burden of chronic diseases, the increasing number of immunocompromised patients, and the resistance of microorganisms to antimicrobials (2). In 2017, the World Health Assembly and the World Health Organization made sepsis a global health priority by adopting a resolution to improve the prevention, diagnosis, and management of sepsis.

Innate immune cells, such as monocytes/macrophages, dendritic cells (DCs), and neutrophils, sense microbial and danger-associated molecular patterns (MAMPs produced by microorganisms, and DAMPs released by injured or stressed cells) through pattern recognition receptors (PRRs). PRRs are grouped into five main families: toll-like receptors (TLRs), NOD-like receptors, C-type lectins, scavenger receptors, RIG-I-like receptors, and intra-cytosolic DNA sensors (3). The interaction between PRRs and MAMPs or DAMPs triggers intracellular signaling pathways that coordinate gene expression, the development of the inflammatory response, the establishment of antimicrobial cellular and humoral responses, and the restoration of homeostasis once pathogens have been contained or eradicated. Sepsis is characterized by an early exacerbation of antimicrobial defense mechanisms, the so-called hyper-inflammatory "cytokine storm," mediating

tissue injury, organ dysfunctions and early mortality, and a concomitant shift toward inflammation resolution and tissue repair. Sepsis-induced immunoparalysis (or immunosuppression) favors the development of secondary infections and long-term immune disabilities accounting for late mortality (4–8).

During the last decades, early goal-directed therapy decreased early mortality from sepsis, which contributed to shift the sepsis ICU population toward a population suffering from chronic critical illness (CCI). Indeed, a subset of ICU patients surviving sepsis develop CCI characterized by long-lasting immunosuppression associated with a persistent, low-grade, inflammation maintained by the continuing release of DAMPs. The underlying inflammation is associated with catabolism and malnutrition. The term persistent inflammation-immunosuppression and catabolism syndrome (PICS) has been proposed to characterize this degraded state. PICS is associated with long-term morbidity, late multiple organ failures and late mortality (9–11).

Clinical trials testing adjunctive therapy to dampen inflammation-related dysfunctions in sepsis have not been conclusive (12). Several reasons may account for these failures, among them the large heterogeneity of the sepsis syndrome. Nowadays, the prevalent view is that restoration of immune capacities using immuno-stimulants might be more efficient than anti-inflammatory therapies. In any case, personalized medicine should be used to define at an individual level whether inflammatory cytokines, immunoparalysis, or metabolism has to be targeted (4, 7, 13–17). In that perspective, significant efforts are devoted to the identification of genetic, molecular, and cellular biomarkers to stratify patients for clinical studies and treatment based on clinical condition and disease stage.

We poorly understand what is responsible for a dysregulated host response and the delay returning to homeostasis in sepsis patients (4–8, 18). Growing interest focuses on a subpopulation of leukocytes called myeloid-derived suppressor cells (MDSCs). MDSCs are involved in the regulation of the immune response in many pathological situations, the best-studied being cancer. A number of comprehensive reviews discusses MDSCs in the context of cancer, autoimmunity and infectious diseases [see for example (19–26)]. Interestingly, recent data suggest that MDSCs are involved in immune dysfunctions observed in sepsis. In this review, we summarize and discuss our current knowledge about the role played by MDSCs during sepsis and the potential of using MDSCs as biomarkers and therapeutic targets of sepsis.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

MDSCs are immature myeloid cells that expand during chronic and acute inflammatory conditions. The premises of MDSC discovery date back more than a century when tumor progression was associated with extra-medullary haematopoiesis and neutrophilia. In the mid-1960s, Lappat and Cawein reported that subcutaneously transplanted A-280 tumor cells generate factors involved in a leucocytosis response that sustains tumor growth (27). Subsequently, leucocytosis was involved in the expansion of cells of myeloid origin with immunosuppressive activity (24). These cells express reduced levels of conventional markers for mature myeloid and lymphoid cells and were named natural suppressor cells, null cells, immature myeloid cells, or myeloid suppressor cells. In 2007, "myeloid-derived suppressor cells" was adopted as a unifying term to minimize the confusion prevailing in the literature (28).

MDSCs are defined primarily by their immunosuppressive functions. Within sepsis, one may predict that MDSCs play a dual role depending on disease progression. On the one hand, MDSCs may be beneficial by limiting hyper-inflammation during the early stages of sepsis, hence protecting from early organ dysfunction. On the other hand, MDSCs may be detrimental by amplifying long-term immunosuppression and contributing to CCI and/or PICS (8, 10). As discussed later, these two facets have been highlighted in experimental models, while clinical studies all pointed to a deleterious role of MDSCs.

Minimal phenotypic characteristics of MDSCs have been proposed, but a definite, consensual phenotyping scheme is lacking (29, 30). Two main subpopulations of MDSCs are usually considered: polymorphonuclear MDSCs (PMN-MDSCs, previously called granulocytic-MDSCs) and monocytic MDSCs (M-MDSCs), so-called because of their morphological and phenotypical homologies with PMNs and monocytes (26, 29–32). In mice, MSDCs are defined as $Gr1^+$ $CD11b^+$ cells (Gr1: granulocyte receptor-1 antigen, consisting of Ly-6G and Ly-6C antigens). PMN-MDSCs are $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ cells and M-MDSCs $CD11b^+$ $Ly6G^-$ $Ly6C^{high}$ cells. In humans, PMN-MDSCs are $CD11b^+$ $CD14^-$ $CD33^+$ ($CD15^+$ or $CD66^+$) cells and M-MDSCs $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{low/-}$ $CD15^-$ cells. PMN-MDSCs overlap phenotypically with mature neutrophils but contrary to PMNs, MDSCs sediment within the PBMC fraction in ficoll gradients after density separation of whole blood. Whether low density gradient (LDGs) PMNs and PMN-MDSCs are the same entity is unclear, albeit the terms is used interchangeably in the literature. The identification of PMN-MDSCs by density gradient is further limited by the rise of not only low-density neutrophils, but also high-density $CD62L^{dim}$ neutrophils that suppress T cells in the blood of healthy humans infused with endotoxin (33). Additional markers are proposed to differentiate MDSCs from monocytes or granulocytes, for example high expression of lectin-type oxidized LDL receptor-1 (LOX-1) by PMN-MDSCs when compared to granulocytes in whole blood (33, 34).

Complicating the picture, other MDSC subsets have been described, among others early-stage MDSCs (e-MDSCs) and eosinophilic MDSCs (eo-MDSCs) (29, 35). In addition, tumor-associated macrophages (TAMs), which unlike their name suggests are present in inflammatory conditions bedsides cancer, can be considered as one of the members making up the MDSC spectrum (36, 37). Finally, MDSCs are highly plastic. They can differentiate into osteoclasts and non-suppressive mature myeloid cells, and M-MDSCs can differentiate into TAMs and PMN-MDSCs (38–41). Overall, to this day, identifying MDSCs based on cell surface phenotyping usually ends up with a mixed population, eventually containing other myeloid cell types, that

does not take into account the hallmark immunosuppressive function of MDSCs.

Adding to the above caveats, improper cell separation through density gradient and freezing whole blood or PBMC samples before flow cytometry analyses affects the detection of MDSCs, especially PMN-MDSCs. Hence, an objective of future studies is to optimize and harmonize sample handling and flow cytometry strategies (labeling, gating, and analyses) to quantify MDSCs in whole blood. This will facilitate the comparison of results from different studies to determine whether MDSCs are reliable disease biomarkers (32, 42). Strategies to identify cell surface markers discriminating MDSCs from other leukocytes using unbiased high discriminating techniques like RNA sequencing and mass cytometry analyses are starting to be used and have not yet improved the immuno-phenotyping of MDSCs (43). To summarize, the analysis of MDSCs and comparing results from different studies is complicated mainly because of: (1) the functional definition of MDSCs, (2) the lack of a defined phenotype(s) of MDSCs, and (3) the plasticity of MDSCs.

MDSCs EXPANSION AND ACTIVATION

Hematopoietic stem cells differentiate into common myeloid progenitors giving rise to immature myeloid cells. An inflammatory environment, as observed in sepsis, stimulates the egress of immature myeloid cells from the bone marrow into the blood stream and the gain immunosuppressive functions (26, 44) (**Figure 1**). The identification of mediators and molecular mechanisms underlying the expansion and the immunosuppressive functions of MDSCs may pinpoint to original therapeutic targets for various diseases. Most of our knowledge comes from disease conditions other than sepsis. In sepsis, most relevant studies analyse the impact of gene specific knockout or the infusion of MDSCs in mice exposed to polymicrobial sepsis induced by cecal ligation and puncture (CLP).

In mice subjected to CLP, MDSCs accumulate in secondary lymphoid organs, in which they represent as much as 10–20% of all leukocytes (45). In the spleen, MDSCs expand within 3–5 days, culminate after 10–14 days and stay high for at least 12 weeks. The rise of MDSCs appears to be a complex and progressive process that involves expansion and activation of immature myeloid cells through many factors. These factors are not specific to sepsis and can be redundant. The expansion of immature myeloid cells is primarily mediated by the action of growth factors (GF) and colony stimulating factors (CSF) [such as vascular endothelial-GF (VEGF), granulocyte-macrophage-CSF, macrophage-CSF (M-CSF) and stem cell factor (SCF)], DAMPs (S100 calcium-binding protein A8/A9, S100A8/9), and possibly chemokines (CXCL1, CXCL2). Activation of pathogenic MDSCs is induced by MAMPs (LPS, staphylococcal enterotoxins), DAMPs (HMGB1), cytokines (IFNγ, IL-1β, IL-4, IL-6, IL-7, IL-10, IL-13, TNF, CXCL3), and acute phase proteins (α2-macroglobulin, serum amyloid A) (26, 42, 46–56). These same factors may induce the maturation of MDSCs, with possible different outcomes. For example, M-MDSCs exposed to R848 (a TLR7/8 agonist), TNF and IFNγ differentiate into inflammatory macrophages that produce TNF and IL-12, while M-MDSCs exposed to Pam_3CSK_4 (a TLR1/2 agonist) differentiate into immunosuppressive macrophages producing IL-10 (47, 57).

Myeloid differentiation primary response 88 (MyD88), glycoprotein 130 (gp130) and nuclear factor I A (NFIA, a transcription factor) control the expansion and the immunosuppressive functions of MDSCs (**Figure 1**). MyD88 is an adaptor molecule that initiates quick nuclear factor-κB (NF-κB) signaling through the IL-1 receptor and all TLRs except TLR3. gp130 is a signal transducer co-receptor for IL-6 family cytokines that cooperates with signal transducer and activator of transcription (STAT3) and C/EBPβ to upregulate MDSCs (45, 54). MDSCs do not expand in $MyD88^{-/-}$ germline mice and in hepatocyte-specific $gp130^{-/-}$ and myeloid-specific $Nfia^{-/-}$ mice subjected to CLP (25, 45, 49, 58, 59). Additionally, $Gr1^+$ $CD11b^+$ MDSCs lacking NFI-A lose their immunosuppressive functions and stop differentiating into mature myeloid cells. The expansion of MDSCs is normal in myeloid-specific $Cebpb^{-/-}$ septic mice, but $Cebpb^{-/-}$ MDSCs produce reduced levels of IL-10 (52, 60). During CLP, triggering of a NF-κB/C/EBPβ/STAT3 axis upregulates the expression of S100A9 (also known as calgranulin B). S100A9 translocates into the nucleus to upregulate the transcription of microRNAs miR-21 and miR-181b that fine tune the expansion and the functions of MDSCs. Mice lacking S100A9 have less splenic and bone marrow MDSCs especially during late sepsis and are protected from death (61, 62). *In vivo* blockade of miR-21 and miR-181 decreases bone marrow MDSCs and improves sepsis survival (63). Recent work suggest that Nfe2l2 (nuclear factor, erythroid derived 2, Like 2; also known as NRF2) contributes to increase the metabolic activity and the expansion of $Gr1^+$ $CD11b^+$ MDSCs during endotoxemia (64).

The molecules mentioned above are not specific to MDSCs, and their genetic ablation can influence other arms of the defenses systems. To bypass this limitation, MDSCs isolated from sepsis mice are infused into wild-type recipient mice subjected to microbial insults. The adoptive transfer of Gr-1^+ $CD11b^+$ MDSCs or PMN-MDSCs harvested from septic donor-mice into recipient mice protects the later from acute endotoxemia, rapidly lethal CLP and *Pseudomonas* airway infection (54, 60, 65–68). Two studies compare the benefits provided by the infusion of Gr-1^+ $CD11b^+$ MDSCs taken either quickly or late after the onset of infection (i.e., 3 vs. 10–12 days post-infection). Interestingly, the transfer of early MDSCs increases while the transfer of late MDSCs decreases or does not change mortality (65, 69). Supported by additional *in vivo* and *in vitro* data (65, 69), this can be explained by the fact that, during the course of sepsis, MDSCs evolve to a more immature and anti-inflammatory state. More work will be required to appraise how much the maturation stage of MDSCs, the timing of expansion and/or infusion of MDSCs and the severity of the infectious models tip the balance toward a beneficial or a detrimental impact of MDSCs on sepsis outcome.

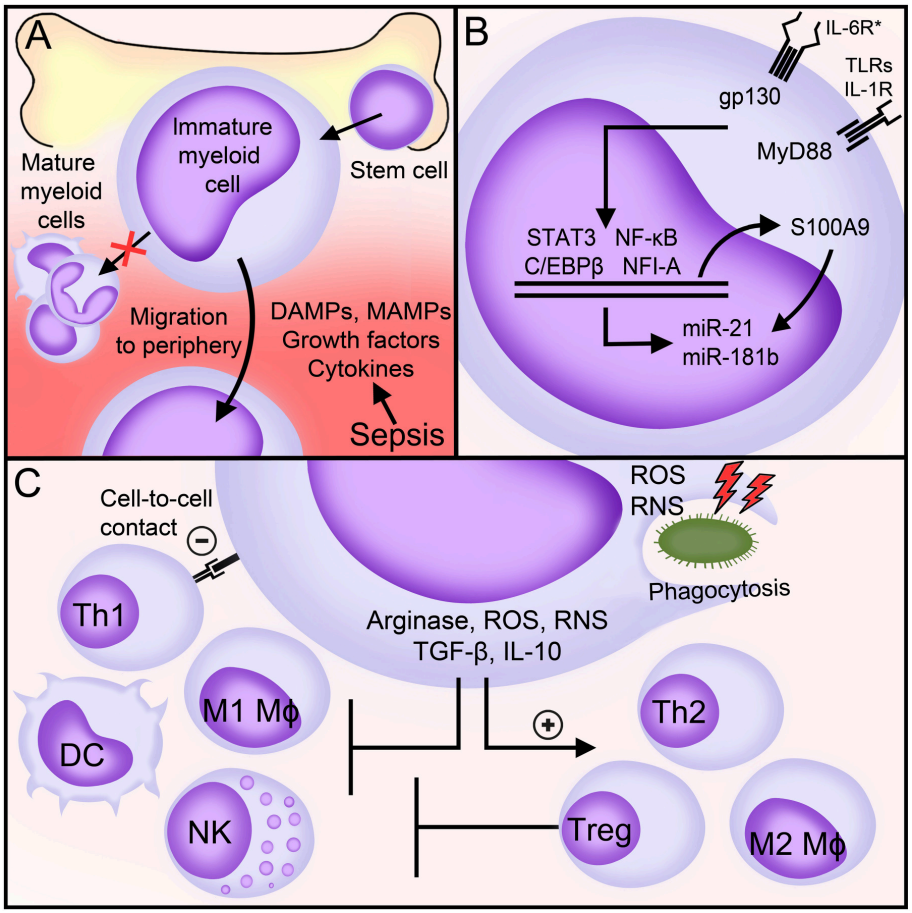

FIGURE 1 | MDSCs in sepsis. **(A)** Factors generated during sepsis induce the expansion and egress of MDSCs from the bone marrow into the peripheral blood. **(B)** Main signaling pathways involved in the expansion and the immunosuppressive functions of MDSCs during sepsis. **(C)** Biological functions of MDSCs during sepsis. See body text for detailed explanations. DAMPs, danger-associated molecular patterns; MAMPs, microbial-associated molecular patterns; IL-6R*, interleukin (IL)-6 receptor family of cytokines (commonly referred to as gp130 cytokines); gp130, glycoprotein 130; TLRs, toll-like receptors; IL-1R, interleukin-1 receptor; MyD88, Myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; NFI-A, nuclear factor I A; STAT, signal transducer and activator of transcription; miR, microRNA; Mφ, macrophage; DC, dendritic cell; Th, T helper; NK, natural killer; Treg, T regulatory; IFNγ, interferon γ; ROS, reactive oxygen species; RNS, reactive nitrogen species; TGF-β, transforming growth factor-β; IL-10, interleukin-10.

As we will see in the last paragraph, the picture is clearer in clinical settings where high proportions of MDSCs indicate a poor prognosis.

The main epigenetic mechanisms, i.e., DNA methylation, histones methylation and acetylation, miRNAs and long non-coding RNAs (LncRNAs), have been implicated in the development of MDSCs with different outcomes (70). For example, inhibition of the DNA methyltransferases (DNMTs) 3a and 3b promotes the suppressive functions of MDSCs while inhibition of the histone methyltransferase SETD1B limits their suppressive function (71, 72). Pan-inhibitors of histone deacetylases (HDACs) 1–11 elicit robust expansion of M-MDSCs (73), in agreement with the observation that HDAC11 itself acts as a negative regulator of expansion and function of MDSCs (74). Interestingly, HDAC2 drives the phenotypic differentiation of M-MDSCs into PMN-MDSCs in tumor bearing mice (75), suggesting that individual HDACs have discrete, specific impact on MDSCs. Remarkably, combination therapies of inhibitors of either DNMTs or HDACs and checkpoint inhibitors (anti-PD-1 or anti-CTLA-4 antibodies) allow the eradication of checkpoint inhibitor resistant metastatic cancers by suppression of MDSCs (76). Finally, miRNAs both positively and negatively regulate the accumulation and functions of MDSCs (for instance miR-9, 17-5p, 21, 34a, 155, 181b, 210, 494, 690 vs. miR-9, 146a, 147a, 185-5p, 223, 185, 424) (70, 77). These observations, obtained in cancer models, are particularly interesting because cancer and sepsis share certain epigenetic features. Therefore, it is no surprise that oncolytic epigenetic drugs have a strong impact on innate immune responses and sepsis development (78–81). Numerous epigenetic drugs are tested in oncologic clinical trials while some are already approved for clinical applications. Altogether, these observations open a fascinating area to test epigenetic drugs targeting the expansion and/or function of MDSCs during sepsis.

IMMUNOSUPPRESSIVE FUNCTIONS OF MDSCs

MDSCs suppress the activity of immune cells through various mechanisms involving the degradation of L-arginine, the production of reactive oxygen and reactive nitrogen species (ROS, RNS), the secretion of anti-inflammatory/immunosuppressive cytokines like IL-10 and transforming growth factor (TGF)-β and the activation of T regulatory cells (Tregs) (**Figure 1**).

L-arginine becomes a semi-essential amino acid during sepsis because of increased usage and reduced production. L-arginine shortage is sustained by the production by MDSCs of arginase that metabolizes L-arginine into L-ornithine and urea (82). L-arginine depletion affects the function of T cells through a decreased expression of the CD3 zeta-chain, which is essential for T-cell receptor (TCR) signaling (50, 83). A lack of arginase also limits the activity of natural killer (NK) cells (84). ROS, RNS, IL-10, and TGF-β skew the polarization of monocytes/macrophages and T cells toward anti-inflammatory/pro-resolving M2, Th2 and regulatory phenotypes (45, 65, 85) and impair TCR and IL-2 receptor signaling, NK cell activity and DC maturation and antigen presentation (86–89) (**Figure 1**). MDSCs suppress Th1 responses though direct cell-to-cell contact, but how precisely this occurs remains to be determined (45, 85). Together with CCL5/RANTES and CCL4/MIP-1β, RNS, IL-10, and TGF-β promote the recruitment and the immunosuppressive activity of Tregs, at least in cancer and in neonates (45, 85, 90, 91). The interaction between MDSCs and Tregs in sepsis is unknown.

Splenic MDSCs harvested from CLP mice early (3–5 days) and late (10 days) after sepsis onset inhibit T cell proliferation. Early MDSCs secrete less S100A9 than late MDSCs (61) and, in response to LPS and IL-6, less TNF, IL-6, IL-10, ROS, and arginase I (65). However, in response to GM-CSF, early MDSCs produce RNS and proinflammatory cytokines while late MDSCs produce arginase, IL-10 and TGF-β (69). Of note, MDSCs can also help fight infections. Indeed, MDSCs efficiently phagocytose *E. coli* and group B streptococci (92) and clear bacteria during late sepsis through a robust production of ROS (65). Thus, MDSCs have diverse biological outputs according to their surrounding milieu and sepsis progression (54, 65). More work is required to fully understand to which extend these biological variations reflect the accumulation or the differentiation of different MDSCs subpopulations during sepsis.

DIAGNOSTIC AND PROGNOSTIC VALUES OF IMMATURE GRANULOCYTES AND MDSCs IN HUMAN SEPSIS

MDSCs make up an important proportion of immature myeloid cells. Thus, we will discuss reports analyzing immature granulocytes (IG) in adult sepsis and then move forward to studies that used more elaborated immuno-phenotyping strategies to identify MDSCs. **Table 1** provides details about the design and the main observations of these studies.

Accumulation of immature myeloid cells is one of the criteria established more than 25 years ago to characterize SIRS (systemic inflammatory response syndrome) and sepsis (107). The assessment of immature cells remained laborious up to the advent of automated cell counters. In an earliest study using automated IG counting on a small number of patients, the percentage of IG was higher in infected than in uninfected patients and was proposed to be a predictor of sepsis (93). Retrospective and prospective observational studies confirmed that IG proportion discriminates between infected and uninfected patients and is associated with disease severity (94–99) (**Table 1**). Automated cell counters can determine a delta neutrophil index (DNI), which reflects the number of immature neutrophils in the blood. A meta-analysis of ten Korean and one Egyptian studies including 1,822 sepsis patients suggests that an elevated DNI (i.e., an increased proportion of immature granulocytes) is associated with mortality (100).

Few reports demonstrate the immunosuppressive functions of immature myeloid cells in relation with sepsis and/or monitor MDSCs subpopulations using advanced flow cytometry. Since cell preparation (whole blood, with and without ficoll purification) and flow cytometry strategies are not standardized, the phenotype of MDSCs, PMN-MDSCs and M-MDSCs differs between studies (**Table 1**).

Gradient density interphase neutrophils arise during sepsis and their proportion correlates with disease severity in ICU patients. Cells isolated from septic shock patients deplete arginine and impair T cell functions *in vitro*, suggesting that they represent PMN-MDSCs (50). High levels of circulating $CD10^{dim}$ $CD16^{dim}$ IG are predictive of clinical deterioration and mortality (101, 102). This population contains a subset of $CD14^{-}$ $CD24^{+}$ myeloid suppressor cells that kill activated T cells *in vitro* (101).

The frequency of PMN-MDSCs (SSC^{high} $CD16^{+}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{high}$ $CD114^{+}$ $CD11b^{+/low}$ LDG) and M-MDSCs (SSC^{low} $CD14^{+}$ $CD11b^{+}$ $CD16^{-}$ $CD15^{+}$) does not differ between non-infectious critical ill patients and sepsis patients (103). However, high levels of MDSCs are linked to nosocomial infections (**Table 1**). In a first study, PMN-MDSCs ($CD14^{-}$ $CD15^{+}$ low-density granulocytes, LDG) representing more than 36% of WBC in ICU patients sampled within 3 days of study inclusion predicts the subsequent occurrence of nosocomial infections (104). Patients that develop nosocomial infections have 2.5 times more PMN-MDSCs than patients that do not. In a second study, a close follow-up of ICU surgical patients (at days 1, 4, 7, 14, 21, and 28 or until discharge of ICU) reveals that patients with continuously high proportions of $CD33^{+}$ $CD11b^{+}$ HLA-$DR^{-/low}$ MDSCs have a longer stay in the ICU, more nosocomial infections and poor functional status at discharge (105). The percentage of total MDSCs in patients with severe sepsis/septic shock raises up to 45% of WBC, and a high proportion of MDSCs at diagnosis is associated with early mortality. Comparing cell-sorted enriched $CD33^{+}$ $CD11b^{+}$ HLA-$DR^{-/low}$ MDSCs from the blood of healthy subjects and septic patients reveals that pathogenic MDSCs dose dependently suppress IFNγ, IL-4, and IL-10 production by T cells more efficiently than MDSCs from

TABLE 1 | Studies investigating immature granulocytes and MDSCs in adults with sepsis.

Subjects	Cells/phenotypes	Observations	References
142 ED patients, 29 uninfected outpatients.	IG (automate-based determination)	Higher % in infected patients, predictor of sepsis.	(93)
70 consecutive ICU patients (51 infected, 19 uninfected).	IG (automate-based determination)	Higher % in infected patients, unrelated to day-21 and in-hospital mortality.	(94)
184 sepsis patients.	IG (automate-based determination)	Increase % associated with severity, but not predictive of mortality.	(95)
136 consecutive ICU patients.	IG (morphology and staining)	Higher % in sepsis than in uninfected patients. Unrelated to mortality.	(96)
35 sepsis and 22 non-septic consecutive burn patients, 19 healthy controls.	IG (flow cytometry)	Increase % post-burn, associated with reduced neutrophil function. Remaining elevated levels (day 7–28) associated with sepsis development	(97)
781 sepsis patients, 20 control outpatients.	IG (flow cytometry)	High % at admission related to organ failure and day-7 and day-28 mortality.	(98)
47 uninfected and 17 infected cardiac surgery patients.	IG (flow cytometry)	Increase % postoperative. Highest levels associated with secondary infection complications.	(99)
Meta-analysis (11 studies) of 1'822 sepsis patients.	Delta neutrophil index (DNI, automate-based determination)	Elevated DNI associated with mortality.	(100)
24 sepsis ICU patients, 12 hospital controls.	Interphase neutrophils (flow cytometry)	Present only in sepsis patients, proportional to sepsis severity. Suppress T-cell activity *in vitro*.	(50)
177 sepsis patients.	IG (flow cytometry)	Increase % at 48 h predictive of clinical deterioration. High % of $CD10^{dim}$ and $CD16^{dim}$ IG correlates with mortality. Kill activated T cells *in vitro*.	(101)
43 septic shock patients, 23 healthy controls.	IG (flow cytometry)	Increased % of $CD10^{dim}$ and $CD16^{dim}$ IG at days 3–4 and 6–8. Patients with lower % have better survival.	(102)
14 sepsis and 8 uninfected critically ill patients, 15 healthy controls.	M-MDSCs: SSC^{low} $CD14^{+}$ $CD11b^{+}$ $CD16^{-}$ $CD15^{+}$ PMN-MDSCs: SSC^{high} $CD16^{+}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{high}$ $CD114^{+}$ $CD11b^{+/low}$	M-MDSCs but not PMN-MDSCs increase at day 13-21 post-sepsis. Similar % of M-MDSCs and PMN-MDSCs in sepsis and non-septic critical ill patients.	(103)
94 sepsis, 11 severity-matched ICU patients, 67 health donors.	M-MDSCs: Lin^{-} $CD14^{+}$ $HLA\text{-}DR^{-/low}$ PMN-MDSCs: LDG $CD14^{-}$ $CD15^{+}$ (Excluding eosinophils)	High % of PMN-MDSCs in sepsis patients. M-MDSCs are higher in gram-negative than gram-positive sepsis. PMN-MDSCs > 36% WBC at entry are associated with higher risk of nosocomial infections. PMN- and M-MDSCs suppress T-cell proliferation *in vitro*.	(104)
67 surgical patients with severe sepsis/septic shock, 18 healthy controls.	MDSCs: $CD33^{+}$ $CD11b^{+}$ $HLA\text{-}DR^{-}$ M-MDSCs: $CD14^{+}$ PMN-MDSCs: $CD14^{-}$ $CD15^{+}$	High % of MDSCs at admission correlates with early mortality. Decreasing levels of MDSCs correlate with short ICU stay. Sustained levels of MDSCs (>30% of WBC) predict nosocomial infections.	(105)
56 sepsis patients and 18 healthy controls.	M-MDSCs: $CD14^{+}$ $CD64^{+}$ $HLA\text{-}DR^{-}$ PMN-MDSCs: LDG $CD33^{+}$ $CD14^{neg/low}$ $CD64^{low}$ $CD15^{+/low}$	High % of M-MDSCs in all sepsis, but particularly in gram-negative sepsis patients. Prominent PMN-MDSCs in gram-positive sepsis. PMN-MDSCs suppress T-cell proliferation *in vitro*.	(106)

ED, emergency department; ICU, intensive care unit; IG, immature granulocytes; LDG, low density granulocytes; Lin, lineage; WBC, white blood cells.

healthy subjects, while healthy and disease MDSCs suppress T cell proliferation alike (105).

The proportion of PMN-MDSCs and M-MDSCs, defined as $CD14^{neg/low}$ $CD64^{low}$ $CD15^{+/low}$ LDG and $CD14^{+}$ $CD64^{+}$ $HLA\text{-}DR^{neg}$ leukocytes, may vary according to causative agent leading to sepsis (**Table 1**). M-MDSCs increase in all sepsis patients, predominantly in gram-negative cases, while PMN-MDSCs increase prominently in gram-positive sepsis (106). A subsequent study confirmed that M-MDSCs (Lin^{-} $CD14^{pos}$ $HLA\text{-}DR^{low/neg}$) are enriched during gram-negative sepsis, but PMN-MDSCs ($CD14^{-}$ $CD15^{+}$ LDG) do not differ according to the gram of the causative bacteria (104). Larger studies are required to ascertain that the microbial origin of sepsis shapes the pattern of MDSCs (108). This is an important parameter since M-MDSCs are more potent immunosuppressive than PMN-MDSCs on a per cell basis (109).

CONCLUDING REMARKS

MDSCs play a dual role during infection and sepsis. MDSCs expanding along emergency erythropoiesis provide a first barrier against microbial invasion by producing high amounts of bactericidal molecules like ROS and RNS and counteract the hyperinflammatory response associated with early organ dysfunctions. However, MDSCs are also detrimental by supporting the establishment and/or the maintenance of a late

protracted immunosuppressive environment. In line with a deletary role of MDSCs, all clinical studies to date associate high proportions of blood MDSCs with clinical worsening, occurrence of nosocomial infections and mortality of sepsis patients. Hence, MDSCs are attractive biomarkers, especially since these cells are barely detectable in healthy subjects. One limitation of clinical studies, not limited to the sepsis field, resides in the uneven phenotypic classification of MDSCs. One important future objective is to harmonize sample handling and flow cytometry strategies. Besides being attractive biomarkers, MDSCs are attractive therapeutic targets for sepsis. Inhibiting MDSCs-mediated immunosuppression or MDSCs trafficking or depleting MDSCs themselves (by normalizing myelopoiesis or inducing the differentiation of MDSCs into mature myeloid cells) would positively influence patient outcome. Interestingly, more than 30 clinical trials are running targeting MDSCs directly or indirectly in cancer patients (22). If ever envisaged for sepsis, these therapies will need specific evaluation since targeting MDSCs aggressively may put critically ill patients at risk of agranulocytosis. The results arising from these oncological studies, added to those from current or future studies in the field of sepsis, will give invaluable information onto whether and how MDSCs might be used to implement sepsis personalized medicine and precision immunotherapy.

AUTHOR CONTRIBUTIONS

IS and TR conceived and structured the manuscript. IS drafted the manuscript and the figure. CT revised the manuscript. TR finalized and edited the manuscript.

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Monocyte HLA-DR Assessment by a Novel Point-of-Care Device Is Feasible for Early Identification of ICU Patients With Complicated Courses—A *Proof-of-Principle* Study

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Background: Critically ill patients, especially following trauma or extensive surgery, experience a systemic immune response, consisting of a pro-inflammatory as well as a counterbalancing anti-inflammatory response. Pro-inflammation is necessary for the initiation of homeostatic control and wound healing of the organism. However, when the counterbalancing mechanisms dominate, a condition of secondary immunodeficiency occurs, which renders the patient susceptible for opportunistic or secondary infections. However, the incidence of this condition is yet illusive.

Methods: For a period of 3 months (May to July 2017), 110 consecutive patients admitted to the surgical ICU of the Heidelberg University Hospital, a tertiary university hospital, were enrolled in the study. Monocyte HLA-DR (mHLA-DR), a long-known surrogate of monocyte function, was assessed quantitatively once on admission utilizing a novel point-of-care flow cytometer with single-use cartridges (Accelix system). Patients were followed up for further 28 days and data on ICU stay, antibiotic therapy, microbiological findings, and mechanical ventilation were recorded. Statistical analysis was performed to evaluate the incidence of immunosuppression—defined by different thresholds—as well as its consequence in terms of outcome and clinical course.

Results: Depending on the HLA-DR threshold applied for stratification (≤8,000/≤5,000/≤2,000 molecules/cell), a large group of patients (85.5/68.2/40.0%) already presented with a robust decrease of HLA-DR on admission, independent of the cause for critical illness. Analyzed for survival, neither threshold was able to stratify patients with a higher mortality. However, both thresholds of 2,000 and 5,000 were able to discriminate patients with longer ICU stay, ventilation time and duration of antibiotic therapy, as well as higher count of microbiological findings. Moreover, a mHLA-DR value ≤2,000 molecules/cell was associated with higher incidence of overall antibiotic therapy.

Conclusion: Single assessment of mHLA-DR using a novel point-of-care flow cytometer is able to stratify patients according to their risk of a complicated course. Therefore, this device overcomes the technical boundaries for measuring cellular biomarkers and paves the way for future studies involving personalized immunotherapy to patients with a high immunological risk profile independent of their background.

Trial Registration: German Clinical Trials Register; ID: DRKS00012348.

Keywords: SIRS, CARS, sepsis, infection, immunosuppression, tolerance, personalized medicine, precision medicine

BACKGROUND

Extensive tissue injury, caused by either major surgery or trauma, induces a transient episode of sterile systemic inflammation, aiming to initiate damage and homeostatic control as well as wound healing (1–3). Simultaneously, a plethora of counterbalancing mechanism like, e.g., the apoptosis of lymphoid cells or the appearance of anti-inflammatory cytokines, occur. In their entirety, those are called compensatory anti-inflammatory response syndrome (CARS) (4). Its assumed evolutionary function is to prevent harm from overshooting inflammation, however, if this reaction is dominating it implies a higher susceptibility toward secondary and opportunistic infections with poor prognosis. In a nutshell, this acquired condition resembles a secondary immunodeficiency. Besides, the response pattern is skewed by the patient's predisposition, involving intrinsic factors as age, gender, and genetics as well as co-morbidities, the concomitant medications and lifestyle (5–7). Although antibiotic treatment can fight most of these arising infections, it cannot approach the fundamental problem of an impaired immunity of the host. Even worse, systemic antibiotic therapy can alter the composition of the body's microbiota, thereby opening niches for the colonization and expansion of opportunistic pathogens like, e.g., *Clostridium difficile* (8, 9). In sum, a vicious cycle between host and pathogens develops, implying tremendous harm to the organism.

An important objective is the development of host-directed therapies, aiming to restore the patient's endogenous immune capacity, especially at the boundaries of gut, skin, and airways (10). Despite a lack of approved drugs, several promising studies (predominantly with septic patients) have already been conducted evaluating the safety and feasibility of available immunomodulating compounds such as interferon-γ (IFN-γ) and granulocyte- or granulocyte/macrophage-colony stimulating factor (G-/GM-CSF) (11, 12). However, to fully exploit the benefit of these treatments and to avoid unnecessary exposure of critically ill patients to drugs and their side effects, patients need to be *a priori* stratified using robust and reliable surrogate biomarkers.

Various parameters have been assessed over the last 30 years, but the most prominent and widely used one for this purpose remains the downregulation of monocyte human leukocyte antigen-DR (mHLA-DR) (13, 14). As part of the heterodimeric major histocompatibility complex class II (MHC II) on the outer cell membrane, HLA-DR represents monocytes' capacity for antigen-presentation and by this means the crosstalk to T helper cells, enabling the activation of the adaptive immune system. Its predictive value concerning nosocomial infections and prognosis has been shown in clinical studies on various conditions, e.g., in patients suffering from sepsis (15), trauma (16, 17), burns (18, 19), or subjected to major surgical procedures such as liver transplantation (20, 21) or coronary artery bypass (22).

However, despite decades of research, mHLA-DR is rarely used in everyday clinical practice, due to the lack of broad access to flow cytometry and the availability of standardized assays. Also, as most previous studies focused on precisely defined groups of patients, there is little knowledge concerning the overall incidence of immunosuppression in the ICU. Consequently, the understanding of how many patients could benefit from personalized immunotherapy is limited. With newly emerging and miniaturized technologies in combination with simplified workflows, flow cytometry is finally coming to bedside and measurements can be facilitated by healthcare professionals around the clock at the point-of-care (POC) without the need of sample logistics and delayed results.

Making use of the Accelix system, a benchtop flow cytometer, our study aimed to determine the incidence of patients already presenting with decreased mHLA-DR already at ICU admission and the consequence of it regarding outcome and clinical course. We consecutively enrolled all patients admitted to a surgical ICU of a tertiary university hospital throughout 3 months. Quantitative mHLA-DR was measured once at admission, and the patients were followed up for further 28 days. As several thresholds have been reported before, we applied these to our cohort to delineate, which one projects best into complicated courses.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; CD, Cluster of Differentiation; G-/GM-CSF, Granulocyte-/Granulocyte/Monocyte-Colony Stimulating Factor; HLA-DR, Human leucocyte antigen DR; ICU, Intensive care unit; IFN-γ, Interferon-γ; MFI, Mean fluorescence intensity; POC, Point-of-care; RCT, Randomized, controlled trial; SOFA, Sequential organ failure assessment score.

METHODS

Study Design and Enrollment

Before enrollment of the first patient, the study protocol was assessed and positively evaluated by the local ethics committee (S-150/2017, Ethical Committee I of the Medical Faculty Heidelberg). Furthermore, the study was registered in the German Clinical Trials Register (ID: DRKS00012348). Over a period of 3 months (May to July 2017), all adult patients admitted to the surgical ICU of the Heidelberg University Hospital were enrolled in the study. Exclusion criteria were prior intensive care unit stays within the same hospital episode and the presence of therapy limitation/palliation at admission. Informed consent was obtained from the patient or, if not possible due to sedation or mental deterioration, from the legal representative. Cases without informed consent ($n = 24$) were excluded from the study's analysis.

On admission, all anamnestic data, as well as clinical scores and laboratory values (PCT, CRP, leucocytes), were obtained. Patients were followed up for a total of 28 days after admission (=day 0) and clinical variables (survival, antibiotic therapy, mechanical ventilation) were prospectively evaluated on a daily basis.

Measurement of HLA-DR

Within 24 h from admission and within 2 h after blood draw, HLA-DR measurements were performed on a novel point-of-care flow cytometer (Accelix®, LeukoDX, Jerusalem, Israel). The system's characteristics and technical validity have been reported before (23). For each measurement, 40 µl of residual anti-coagulated blood drawn from the patient for routine blood gas analysis was applied onto the inlet port of a single-use cartridge containing antibodies as well as all reagents for cell preparation. After blood aspiration, the cartridge was closed and inserted into the Accelix® system for automated analysis within 25 min. The final HLA-DR value (average number of monoclonal antibodies bound per monocyte) was directly reported by the system after measurement. To facilitate this, an internal 4-point bead-based calibration curve was measured for each sample. Furthermore, as each anti-HLA-DR antibody is conjugated to only one molecule of fluorophore, the number of bound antibodies equals the number of molecules per cell.

In one case of a patient with severe neutropenia (0.3 leucocytes/nL), the system was not able to perform the measurement due to system-inherent algorithm-based quality rules, requiring a certain number of cellular events per second. The case was therefore excluded from the final analysis.

Statistical Analysis

All statistical analysis and visualizations have been performed using SPSS Statistics (Version 25.0.0.1, IBM, Armonk, USA) with the exception of the scatter plot for individual HLA-DR values, which was generated in GraphPad Prism (Version 6.0c, GraphPad Software Inc., La Jolla, USA). Kaplan-Maier procedure was used for analysis of survival time and incidence of antibiotic therapy. Patients were grouped according to different thresholds of HLA-DR and groups were subsequently compared using the Log-rank test. Patients transferred or discharged from hospital were censored from the analysis of incidence of antibiotic therapy (detailed information of each patients' therapy was not legally permitted in case of treatment in other hospitals), but were maintained in the survival analysis, assuming no discharge in critical condition. For group comparisons of continuous variables (ICU stay, ventilation time, time under antibiotic therapy, microbiological findings), non-parametric Mann-Whitney *U* test was performed to compare different threshold groups. For the comparison of categorical variables, Chi-square test was performed. A *p*-value of ≤ 0.05 was accepted as significant for all comparisons. To assess the prognostic performance of HLA-DR *Area Under Receiver Operator Characteristic* (AUROC) analysis was performed regarding the variables "antibiotic therapy" and "28-day-mortality." Area under curve (AUC) and the 95% confidence interval are reported as global indicators of discriminatory performance. To identify the cut-off value corresponding to the best combination of sensitivity and specificity, Youden index was calculated [(Sensitivity + Specificity)−1] and the maximum value selected.

RESULTS

The Study Cohort and Incidence of Immunosuppression

Overall, 135 critically ill patients consecutively admitted to a single surgical ICU of an academic hospital for any reason were enrolled. Of those, 110 were available for final analysis (24 patients dropped out due to inability to gain informed consent, one patient suffered from leucopenia and no POC HLA-DR assessment was possible) (**Figure 1A**). The median age of the study cohort was 63 years (range 20–92), with a majority of 83 male patients (75.5%) (**Table 1**). With nearly two thirds of patients grouped into ASA class III, the cohort exhibited a high burden of co-morbidities, especially of the cardiopulmonary system, and a high degree of illness on admission, as depicted by a median SOFA score of 5 (range: 0–17) and APACHE II score of 19 (range: 2–41) (**Table 2**). HLA-DR values only weakly, but yet significantly correlated with these scores (**Supplementary Table 1**). Only one patient after esophagectomy was admitted to the ICU in an elective manner, while the large majority of patients presented with either complications in the course of surgical treatment [unclear clinical deterioration: $n = 42$ (38.2%), surgery-associated infections: $n = 19$ (17.3%), bleeding complication: $n = 9$ (8.2%)], or came as external emergencies [$n = 26$ (23.6%)]. Multi-visceral resection [$n = 28$ (25.5%)], vascular as well as aortic surgery [$n = 19$ (17.3%); $n = 13$ (11.8%)] represented the most abundant procedures during the current hospital episode.

On admission, 54 patients (49.1%) depended on mechanical ventilation, mainly due to lung failure [$n = 41$ (41.8%)] and half of the patients ($n = 55$) already received antibiotics when arriving in ICU. Sepsis ($\geq$2 SIRS criteria + antibiotic therapy) was present in 23 patients (20.9%).

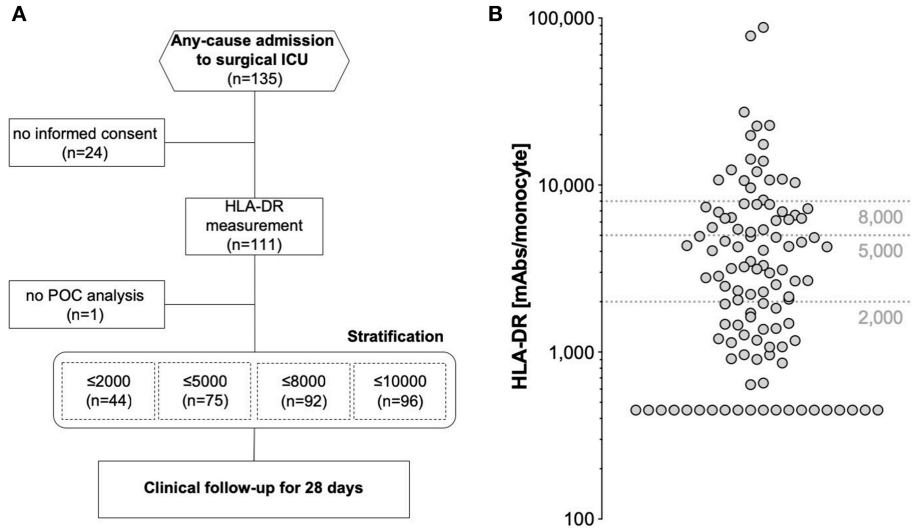

FIGURE 1 | (A) Study flowchart according to STROBE and **(B)** distribution of HLA-DR measurements in the analyzed study cohort of 110 patients. Dashed horizontal lines depict threshold values indicated in earlier studies.

Quantitative monocyte HLA-DR expression was measured once after admission and obtained HLA-DR values ranged from below 450 HLA-DR molecules/monocyte (the system's lower limit of detection) to 87.768 molecules/monocyte (**Figure 1B**). We applied different thresholds reported earlier in literature to our results for further stratification: 94 patients presented (85.5%) with ≤10.000 molecules/cell [assumed as severe immunodepression (24)], 92 patients (83.6%) with ≤8.000 molecules/cell (25), 75 patients (68.2%) with ≤5.000 molecules/cell [postulated as threshold of immunoparalysis (24)], and finally 44 patients (40%) presented with ≤2.000 molecules/cell (26). In summary, our study involved unselected critically ill patients with a high degree of morbidity and with the majority of subjects already showing a lowered HLA-DR expression on monocytes on admission.

Different HLA-DR Thresholds do Not Predict Survival

We applied different HLA-DR threshold (2.000/5.000/8.000) to our cohort and analyzed their association with survival. Overall, 11 patients (10%) died within the observational time frame. Concerning significance, none of the thresholds was able to stratify our cohort into groups of different survival outcome (**Figures 2A–C**). However, a threshold of 2,000 reached the best separation, with 13.6% (6 of 44) of patients below threshold dying within 28 days compared to only 7.6% of patients (5 of 66) with HLA-DR above threshold. A *de novo* AUROC analysis also found no HLA-DR cut-off with a predictive value for mortality in our cohort (**Supplementary Figure 2**).

HLA-DR Is Able to Predict Complicated Clinical Courses

Next, we applied the threshold and compared clinical variables between the groups. Patients with HLA-DR values ≤2,000 as well as ≤5,000 had a longer median ICU stay (6d (range: 2–29) vs. 3d (range: 1–29), $p = 0.004$; 4d (1–29) vs. 3d (1–29), $p = 0.029$) (**Figure 3A**, **Supplementary Table 2**). Similarly, duration of mechanical ventilation was longer in patients ≤2.000 and ≤5.000 HLA-DR: 3d (0–29) vs. 1d (0–29) ($p < 0.001$) and 2d (0–29) vs. 0d (0–29) ($p = 0.022$), respectively (**Figure 3B**, **Supplementary Table 2**). Severe infections are the superior threat on ICUs and we, therefore, analyzed the median antibiotic exposure time between HLA-DR stratified groups. Patients with HLA-DR ≤2,000 received systemic therapy for 14d (0–29) compared to 8d (0–29) ($p < 0.001$) and the patient group of HLA-DR ≤5,000 for 10d (0–29) compared to 6d (0–29) ($p = 0.02$) (**Figure 3C**, **Supplementary Table 2**). Not surprisingly, these results are corroborated by a higher number of total microbiological findings (for overview, see **Supplementary Table 3**) in the groups below thresholds [≤2,000: 4 (0–22) vs. 1 (0–21), $p = 0.001$; ≤5,000: 3 (0–22) vs. 1 (0–21), $p = 0.002$)] (**Figure 3D**, **Supplementary Table 2**). Surprisingly, when only blood cultures were considered, no difference was observed. In general, applying a threshold of 8,000 did not yield group of significant different outcomes. Importantly, disease severity as indicated by common ICU scores differed between the HLA-DR stratified groups, irrespective of the applied threshold (**Supplementary Table 2**). In conclusion, lower thresholds of 2,000 and 5,000 are capable to stratify patients into groups with longer ICU stay and ventilation time, longer antibiotic exposure and a higher number of microbiological findings.

Lower HLA-DR Is Associated With Higher Incidence of Antibiotic Therapy

Based on our finding of longer duration of antibiotics we asked about the fraction of patients needing antibiotic therapy within the observation period of 28 days. In line to the analysis reported before, we grouped the patients according to their HLA-DR value and different thresholds. We found an increased incidence

TABLE 1 | Baseline demographic and medical information of study population.

	ICU patients (n = 110)	
DEMOGRAPHY		
Age (years)	63	(20–92)
Sex (male)	83	75.5
ASA CLASSIFICATION		
I	6	5.5
II	22	20.0
III	69	62.7
IV	12	10.9
V	1	0.9
BMI (kg/m^2)	25.45	(16.1–45.9)
REASON FOR ICU ADMISSION		
Preclinical emergency	26	23.6
Unclear clinical deterioration	42	38.2
Elective post-surgery	1	0.9
Infection (surgery-related)	19	17.3
Infection (other)	4	3.6
Bleeding	9	8.2
Internistic condition	4	3.6
Thromboembolic event	3	2.7
Other	1	0.9
SURGICAL PROCEDURES		
Esophageal resection	7	6.4
Gastrectomy	1	0.9
Small bowel resection	7	6.4
Colectomy	5	4.5
Liver resection	8	7.3
Pancreatic resection	1	0.9
Multivisceral resection	28	25.5
(Partial) Kidney resection	3	2.7
Bladder resection	1	0.9
Prostate resection	1	0.9
Liver transplantation	7	6.4
Aortic surgery	13	11.8
Vascular surgery	19	17.3
Polytrauma/Damage control surgery	20	18.2
Orthopedics	10	9.1
Other	40	36.4
COMORBIDITIES		
Diabetes mellitus	26	23.6
Coronary heart disease	20	18.2
Renal insufficiency	11	10.0
Liver cirrhosis	11	10.0
Inflammatory bowel disease	4	3,6
Leukemia	1	0.9
Hepatitis B	3	2.7
Hepatitis C	2	1.8
Peripheral arterial disease	7	6.4
Arterial hypertension	55	50.0
Atrial fibrillation	14	12.7
Dyslipoproteinemia	12	10.9
COPD	12	10.9
Asthma	2	1.8
Thyroid disease	15	13.6
Tumor	52	47.3
Other, cardiologic	10	9.1
Other	64	58.2

All values represent number (%), except for age, and BMI, where median (min–max) is given. BMI of two cases could not be extracted from the medical records. ASA, American Society of Anesthesiologists; BMI, Body mass index; COPD, Chronic obstructive pulmonary disease.

TABLE 2 | Laboratory parameters, scores and outcome of study population.

	ICU patients (n = 110)	
LABORATORY PARAMETERS		
Leucocytes (1/nL)	10.17	(1.58 – 39.4)
CRP (mg/L)	81.4	(1.9 – 428)
PCT (ng/mL)	1.37	(0.06 – 284.8)
SCORES		
APACHE II	19	(2 – 41)
SAPS II	33	(0 – 88)
SOFA	5	(0 – 17)
OUTCOME		
Antibiotic therapy on admission	55	50
Antibiotic therapy on day 1	60	54.6
Sepsis on admission	23	20.9
Length of ICU stay (d)	4	(1 – 29)
Ventilation time (d)	2	(0 – 29)
Cumulative antibiotic therapy (d)	9	(0 – 29)
Mortality (28-day)	11	10
Discharged (within observation time)	51	46.4

All values represent median (min–max), except "Antibiotic therapy on admission/day 1," "Sepsis on admission," "Mortality," and "Discharged," where numbers (%) are given. CRP measurements are available of 109 cases and PCT of 60 cases. APACHE II, Acute Physiology And Chronic Health Evaluation II; CRP, C-reactive protein; PCT, procalcitonin; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential organ failure assessment score; ICU, Intensive care unit.

when comparing patients below and above a threshold of 2,000 molecules/cell (95.5% (42 of 44 patients) vs. 74.2% (49 of 66 patients) (**Figure 4A**). For the thresholds of 5,000 and 8,000, no differences could be shown (**Figures 4B,C**). As half of the patients already received antibiotic therapy when admitted to ICU, we performed another analysis only including patients being antibiotic naïve on the admission day. Comparably, the lowest threshold of 2,000 significantly stratified patients according to their overall incidence of antibiotic therapy (88.9% (16 of 18 patients) vs. 54.1% (20 of 37 patients), while the others did not (**Supplementary Figure 1**). Aiming to evaluate the specific HLA-DR cut-off in our cohort, we conducted an AUROC analysis. We found a value of 4,266 to be of best predictive value [AUROC: 0.692 (0.573–0.811)] (**Supplementary Figure 2**), further substantiating the former results.

DISCUSSION

We report here the results of a proof-of-principle study on critically ill patients, using for the first time a novel point-of-care flow cytometer for easy and rapid assessment of mHLA-DR expression. We evaluated the incidence of decreased HLA-DR in an unselected cohort of patients immediately after ICU admission and found, independent of the reason for admission, a large percentage of patients presenting with dysfunctional monocytes, as defined by HLA-DR values below 2,000 or 5,000. Furthermore, those low values projected into the clinical course with "low HLA-DR" patients exhibiting a

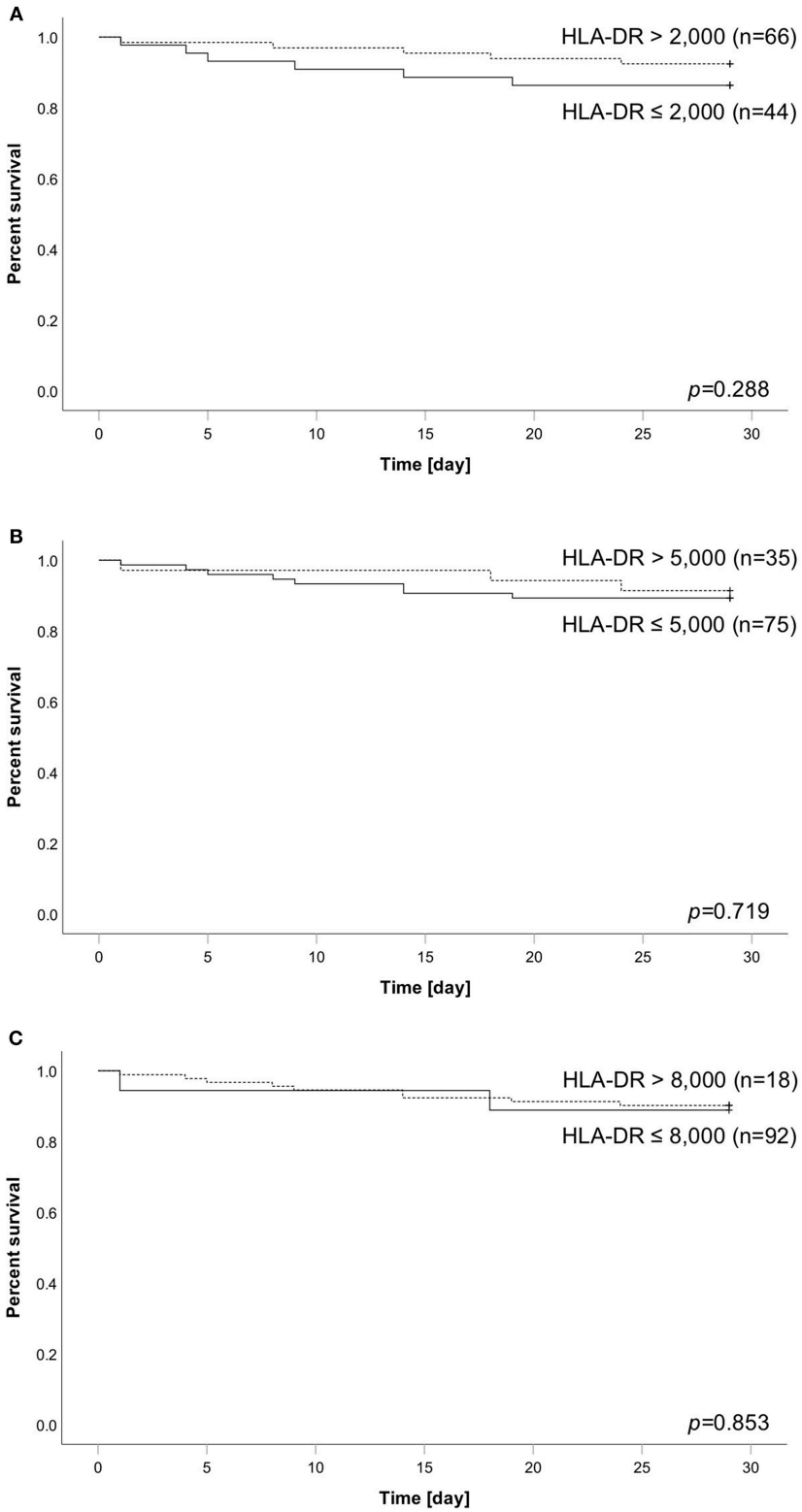

FIGURE 2 | Analysis of patient survival over 28 days stratified according to HLA-DR thresholds of **(A)** 2,000, **(B)** 5,000, or **(C)** 8,000. Dashed line always indicates subgroup above corresponding threshold, whereas solid line indicates the group of patients below. Number in brackets equal subgroup size, a total of 110 patients has been analyzed. Group comparison was performed using logrank test and calculated *p*-values are given within the subpanels.

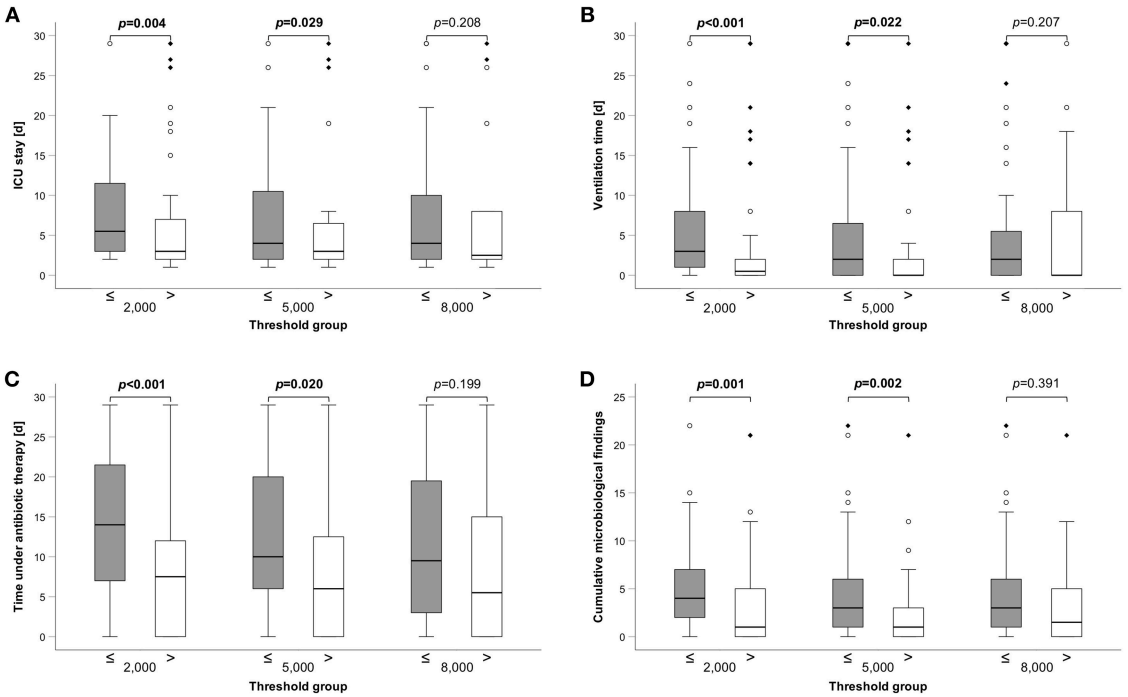

FIGURE 3 | Group comparisons of clinical variables between different HLA-DR thresholds. **(A)** Length of ICU stay, **(B)** Ventilation time, **(C)** Time under antibiotic therapy (all 3 in days), and **(D)** Cumulative microbiological findings are shown. A total of 110 patients has been analyzed. Box edges represent quartiles with median given as horizontal line within, whiskers span the 95% confidence interval. Open circles and diamonds indicate outliers. Group comparisons were performed by Mann-Whitney *U*-test and *p*-values are given above the compared groups. Bold type indicates a $p \leq 0.05$, assumed as significant.

longer ICU stay and prolonged mechanical ventilation as well as antibiotic therapy.

Three decades ago, mHLA-DR entered the stage for the outcome prediction of trauma patients. At that time, the percentage of HLA-DR$^+$ monocytes was incorporated into a score as a weight factor (27). Since then, more than 130 clinical studies conducted on a variety of patient cohorts have described the ability of mHLA-DR for prediction of outcome or secondary infection. However, until today, this parameter has not found its way into clinical practice for several reasons. Due to the lack of a standardized assay, mHLA-DR was reported in early studies either as "mean fluorescence intensity" (MFI; a raw parameter of cytometry) or as "HLA-DR$^+$ monocytes." Both parameters are largely influenced by a plethora of variables, including the preanalytic sample handling, antibodies, and protocols used for cell staining, and not finally the flow cytometer and applied settings. Not surprisingly, this heterogeneity hampers the comparability between the studies and, overall, the generalizability of the results. Despite this, interventional trials using IFN-γ were initiated very soon to correct for the low HLA-DR phenotype. Polk and colleagues examined in their RCT the efficacy of IFN-γ treatment in patients after trauma (28). They were able to show an increase in mHLA-DR$^+$ monocytes, but no decrease in the incidence of major infections or death. Similarly, in a case series of nine patients with sepsis and <30% HLA-DR$^+$ monocytes, IFN-γ treatment rapidly expanded this cell population (29).

Ten years later, this approach was repeated in a second case series of comparable patients (sepsis and MFI <150), proving the ability of GM-CSF to reconstitute mHLA-DR (30). In 2005, a quantitative assay consisting of two antibodies (anti-CD14 and anti-HLA-DR; the latter conjugated in a fluorophore to antibody ratio of 1:1) and a separate bead-based calibration curve was developed, which enables the reproducible and platform-independent quantification of mHLA-DR as molecules per cell (24). Importantly, a comparable assay has been incorporated into the Accelix system used in our study, enabling technical comparability to other studies and machines (23). Döcke et al., did not solely develop the assay, but also reported two essential aspects: First, standard values of 100 healthy volunteers were reported, ranging from 13,200 to 42,500 for females (95% CI; median 26,200), and 15,300–40,100 for males (95% CI; median: 25,300). Those ranges were confirmed in a small cohort of 32 healthy donors, yielding a range of 13,255–20,890 (min–max; median: 16,884) (15). Similar to our study, they report a profoundly reduced expression of mHLA-DR in all subgroups of critically ill patients within 3 days of admission. This broad time frame to the first measurement was a result of limited laboratory availability, an archetypical barrier of implementation. In contrast, we assessed HLA-DR within 24 h after admission with no recruitment gaps. Astonishingly, if we would apply the

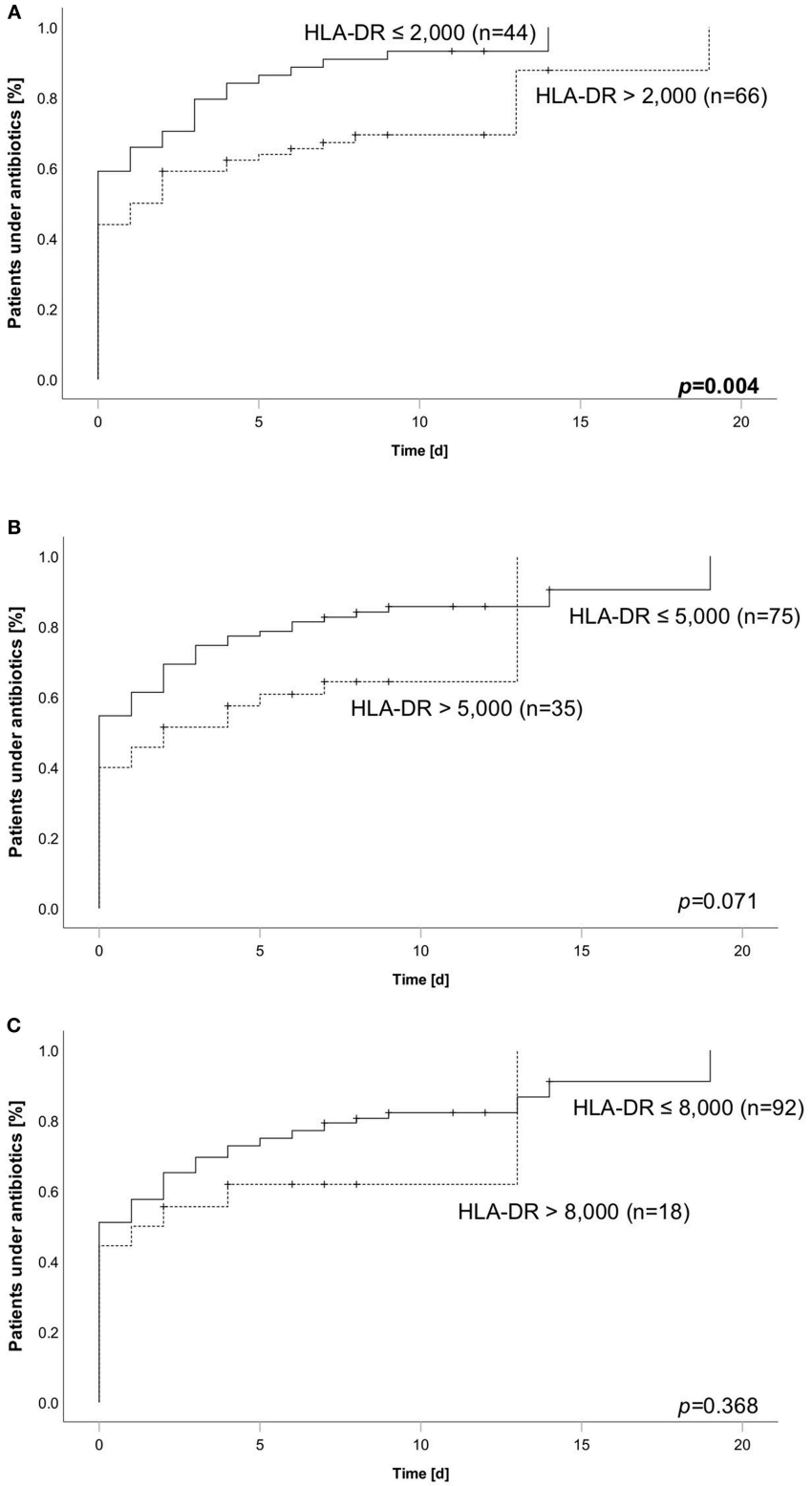

FIGURE 4 | Cumulative incidence of antibiotic therapy over 28 days stratified according to HLA-DR thresholds of **(A)** 2,000, **(B)** 5,000, or **(C)** 8,000. Dashed line always indicates subgroup above corresponding threshold, whereas solid line indicates the group of patients below. Number in brackets equal subgroup size, a total of 110 patients has been analyzed. Group comparison was performed using logrank test and calculated *p*-values are given within the subpanels. Bold type indicates a $p \leq 0.05$, assumed as significant.

standard values given above, only 9 of our 110 patients would possess "normal" mHLA-DR levels. Importantly, these numbers also clearly indicate that just being below standard range might not necessarily implicate an elevated risk for the individual patient. In the study of Lukaszewicz et al., a lack of HLA-DR reconstitution over time, assessed by several measurements, was predictive for secondary infection, but no association to mortality was observable. These core findings closely match our observations, despite the substantial differences in the study design. In line, Trimmel et al., were also not able to extrapolate outcome information from mHLA-DR assessment (31).

The second important achievement of Döcke et al., is the transfer of the old thresholds for "HLA-DR$^+$ monocytes" (e.g., <30%) into their newly developed quantitative assay, thereby establishing a threshold of 5,000 molecules/cell indicative for an immunoparalysis. The implicated clinical risk for infection has been proven in a cohort of patients undergoing cardiac surgery with cardiopulmonary bypass (22). Overall, the informational content of mHLA-DR largely depends on the examined cohort: in contrast to total ICU patients, Wu et al., can delineate surviving from non-surviving patients with sepsis by comparing changes of HLA-DR$^+$ monocytes over time (32). The rationale for this might lie in the bold difference of mortality between patients with sepsis and general ICU patients and the implicated effect size. In our cohort, we observed a 28-day mortality of 10%, in line with other reports (15). Compared to studies reporting overall ICU mortality (33), this value seems low and might be a consequence of delayed death beyond our observation time. Despite not significantly different, one could propose that with a larger sample size in our study, the threshold of 2,000 would have revealed slight mortality differences between the groups. However, when using a cellular biomarker of immune function for stratification of general ICU patients, the key question remains whether mortality is the relevant endpoint to look for or if endpoints of closer causality like, e.g., secondary infection, might be of higher interest.

In 2009, a small hallmark RCT on GM-CSF therapy for patients with sepsis used mHLA-DR values below 8,000 molecules/cell (for 2 subsequent days) as inclusion criteria and found GM-CSF to be able to reconstitute immune function as well as to decrease duration of mechanical ventilation (25). Despite a considerable heterogeneity of patients in our cohort containing only 23 patients with sepsis, we can readily observe a shorter time of mechanical ventilation in patients with mHLA-DR above thresholds of 2,000 as well as 5,000. Interestingly, a threshold of 8,000 in our cohort was not applicable for stratification. A technical and systematic bias can explain this: a value of 5,000 measured by Accelix is comparable to 8,000 molecules/cell on a conventional cytometer (23). The comparator of conventional cytometry was performed using the assay of Döcke et al., commercially available from Becton Dickinson under the brand name QuantiBrite. Considering the technical bias, it might be time to promote this assay from its informal status to "the" gold standard. This will enable to harmonize readouts of emerging systems like the Accelix in the future. Above this issue to be solved, one question remains: Which is the threshold of mHLA-DR to consider for patient stratification?

This dilemma can be symbolically pictured by two consecutive studies, which examined the usefulness of regulatory T cells, CD88 expression on neutrophils and mHLA-DR alone or in combination to predict infections in critically ill patients (26, 34). Both studies incorporated ROC analysis to find the optimal cut-off for prediction and while one of the studies revealed an optimal mHLA-DR cut-off of 10,000 molecules/cell, the concomitant INFECT study found a value of 2,009 to work best. Again, technical changes have been proposed as the underlying reason for this discrepancy. Our intention was not to identify novel thresholds, but we primarily made use of the previously reported ones [2,000 from (26), 5,000 from (15), and 8,000 from (25)] and applied it on Accelix-based measurements. We can clearly show that thresholds of 2,000 and 5,000 can identify patients of complicated clinical courses, most likely caused by nosocomial infections. However, if applying *de novo* AUROC analysis to our cohort, a cut-off of 4,266 works best to predict future antibiotic use in our patients. This approach further underlines the feasibility of the present cut-offs. As a limitation, our study did not approach the item "infection" directly as other studies did before. The reason for this is the challenge to group highly complex patients into this binary basket. Instead, we used "antibiotic therapy" as a surrogate. We anticipated that antibiotics would only be delivered to patients with clinically relevant infections (and stopped when feasible), as judged by the treating intensivists in daily practice. Nevertheless, we think our approach might even be more conservative, as our analysis for the incidence of antibiotic therapy does only consider the first initiated treatment and might mask further episode initiated later on. We can also clearly observe that subgroups of patients below threshold possess a higher severity of illness (e.g., SOFA score). HLA-DR might be proposed to serve as a pathophysiological bridge between critical illness *per se* and predisposition for infection.

In line with others, we propose low HLA-DR to be a good predictor for infection and complicated courses, but we should not anticipate that (secondary) infection necessarily projects into mortality when considering a general ICU population with high heterogeneity. Our results prove that immunosuppression is apparently a common feature of ICU patients and not an exclusive condition of sepsis or trauma patients. This concept is substantiated by a large-scale cohort study, which assessed the incidence of secondary infections between patients admitted either for sepsis or other reasons and found no differences (35). However, patients with sepsis and secondary infections exhibit tremendously higher mortality compared to non-sepsis patients, indicating the urgent need for risk mitigation.

Results of recent studies also indicate a potential value of HLA-DR for diagnosing sepsis in difficult-to-diagnose cohorts of patients in the emergency department or presenting with SIRS (36, 37). Importantly, the study by Parlato and colleagues did measure HLA-DR expression by quantitative PCR of whole blood samples, not by flow cytometry. This alternative approach has been reported before to be highly comparable to flow cytometry and if used on a digital PCR platform, to exhibit superior diagnostic performance to conventional biomarkers {Winkler:2017kl}{Almansa:2019ke}.

However, if this approach is easier for real-life adoption and how it can be operationalized in terms of thresholds, especially in the context of immunosuppression, needs to be further elucidated.

Furthermore, the results of a recently completed confirmatory French RCT for GM-CSF treatment of sepsis patients with low mHLA-DR will finally evaluate the value of HLA-DR as a theranostic marker for immunotherapy (ClinicalTrials.gov identifier NCT02361528). Importantly, the primary endpoint of this trial is the incidence of secondary infection and, therefore, in case of a positive result, might be transferable to non-sepsis patients. To this end, the measurement of a biomarker makes only sense if it delivers actionable results to the clinician. However, when it comes to immunotherapy, many open gaps in knowledge remain to be filled. Which drug works best in which patient cohorts? For how long does a patient need to be immunosuppressed to involve a risk? And must it be an expensive and risk-associated intervention like pharmacological immunomodulation or might organizational and extended hygiene measures (e.g., strict access barriers) might already provide a patients' benefit?

In conclusion, assessment of mHLA-DR using the Accelix system, a novel point-of-care flow cytometer, is easy and reveals a broad incidence of immunosuppression in ICU patients. Furthermore, it is able to stratify patients according to their risk of a complicated course, including infection. Bedside systems can take away the work burden from central laboratories and by enabling rapid measurements on ward to circumvent HLA-DR-specific preanalytic problems that arise e.g., with extended storage of the blood. This class of devices is setting the future stage for stratification of patients into risk and therapy groups, enabling healthcare professionals to close the theranostic circle of immunotherapy.

DATA AVAILABILITY

Data is available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

ST designed the study and study protocol, obtained ethics approval, performed patient recruitment and informed consent, data collection, data analysis and discussion, and wrote the manuscript. JK performed measurements and data collection, and wrote the manuscript. TB performed patient recruitment and informed consent, and results discussion. MW designed the study and study protocol, obtained ethics approval, recruitment and informed consent, results, and discussion. FU designed the study and study protocol, obtained ethics approval, established laboratory methodology, data collection, analyzed the data, and wrote the manuscript. All authors read the manuscript draft and agreed upon its submission.

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REVIEW
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The Long Pentraxin PTX3 as a Humoral Innate Immunity Functional Player and Biomarker of Infections and Sepsis

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The first line of defense in innate immunity is provided by cellular and humoral mediators. Pentraxins are a superfamily of phylogenetically conserved humoral mediators of innate immunity. PTX3, the first long pentraxin identified, is a soluble pattern recognition molecule rapidly produced by several cell types in response to primary pro-inflammatory signals and microbial recognition. PTX3 acts as an important mediator of innate immunity against pathogens of fungal, bacterial and viral origin, and as a regulator of inflammation, by modulating complement activation and cell extravasation, and facilitating pathogen recognition by myeloid cells. In sepsis, PTX3 plasma levels are associated with severity of the condition, patient survival, and response to therapy. In combination with other established biomarkers, PTX3 could improve stratification of sepsis patients and thus, complement the system of classification and monitoring of this disease.

Keywords: pentraxin, pentraxin 3 (PTX3), inflammation, innate immunity, sepsis

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INTRODUCTION

The first line of defense against invading pathogens is provided by the innate immune system that comprises both cellular and humoral arms. In contrast with adaptive immunity, the innate immune receptors are germ-line encoded receptors called pattern recognition molecules (PRMs), which recognize conserved structure on the surface of pathogens, the so-called pathogen-associated molecular patterns (PAMPs). Based on their localization, PRMs have been divided into cell-associated receptors and soluble molecules. Cell-associated PRMs are located in different cellular compartments and include endocytic receptors (e.g., scavenger receptors), signaling receptors such as Toll-like receptors (TLRs), the NOD-like receptors and RIG-like receptors (1). Fluid-phase molecules contribute to the humoral arm of innate immunity, and function as the ancestor of antibodies. They are diverse in terms of structure, expression, and specificity, but share basic functions, including regulation of complement activation, opsonisation of pathogens and apoptotic cells and regulation of inflammation (1). Fluid-phase PRMs, which include complement components, mannose-binding lectin (MBL), surfactant protein, ficolins, and pentraxins (PTXs), are expressed by different cell types including myeloid, epithelial and endothelial cells (1).

Pentraxins are a superfamily of phylogenetically conserved proteins, sharing an ∼200 amino acid long domain characterized by the presence of the so-called "pentraxin signature" in their carboxy-terminal, which is a conserved 8 amino acid long-sequence

(His-x- Cys-x- Ser/Thr-Trp-x- Ser, where "x" represents any amino acid). Based on the length of the N-terminal region, these multifunctional proteins are divided into short and long pentraxins. Short pentraxins include C-reactive protein (CRP) and serum amyloid P (SAP), whereas PTX3, PTX4, neuronal pentraxin 1 (NP1) and NP2 belong to the long family (2).

The short pentraxins CRP and SAP are ∼25-kDa proteins produced by hepatocytes in response to pro-inflammatory signals, such as IL-6, and constitute the main acute phase proteins in human and mouse, respectively. They have a peculiar quaternary structure with five (CRP) or ten (SAP) identical subunits arranged in pentameric symmetry with 51% amino acid sequence identity. In human, plasma level of CRP is low in healthy adults (below 3 mg/L), but it can increase as much as 1,000 folds in 48 h during inflammation. In contrast, SAP plasma level is constitutively 30–50 mg/L even during the acute phase response in human (1).

CRP is the first identified PRM and its name derives from its capacity to recognize C-polysaccharide of *Streptococcus pneumoniae*. The CRP gene is located on chromosome 1q23.2 locus in human. The human SAP gene is located on chromosome 1 and shares with CRP the exon/intron structure (2).

CRP and SAP share functional properties including recognition of pathogens, regulation of complement and interaction with Fcγ receptors (FcγR), resulting in enhancement of pathogen phagocytosis (1).

PTX3 (also named TSG-14) was identified during the early 1990s as a secreted protein containing a C-terminal pentraxin domain (2). The human PTX3 gene is located on chromosome 3q25 and is organized in three exons coding for the leader signal peptide, the long N-terminal domain (amino acids 18–178) and the C-terminal pentraxin domain (amino acids 179–381) (1). In contrast to the C-terminal, the N-terminal region of PTX3 is unrelated to any known protein domain (1). Human and mouse PTX3 display 92% amino-acid conservation and 82% of these amino-acid residues are identical, suggesting a strong evolutionary pressure to maintain both its structure and function.

PTX3 has a complex quaternary structure characterized by two tetramers linked together by covalent bonds to form an octamer of 340 kDa that is the main form of the molecule and shows greater functional activity, comparing with the tetrameric form (3).

Human and murine PTX3 gene promoters are characterized by numerous potential binding sites for inflammatory transcription factors, including PU.1, AP-1, NF-κB, SP1, and NF-IL-6 sites (2).

While CRP and SAP are mainly produced by the liver upon stimulation by IL-6, PTX3 is secreted by different cell types, including dendritic cells (DCs), monocytes, macrophages, fibroblasts, synovial cells, chondrocytes, adipocytes, epithelial cells, vascular endothelial cells, smooth muscle cells, mesangial cells, granulosa cells, and glial cells in response to TLR agonists, inflammatory cytokines (such as IL- 1β and TNFα), microbial moieties [LPS or outer membrane protein A (OmpA)], and microorganisms (**Figure 1**) (1, 4). Finally, neither T and B lymphocytes or NK cells express PTX3 mRNA (2), while PTX3 expression in mature neutrophils is still debated. Jaillon et al. reported that immature neutrophil precursors express PTX3 transcripts and synthesize the protein during differentiation in the bone marrow, while mature peripheral blood neutrophils were not able to express PTX3 mRNA. PTX3 is stored as a reservoir in lactoferrin- positive granules in a ready to use form and is promptly released after microbial recognition and inflammatory signals (5). In contrast with the study by Jaillon et al. (5) and Imamura et al. (6) reported the expression of PTX3 mRNA in freshly isolated mature neutrophils before and after stimulation with LPS (5, 6). In addition, it has been recently reported that splenic B helper neutrophil cells (NBh cells) can express PTX3 transcript in response to GM-CSF and LPS (7). PTX3 produced by NBh cells bound marginal zone B cells and promote IgM to IgG class switch recombination, thus representing a bridge between the humoral arms of the innate and the adaptive immune system.

Based on its quaternary structure, PTX3 interacts with various ligands, exerting different biological activities. In the context of innate immune responses, PTX3 binds different complement components and modulates complement activity (2). In addition, PTX3 binds P-selectin and tunes recruitment of neutrophils limiting inflammation (8). PTX3 also binds to a wide range of microorganisms, including fungi, bacteria, and viruses and is involved in resistance to selected infectious diseases (2). In addition, by interacting with a series of ligands and tuning inflammatory responses, PTX3 has various roles in different settings, such as wound healing and tissue remodeling, cardiovascular diseases, fertility, and cancer (1).

In line with the behavior of CRP as inflammatory biomarker, PTX3 has been developed as a novel marker for infectious or inflammatory disease severity.

Here, we will present the experimental evidence showing the functional roles of PTX3 and its potential as biomarker focusing on infections and sepsis.

ROLE OF PTX3 IN INNATE RESISTANCE TO INFECTIONS

Since its discovery, PTX3 has been described to modulate innate immune mechanisms involved in protection against infectious diseases (**Figure 1**). Several studies using *Ptx3*-deficient mice showed an increased susceptibility to fungal, bacterial and viral pathogens such has *Aspergillus fumigatus*, *Pseudomonas aeruginosa*, *Shigella flexneri*, uropathogenic *Escherichia coli,* Influenza virus and murine cytomegalovirus (9–13). In contrast, a recent study showed a deleterious effect of PTX3 during Ross River virus (RRV) infection in mice (14). In the context of RRV infections, it has been shown that PTX3 increases viral replication and entry in target cells (14). Furthermore, the transgenic overexpression of PTX3 in mice is responsible of resistance against *Klebsiella pneumoniae* respiratory infection and increased phagocytosis of *Paracoccidioides brasiliensis* by macrophages (15, 16). The protective effect of PTX3 has been investigated treating infected animals with the recombinant protein. Indeed, PTX3 treatment alone or in combination with antifungal compounds, showed a protective effect in *A. fumigatus* infections,

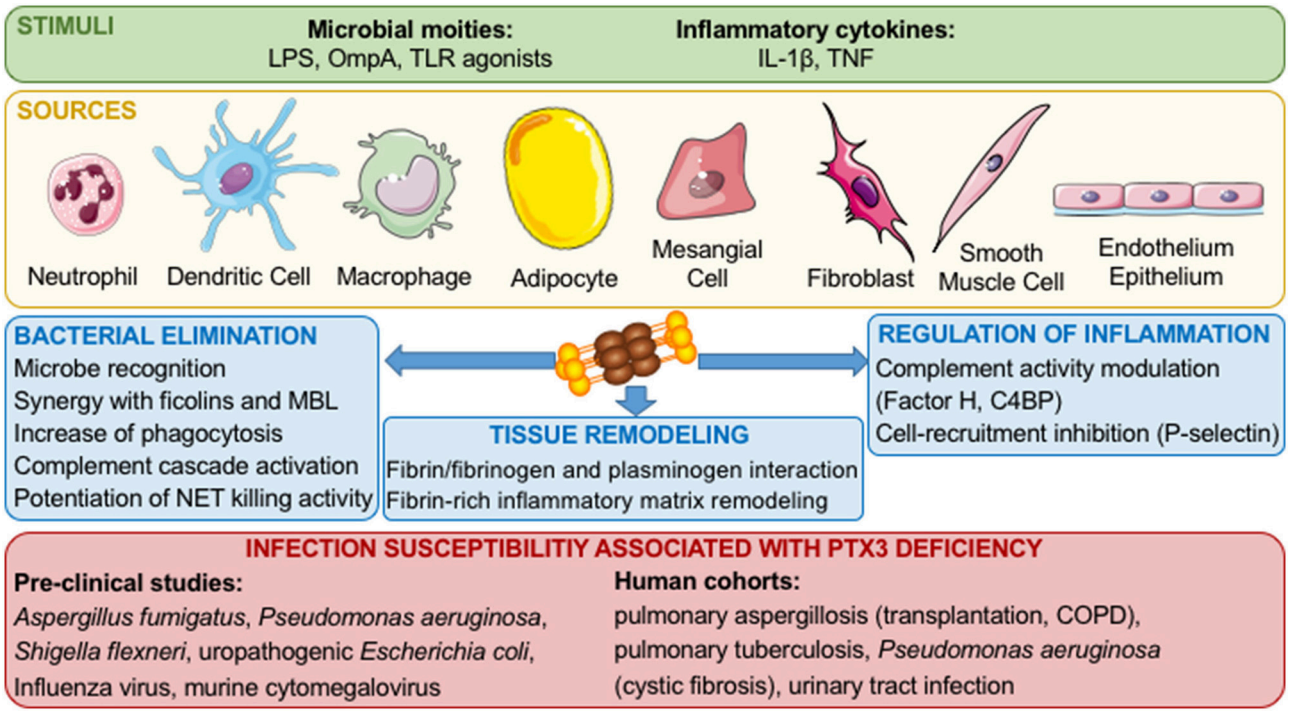

FIGURE 1 | Role of PTX3 in innate immune responses and inflammation. Inflammatory stimuli and cellular sources of PTX3 are shown. Once released, PTX3 interacts with microorganisms, complement components, phagocytosis receptors, P-selectin, and components of the haemostatic system and fibrinolytic cascade, thus promoting pathogen clearance, tuning inflammatory responses and promoting tissue remodeling.

(17–20). In addition PTX3 administration is protective also against infections with Influenza virus, murine cytomegalovirus, *Neisseria meningitidis*, and *P. aeruginosa* in neonates and during chronic infections (10, 11, 21–24). Interestingly, in addition to being effective against opportunistic bacteria known to be responsible of sepsis (e.g., *K. pneumoniae* and *P. aeruginosa*), PTX3 was shown to have protective effect by reducing the mortality in mouse models of sepsis induced by histone infusion, LPS-induced endotoxemia and cecal ligation and puncture, through its N-terminal domain (25, 26).

Antimicrobial Mechanisms

Similar to short pentraxins, PTX3 binds a number of selected bacteria, fungi and viruses (2). The capacity of PTX3 to bind microorganisms has been described first with *A. fumigatus* conidia: in this context PTX3 was shown to increase the internalization and killing of conidia by alveolar macrophages (5, 9, 27). This opsonic activity depends on the complement system, since PTX3 enhances neutrophil phagocytic activity in the presence of normal human serum, but this effect is abolished with heat-inactivated serum. Interestingly, the same study demonstrated that the opsonic activity of PTX3 is Ig-independent, since PTX3 enhanced phagocytosis in the presence of Ig-depleted serum (27). In addition, by using complement-depleted sera (Factor B and C3) and reconstituted with purified complement components (C3, Factor B, Factor H, Factor D and Factor I), Moalli et al. showed the main involvement of the alternative complement pathway in the pro-phagocytic activity exerted by PTX3. Finally, with integrin and FcγR blocking antibodies, they demonstrated that FcγRIIa (CD32) and complement receptor 3 (CD11b/CD18) are required for the interaction with PTX3-opsonized *A. fumigatus* conidia, are recruited into the phagocytic cup and cooperate in the phagocytosis process amplified by PTX3 (27).

This opsonic capacity has been extended to others pathogens such as uropathogenic *E. coli* and *P. aeruginosa* (12, 21). PTX3 is also able to bind Influenza virus, murine cytomegalovirus, *P. brasiliensis*, encapsulated *N. meningitidis*. An interaction was described with outer membrane vesicles or selected antigens from *N. meningitidis* or with OmpA of *K. pneumoniae* (10, 11, 15, 24, 28, 29).

The formation of neutrophil extracellular traps (NETs) by neutrophils releasing DNA, histone and bactericidal compounds is an important pathway to control infections. Jaillon et al. observed the localization of PTX3 in human neutrophil granules and the secretion after bacterial and PRR stimulation. In this study they also showed PTX3 localized in NETs formed after neutrophil activation (5). Proteomics analysis revealed that PTX3 forms complexes with two anti-microbial proteins [azurocidin (AZU1) and myeloperoxidase (MPO)] associated to NETs (30). More recently, PTX3 localization in NETs has been confirmed, and the colocalization with AZU1 and MPO has been defined more accurately (31). Further investigation will be needed to understand the involvement of PTX3

interaction with AZU1 and MPO in their antibacterial role during NET formation.

Regulation of Complement Activation

PTX3 interaction with microorganisms is not restricted to directly increase phagocytosis. PTX3 can be used as a sensor by complement to rapidly recognize microbial patterns and enhance its activation and its antimicrobial activity. Once bound, PTX3 recruits the complement component C1q and activate the classical pathway of the complement cascade, leading to C3 deposition on apoptotic cells (32). In contrast, the interaction between PTX3 and C1q in the fluid phase prevents complement deposition onto apoptotic cells and their clearance by dendritic cells (33). PTX3 also interacts and activates the lectin pathway of complement. Indeed, Ma et al. demonstrated that PTX3 bound on *A. fumigatus* conidia interacts with ficolin-2 and leads to an increase of complement deposition on *A. fumigatus* (34). Later on, this group showed that the interaction of PTX3 with ficolin-1 on the cell surface of apoptotic cells leads to an increase of their phagocytosis by human monocyte–derived macrophages, but this does not occur for *A. fumigatus* conidia (35). Ma and co-workers also described the interaction of PTX3 bound to *Candida albicans* with mannose-binding lectin (MBL), and the consequent enhancement of C3 and C4 deposition on the yeast and its phagocytosis by neutrophils (36).

In addition to the ability to enhance complement activation, PTX3 has been described to interact with complement regulators. Deban et al. described the interaction of PTX3 with factor H (FH), the main regulator of the alternative complement pathway. The interaction between apoptotic cell surface bound-PTX3 with FH enhanced iC3b deposition on the cells, thus preventing the cleavage of C3b and its deposition on the apoptotic cells (37, 38). Moreover, another study described the direct interaction of PTX3 with C4-binding protein (C4BP), another regulator of complement activation. In this study, the authors showed that PTX3 recruits C4BP on late apoptotic cells determining C4b cleavage and reduction of the formation of the final complement lytic complex C5b-9 (39). Thus, these studies demonstrate that PTX3 can recruit the two complement regulators FH and C4BP to limit excessive complement activation.

Modulation of the Inflammatory Response

The development of the inflammatory response and the control of inflammation during infections need to be tightly regulated. PTX3 has been described to control cell recruitment at inflamed sites by binding P-selectin (8). In their study, Deban et al. observed that *Ptx3*-deficiency is associated with an increase of leukocyte rolling interactions and suggested that PTX3 binding to P-selectin would limit leukocyte rolling and thus inflammatory cell infiltration. The involvement of P-selectin has been confirmed in a model of post-ischemic renal injury. In this model, *Ptx3*-deficient mice exhibit a higher leukocyte recruitment compared to wild type mice that is inhibited by a P-selectin blocking antibody (40). More recently, in a model of chemical carcinogenesis, the lack of PTX3 has been associated with an exacerbated cancer-related inflammation explained by a defect of complement regulation, as indicated by excessive C3 deposition and a higher amount of the highly inflammatory compound C5a in *Ptx3*-deficient compared to —competent mice (41) In murine models, until now, only one study has associated the alteration of inflammatory control by PTX3 with the susceptibility to *K. pneumoniae* infections (16). In this study, PTX3 was shown to regulate the production of TNFα and nitric oxide, and depending on the intensity of the inflammatory response induced by a given inoculum, promote or inhibit neutrophil influx and the susceptibility to pulmonary infection with *K. pneumoniae*.

Orchestration of Tissue Repair

Recent i*n vitro* and *in vivo* studies demonstrated that PTX3 interacts with components of the haemostatic system and fibrinolytic cascade, in particular with fibrinogen/fibrin and plasminogen, at acidic conditions, which occur in damaged tissues. This interaction promoted remodeling of the fibrin-rich inflammatory matrix ensuring a normal tissue repair in different experiment models (42). The interaction between PTX3, fibrin and plasminogen is potentially relevant during specific infections or in septic conditions, but further studies are needed to address this aspect.

PTX3 IN HUMAN INFECTIONS

The homology with CRP, that was first discovered in the serum of patients with acute pneumococcal pneumonia and is upregulated in the context of inflammation and infections, prompted investigations on the involvement of PTX3 as biomarker of infections. In inflammatory or infectious diseases, PTX3 behaves as an acute phase response protein and the basal blood levels observed in normal condition (25 ng/ml in the mouse and <2 ng/ml in human) rapidly increase during the course of pathological conditions, reaching in humans 100–1,000 ng/ml, depending on the severity (43). Cardiovascular diseases, cancer and infections, all characterized by an inflammatory origin, are the most relevant pathologies showing upregulated plasma PTX3 levels (43–48). In general, PTX3 increase over basal levels could be already appreciated beginning from 6–8 h after insult, while CRP needs longer times and levels start to be significantly altered only after 24–30 h. This is likely the result of local production of PTX3 following detection of bacterial moieties or tissue damage by innate immune cells, combined with the production of the primary pro-inflammatory cytokines IL-1β and TNFα, main inducers of PTX3. By contrast CRP is systemically produced by the liver in response to IL-6, with a process that requires longer time.

Focusing on the context of infective diseases, PTX3 has been characterized as a biomarker of severity and outcomes in different infections caused by bacteria, fungi or viruses (**Table 1**). Patients with pulmonary aspergillosis, tuberculosis, dengue virus infection, meningococcal disease leptospirosis and shigellosis have increased PTX3 plasma levels that correlate with disease severity and could act as predictor of unfavorable outcomes (13, 49–51, 59, 61). PTX3 levels in plasma and bronchoalveolar lavage fluids (BALF) discriminate patients with invasive aspergillosis from those with other conditions, including

TABLE 1 | Clinical studies reporting the use of PTX3 as biomarker of infectious diseases or sepsis/shock.

	Pathology*	Main results	References
Bacterial infections	• *Mycobacterium tuberculosis* infection	Correlation with disease severity & hospital stay; predictor	(49)
	• Leptospirosis	of bloodstream infections, response to treatment &	(50)
	• Meningococcal disease	mortality; biomarker of bacteria-associated exacerbation in	(51)
	• Shigellosis	COPD; early indicator of shock.	(52)
	• Community acquired pneumonia		(53)
	• Pyelonephritis		(54)
	• Bacteremic patients		(55)
	• Pneumonia in intubated patients		(56)
	• Bacterial infections in COPD[a]		(12)
	• Febrile patients		(57)
	• Parapneumonic effusion		(58)
			(13)
Viral infections	• *Dengue* virus	Correlation with disease severity	(59)
	• Lower respiratory tract infections (mostly RSV[c], IV[d] and hRhV[e])		(60)
Fungal infections	• *Aspergillus fumigatus* (proven/probable)	Monitoring of fungal infection	(61)
			(62)
			(63)
SIRS, sepsis or septic shock		Correlation with organ dysfunction & disease severity;	(64)
		predictor of mortality; associated to amputation in NSTI[b]	(65)
		patients and with lower APGAR scores in preterm	(66)
		neonates.	(67)
			(68)
			(69)
			(70)
			(71)
			(72)
			(73)
			(74)
			(75)
Infections/sepsis in acute decompensated cirrhosis		Predictor of disease severity and case fatality	(76)
Cardiogenic shock		Predictor of mortality	(77)

[a] *COPD, Chronic Obstructive Pulmonary Disease.*
[b] *NSTI, Necrotizing Soft Tissue Infections.*
[c] *RSV, Respiratory Syncytial Virus.*
[d] *IV, Influenza Virus.*
[e] *hRhV, human rhinovirus.*
**Studies focused on microbial infections are reported in the bacterial, fungal or viral infection groups. Studies on sepsis/shock irrespective to the causing agent(s) are reported in the SIRS, sepsis or septic shock group.*

lung cancer, community-acquired pneumonia (CAP), pulmonary cryptococcosis and *Aspergillus fumigatus* colonization (62, 63). Circulating levels of PTX3 were associated with SOFA (sequential organ failure assessment) score and case fatality in patients with bacteremia caused by *Staphylococcus aureus, Streptococcus pneumoniae,* β-hemolytic streptococcae or *Escherichia coli* (53).

PTX3 was shown to predict bloodstream infection and severe disease in febrile patients admitted to emergency departments (52), indicated acute respiratory distress syndrome (ARDS) in critically ill patients (78), and correlated with pneumonia severity and length of hospital stay in adults with CAP (54). Serum and urinary PTX3 levels are increased in pyelonephritis patients and correlated with parameters of disease severity (12). In patients with chronic obstructive pulmonary disease (COPD), levels of PTX3 in sputum samples are associated with bacterial infections and can potentially be a marker of exacerbation of the disease (58). On the opposite, in children with lower respiratory tract infections, mostly of viral origin, PTX3 levels were increased irrespective of the causative agent, but were indeed correlating with the febrile peak and reflected disease severity (60).

The diagnostic accuracy of PTX3 as local marker of infection has been strengthened by the observation that its levels can identify microbiologically confirmed pneumonia in BALF and plasma of mechanically ventilated patients (55, 57). The accuracy of PTX3 was compared to other biomarkers used to follow critically ill patients, namely procalcitonin (PCT) (79, 80) and soluble triggering receptor expressed on myeloid cells 1 (sTREM-1). PCT, released by thyroid C cells in healthy subjects, is induced in multiple tissues by LPS and proinflammatory cytokines during infections. Circulating levels of PCT are a reliable biomarker of bacterial infection and provide a guide to follow antibiotic therapy in critical ill patients with suspected infections (81). sTREM-1 is expressed by myeloid cells in BALF and is useful to make diagnosis of pneumonia infection in intubated patients (82). Area under the curve-receiver operating characteristics (AUC-ROC) analysis indicated that the diagnostic accuracy of PTX3 in BALF was better than the other biomarkers currently

used, namely PCT, CRP, and sTREM-1. More in general, PTX3 levels in pleural fluids can discriminate parapneumonic exudative effusion of infectious origin from malignant or other cause pleural effusion (56).

Monitoring the level of PTX3 has not only been described to be a good biomarker for detecting infections, but also to follow the response to therapy. Indeed, in a population of 220 newly diagnosed patients with *Mycobacterium tuberculosis* infections, PTX3 plasma levels were significantly decreased in individuals who responded to therapy compared to patients with treatment failure (49). After antibiotic treatment PTX3 levels were reduced in plasma of patients with CAP (54), or in urine of pyelonephritis patients (12).

Impact of Genetic Polymorphisms

In human, PTX3 is not only interesting for its biomarker features. Indeed, its deficiency, as described in the preclinical studies above, has been associated with an increased susceptibility to infections. In a first cohort of patients undergoing hematopoietic stem-cell transplantation, three single-nucleotide polymorphisms (SNPs) within the *PTX3* gene were identified that were consistently associated with a defect in PTX3 expression in BALF, lung-biopsy specimens, and innate immune cells (83, 84). The defective expression of PTX3 is mainly observed in neutrophils and was proposed to be due to messenger RNA instability, but the exact mechanism is presently unknown. For these patients, genetic variants of the *PTX3* gene of transplant donors were associated with the susceptibility to invasive aspergillosis (IA). Several studies confirmed the association of these genetic polymorphisms to pulmonary aspergillosis and other fungal infections in hematopoietic stem cell transplantation and solid organ transplantation, in particular lung, (83–87). One study has also shown a significant association between PTX3 SNPs and the susceptibility to pulmonary aspergillosis in patients with Chronic Obstructive Pulmonary Disease (COPD) (88). Clinical studies also support the relevance of the preclinical models. Indeed, PTX3 SNPs have been associated with increased susceptibility to pulmonary tuberculosis in West Africa patients, *P. aeruginosa* infections in cystic fibrosis Caucasian patient and urinary tract infection in Swedish patients (12, 89, 90).

The higher susceptibility to IA associated with a particular haplotype of PTX3 that is likely related to lower production of the protein, further supports the non-redundant role of PTX3 in host defense against *A. fumigatus* and opens up the prospective for a therapeutic use of this long pentraxin in infections.

PTX3 AS BIOMARKER IN SEPSIS

A deregulated host response to infections results in a systemic inflammatory response that activates a cascade of events known as systemic inflammatory response syndrome (SIRS), eventually leading to sepsis. The induction of PTX3 by primary pro-inflammatory cytokines and microbial components prompted to analyse the circulating levels of this protein in sepsis and its complications. In a small cohort of critically ill patients, Mueller and co-workers observed increased PTX3 plasma levels compared to healthy control donors, with a gradient from SIRS to sepsis and septic shock, and a significant correlation with disease severity as assessed by clinical scores (64). Most important, PTX3 levels on admission or day 2 were significantly associated to mortality.

Since this first observation, several papers analyzing PTX3 plasma levels in patients with sepsis and its complications were published (see **Table 1** for a summary). In a group of critically ill patients, persistently high PTX3 levels from the first days after diagnosis were significantly associated with poor outcome. Levels of PTX3 were correlated with disease severity, organ dysfunction and markers of coagulation activation and, when compared with other biomarkers (i.e., IL-6, TNFα and CRP) showed a stronger correlation with clinical parameters (65). Bastrup-Birk et al. analyzed PTX3 levels in patients admitted to ICU for SIRS and showed a significant correlation with Simplified Acute Physiology Score 2 (SAPS2). In this group of patients, Cox regression analysis revealed a significant association between PTX3 levels and 90-day mortality, in contrast with CRP levels (66). In a group of 112 patients admitted to the ICU with septic shock, baseline PTX3 levels were the only independent risk factor for 28-day mortality, in contrast with CRP and PCT (72). Similarly, in a group of more than 500 patients admitted to emergency room with suspected infection, PTX3 levels predicted severe sepsis and mortality while CRP was not associated to case fatality (67). In patients with necrotizing soft tissue infections, PTX3 plasma levels were associated with amputation and were higher in individuals with sepsis (69). PTX3 was compared with PCT and CRP in a small group of patients undergoing early goal-directed therapy and initial resuscitation (73). In this cohort, PTX3 was the only biomarker significantly different between survivors and non-survivors in all the time points considered (day 0, 3, and 7). Despite a correlation with PCT or CRP, plasma PTX3 measured at day 0 was the only independent marker of 28-day all-cause mortality. PTX3 levels were consistently higher in patients with sepsis or septic shock and were predictor of mortality also when evaluated according to the latest Sepsis-3 definition (70, 74, 91). In addition, PTX3 correlated with disease severity and degree of organ dysfunction. Finally, a study in a small group of preterm infants showed a correlation between PTX3 levels in newborns and overall worsen neonatal outcome (i.e., lower APGAR score, elevated respiratory distress syndrome rate, clinical sepsis, and prolonged NICU stay) (68).

Severe systemic inflammation, organ failure and septic shock are major life-threatening complications in different pathological conditions. A significant percentage of cirrhotic patients develops bacterial infections that can trigger systemic inflammation, organ failure and septic shock. Circulating levels of PTX3 are increased in these patients, compared to well-compensated cirrhotic patients, and predict disease severity and risk of mortality (76). Similarly, plasma PTX3 levels are increased in patients with cardiogenic shock and predict 3-month mortality, but levels over the time course are not associated to the presence of infections (77).

A major limitation of the studies mentioned above is the heterogeneity and limited numbers of patients enrolled. To overcome this point, PTX3 was recently measured in a large group of patients enrolled in a biomarker substudy of the

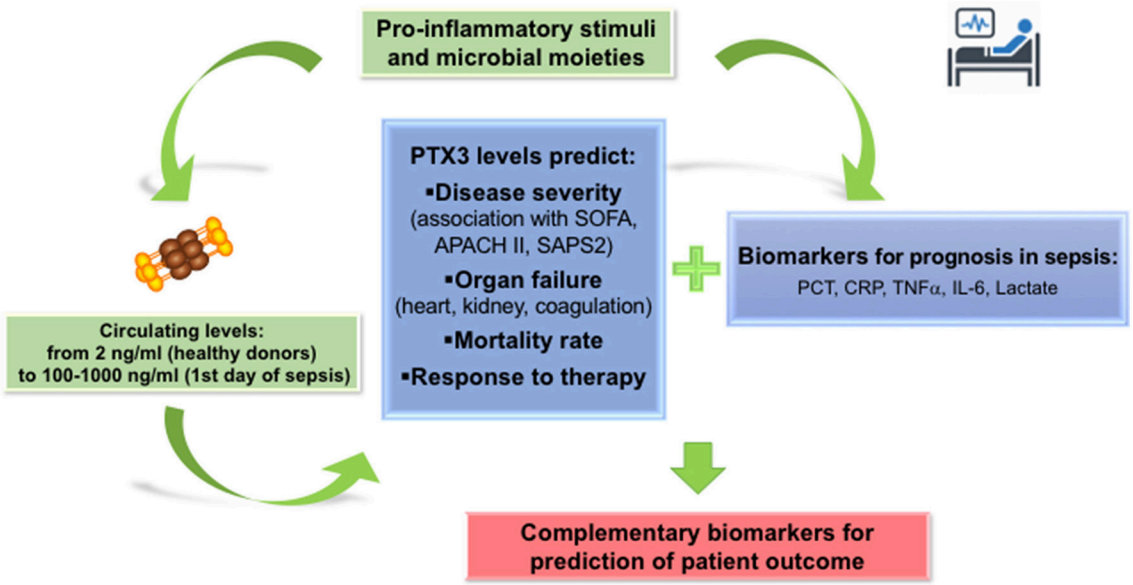

FIGURE 2 | PTX3 in the prognosis of sepsis. PTX3 can be considered as a reliable biomarker for prognosis of sepsis patients, predicting severity of disease, incidence of new organ failures and mortality risk. Addition of PTX3 levels measured on the first day in ICU or ER to the panel of markers routinely used in Intensive care units and emergency room units, may increase the accuracy of prognosis in sepsis patients.

Albumin Italian Outcome Sepsis (ALBIOS) trial, a multicentre open-label randomized controlled trial that enrolled patients with severe sepsis or septic shock [NCT00707122] (92). Within the total population of 1,818 patients enrolled, PTX3 levels were measured in 958 patients at day 1, 2, and 7 after ICU admission. Results obtained in this large cohort demonstrated that PTX3 levels on day 1 were higher compared to levels in healthy population and were correlated with severity. In addition, PTX3 levels on day 1 predicted development of novel organ dysfunctions, including cardiovascular and renal dysfunction (71). In each time point analyzed, PTX3 levels were consistently higher in patients who died than in survivors, and levels on day 7 showed a significant predicting value of 90-day mortality. Similarly, slower decreases in PTX3 levels from day 1 to day 2 were independently associated with higher mortality (71). In addition, this study showed that PTX3 levels at day 1 were inversely associated with platelets count and predicted coagulation dysfunctions. These results are in line with results obtained in preclinical studies, showing a link between PTX3 and haemostasis and fibrinolysis (42), and suggest that PTX3 could be a useful marker to stratify patients with coagulation dysfunctions. In conclusion, this study confirmed in a large and controlled population that PTX3 levels are highly increased in severe sepsis and even more in septic shock, and that impaired normalization or reduction of PTX3 levels in the first days predicts multiorgan dysfunction and risk of mortality.

The latter study, and many others, showed that PTX3 concentration dropped immediately following effective treatment during ICU stay, suggesting that monitoring PTX3 levels could be useful to follow responsiveness to therapy (67, 70–72).

While there is a general consensus on the increase of PTX3 plasma levels in septic patients, and the correlation with severity from SIRS to sepsis and septic shock, the diagnostic superiority of PTX3 over other biomarkers, such as PCT, IL-6, CRP and lactate, is still under debate. A possible strategy to increase the diagnostic accuracy is to analyse simultaneously a combination of different biomarkers (**Figure 2**). In a prospective analysis, the changes in plasma levels of PTX3, PCT and lactate during the first week in ICU stay were compared to severity scores of sepsis (SOFA and APACHE II). All the biomarkers correlated with SOFA and APACHE II and were associated with 28-day mortality by univariate and multivariate Cox regression analysis (75). A model combining these three biomarkers improved significantly mortality prediction of patients with sepsis (75). Similarly, PTX3 in combination with IL-6 improved the risk stratification of patients with sepsis or septic shock as classified using the updated sepsis-3 definition (70).

Limitation

Physiological concentration of plasma PTX3 are influenced by several factors, including sex, age, pregnancy, triglyceride levels and body mass index (93, 94), as well as by PTX3 allelic variants (83, 95), which induce variation of PTX3 levels in the range of about 1 ng/ml. In contrast, during severe infections and sepsis PTX3 levels pass from 1–2 ng/ml to 100–600 ng/ml, suggesting that variations due to these factors are not a major limitation for the use of PTX3 as a biomarker in sepsis. However, several pathological conditions, such as cardiovascular diseases and kidney diseases (96–98) induce increased PTX3 circulating levels and must be taken into consideration as potential confounding factors in

the stratification of septic patients with these underlying conditions. Along the same line, pregnancy disorders, such as intrauterine growth restriction (94), lead to increased PTX3 levels in neonates and may be confounding factors in neonatal sepsis (68).

CONCLUDING REMARKS

Sepsis and its complications are a major cause of death in ICU, with a mortality rate still ranging from 10% in the systemic inflammatory response syndrome (SIRS), to 60% in septic shock, while the short term mortality remains around 20% (99–101). At the moment validated diagnostic tests are not available, thus diagnosis is still largely based on clinical evaluation of the patient (91). Identification of reliable circulating biomarkers that can predict severity and mortality of disease, improve risk stratification and help in defining optimal treatment, could be thus highly valuable in the clinical practice. So far various biomarkers have been investigated, but none of them has appropriate specificity or sensitivity to be routinely applied in the clinical practice (99, 102).

PTX3 is a soluble pattern recognition receptor rapidly produced in response to primary pro-inflammatory signals and microbial recognition. Data collected over the years demonstrated that circulating PTX3 levels increase rapidly in response to infections and play important regulatory roles on inflammation, regulating complement activation, cell extravasation and pathogen recognition by myeloid cells. In sepsis and septic shock, PTX3 discriminates from healthy controls, and non-survivors consistently show levels higher than survivors. In addition, PTX3 levels at early time points and/or a lower decrease in response to first therapies are an independent predictor of mortality. Several recent data demonstrate that PTX3, in combination with other established biomarkers, could be useful to improve stratification of patients with sepsis or septic shock. Thus, PTX3 could complement the system of classification of the disease, contributing to define a group of useful biomarkers and strengthening their use in the diagnosis and monitoring of sepsis and septic shock.

AUTHOR CONTRIBUTIONS

RéP, SD, FA, and RaP did the literature search and wrote the manuscript. AM, CG, and BB critically revised the manuscript and approved the final version.

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REVIEW
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Challenge to the Intestinal Mucosa During Sepsis

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Sepsis is a complex of life-threating organ dysfunction in critically ill patients, with a primary infectious cause or through secondary infection of damaged tissues. The systemic consequences of sepsis have been intensively examined and evidences of local alterations and repercussions in the intestinal mucosal compartment is gradually defining gut-associated changes during sepsis. In the present review, we focus on sepsis-induced dysfunction of the intestinal barrier, consisting of an increased permeability of the epithelial lining, which may facilitate bacterial translocation. We discuss disturbances in intestinal vascular tonus and perfusion and coagulopathies with respect to their proposed underlying molecular mechanisms. The consequences of enzymatic responses by pancreatic proteases, intestinal alkaline phosphatases, and several matrix metalloproteases are also described. We conclude our insight with a discussion on novel therapeutic interventions derived from crucial aspects of the gut mucosal dynamics during sepsis.

Keywords: sepsis, innate immunity, gut-barrier dysfunction, perfusion disturbances, enzymatic response, microbiome

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INTRODUCTION

The mucosa is a highly organized and compartmentalized structure, which lines our body cavities for example, the respiratory, urogenital and intestinal tracts. It provides an interface between the external environment and the host tissues (1), possessing various functions including absorption of water, nutrients and gases, secretion of molecules, clearance of waste, improvement of bio-mechanical features and maintenance of immunity. Therefore, it is not surprising that the combined surface area of the digestive and respiratory tracts by far exceeds the surface dimension of our largest organ, the skin (2). These functions also necessitate a unique immune system which is tightly regulated and this is termed as the mucosal immune system (MIS) (2). The MIS in the gut is capable of distinguishing between regular nutrient flux, self-antigens, a diverse milieu of commensal bacteria and invading pathogenic microbes (3–5). Lymphoid compartments, commonly known as the mucosa-associated lymphoid tissue (MALT), are integrated into the mucosa and perform immune-associated activities. Organized MALT has been found not only in the gut (GALT), but also in a number of other sites, like in the nasopharynx, salivary-gland and duct, larynx, bronchus and urogenital tissues (6). In the intestine, the structural organization of the coexisting symbiotic bacteria is unique, where the large intestine alone houses 10^{11}–10^{12} bacteria/gram feces, the highest concentration in the entire intestinal tract (2). However, an imbalance of the co-inhabitation of the intestinal microbiome with the host can potentially threaten well-being (1, 5, 7, 8).

The homoeostatic status quo is essentially supported by the maintenance of the gut barrier integrity. In principle, any infection or severe extra intestinal trauma can cause significant

alterations of the gut barrier homeostasis, which may result in a profound generation and secretion of intestinal proteolytic enzymes, alterations in mucus layer formation and composition (9, 10), increased epithelial cell permeability and damaged intestinal cells with subsequent inflammatory signaling (5, 9, 10). These distinct pathophysiological changes are frequently found in septic patients. Previously, sepsis was defined as a systemic inflammatory response (SIRS) with an underlying primary infectious cause (11). Declared as a "silent killer" in critical care units and with high global mortality rates, sepsis has been recently redefined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" (12, 13). In the clinical setting, diffused and hidden symptoms frequently make the diagnosis of sepsis difficult. To help define septic conditions, clinicians and clinical scientists can utilize the sequential (sepsis-related) organ-failure assessment (SOFA) Score, which allows more precise detection of sepsis-associated organ dysfunction compared to the SIRS-criteria (12–15). The alarming pace of sepsis with possible development of multiple organ dysfunction syndrome (MODS) frequently includes disseminated intravascular coagulopathy (DIC), making sepsis patients a colossal challenge for both clinicians and researchers.

Years of research have focused on the various intricacies, from the underlying pathology to clinical targets that could help treat sepsis patients. In the scope of our review, we consider the effects of sepsis on the intestinal mucosa regarding the main immunological mechanisms that yield a dysregulated intestinal mucosal system and the scope of associated promising therapeutic strategies.

Abbreviations: AMP, antimicrobial peptide; APC, antigen presenting cell; APACHE-II, Acute Physiology and Chronic Health Evaluation- II Score; Bcl-2, B-cell lymphoma gene-2; C-BF, cathelicidin-BF; C3, complement factor 3; C3a, activated complement factor 3; C5, complement factor 5; CD4, cluster of differentiation 4; CLP, cecal ligation and puncture; COX-2, cyclooxygenase-2; CRP, C-reactive protein; CSF, cerebrospinal fluid; DAMP, danger-associated molecular pattern; DAO, Diamine Oxidase; DC, dendritic cell; DIC, disseminated intravascular coagulopathy; DNA, deoxyribonucleic acid; EF, ejection fraction; FXIII, clotting factor XIII; γ-EV, dietary dipeptide gamma-l-glutamyl-l-valine; GALT, gut-associated lymphoid tissue; HMGB1, high-mobility-group-protein-B1; IAP, intestinal alkaline phosphatase; IBD, inflammatory bowel disease; ICAM, intercellular adhesion molecule; ICU, intensive care unit; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; I-FABP, intestinal fatty acid-binding protein; IFN, interferon; IgA, immunoglobulin A; IL, interleukin; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; i. v., intravenous; LPS, lipopolysaccharide; LGG, Lactobacillus GG; M-cells, microfold cells; MALT, mucosa-associated lymphoid tissue; MIS, mucosal immune system; MLCK, myosin-light chain kinase; mmHG, millimeter of mercury; MMP, matrix metalloprotease; MODS, multiple organ dysfunction syndrome; MOF, multi-organ failure; MUC, mucin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; NOD, nucleotide-binding oligomerization domain; PAI-1, plaminogen activator inhibitor-1; PAMP, pathogen-associated molecular pattern; PG, proteoglycan; PRR, pattern recognition receptor; rDNA, ribosomal deoxyribonucleic acid; RNA, ribonucleic acid; ROS, reactive oxygen species; SATI, short acting thrombin (factor II) and factor Xa (FXa) inhibitor; SIRS, systemic inflammatory response syndrome; SIRT2, NAD-dependent deacetylase sirtuin 2; SOFA, sequential organ-failure assessment; SP, surfactant protein; sTNF, soluble TNF; TAB, TAK1-binding protein; TCR, T-cell receptor; TED, transepithelial dendrites; TF, tissue factor; Th, T helper lymphocyte; TJ, tight junction; TLR, Toll-like-receptor; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor; VCAM, vascular cell adhesion protein; ZO-1, zonulin-1.

Structure-Function Relationship of the Intestine for the Maintenance of Immune Defense

The surface of the *small intestine* is formed by a monolayer of highly prismatic epithelia, which are modified into structures like plications, villi (0.2–1 mm), crypts, and microvilli. Crypts contain stem cells, which generate intestinal epithelial cells (IECs). Paneth cells within the crypts secrete antimicrobial peptides (AMPs), for example, α-defensin and lysozyme, to confer intestinal protection from pathogenic insults (16, 17). The IECs in villi reabsorb nutrients and are interconnected by tight junctions (TJs) (e.g., occludins, claudins) that form apical paracellular seals thus preventing the flux of hydrophilic molecules (18). Further along the IECs lie adherens junctions (e.g., cadherins) and gap junctions (e.g., connexins), all of which determine the cellular polarity and regulate cell-cell communication and exchange of substances. The epithelium can also secrete pro-inflammatory cytokines and reactive oxygen species (ROS) in response to pathogens and metabolic stress (19). Goblet cells in the villi produce mucus, a key component of the gut barrier. A single unattached mucus layer is present superficially on the surface of the small-bowel epithelia (20, 21). Mucus contains soluble glycoproteins termed mucins, which are normally negatively charged, consisting of a core protein to which multiple polysaccharide moieties are attached, capable of binding water molecules (22). In addition to the predominant mucin-2 (MUC2), other bioactive molecules, for example, membrane-bound mucins, like MUC1, MUC3, and MUC17 and peptides, like Fc-γ binding protein and intestinal trefoil factor peptides, are secreted by goblet cells (22, 23). These play a major role in maintaining mucosal homoeostasis, mainly by limiting contact between commensals/pathogens and IECs (23). The *large intestinal mucosa* comprises crypts without any villi, with significantly greater numbers of goblet cells in comparison to the small bowel. The colon functions mainly as a reabsorbing organ for water and electrolytes and additionally produces mucus. One important distinction is the double layer of mucus on the colonic epithelial cell surface, where the inner layer is immediately above the epithelium, is mostly immobile and is thinner than the outer mucus layer, which is not attached to the colon wall (24). Both layers consist of gel-forming MUC2, but the glycoproteins of the inner layer form a large and dense net, whereas the outer layer consists predominantly of MUC2 cleavage products (25).

Regarding cellular immunity in the intestine, there is a well-regulated interplay between antigen-presenting dendritic cells (DCs), intestinal macrophages and adaptive immune cells. After recognition of antigens and/or pathogen-associated-molecular-patterns (PAMPs) via pattern recognition receptors (PRR), including Toll-like-receptors (TLRs) and NOD-like-receptors, intestinal DCs regulate the immune response by enhancing or suppressing T-cell activity. To achieve this, dendrites of DCs penetrate intercellular spaces through the intestinal TJs while maintaining barrier integrity (26). DCs, via these dendrites sense and bind luminal PAMPs and bacteria and present processed antigens to immune cells located in lymphoid follicles found in the connective tissue and the lamina propria. Intestinal

macrophages (type CX3CR1^{hi}) can also sense PAMPs by forming transepithelial dendrites (TEDs). Of note, this specific type of macrophage has only been observed in the murine ileum and the importance of the TEDs remains uncertain (27). Another means to reabsorb antigens is accomplished by villous microfold cells which offer antigens a channel to lymphoid tissue, where antigen presenting cells resorb the molecules and present them to CD4^{+}T-cells via Major-Histocompatibility-Complex II (28). Moreover, DCs selectively induce a pro- or anti-inflammatory immune response by interacting with T- and B cells. IgA^{+}-B cells colonize in the lamina propria and secrete IgA into the lumen via transcytosis (29–31) (**Figure 1**). This complex intestinal organization is subject to activation and dysregulation during sepsis.

Gut Barrier Dysfunction and Systemic Consequences During Sepsis

A major pathophysiological mechanism of sepsis harnesses recruitment of inflammatory cells and generation of an overwhelming pro-inflammatory response. PAMPs, for example, lipopolysaccharide (LPS), peptidoglycan and bacterial DNA among others and damage-associated molecular patterns (DAMPs), including mitochondrial DNA, High-Mobility-Group-Protein-B1 and serum amyloid A, result in the upregulation of adhesion molecules on the intestinal endothelium followed by the recruitment of neutrophils and macrophages (43). Upon migration to the intestinal tissue, these cells of the first line of defense produce pro-inflammatory cytokines, clinically manifested as classical signs of local and systemic inflammation (32, 44). Cell-wall components from gram-negative and gram-positive bacteria activate PRRs like TLR4 and TLR2, respectively, resulting in a "cytokine storm" of pro-inflammatory mediators generated mainly via the mitogen-activated protein kinase and NF-κB pathways (32). Of note, pro-inflammatory responses are interspersed with anti-inflammatory responses, also termed the compensatory anti-inflammatory response syndrome (45–47), where patients with sepsis undergo a reprogramming of their defense strategies and frequently fail to eliminate primary infection, thus being unable to prevent secondary infection development (33). However, an initial hyper-inflammatory response might dominate to beneficially isolate local infectious foci and limit systemic spillover (33). Gut barrier dysfunction can be considered both a result and a cause of sepsis development, characterized by enhanced mucosal layer permeability (5, 9, 10, 23, 48–51), disturbed mucosal perfusion (38, 52–54), development of tissue edema, coagulation-associated local dysregulation (36, 37), bacterial translocation (48, 55, 56) and a shift in the gut microbiome (57, 58). Furthermore, apoptotic and necrotic mechanisms damage the mucosal epithelia, resulting in a vicious cycle of further release of DAMPs, feeding into inflammatory responses combined with the development of ulceration and hemorrhage and exacerbation of mucosal homeostatic imbalance (**Figure 1**) (50, 59). The causes and consequences of gut barrier dysfunction have been described in literature extensively.

Disturbances in Vascular Tonus and Perfusion

Hypoperfusion in the splanchnic region is considered one of the main reasons for mucosal gut barrier breakdown during sepsis (38). The splanchnic vasculature system normally receives about 25% of the total cardiac output, which increases up to 35% during digestion (60, 61). Perfusion is mainly controlled by local mediators, including nitric oxide and prostaglandin derivatives, but also by systemic mediators, like vasoactive substance P and by the sympathetic innervation (61). Splanchnic hypoperfusion converts the gut into a cytokine-generating organ, which releases a "toxic fluid," containing pro-inflammatory agents and induces MODS via the circulation (48). Hypovolemia and cardiac depression during sepsis are associated with a robust inflammatory response of cytokines and other inflammatory mediators (39). Blood cells, endothelium and vascular smooth musculature are potential targets of these pro-inflammatory cytokines leading to vasodilatation, high capillary leakage, increased venous capacity and decreased venous return, all of which result in a decrease in cardiac output and tissue perfusion (39, 40). In turn, the renin-angiotensin-aldosterone-system is stimulated and increasingly generates vasoconstrictive agents, which also adds to local hypoperfusion thus developing both micro- and macro-circulatory disturbances (39, 52, 62–64). As an overall consequence, gut mucosal perfusion is reduced during sepsis, which results in further hypoxia and consequent destruction of the mucosal barrier (38). Studies using laser Doppler measurements have also revealed that CLP-induced sepsis in normotensive rats caused a decrease in the number of perfused capillaries in the small gut mucosa (65). In this context, it is also known that mucosal blood flow is dependent on inflammatory processes (52). As a result of sepsis-associated excessive inflammation, the microvasculature loses its capacity to regulate blood flow and oxygen distribution mainly based on the generation of ROS (39, 40, 66). As a consequence, increasing the blood flow by vasodilatation during hypoperfusion is not possible (53, 54), explaining why maximal O_2 extraction cannot be accomplished in sepsis (**Figure 1**). A further process that results in an impairment of the local vasodilatory response is the pathological opening of arteriovenous shunts that alters the blood flow between hypoperfused and perfused areas (39, 67).

There exist further sepsis-induced alterations in perfusion, including increased intercapillary distances due to edema (39) and greater diffusion distances (39, 63). However, the pathophysiological details are beyond the scope of this review.

Increased Intestinal Permeability

It is well-established that sepsis results in a dysfunction of the intestinal barrier with increased permeability (5, 9, 10, 23, 48, 49, 51, 68, 69). Locally transmigrated bacteria and endotoxin exposure lead to a local activation of the MIS and in turn to the production of various pro- and anti-inflammatory cytokines by IECs and intestinal immune cells. This cellular response may also contribute to the systemic response (**Figure 2**). Furthermore, activation of intestinal immune cells results in a further increase in gut permeability by altering TJs (68, 69, 84, 85). Induction

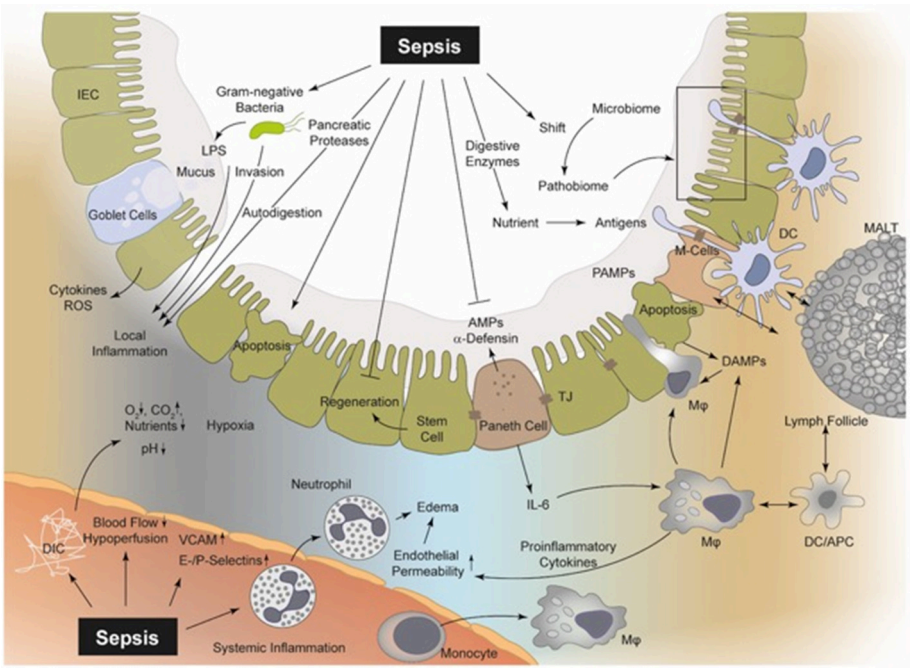

FIGURE 1 | Sepsis is involved in several pathophysiological processes regarding the intestinal epithelial integrity, perfusion, coagulation, enzymatic response, and MIS. In sepsis, bacteria and their products (PAMPs), including LPS, PG, and bacterial DNA, can be recognized by PRRs (e.g., TLR2 and TLR4) upon the surface of macrophages, neutrophils, DCs, and even IECs (19, 32). Thereby, intestinal macrophages and DCs as part of the MIS can detect luminal PAMPs via transepithelial dendrites (TEDs) (26, 27, 33). Consequently, PAMPs induce a "cytokine storm" of pro-inflammatory mediators, which drive the local intestinal and systemic inflammation (32). The released mediators can lead to an upregulation of endothelial adhesion molecules (e.g., ICAM, VCAM, E-, and P-selectin), resulting in increased recruitment of neutrophils and monocytes and in turn to increased levels of pro-inflammatory cytokines and ROS (34, 35). These cellular responses aggravate vasodilatation and induce a high level of capillary leakage with the development of interstitial edema. Local DIC is frequently observed during sepsis with a decreased supply of oxygen and nutrients, but increased carbon dioxide concentration (36, 37). Hypoxia in turn leads to increased apoptosis and necrosis of IECs and the regeneration of these IECs is suppressed during sepsis (38–40). Furthermore, the IEC integrity is disrupted and bacterial translocation may be facilitated. Pancreatic proteases are capable of autodigestion and potentiation of MOF and self-digestion leads to an increased release of further DAMPs (10, 19, 41, 42). MIS, mucosal immune system; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; LPS, lipopolysaccharide; PG, proteoglycan; PRR, pattern-recognition-receptors; TLR, toll-like receptor; DCs, dendritic cells; IECs, intestinal epithelial cells; TEDs, transepithelial dendrites; DIC, disseminated-intravascular-coagulation; MOF, multi-organ failure; ICAM, intercellular adhesion molecule 1; VCAM, vascular cell adhesion protein 1; ROS, reactive oxygen species; M-cells, microfold cells; AMPs, antimicrobial peptides; APC, antigen presenting cells; TJ, tight junctions; MALT, mucosa-associated-molecular pattern.

of experimental sepsis leads to a redistribution of the TJ proteins occludin and claudin-1, 3, 4, 5, and 8 (68, 85). In agreement with this, murine endotoxemia resulted in a disrupted ultrastructure of occludin and zonulin-1 (ZO-1) in the intestinal epithelium (86). Of note, these changes could be corrected by vagal nerve stimulation (86) or even by treatment with plant products, including berberine (87). Berberines are known to decrease downstream myosin-light chain kinase (MLCK) and NF-κB activity, representing mechanistic intermediates that may modulate TJ organization. Elaborating on this mechanism, it was found that MLCK phosphorylates myosin light chain which causes cytoskeletal contraction and junction disruption (88). Furthermore, β-catenin, another TJ organization protein, was found to be irregularly distributed in LPS-treated rats while platelet activating factor appeared to attenuate this disorganization (89). More recently, increased plasma ZO-1 levels have been found during experimental sepsis and an elevated plasma zonulin concentration, a regulator of TJs, in patients with sepsis (90). Cyclooxygenase-2 (COX-2) and particularly its product prostaglandin D2 appear to play an important role in the maintenance of epithelial TJs and barrier function, because the absence of COX-2 led to an increased permeability of the murine ileum and to a reduced expression of TJ proteins (91). Consequently, reinforced bacterial translocation and a higher mortality rate were observed in septic COX-2-knockout mice after cecal ligation and puncture (CLP) (91). With enhanced permeability, what becomes entirely imminent is the translocation of bacteria, a highly probable threat to the intestinal mucosal system.

Bacterial Translocation via the Mucosa

Gut permeability theoretically prompts the possibility of local bacterial translocation, supported by several pieces of evidence. For example, TLRs play a role in directing the response via MLCK activation, as shown in morphine-treated animals, which in turn facilitate bacterial translocation and even cause infection or sepsis of gut origin (92). Other factors like insulin growth factor-1 promote bacterial translocation, which induces intestinal cell apoptosis (93), or increased pneumoperitoneal pressure (during laparoscopic surgery) (94). Sepsis development has also been

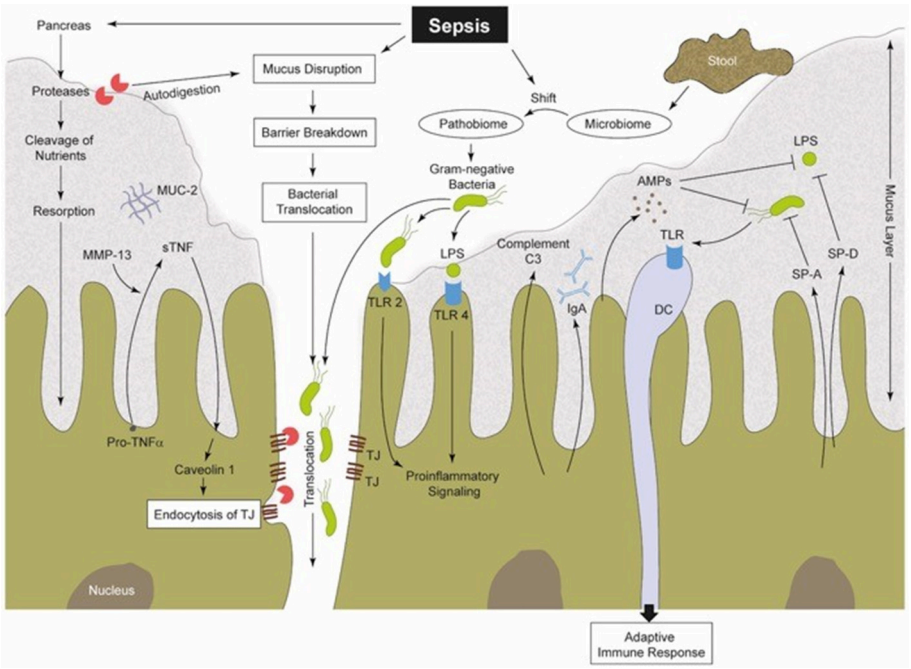

FIGURE 2 | Sepsis-induced alterations in perfusion, vascular tonus and coagulation lead to a hypoxic microenvironment of the intestinal tissue (38). Therefore, the protective gel-forming MUC2 mucus layer becomes disrupted (22). Both, bacteria (products) and (pancreatic) proteases gain access into intestinal epithelia, inducing damage followed by increased pro-inflammatory signaling (41, 48, 70, 70, 71). Furthermore, MMPs (like MMP13) are able to cleave membrane-bound pro-TNF into sTNF, which in turn is able to stimulate caveolin-1-dependent endocytosis of TJs (72, 73). Gut barrier breakdown and dysfunction is one consequence (72). Intestinal commensal microbes regulate the maturation of the MIS and support local mucosal immunity (8, 74). During sepsis, the well-regulated interplay between the commensal microbiome, IECs and mucosal immune cells becomes imbalanced. There is a sepsis-induced shift from a physiological microbiome to a "pathobiome," which is able to dysregulate the immune system by activating PRRs (32, 58). SP-A and SP-D can be synthetized by IECs (75, 76). These SPs are capable of increasing the permeability of bacterial membranes and in turn reduce the bacterial burden (77). IECs produce AMPs (e.g., α-defensin and lysozyme), to confer intestinal protection from pathogenic insults. Thereby, AMPs can act two ways, on the one hand directly by antimicrobial killing and on the other hand by innate immune modulation (78). Complement factors are mainly produced in the liver (79, 80), but also IECs were identified to synthesize and secrete C3 into the intestinal lumen (81), and thus may also play a role in intestinal immunity (82, 83). IECs, intestinal epithelia cells; MUC2, mucin-2; MMPs, matrix metalloproteinases; TNF, tumor necrosis factor; sTNF, soluble TNF; TJs, tight junctions; MIS, mucosal immune system; PRR, pattern recognition receptors; TLR, toll-like receptor; SP, surfactant protein; AMPs, antimicrobial peptides; C3, complement factor 3; LPS, lipopolysaccharide; DC, dendritic cell; IgA, immunoglobulin A.

reported as a secondary effect of pneumonia due to mucosal and microvascular injury in the gut (95). In turn, bacterial translocation may occur as a common process, secondary to a primary infection, severe trauma, or major surgery, giving rise to sepsis and consequently supporting the "gut origin hypothesis of sepsis" (96).

There are further assumptions about the driving force of sepsis and MODS. Translocating bacteria and endotoxin from the gut lumen may not directly enter into the systemic circulation, but rather induce an immune response in the local GALT or draining lymph nodes, which results in significant systemic effects, for example, via "toxic lymph" (**Figure 2**) (48, 70). The mesenteric lymph contains several different proteins and lipid factors, including a modified albumin species (97), which could cause cellular damage and the activation of TLR4, resulting in priming of neutrophils and inducing remote lung injury (98). In agreement with this, a correlation between gut barrier dysfunction and secondary lung injury has been found (48, 49). Because novel techniques on the nanoscale in bacterial (product) detection have been developed over the last decade, further clinical studies may help to re-evaluate and elucidate the "bacterial translocation" paradigm and its mechanisms.

Coagulation and Its Factors Modulating Mucosal Dysfunction

The mechanisms of sepsis-induced consumptive coagulopathy are manifold. The procoagulant tissue factor (TF), which is produced by the liver, monocytes, neutrophils and endothelial cells, is significantly increased after exposure to endotoxin or PAMPs (36, 99, 100). Synchronous inhibition of fibrinolysis occurs by an enhanced production of plasminogen activator inhibitor (PAI-1) and the downregulation of the protein-C pathway, which are important in the initiation and progression of coagulopathy with clinical manifestation of both thrombosis and DIC (36, 37). While intestinal microcirculatory disturbances are common during sepsis-induced DIC, various clotting factors may directly or indirectly affect intestinal physiology and immune-cell recruitment. For example, septic rats displayed a decrease in functional capillary density, indicating a reduction in microvascular perfusion, which could be corrected when

these animals were treated with factor XIII (FXIII) (101). In addition, factor-XI deficiency could confer a survival advantage on mice with peritoneal sepsis (102). In agreement with this, microcirculatory disturbances were found in the intestinal epithelium of CLP rats associated with high intestinal TF levels, all of which could be improved by sodium tanshinone IIA sulfonate, a substance recently proposed to exhibit protective effects against coagulatory disturbances (103). Similar effects were shown for the thrombin inhibitor Argatroban (104). Dual pharmacological inhibition of factor II and factor Xa by SATI resulted in preserved activation of coagulation with no bleeding complications and protection of organ function during experimental sepsis in baboons, representing a promising tool against sepsis-induced DIC (105). Furthermore, treatment with recombinant human antithrombin has been shown to ameliorate leukocyte adhesion in mesenteric venules and to reduce intestinal injury in endotoxemic rats (106) and concomitantly improved the 28-day mortality rate in septic patients (107). Nevertheless, application of PAI-I (108) or recombinant human thrombomodulin (109) failed to reveal beneficial effects in septic patients. Linking mucosal immunity to coagulation, mucosal M2 macrophages have been recently shown to contain intracellular FXIII stores. The cell number of this subtype is decreased in inflamed mucosa in the setting of ulcerative colitis (110). Previously, macrophage procoagulant activity was found to be increased in rats with depleted intestinal microflora and orally fed with streptomycin-resistant *E. coli*, implying that the gut is a focal point from which systemic inflammation arises (111). Deficiency of carboxypeptidase B2, an enzyme able to cleave both fibrinogen and the central complement components C3 and C5, was shown to confer survival advantage to mice, which was mainly mediated by C3a-induced peritoneal macrophage recruitment (112). Although there is evidence of an intensive crosstalk between coagulation and the innate immune response in driving inflammation during sepsis, the exact underlying mechanisms still need to be defined.

Apoptosis as a Central Driver of Intestinal Damage

Apoptotic events play a critical role in the development of sepsis. Interestingly, in murine sepsis models and in autopsy studies of septic humans, there were barely any significant histological changes except for increased gut epithelial/lymphocyte apoptosis in comparison with non-septic deceased patients (113). Nevertheless, experimental prevention of apoptosis in sepsis models increased the survival rate (33, 113) and therefore, the hypothesis of immune cell apoptosis as a relevant pathological mechanism in sepsis could also be of special interest for mucosal immunity (114). Sepsis induced by *Pseudomonas aeruginosa* pneumonia was, for example, caused by apoptotic intestinal epithelia associated with reduced epithelial proliferation (45). Mechanistic investigation of intestinal cell apoptosis during sepsis identified gene overexpression of interleukin (IL)-1β-converting enzyme, which may play an important role during experimental sepsis (115). In addition, when anti-apoptotic proto-oncogene Bcl-2 was gut-specifically overexpressed, a decrease in sepsis-induced intestinal epithelial apoptosis was found in murine models (45, 116, 117). MicroRNA 195, a regulator of Bcl-2 gene expression, which assists in maintaining the pro/anti-apoptotic balance, has been shown to be upregulated in murine sepsis and its inhibition could prevent apoptosis and even the development of MODS (118). Therefore, new approaches to improve gut barrier function during sepsis could be represented by application of silencing microRNAs regulating intestinal apoptosis (117, 118).

Apart from generic apoptosis related transducers, other molecules have also been implicated to play a role in sepsis-associated apaptotic mechanisms. Cytokine IL-15 was identified to be capable of preventing apoptosis and of immune suppression as well. In sepsis, IL-15 attenuated the apoptosis rate of intestinal epithelia and increased Bcl-2 and IFN-γ expression in IECs as well as the natural killer cell population, which produced further IFN-γ (119, 120). Of note, the lung surfactant proteins SP-A and SP-D have additionally been found to be generated by epithelial cells of the small and large intestines and in gastric cells (75, 76), and the absence of SP-A and -D resulted in increased LPS-induced apoptosis of primary IECs (77). Nonetheless, to what extent surfactant molecules may therapeutically protect the gut barrier remains to be investigated.

Intestinal Microbiome as an Actor and Target

During the last decade, the commensal microbiome has been defined to play a key role in intestinal immunity because microbes regulate the maturation of the MIS (8, 74), support local mucosal immunity (7, 8) and regulate cellular growth and maintenance of epithelial barrier function (1, 5). It is likely that the human immune system not only controls bacteria, but that the microbiome also regulates the immune cell function, particularly on mucosal surfaces (8, 121). It putatively modulates neonatal immunity and determines susceptibility to infection depending on the mode of childbirth (122–125). Alterations of the lung microbiota due to colonization by gut microbes has also been shown in animal studies, which to some extent may explain the frequent simultaneous appearance of acute respiratory distress syndrome with sepsis (126). If the symbiosis between commensal bacteria and the human host becomes imbalanced, the innate and adaptive immune systems are disturbed (**Figure 2**) (121, 127). A decline or even a loss of protective anaerobes in fecal specimens has been observed in patients with severe sepsis (57, 58) and hypothetically this "pathobiome" is able to manipulate and dysregulate the immune system in critically septic and ill patients (58). Moreover, commensal bacteria are involved in the regulation of $CD4^+$ T-cell immunity though the exact mechanisms remain unknown (128). Indicating the harmful effect of opioid analgesics in treating critical care patients, murine polymicrobial sepsis with opioid treatment selectively influenced gram-positive gut microbiome translocation and dissemination, inducing its pro-inflammatory effects through IL-6 and IL-17A cytokines (129). Furthermore, the function and aging of neutrophils as first cellular line of defense were also shown to be regulated by the microbiome during sepsis (130, 131). Overall,

it is tempting to speculate that therapeutic interventions on the altered microbiome might improve barrier, immune and organ function as well as sepsis outcome.

Intestinal Enzymatic Response Induces Self-Destruction

The underlying mechanisms of the interplay between pancreatic enzymes, sepsis, and septic shock remain unclear, although Schmid-Schönbein and colleagues had already hypothesized in 2005 that pancreatic enzymes are capable of self-digestion and potentiation of multi-organ failure (MOF) (41). In the case of sepsis-induced hypoperfusion/ischemia of the intestine, autodigestion processes can affect the mucosal barrier (10, 42). Such self-digestion may lead to an increased release of DAMPs and enhance the systemic response due to the release of pro-inflammatory mediators by stressed IECs (**Figure 2**) (19, 42, 71). The inhibition of pancreatic enzymes with subsequent prevention of gut-specific autodigestion indeed improved the outcome of septic mice (132). In this regard, inhibiting pancreatic proteases with tranexamic acid reduced inflammation and could also be exploited as a future sepsis treatment beyond its application in treating traumatic coagulopathy (132). Intestinal alkaline phosphatase (IAP) is another enzyme that protects the intestinal brush borders, particularly against intestinal bacterial invasion (133). Some of the major functions of IAP are duodenal surface pH regulation (via HCO_3^- secretion), mitigation of intestinal inflammation by PAMPs and gut microbiome control (134). IAP-mediated inactivation of bacterial products, including LPS, decreases their binding to TLR4 and reduces the resultant inflammatory responses. Interestingly, in the absence of bacteria, a lack of IAP expression results in the loss of mucosal protection (135). Mice treated with IAP after exposure to a lethal dose of *Escherichia coli* had an improved survival rate of 80%, compared to 20% in the control sepsis group (135, 136). In conclusion, loss of IAP expression or function increased intestinal inflammation, dysbiosis, and bacterial invasion, culminating in systemic inflammation (134).

LPS can furthermore induce matrix metalloprotease 7 (MMP7) expression and degranulation of Paneth cells, leading to increased intestinal permeability (17). MMP7 was observed as an amplifier of inflammation; MMP7-deficient mice displayed an attenuated intestinal inflammatory response (137). MMP7 is able to activate α-defensin, which in turn stimulates IL-6 release by macrophages and ileal epithelia, thereby enhancing local intestinal inflammation and damage (137). Moreover, MMP7 has also been correlated with the loss of intestinal barrier integrity, enhanced bacterial translocation and MOF development (137). Similarly, MMP13 has been described to play a role in inflammatory bowel diseases (IBD) and during sepsis (72). It is able to cleave membrane-bound pro-TNF into soluble bioactive TNF, which can affect TJs through caveolin-1-dependent endocytosis (72). The consequences are the loss of TJs, increased intestinal permeability and the creation of a new pathway for migrating bacteria, which induces further inflammation (72, 73, 138). MMPs are also present in the large intestine and play a similar role in sepsis progression through similar mechanisms. MMP-1, 2, 3, and 9 were detected in the human colon mucosa and were also increased during IBD (139, 140). However, their exact role in sepsis is yet to be investigated.

Metabolic Response Within the Intestinal Mucosa

While intestinal permeability is enhanced, amino-acid absorption by the intestine is affected as early as 24 h after sepsis onset (141). In this regard, *in vivo* and *in realiter* studies revealed that gut glutamine absorption and metabolism decreased during sepsis because of suppressed glutaminase activity (142). By contrast, glutamine supplementation improved other effects of sepsis: it reduced bacterial translocation, restored permeability and microcirculatory characteristics (143–146), and even increased the number and survival of intestinal epithelia while blocking inflammatory cytokine secretion by CD8αα(+) TCRαβ(+) IEL cells (147) and γδT-IELs (148). As a further risk factor, a high-fat diet was detrimental for sepsis outcome and worsened endotoxemia in mice by disrupting the *Bifidobacterium spp*. colony. Correction of the dysbiosis and its consequences by feeding prebiotic oligofructose resulted in reduced systemic inflammation in experimental (149) and clinical sepsis (150). This preliminary evidence suggests that the intricate repertoire between metabolic intermediates, gut microbiome and inflammatory responses following sepsis requires further investigation and represents a promising therapeutic potential.

Therapeutic Approaches to Improve Sepsis-Associated Mucosal Immunopathology

In the era of resurrection from the "therapeutic graveyard of sepsis," novel pharmacological approaches address crucial aspects of gut mucosal dynamisms. For example, the dietary dipeptide gamma-l-glutamyl-l-valine (γ-EV), which leads to decreased pro-inflammatory cytokines in both plasma and the small intestine, is also effective against bacterial infections (151). γ-EV stimulates the interaction of β-arrestin-2 with toll-interleukin-1-receptor signaling proteins, including TRAF6, TAB1, and IκBα, which further suppress the inflammatory response in the small intestine (151, 152). Similar results in murine IBD models have been shown for γ-glutamyl-cysteine, which inhibits TNF signaling in intestinal epithelia (152). In other studies, the small peptide hormone ghrelin was identified to be protective by inducing autophagy in the case of tissue hypoxia. Thereby, ghrelin appears also able to protect IECs in the small intestine in the early stage of sepsis (153). Application of deacetylase sirtuin-1, a signaling intermediate that is decreased in obesity and results in enhanced microvascular inflammation within the small intestine, reduced the mortality rate in the early stage of sepsis (154, 155). Treatment with resveratrol increased the expression of sirtuin-1 in obese septic mice and the inflammatory response thereafter was diminished (154). Sirtuins also play a major role during the late onset of septic "hypo-inflammation"; SIRT-2 inhibition in obese septic mice preserved a decreased microvascular inflammation and protected against thrombotic events (155).

The antimicrobial peptide cathelicidin-BF (C-BF) has been observed as a protective molecule, which can safeguard LPS-induced septic rodents from the development of small intestinal barrier dysfunction (156). C-BF prevented LPS-induced TJ breakdown and reduced IEC apoptosis by attenuated expression and secretion of TNF and suppression of the underlying NF-κB pathway (156). Ulinastatin is another drug able to increase the survival rate and to reduce injury of the small intestine, for example, through diminished IEC apoptosis (142). Post-treatment IL-6 and TNF plasma levels were decreased, suggesting an interesting strategy for sepsis (59, 157).

Stem-cell therapy could also represent a potential treatment approach for sepsis. In murine CLP-sepsis, human adipose-derived mesenchymal stem cells were able to modulate sepsis by downregulation of Th1-cell responses, associated with lower levels of pro-inflammatory cytokines (TNF, IL-1β, IL-6, IL-12, IFNγ) and higher levels of anti-inflammatory IL-10 derived from macrophages (158). Application of (mesenchymal) stem cells may, therefore, protect septic mice by reducing inflammatory cell infiltration and pro-inflammatory responses and enhancing anti-inflammatory signals (158). Nevertheless, to what extent stem cells or their cellular structure or secretome will modulate mucosal immunity during sepsis has to be clarified in future studies.

Defining the undisputed role of the microbiome in shaping sepsis-associated immunopathology is gradually gaining momentum, discussed in detail in several reviews (159–161). A dysregulated gut microbiome is a common causation of sepsis (162), like in late-onset sepsis development of preterm neonates (163). Conversely, burn-injury associated altered gut microbial community and leakiness of the gut have been implicated in sepsis development (164). A common approach undertaken to manage sepsis patients involving therapy with antibiotics can impair the diversity of microbes in the intestine and reduce the protective role of bacteria, which in turn leads to increased inflammation in murine models of Gram-positive as well as Gram-negative pneumosepsis (7, 165, 166). Thus, the loss of microbiome diversity was indeed identified as a predictive factor for the length of hospitalization of patients in the ICU (166, 167). Though a recent study disproved that antibiotics-mediated disrupted microbiota modulates innate immune system in endotoxemic patients (168), the exact role of how the immune system is modulated is left to be delineated. Nevertheless, to correct this ensuing dysbiosis, treatment options have included procedures like fecal microbiota transplantation (FMT) with combined usage of antibiotics in the clinical management of sepsis (169–171). In the 1950s, FMT had been developed to treat *Clostridium difficile* associated pseudomembranous colitis and has since subsequently proven to be an effective treatment modality in the management of *C. difficile* infection (172–174). Recently, the US Centers for Disease Control indicated that "death rates from sepsis following infections (e.g., *C. difficile*) have surged (175). Therefore, a perfect premise to facilitate new treatment approaches, FMT is a potentially effective treatment route, which could counterbalance dysbiosis, support the gut microbial barrier and improve the outcome of sepsis (171, 172, 176). Microbial dysbiosis of the gut leads to changes in the metabolism of bacteria and as a consequence to an impaired interaction between microbes, immune cells and IECs (1, 5, 7, 8). During sepsis, the exact mechanism of action for the use of FMT on the intestine is still unknown. Recolonization of the intestinal microflora has been beneficial as well, where 16 days post-FMT, improved symptoms were observed in two separate patient studies involving stroke (176) and post-surgical sepsis development (171). FMT proves to be a viable future treatment option for sepsis and further human clinical research is needed to evaluate its effectiveness in critically ill patients.

Further therapeutic approaches have diversified to include supplementing antibiotics with probiotics, prebiotics and synbiotics. Probiotics are live beneficial microorganisms, which can improve the health of hosts (170), prebiotics are non-viable and non-digestible dietary ingredients e.g., fructooligosaccharides which stimulate the growth and/or activity of a limited number of bacteria in the large intestine (170, 177) and synbiotics refer to combined usage of prebiotics with probiotics (178). Studies have shown that supplementation of *Bifidobacterium breve* strain Yakult and *Lactobacillus casei* strain Shirota as probiotics and galactooligosaccharides as prebiotics can reduce the incidence of infectious complications, e.g., enteritis, pneumonia and bacteremia in patients with severe SIRS compared to those who did not receive synbiotics (179, 180). The administration of synbiotics could maintain the gut flora and reduce septic complications in patients with severe SIRS by enhancing the levels of beneficial bacteria in the intestine. A further study suggests that the orally consumed synbiotics (*Lactobacillus planatrum* and fructooligosaccharide) in newborn infants improve the primary outcome (complication of sepsis or death) as well as lower respiratory tract infections compared to newborn infants with placebo treatment (181). In contradiction to these studies, other studies have suggested no difference in incidence of late-onset sepsis and mortality rate in preterm infants (182, 183). Similar results postulated that prophylactic administration of *B. clausii* to preterm neonates do not reduce the burden of late-onset sepsis compared to placebo (184). Further, research may be focused on dysbiosis of the gut microbiome and resultant immunosuppression as one consequence of sepsis restored by gut commensals through administration of probiotics, to reduce the incidence of late infections and the sepsis mortality rate (185, 186). Synbiotics also seem to be a potential treatment option for sepsis patients. The complications of enteritis and ventilation-associated pneumonia were significantly lowered in the patients who have been treated with synbiotics, compared to those without synbiotic administration, although the incidence of bacteremia and the mortality rates did not differ between the groups (169). The process of bacterial translocation from the intestinal lumen to systemic circulation as described in section Bacterial Translocation via the Mucosa is another interesting premise to consider as a treatment target. There are some clinical correlations showing bacterial translocation as one cause for subsequent sepsis, or in reverse, induce late onset-sepsis complications. In patients with acute pancreatitis, septic complications as a result of pancreatic necrosis is a major cause of

TABLE 1 | Table with ongoing and/or completed clinical trials targeting the gut in sepsis patients.

Trial registration number and Name	Study Population and inclusion criteria	Short description of intervention and duration of treatment	Study duration, study endpoints
NCT02127749 The role of the gut microbiota in the systemic immune response during human endotoxemia (MISSION-2) (168)	16 persons • Healthy • Caucasian • Non-smoking • Male • Age 18–25	Treatment of healthy male candidates with broad-spectrum antibiotics (ciprofloxacin, vancomycin, metronidazole) or placebo for 7 days. After a 36 h wash-out period, an i.v. bolus infusion of LPS given.	• Day 0 = 1 day before antibiotic treatment • Day 9 = 1 day after finishing treatment with broad-spectrum antibiotics, before i.v. LPS administration • Day 10 LPS administration ($t = 0$) • Blood sampled at $t = 0{,}5; 1; 1{,}5; 2; 3; 4; 6; 8$ h after LPS injection.
"*Bacillus clausii* for Prevention of late-onset sepsis in preterm infants: a randomized controlled trial" (184)	• Preterm neonates <34 weeks • 120 neonates grouped under "extreme preterm" • 124 grouped as "very preterm."	Neonates of each group were randomized to receive prophylactic probiotic supplementation with *Bacillus clausii* or placebo till 6 weeks of postnatal age.	• Study started on 01.03.2012 and ended on 28.02.2014 • Study endpoint: End of 6 weeks supplementation of probiotics, or till discharge, or death, or occurrence of late-onset sepsis.
CTRI/2009/000945 Effect of oral glutamine supplementation on gut permeability and endotoxemia in patients with severe acute pancreatitis: a randomized controlled trial (191).	80 persons (age 18–80 years) $n = 41$ treatment $n = 39$ for placebo • Patients with acute severe pancreatitis • 3-fold increase in the levels of serum amylase. • Confirmed 1 or more organ failure and/or APACHE II > 8 and/or CT severity index> 7	Oral glutamine administration for 7 days. Objective of the study was to check for the effect of glutamine supplementation on endotoxemia and gut permeability following severe acute pancreatitis.	• Started on November 2009 and ended on October 2012. • Duration of intervention has been 7 days • Day 0 = start with the intervention • Endpoint day 7 = end of intervention with glutamine administration
ISRCTN61157513 Modulation of gut function using Gut Specific Nutrients in the critically ill (192).	50 persons $n = 25$ treatment $n = 25$ for placebo Critically ill surgical patients with inadequate gut function.	Treatment with a gut-specific nutrients cocktail or Placebo-cocktail for 30 days	• Restoration of gut function to normalcy was the primary endpoint. • Patients reviewed twice daily for 1 month of their hospital stay then followed-up till 3 months.
NCT00518596 Prevention of infection in Indian neonates—phase II probiotics study (181).	4,556 infants as $n = 2{,}278$ synbiotic; $n = 2{,}278$ placebo • Neonate >24 h and <96 h old • Birth weight >2,000 g • Breastfeeding begun by 24 h of life • Ability to tolerate oral feeds.	Daily one-time oral synbiotic treatment with 10 billion *Lactobacillus plantarum* and 150mg of fructoologiosaccharide for 7 days from day 2-4 of post-natal life.	Study started on October 2008 and ended on May 2012 Duration of clinical surveillance was 60 days of post-natal life with daily evaluation of the infants
R000007633 Synbiotics modulate gut microbiota and reduce enteritis and ventilator-associated pneumonia in patients with sepsis: a randomized controlled trial (179).	72 Patients; $n = 35$ synbiotics; $n = 37$ placebo • Age >16 years • > 3 days of mechanically ventilated sepsis patients	Daily oral administration of synbiotics (*Bifidobacterium breve* strain Yakult, *Lactobacillus casei* strain) Shirota and (galactooligosaccharide) 3 days post hospital admission.	4 weeks after admission to hospital, study was ended. • **Primary outcome** within 4 weeks: ventilator induced pneumonia, bacteremia and enteritis. • **Secondary outcomes**: fecal bacterial numbers, concentration of organic acid and mortality within 4 weeks.
"Synbiotics decrease the incidence of septic complications in patients with severe sirs: a preliminary report (193)."	55 patients $n = 29$ synbiotics $n = 26$ non-synbiotics • Severe SIRS • C-reactive protein >10 mg/dl.	Oral treatment with synbiotics, *Bifidobacterium breve* (3×10^8/day), *Lactobacillus casei* and galactooligosaccharide (10 g/day) or placebo on day of admission.	• Study started on July 2004 and ended on March 2005 • Study endpoint has been defined as the last collection of fecal samples on day 40 after admission.
NCT00835874 Probiotic administration to mothers of preterm infants to prevent necrotizing enterocolitis and sepsis. (194)	57 participants • Mothers of preterm infants Pumping breast milk	Oral treatment with probiotics *Lactobacillus acidophilus, Bifidobacterium lactis* once daily	**Primary Outcome Measures**: 3 month evaluation of all-cause mortality, incidence and severity of necrotizing enterocolitis and appearance of bacterial or fungal infection (blood, csf, or urine cultures).

(Continued)

TABLE 1 | Continued

Trial registration number and Name	Study Population and inclusion criteria	Short description of intervention and duration of treatment	Study duration, study endpoints
NCT01100996 Anti-inflammatory effects of enriched enteral nutrition during human experimental endotoxemia (VIHE) (195).	36 participants • Age >18 and <35 • Male Non-smoking	Triggering of human endotoxemia by LPS infusion. Then: • No intervention—fasted control or, • Placebo comparator—control feeding (20% fat, 16% protein and 49% carbohydrates) 1 h after LPS administration until 6 h after LPS or, • Active comparator— enriched feeding (46% fat, 24% protein and 30% carbohydrates and phospholipids) 1 h and until 6 h after LPS administration.	• **Primary Outcome Measures:** Circulating cytokines (Time Frame: several time points from LPS administration until 24 h) • **Secondary Outcome Measures:** Markers for sub-clinical organ damage (kidney, endothelium, intestine) (Time Frame: several time points from LPS administration until 24 h)
NCT02768324 The effect of gut microbiota on the prognosis of sepsis. (180)	30 participants • Age >18 and <65 Sepsis	No treatment, only observation of septic patients and collecting stool and blood samples.	Primary Outcome: Discharged from ICU; Time Frame 7 days
NCT03472170 Clinical trial for valuation of the effectiveness of lactoferrine in the prevention of sepsis in new premature born. bimonitorization of the antinflammatory mechanisms, antioxidants and the intestinal microbiote. (LACTOPREM)	26 participants • Male or female children born preterm • Birth weight <1,500 g and or EG <32 weeks	Enteral administration of bovine lactoferrin or treatment with placebo for 4 weeks (exceptional extending for 6 weeks in newborns EG <28 weeks or birth weight <1,000 g)	Primary Outcome: Incidence of proven and probable late sepsis (12 months)
NCT03488940 Effect of different feeding method on gastrointestinal function of septic patients (DFM-GF Trial) (DFM-GF)	60 participants • Sepsis • APACHE-II Score >15 points	Patients were allocated into 3 cohorts • 24 h group • 16 h group • intermittent group Each group refers to the time upto which nutrition preparation is enterally pumped. The duration of the treatment is 7 days.	• **Primary Outcome Measures:** The mean time (hours) that reach to the caloric goal in every group (7 days after intervention) • **Secondary Outcome Measures:** The rate of onset Gastric residual (%) (baseline and first 7 days after intervention); Abdominal pressure mmHg; Level of plasma motilin (pg/ml); Rate of new onset pneumonia.
NCT03861325 acute intestinal failure in critically ill patients and microbial translocation (IGA-TM)	50 participants • Age >18 • Septic shock (as defined by the 3rd International Conference for the definition of sepsis and septic shock)	Observation for 7 days—no treatment	• **Primary Outcome Measures:** Incidence of microbial translocation in critical illness by measuring 16S rDNA and 18S rDNA plasma levels (at admission, 12 h, 1 d, 2 d, 3, and 7 d after admission to hospital) • **Secondary Outcome Measures:** Plasma I-FABP and zonulin (at admission, 12 h, 1 d, 2 d, 3, and 7 d after admission to hospital); Correlation between titer of I-FABP, zonulin, 16S rDNA, 18S.
NCT02306239 The effect of terlipressin on intesitnal function in septic shock patients	40 participants • Age >18 and <80 • Septic shock	Treatment with Terlipressin or Norepinephrine	**Primary Outcome Measure:** Evaluation of the intestinal function based on abdominal distension, intestinal bleeding, peritonitis, plasma DAO level, and enteral nutrition (Time Frame 7 days).
NCT00378586 Barrier function and production of inflammatory cytokines in the rectal mucosa in patients with septic shock	30 participants • Age >18 • Septic shock	Extraction of a rectal biopsy	**Primary Outcome Measure:** • Measurement of IL-6 and TNF levels in the rectal mucosa • Measurement of rectal lactic acid

Various clinical trials have enabled active application of prebiotic, probiotic and synbiotic treatment modalities incorporated into traditional therapeutic approaches. Here is a comprehensive tabular representation of all clinical trials that have employed such alternative treatments in the context of sepsis related modulation of the gut. Many of these clinical trials have been instrumental in understanding the ensuing gut pathophysiological changes, its effect on SIRS and how the altered microbiome is a robustly affected cause and effect thereof. i.v., intravenous; LPS, lipopolysaccharide; LGG, Lactobacillus GG; ICU, intensive care unit; SIRS, systemic inflammatory response syndrome; CRP, C-reactive protein; SOFA, sequential organ-failure assessment; IL-6, interleukin 6; APACHE-II score, Acute Physiology And Chronic Health Evaluation- II Score; mmHG, millimeter of mercury; DAO, Diamine Oxidase; I-FABP, intestinal fatty acid-binding protein; rDNA, ribosomal deoxyribonucleic acid; TNF, tumor necrosis factor; csf, cerebrospinal fluid; EF, ejection fraction.

death. Therefore, bacterial overgrowth and subsequent bacterial translocation can be prevented by administration of selected probiotics, because the usage of these bacterial supplementation have been shown to reduce infectious complications in patients with severe acute pancreatitis (187). The oral administration of *Lactobacillus plantarum* in combination with enteral feeding improved gut permeability and led to a significantly better clinical outcome (188). Treatment of severe acute pancreatitis could be adjusted by enteral nutrition (EN) and ecoimmunonutrition (EIN), because alone as well as in combination, both decrease the expression of plasma endotoxin, TNF, IL-6, bacterial translocation and pancreatic sepsis (189). As described in this review, synbiotics could prevent dysbiosis of the human gut, but administration of synbiotics may not affect the intestinal permeability in critically ill patients (150). A further clinical study with 72 patients have found the influence of pro- and synbiotics (termed as Synbiotic 2000FORTE) (*P. pentoseceus* 5-33:3, *L. mesenteroides* 32-77:1, *L. paracasei ssp*. 19, *L. planatarum* 2362) on the immune response in patients with multiple injuries (190). A significant decrease ($p = 0.028$) have been shown in the incidence of septic events as well as the occurrence of ventilation associated pneumonia by *Acinetobacter baumannii*. The risk of sepsis as a consequence of bacteremia was significantly decreased and even the treatment with the specific synbiotics prolonged the time of progression of primary bacteremia, compared to the placebo group (190). In the molecular level, white blood cell counts and serum C-reactive protein were significantly lower in patients treated with Synbiotic 2000FORTE compared to the placebo cohort and it could reduce the incidence of death caused by MODS (190).

CONCLUSION

The impact of sepsis on the gut is manifold, e.g., sepsis mediated alteration of the gut-blood barrier and increase in the intestinal permeability, which may correlate with the phenomena of bacterial translocation and lymphatic activation ("toxic-lymph"). Systemic consequences of sepsis are widespread and concern to the coagulative system, the microbiome as well as enzymes, such as pancreatic proteases, MMPs and IAPs. Nevertheless, the therapeutic approaches for modulating the mucosal immune system are still rarely effective in daily routine. Recent published studies showing that treatment with FMT, probiotics and synbiotics are new concepts for gut-specific therapeutic prevention of sepsis (**Table 1**). Since the past decade, several clinical trials have been completed and are underway to comprehensively actualize the currently understood putative effectiveness of targeting the gut during sepsis. This has been presented in **Table 1**, enlisting all completed published, completed unpublished and ongoing trials so far. One exemplary study was proven to be an effective synbiotic treatment of fructooligosaccharides and *Lactoacillus plantarum* to preterm neonates which prevented sepsis and mortality in the treatment group (181). However, these promising therapeutic approaches are yet to be appraised as accepted therapeutic options. More clinical investigations could help substantiate these findings and extend them into becoming alternative treatment options. This also brings into light the importance of understanding the gut mucosal immune system, where further investigation is required to evaluate unknown sepsis-induced intestinal pathophysiological processes. Scarce as of now, nonetheless, investigations to understand the MIS would prove additionally beneficial so as to identify added novel therapeutic modalities.

AUTHOR CONTRIBUTIONS

FH and MH-L conceptualized the review. FH and SC performed the literature search for the review. FH wrote the review as a first author, with written contribution by SC as the second, RH as the third and MH-L as the senior author. The table was compiled and prepared by FH and SC. Figures were prepared with the help of Dr. Stephanie Denk as mentioned in Acknowledgments.

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Evaluation of Mannose Binding Lectin Gene Variants in Pediatric Influenza Virus-Related Critical Illness

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Background: Mannose-binding lectin (MBL) is an innate immune protein with strong biologic plausibility for protecting against influenza virus-related sepsis and bacterial co-infection. In an autopsy cohort of 105 influenza-infected young people, carriage of the deleterious MBL gene *MBL2*_Gly54Asp("B") mutation was identified in 5 of 8 individuals that died from influenza-methicillin-resistant *Staphylococcus aureus* (MRSA) co-infection. We evaluated *MBL2* variants known to influence MBL levels with pediatric influenza-related critical illness susceptibility and/or severity including with bacterial co-infections.

Methods: We enrolled children and adolescents with laboratory-confirmed influenza infection across 38 pediatric intensive care units from November 2008 to June 2016. We sequenced *MBL2* "low-producer" variants rs11003125("H/L"), rs7096206("Y/X"), $rs1800450_{Gly54Asp}$("B"), $rs1800451_{Gly57Glu}$("C"), $rs5030737_{Arg52Cys}$("D") in patients and biologic parents. We measured serum levels and compared complement activity in low-producing homozygotes ("B/B," "C/C") to HYA/HYA controls. We used a population control of 1,142 healthy children and also analyzed family trios (PBAT/HBAT) to evaluate disease susceptibility, and nested case-control analyses to evaluate severity.

Results: We genotyped 420 patients with confirmed influenza-related sepsis: 159 (38%) had acute lung injury (ALI), 165 (39%) septic shock, and 30 (7%) died. Although bacterial co-infection was diagnosed in 133 patients (32%), only MRSA co-infection ($n = 33$, 8% overall) was associated with death ($p < 0.0001$), present in 11 of 30 children that died (37%). *MBL2* variants predicted serum levels and complement activation as expected. We found no association between influenza-related critical illness susceptibility and *MBL2* variants using family trios (633 biologic parents) or compared to population controls. *MBL2* variants were not associated with admission illness severity, septic shock, ALI, or bacterial co-infection diagnosis. Carriage of low-MBL producing *MBL2* variants was not a risk factor for mortality, but children that died did have higher carriage of one or more B alleles (OR 2.3; $p = 0.007$), including 7 of 11 with influenza MRSA-related death (vs. 2 of 22 survivors: OR 14.5, $p = 0.0002$).

Conclusions: *MBL2* variants that decrease MBL levels were not associated with susceptibility to pediatric influenza-related critical illness or with multiple measures of critical illness severity. We confirmed a prior report of higher B allele carriage in a relatively small number of young individuals with influenza-MRSA associated death.

Keywords: MBL, influenza, pediatric, methicillin-resistant *Staphylococcus aureus*, critical illness, sepsis, mortality

INTRODUCTION

Severe sepsis is the most common cause of death in infants and children across the world (1). Influenza virus is a common global pathogen causing severe sepsis, and annually leads to 100–350 deaths and over 25,000 hospitalizations in North American children (2–4). Influenza suppresses the immune system, allowing respiratory tract colonizers to invade and cause bacterial co-infection, a major contributor to influenza-related morbidity and mortality (5). In 2003, methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in the United States as a major co-infecting bacterial organism in children with influenza virus infection and an independent predictor of death (6). In comparison to healthy adults, children are heavily reliant on innate immunity for protection against influenza virus and bacterial pathogens as they have limited adaptive immunity and fewer years of exposure to develop anti-microbial antibodies (7).

The wide spectrum of influenza virus-related disease severity is likely influenced by host genetics. Novel primary immunodeficiencies to influenza and other common viruses have been identified in children previously thought to be healthy (8–10). Most are in interferon regulatory genes, essential for innate immunity to viruses (11). However, other gene pathways may influence disease severity, particularly in relation to bacterial co-infection. Mannose-binding lectin (MBL), a key innate immunity pattern-recognition protein, activates the lectin complement pathway. MBL has strong biologic plausibility as an innate immunity candidate protein that could protect against influenza-related sepsis with and without bacterial co-infection (12). MBL binds to microbial surface glycosylation residues and targets influenza virus via direct neutralization, by recognition of influenza hemagglutinin surface proteins on infected cells, and can also ameliorate severity by defending against bacterial pathogens (13–15). Additionally, influenza virus uses a glycan-binding entry mechanism to invade host cells, and lectins such as MBL may interfere directly with entry of the pathogen into the cell (16).

MBL serum levels and functional activity are strongly influenced by five single nucleotide polymorphisms (SNPs) in the *MBL2* gene. As shown in **Figure 1A**, three *MBL2* missense mutations in Exon 1, "B" (rs1800450_A; codon 54 Gly to Asp), "C" (rs1800451_A; codon 57 Gly to Glu), and "D" (rs5030737_T; codon 52 Arg to Cys), combine with *MBL2* promoter polymorphisms "H/L" and "Y/X" to form low-, intermediate- and high-producing MBL haplotypes (17, 18) (see **Figure 1B**). Although all 3 exon mutations are associated with the lowest levels of MBL, only the B variant Asp residue has been reported to destabilize circulating MBL oligomers having a dominant effect when present, further decreasing functional activity (15, 19, 20). MBL deficiency is common, and may occur in 30% of the population depending on what MBL level cutoff is used for defining it; deficiency has been defined variably as <1,000, <500, <200, or <100 ng/mL (21). In critically ill patients, serum levels may be influenced by inflammation, diluted by fluid resuscitation, or raised via fresh frozen plasma transfusion, so genotype has been used as an inexact proxy to estimate pre-illness MBL deficiency (17, 22).

Many studies have evaluated the association between MBL genotypes and sepsis, with most comparing carriage of low-MBL producing mutations in B, C or D mutation in exon 1 (termed "O") to wild-type ("A"), but the studies have shown conflicting and inconclusive results (23). A limited number of small studies have evaluated associations between MBL and influenza-related critical illness. Herrera-Ramos *et al* reported no increased frequency of low-producing MBL genotypes in 93 adult Spanish inpatients and outpatients infected with 2009

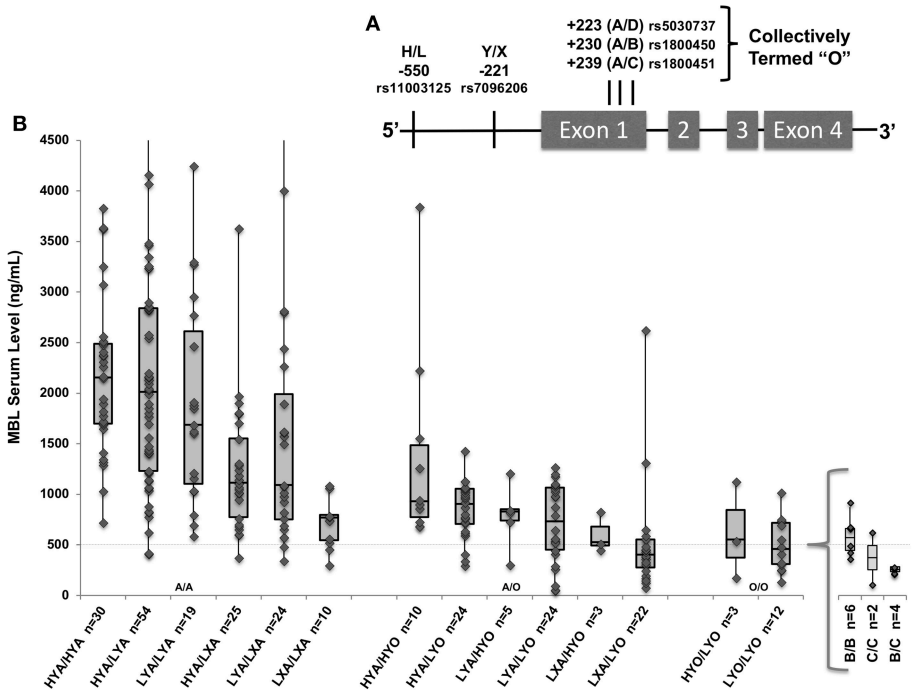

FIGURE 1 | (A) *MBL2* gene with known low-producer variant polymorphisms. Marked positions denote promoter variants H/L and Y/X, and exon 1 variants "B," "C," and "D" (collectively termed "O"). **(B)** The gene-to-level analysis ($n = 265$) included all genotyped patients who also had measured MBL levels. Box and whisker plots indicate serum level quartiles: 1st quartile (bottom line) 2nd/3rd quartile (box) with median at line, and 4th quartile (top line). *MBL2* haplotypes combined into diplotypes with associated serum MBL levels (n = number of patients included); wild type (A/A), heterozygote (A/O), and homozygotes (O/O) with additions of low-producing promoters from left-to-right within each X-axis group. Specific variants within LYO/LYO homozygotes are shown in insert; there were not significant differences in levels among B/B, C/C, and B/C homozygotes. Four patients (3 HYA/LYA, 1 LYA/LXA) had values > the Y axis set maximum of 4,500 ng/mL**.** Dashed line indicates 500 ng/mL throughout.

pandemic H1N1 ($H1N1_{pdm}09$) (24) Higher MBL serum levels were associated with mortality in 27 influenza $H1N1_{pdm}09$-infected critically ill adults (25) whereas 12 critically ill pediatric patients had lower levels compared to ward patients (26). A case series comparing *MBL2* genotypes in 100 fatal pediatric influenza cases (from autopsy samples) with a pediatric population control cohort did not find differences in the frequency of *MBL2* variants known to influence MBL levels after stratifying by ethnicity, but fatal influenza cases with MRSA co-infection were more likely to carry the B mutation (27).

The 2017 World Health Organization Public Health Research Agenda emphasized that identifying host genetic factors influencing influenza susceptibility and severity is paramount for targeting prevention and identifying novel therapeutics (28). MBL repletion is feasible (29) for influenza-infected children predicted to have low or deficient levels. Identification of associations between MBL variants known to influence levels, influenza-related sepsis, and bacterial co-infection could allow opportunities for precision diagnostics and interventions. Therefore, we evaluated associations between *MBL2* variants and overall influenza susceptibility, severity, and bacterial co-infection in a multicenter prospective cohort of critically ill children and adolescents in the Pediatric Intensive Care Influenza (PICFLU) Study.

MATERIALS AND METHODS

From November 2008 through June 2016, the PICFLU Study prospectively enrolled patients (<21 years of age) admitted to 38 PICUs in the Pediatric Acute Lung Injury and Sepsis Investigator's (PALISI) Network with suspected or confirmed community-acquired influenza infection. Details of the PICFLU Study design have been previously published (7, 11, 30, 31). Beginning in fall 2010, patients with known risk factors for becoming severely ill with influenza virus such as immunodeficiency, severe chronic lung or heart conditions, were excluded to enrich for identification of genetic susceptibility factors. Testing for influenza virus and other viral pathogens was performed at the enrolling site and in collected respiratory samples using sensitive PCR testing (30, 31). Bacterial co-infection was defined as a diagnosis at the clinical site with microbiologic identification of the pathogen within 72 h prior to or after PICU admission (to exclude hospital-acquired infection). Cultures had to come from a sterile site: endotracheal or bronchoscopic specimen, bloodstream or pleural fluid (31). Patients were defined as previously healthy if they had no underlying comorbidities and were on no chronic medications. Sepsis was defined using the 2005 International Consensus Conference on Pediatric Sepsis criteria (32). Illness severity was

assessed by the Pediatric Risk of Mortality (PRISM) III Score (33). The American European Consensus Conference Criteria were used for diagnosis of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (34). We collected samples from pediatric patients (blood) and their parents (saliva). Blood was collected as close to admission as possible and used both for DNA extraction and MBL serum level measurements. Each enrolling site received site Institutional Review Board approval. Written informed consent was obtained from a parent or legal guardian for patients and for each parent contributing their own sample for DNA. The population control cohort included healthy adolescents (12–19 years old) from the National Health and Nutrition Examination Survey (NHANES) (35) who had *MBL2* genotyping available (27).

Serum MBL Evaluation, MBL2 Genotyping, and MBL Functional Assessment

Genomic DNA from peripheral blood was extracted using the Gentra Puregene Blood kit (Qiagen). Saliva samples were collected using Oragene Saliva kits (DNA Genotek Inc., Ontario, Canada). DNA extraction followed manufacturer's recommendations. We used TaqMan assays to genotype samples for single nucleotide polymorphism (SNPs) using the TaqMan OpenArray® SNP Genotyping Platform (Applied Biosystems, Foster City, CA). We genotyped the following SNPs known to influence MBL levels from both patients and biologic parents: rs7096206 ("Y/X"), rs11003125 ("H/L"), rs1800450_$A_{Gly54Asp}$ (allele "B"), rs1800451_$A_{Gly57Glu}$ (allele "C"), and rs5030737_$T_{Arg52Cys}$ (allele "D") (see **Figure 1A**).

To identify rare deleterious variants in *MBL2*, we performed targeted re-sequencing of the exons and nearby regions using Illumina TruSeq Custom Amplicon kit (TSCA, Illumina, San Diego, CA) as described previously (30), and this was compared with Taqman genotyping. In samples where genotyping remained inconclusive, Sanger sequencing for the 5 SNPs in *MBL2* was performed.

For patients enrolled prior to May 2014, MBL serum level measurements were done at the Cytokine Reference Laboratory using a commercial enzyme-linked immunosorbent assay kit from R&D Systems (Minneapolis, MN).

MBL protein activity was evaluated by measuring lectin pathway complement fixation in the sera of homozygote patients, either "B/B" ($n = 4$) or "C/C" ($n = 2$), and compared to wild type controls (HYA/HYA; $n = 6$). There were no "D/D" homozygotes for evaluation. Dilutions of patient and control sera were incubated on mannan (Sigma M7504) coated plates in Tris buffered saline with Tween and 5 mM CaCl2 (Boston Bioproducts). After incubation at 37°C and rinsing, deposited C3 fragments were detected with HRP-labeled polyclonal sheep anti-human C3c (BioRad 2222-6604P) and measured by ELISA. Lectin pathway activity was determined by comparing absorbance at 450 nm of C3c bound to mannan from patient sera as previously described (36, 37).

Statistical Analyses

MBL2 genotypes were correlated with MBL serum levels to ensure that previously described (17, 38) relationships between *MBL2* variants and serum MBL levels were present in the PICFLU cohort. A multiple linear regression model, adjusting for gender, bacterial co-infection, age, race, and influenza status, was used to evaluate associations between variant alleles and serum levels. Frequencies of allele distribution were also compared between influenza virus subgroups ($H1N1_{pdm}09$ versus non-$H1N1_{pdm}09$; influenza A vs. B) and racial groups (all races, white only, white non-Hispanic). Categorical variables were compared using Chi-square test or Fisher's Exact Tests and continuous variables with the Wilcoxon-Mann-Whitney test.

MBL2 variant frequencies vary by ethnicity and race. To control for possible confounding by population substructure, we used family-based analyses (PBAT/HBAT) (39–41) based on the Transmission Disequilibrium Test (TDT) in family trios. When analyzing family trios, only heterozygous parents are included, expected to transmit either alleles 50% of the time to their children. Significantly increased/decreased allele transmission above 50% is defined as transmission disequilibrium. A positive TDT test suggests that the gene itself influences susceptibility to the disease or is in tight linkage with the disease-predisposing gene (39, 41). The software (www.hsph.harvard.edu/fbat) was used to analyze the pedigrees using the PBAT test for individual variants and statistical power estimation and HBAT for *MBL2* haplotypes (minimum informative families = 10), first testing the additive model, with follow-up testing of dominant and recessive models.

We compared *MBL2* variant frequencies in PICFLU to a population control cohort of healthy children (12–19 years old), from the National Health and Nutrition Examination Survey (NHANES) (35) who had MBL genotyping available (27). We stratified the comparison by the racial groups reported in NHANES (white non-Hispanic, Black non-Hispanic and white Hispanic). We tested for Hardy Weinberg equilibrium in each subgroup and compared frequencies using the allelic test (using Rx64 3.2.1 software package) (42).

In the severity analysis, we a utilized a whole genome association analysis toolset called PLINK (43) (available at https://pngu.mgh.harvard.edu/~purcell/plink/) that uses Chi-square or Fishers Exact tests to evaluate allele and genotype associations. We evaluated associations between the five individual *MBL2* alleles and severity phenotypes using the allelic test (42). Data were analyzed in the entire genotyped cohort, as well as in the major racial ethnic subgroup (white non-Hispanics) to exclude confounding by population admixture. Severity markers included the continuous PRISM-III admission illness severity score (33) (untransformed and Log transformed) and the dichotomous outcomes of ALI/ARDS, extracorporeal life support (ELS), bacterial co-infection, septic shock, and hospital mortality.

To control for multiple comparisons, we used a Bonferroni corrected p-value critical value of 0.0083 (0.05/6) adjusted for 5 *MBL2* variants making 6 haplotypes that were tested. Although this adjustment may not be sufficiently stringent given the number of phenotypes tested, the severity phenotypes were not independent.

A priori, we planned to test for interactions with other reported genetic associations in PICFLU, which was with

IFITM3 rs34481144 and mortality (11). IFITM3 rs12252 was not associated with influenza susceptibility or severity in PICFLU (30). We also recently reported higher mortality in children with influenza-MRSA co-infection with vancomycin monotherapy vs. dual anti-MRSA coverage (31). So we used Gaussian linear modeling to check for interaction with antibiotic therapy and associated alleles using the lm function in the R basic statistical library (**Supplemental Table 3**). We did not correct for multiple comparisons in these secondary analyses.

RESULTS

We enrolled and genotyped 420 children with confirmed influenza-related critical illness; 333 (80%) had influenza A infection (41% $H1N1_{pdm}09$, 15% H3N2, 23% other), and 81 (19%) had influenza B infection. **Table 1** lists the demographic characteristics and clinical course of the patients, including comorbid conditions and complications. Of the 420 children, 159 (38%) developed ALI/ARDS, 165 (39%) developed septic shock requiring vasopressors and 30 patients died (7%). Influenza-MRSA co-infection was present in 33 of 420 children in the cohort (8%) and was a risk factor for mortality ($p < 0.0001$) present in 37% of fatalities but only 6% of survivors. Influenza-MSSA co-infection was identified in 41 children (10%), but was present approximately equally (∼10%) in deaths and survivors ($p = 0.1$)

The subgroup of 265 patients enrolled prior to May 2014 with available MBL levels had similar demographics and clinical characteristics to the full cohort. The median MBL level was 1,076 ng/ml (interquartile range [IQR] 666–1,894 ng/mL; range 71–7,028). Forty-four patients had levels <500 and 3 patients had levels <100 ng/ml. As shown in **Figure 1B**, as would be expected wild type (HYA/HYA) diplotypes had the highest levels (median 2,155; IQR 1,698–2,488) and homozygotes for an "O" (B, C, or D) allele had the lowest levels (17, 38). Using multiple linear regression adjusted for sex, race and age, on average, each promoter L variant carried was predicted to decrease MBL levels by 12% ($p = 0.04$), each promoter X by 35% ($p < 0.001$), and each O allele (B, C, or D) by 56% ($p < 0.001$, full regression model shown in **Supplemental Table 1**).

The results of the *in vitro* complement fixation analysis are shown in **Figure 2**. Homozygote B/B or C/C serum had minimal fixation of C3c complement, including those patients with measured serum levels of >500. There were no D/D homozygotes to test. High MBL producing (wild type) controls (HYA/HYA) fixed complement at expected dilutions (17, 36, 37).

Targeted re-sequencing of the *MBL2* exons identified a rare deleterious variant in a white Hispanic patient who had an MBL serum level below the assay's detectable limits (<3.2 ng/mL). This patient was excluded from the gene-to-level analyses. The patient had an HYA/LYA genotype so was predicted to be a high-MBL producer (see **Figure 1B**). However, he was homozygous for rs74754826 which creates a premature stop codon at aa210 leading to null production of MBL. According to the gnomAD database (gnomAD r2.0.2), the variant is not usually identified in populations of European or Hispanic origin and is overall rare (frequency 0.0006, http://gnomad.broadinstitute.org/variant/10-54528016-C-A) with a relatively higher frequency in populations of African origin (MAF = 0.006). The patient was born prematurely and the respiratory viral culture grew cytomegalovirus at time of influenza diagnosis. The patient had recurrent upper and lower respiratory infections before admission and after discharge. We identified no other individuals with rare, potentially deleterious mutations in *MBL2*.

The results of the individual SNP (PBAT) and haplotype (HBAT) family-based analyses are shown in **Table 2**. Estimated statistical power in PBAT was acceptable for low MBL-producing variants (X, B, C, and D) but not for the L variant, which is in tight linkage with the other low-producing variants and has minimal influence on MBL levels by itself. From 633 available parents there were 252 nuclear families. Due to high linkage disequilibrium, the five variant alleles combined into six common (≥5% frequency) haplotypes with the X, B, C, and D alleles each represented by one haplotype (17, 38). No transmission disequilibrium was detected for MBL variants or haplotypes ($p \geq 0.06$ for all analyses).

Figure 3 shows the *MBL2* variant frequencies in the PICFLU cohort (for whom self-reported race and ethnicity were available, $n = 357$) compared to the NHANES pediatric population ($n = 1{,}142$) sub-grouped by race and ethnicity (data provided in **Supplemental Table 2** and individual patient data available upon request). No significant differences were identified (all $p > 0.05$). HWE was $p > 0.05$ in all subgroups except the X allele was out of HWE in the white Hispanic NHANES controls.

In the severity analyses, none of the individual *MBL2* variants were associated with overall illness severity (PRISM III score), or with frequency of shock requiring vasopressors, ALI, or bacterial co-infection (all $p > 0.05$). Carriage of the B missense mutation was higher in children that died. As shown in **Figure 4A**, in children that died 47% (14/30) carried at least one B mutation compared to 26% (100 of 390) survivors (OR 2.3, $p = 0.007$ overall; white non-Hispanics $n = 217$, OR 2.9, $p = 0.007$). The B mutation was not associated with the frequency of MRSA co-infection ($p = 0.65$, **Figure 4B**). MRSA co-infection was a risk factor for death, however, and 64% ($n = 7/11$) of children with influenza-MRSA co-infection that died carried at least one B mutation compared to 9% (2/22) survivors with influenza-MRSA co-infection (OR 14.5; $p = 0.0002$; see **Figure 4C**). In influenza-infected children who died without MRSA co-infection, 37% ($n = 7/19$) carried at least one B mutation compared to 27% ($n = 98/368$) of survivors (OR 1.3, $p = 0.49$, see **Figure 4D**).

The characteristics and clinical course of the 11 children that died with influenza-MRSA co-infection are shown in **Table 3**. All children received fluid resuscitation for severe shock and required vasopressors. Ten (91%) were supported via extracorporeal life support (ELS) before death. Although older age was associated with mortality (see **Table 1**), age was not associated with B allele carriage ($p = 0.48$) so it was not a confounder. The B allele was also not associated with *IFITM3* variant rs34481144 which we previously reported was associated with mortality in PICFLU (11).

TABLE 1 | Demographic and clinical characteristics, clinical course, and outcomes of the critically ill children in the PICFLU cohort including a comparison of those with fatal vs. non-fatal infection.

	Total	Fatal	Survived	*P*-Value^
	(N = 420)	(N = 30)	(N = 390)	
Female, N (%)	163 (38.8)	14 (46.7)	149 (38.2)	0.4
Hispanic, N (%)	113 (26.9)	11 (36.7)	102 (26.2)	0.2
Race, N (%)				0.3
White	305 (72.6)	22 (73.3)	283 (72.6)	0.9
African-American	64 (15.2)	2 (6.7)	62 (15.9)	
Asian	14 (3.3)	1 (3.3)	13 (3.3)	
Mixed or Other	37 (8.8)	5 (16.7)	32 (8.2)	
Age, years, med (IQR)	6.5 (2.4, 11.2)	10.1 (1.7, 14.2)	6.3 (2.4, 10.9)	0.09
Baseline Health Status*, N (%)				
Previously Healthy	226 (53.8)	17 (56.7)	209 (53.6)	0.7
Respiratory*	140 (33.3)	4 (13.3)	136 (34.9)	
Immune Compromised*	6 (1.4)	3 (10.0)	3 (0.8)	
Other*	177 (42.1)	13 (43.3)	164 (42.1)	
Support and Complications, N (%)				
Acute Lung Injury	159 (37.9)	23 (76.7)	136 (34.9)	**<0.0001**
Myocarditis	11 (2.6)	3 (10.0)	8 (2.1)	0.04
Encephalitis	10 (2.4)	0 (0)	10 (2.6)	1.0
Shock Receiving Vasopressors	165 (39.3)	138 (35.4)	27 (90.0)	**<0.0001**
Extracorporeal Life Support	46 (11.0)	17 (56.7)	29 (7.4)	**<0.0001**
PRISM Score, median (IQR)	5 (2, 11)	26.5 (11, 34)	5 (2, 10)	**<0.0001**
Bacterial Co-infection, N (%)**	133 (31.7)	18 (60.0)	115 (29.5)	**0.0005**
Methicillin-resistant *S. aureus*	33 (7.9)	11 (36.7)	22 (5.6)	**<0.0001**
Methicillin-resistant *S. aureus*	41 (9.8)	3 (10.0)	38 (9.7)	1.0
Streptococcus pneumoniae	16 (3.8)	1 (3.3)	15 (3.9)	1.0
Other Bacteria	50 (11.9)	3 (10.0)	47 (12.1)	1.0
Viral Co-infection, N (%)	94 (22.6)	6 (20.0)	88 (22.6)	0.7
Influenza Type, N* (%)**				
Influenza A	333 (79.7)	22 (73.3)	311 (80.2)	0.4
$H1N1_{pdm}09$	170 (40.5)	12 (40.0)	158 (40.5)	
H3N2	68 (16.2)	4 (13.3)	64 (16.4)	
Other or no subtype	95 (22.6)	6 (20.0)	89 (22.8)	
Influenza B	81 (19.3)	8 (26.7)	73 (18.7)	0.3
Influenza A and B	2 (0.5)	0 (0)	2 (0.5)	

*Children could have multiple underlying medical conditions and **multiple bacterial co-infections.

***4 patients were Influenza positive with subtype unknown.

^p-values compare Fatal vs. Survived groups. Bolded values are statistically significant.

We previously published an association between vancomycin monotherapy and death in influenza-MRSA co-infected patients (31), therefore in a secondary analysis we evaluated antibiotic monotherapy as a potential explanation for the association of the B mutation carriage and death. There was no association between carriage of a B mutation and the number (monotherapy vs. two or more) of anti-MRSA antibiotics received in the first 24 h of PICU admission ($p = 0.44$). Multiple logistic regression confirmed an association between anti-MRSA monotherapy and increased mortality ($p = 0.007$) and an independent mortality association with carriage of one B mutation ($p = 0.0007$). There were only two BB homozygotes with MRSA co-infection and both died, precluding comparison.

DISCUSSION

In North American children and adolescents admitted to the PICU with influenza virus infection during the 2008 to 2016 influenza seasons, *MBL2* variants predicting low MBL levels did not explain disease susceptibility. The four *MBL2* low-producing variants did influence MBL serum levels and the B and C alleles decreased MBL functional activity as previously described (17, 18, 38). Carriage of low-producing *MBL2* variants was not associated with higher disease severity on admission, with development of ALI or shock, or with diagnosis of bacterial co-infection. MBL deficiency does not appear to confer higher risk for severe influenza infection or influenza-related complications.

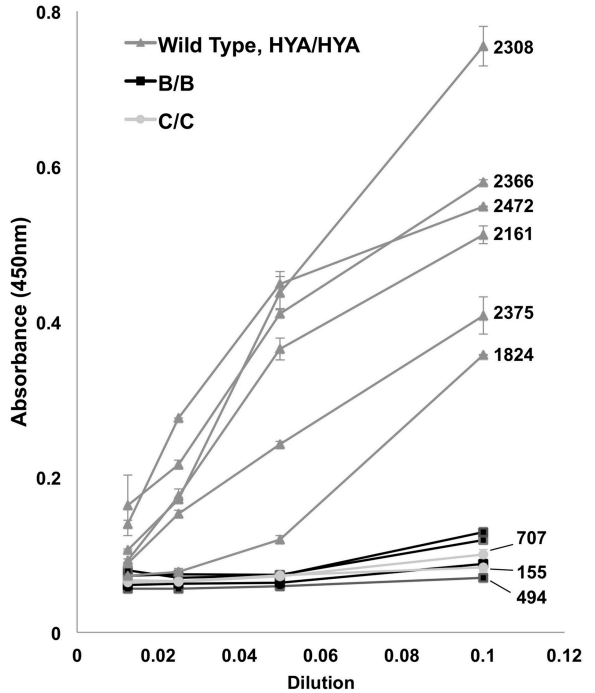

FIGURE 2 | Complement (C3c) fixation by ELISA. Controls (HYA/HYA) fixed complement at typical absorbance (450 nm) measurements despite serial dilutions. Homozygotes [$rs1800450_{Gly54Asp}$(B/B) and $rs1800451_{Gly57Glu}$(C/C)] had markedly reduced complement activation. Bars above and below data points indicate standard deviation. Patients with measured serum levels available have numerical (ng/mL) levels labeled on right. There were no $rs5030737_{Arg52Cys}$(D/D) homozygotes in the population.

TABLE 2 | Results of the Family Based Association Test Analysis in children with influenza using PBAT for individual SNPs and HBAT for haplotypes.

SNP (allele)	Frequency	Families	*Z*-Score	*P*-value^	Power*
H (rs11003125)	0.39	166	−1.74	0.08	0.05
L	0.61		+1.74		
Y (rs7096206)	0.84	126	−0.34	0.73	1.00
X	0.16		+0.34		
B (rs1800450)	0.14	104	+1.66	0.10	1.00
Wild Type	0.86		−1.66		
C (rs1800451)	0.05	36	−1.76	0.08	1.00
Wild Type	0.95		+1.76		
D (rs5030737)	0.05	51	−0.14	0.89	1.00
Wild Type	0.95		+0.14		
Haplotype	**Frequency**	**Families**	***Z*-Score**	***P*-value^**	
HYA	0.34	158	−1.73	0.08	–
LYA	0.25	151	0.75	0.45	–
LXA	0.16	121	0.53	0.60	–
LYB	0.14	97	1.88	0.06	–
LYC	0.05	31	−1.72	0.09	–
HYD	0.05	45	0.17	0.87	–

**Calculated using the Additive Model in PBAT.*
^p-values are using the Additive Model.

We found MBL did not increase susceptibility to severe influenza infection in pediatric patients. Overall, the literature on whether low MBL predisposes to severe infections is conflicting. A systematic review and meta-analysis of studies published before 2013 (23) reported increased risk of sepsis susceptibility in children carrying an "O" (B, C, or D) variant and in white adults carrying a B mutation (23). Subsequently, a large study of 1,839 European adults with sepsis from community acquired pneumonia (viral and/or bacterial) or peritonitis showed no association between low-producing *MBL2* variants and infection susceptibility or severity (44). Similarly, a meta-analysis of 5 pediatric studies showed that the B allele was not a risk factor for recurrent respiratory infections in children (45). A meta-analysis of 7 neonatal studies (mostly premature infants) associated low MBL, using levels and predictive variants, with development of sepsis (46). Population-based birth cohort studies showed no increased risk of invasive (bacteremia or meningitis) meningococcal (47) or pneumococcal (48) disease in Danish children carrying low-producing *MBL2* variants. A large cohort of hospitalized children with meningococcemia also did not have higher carriage of O alleles (49). In single center nested case-control PICU studies, we reported no differences in carriage of low-producing *MBL2* variants in patients with severe infections (22) but others reported a 2-fold overall increase in carriage of the O allele with an increase in homozygotes (50). Although *MBL2* haplotypes associated with deficiency appear to be a risk factor for a range of infections in neonates or other immune compromised patients, our study adds to the literature that carriage of low-producing *MBL2* variants does not increase risk of severe viral infections (51). We did identify one child carrying an extremely rare, highly deleterious mutation in *MBL2*, further adding to the literature that rare variants play some role in severe influenza infection susceptibility (8).

We report an association with influenza virus-related mortality and increased carriage of the B mutation in PICFLU, primarily limited to children with MRSA co-infection. The study that stimulated us to evaluate MBL was a pediatric autopsy series reporting of increased carriage of the B mutation in children dying from influenza-MRSA co-infection (27). The autopsy cohort had no survivors for comparison, but 56% (n = 5/8) of children dying from influenza-MRSA carried a B mutation (27), significantly higher than the ∼14% expected across the population. Similarly, in PICFLU 64% of children dying from influenza-MRSA co-infection carried the B allele (n = 7) compared to 9% of children who survived the co-infection. However, the number of influenza-MRSA deaths carrying the B allele across both studies is only 12, precluding any strong conclusions. The association should not be specific to MRSA. Carriage of the B allele was not higher overall in children with influenza-MRSA co-infection. Prior reports of the association of MBL deficiency and death have not been replicated. Eisen et al. combined individual patient data from 4 studies in a meta-analysis, using <500 ng/uL to define deficiency, and reported that deficiency increased the risk of death from *Streptococcus pneumoniae* (52). A subsequent study did not replicate this finding (44). The complement system is redundant, which may lead to contradictory results from evaluation

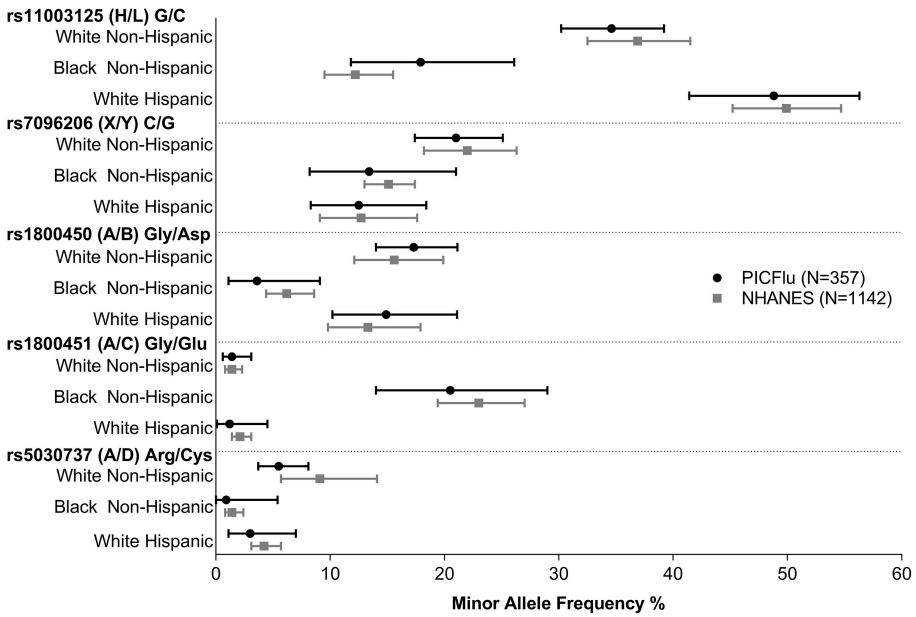

FIGURE 3 | PICFLU MBL variant frequencies, compared with NHANES pediatric population reference, by ethnicity/racial subgroups. Frequencies of all variants were similar ($p > 0.05$ for all comparisons). The calculated 95% confidence interval around the allelic point estimate is shown.

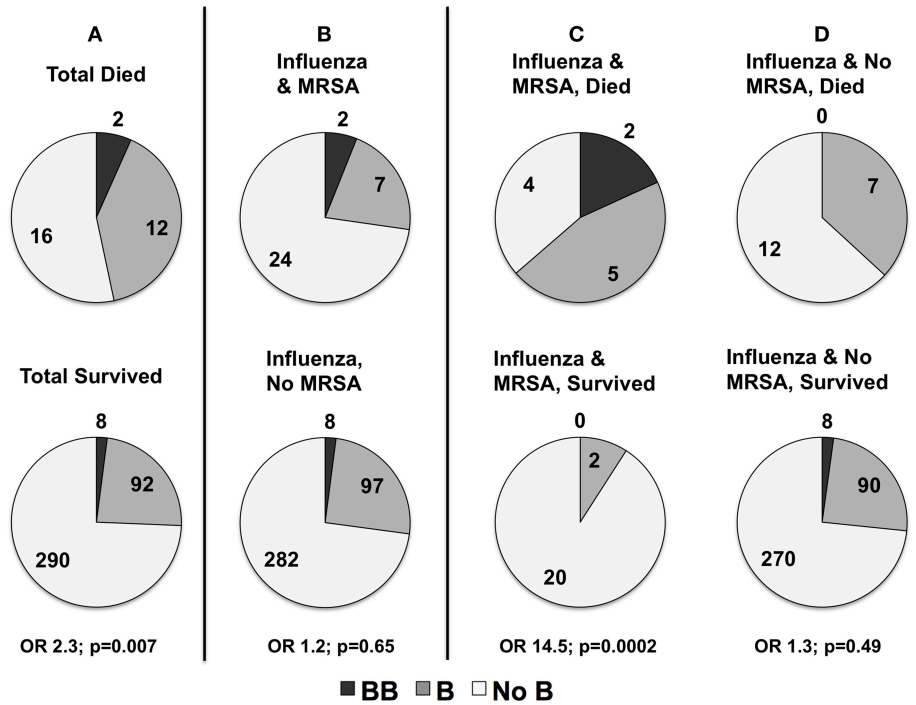

FIGURE 4 | (A) Distribution of rs1800450$_{Gly54Asp}$(B) in children with influenza critical illness who died or survived (numbers shown) to hospital discharge in all children ($n = 420$). Mortality was higher in all children carrying at least one copy of the rs1800450$_{Gly54Asp}$(B) allele ($p = 0.007$). **(B)** Distribution of rs1800450$_{Gly54Asp}$(B) in children with and without methicillin-resistant *Staphylococcus aureus* (MRSA) co-infection. There was no association with risk of contracting MRSA co-infection ($p = 0.65$). However, sub-analysis of deaths in patients with MRSA co-infection compared to those without MRSA co-infection showed a strong association of rs1800450$_{Gly54Asp}$(B) with mortality in the patients with MRSA co-infection. **(C)** rs1800450$_{Gly54Asp}$(B) Association with MRSA Co-infection Died vs. MRSA Co-infection Survived ($p = 0.0002$). **(D)** No MRSA Died vs. No MRSA Survived ($p = 0.49$).

TABLE 3 | Characteristics and *MBL2* genotype and MBL level (when available) of children with fatal influenza MRSA co-infection in the PICFLU cohort.

Age group/gender	Influenza type	*MBL2* haplotype	*MBL2* mutation	MBL level	Risk factors^
13–18 yrs/Male	Influenza B	HYA/LYB	rs1800450 (B)	1063*	None
13–18 yrs/Female	Influenza A H3	HYA/LYB	rs1800450 (B)	Not available	None
13–18 yrs/Male	Influenza B	LXA/LYB	rs1800450 (B)	666*	None
13–18 yrs/Male	Influenza B	LXA/LYB	rs1800450 (B)	570	None
13–18 yrs/Male	Influenza B	LYB/LYC	rs1800450 (B), rs1800451 (C)	338*	None
5–12 yrs/Male	Influenza A(H1N1)pdm09	LYB/LYB	rs1800450 (B/B)	1025*	None
13–18 yrs/Male	Influenza A(H1N1)pdm09	LYB/LYB	rs1800450 (B/B)	Not available	None
13–18 yrs/Male	Influenza A H3	LYC/LYC	rs1800451 (C/C)	707	None
13–18 yrs/Female	Influenza B	HYA/HYA	none	1417*	None
5–12 yrs/Male	Influenza A(H1N1)pdm09	LYA/LYA	none	1042**	None
13–18 yrs/Male	Influenza A(H1N1)pdm09	LYA/LYA	none	708*	Asthma

Carriage of one or more copies of the rs1800450(B) allele was present in 7 of the 11 children.

^All patients were intubated, and met criteria for both shock and ARDS. All patients except one received extracorporeal life support (ELS) prior to death.

**MBL levels measured after initiation of ELS or **unknown when MBL level measured in relationship to ELS initiation.*

of MBL2 variants across different populations. Contradictory results could also be because increased inflammation from complement activation from high-MBL production could make low-producing MBL2 genotypes protective (53). However, in contrast to reports in adult patients, we saw no evidence of increased lung disease severity (25, 54). or organ dysfunction (55) in children and adolescents with higher MBL. Overall, MBL levels in our population were higher than expected for carriers of an O allele (56), which are usually predicted to be <200 ng/ml (21). This may be secondary to fresh-frozen plasma administration (29) during critical illness or from inflammatory phase elevation.

The PICFLU cohort is a major strength of this study, including children and adolescents with influenza critical illness from multiple centers with rigorous diagnostic testing and phenotyping. The majority of children were previously healthy, including almost all of those that died, increasing the ability to identify genetic influences in severe influenza infection. The inclusion of parental DNA facilitated family-based association testing. Use of sera from the influenza-infected homozygote patients allowed us to verify that variant MBL markedly decreased complement activity. We used a rigorous diagnosis of bacterial co-infection requiring microbiologic confirmation and clinical diagnosis. Although we did not have sufficient sera to demonstrate decreased anti-influenza activity in low-MBL producer variants, this has been shown previously (13). This study was *a priori* designed evaluate the association of MBL and other candidate genes with influenza infection, and we did not have consent to perform whole genome or whole exome sequencing (11, 30). We also did not evaluate other proteins in the lectin pathway of compliment activation (57). Because most PICFLU patients were white and not Hispanic, our findings are generalizable mainly to that group. Unfortunately, we were unable to identify a similar prospectively enrolled cohort of children with influenza-related critical illness for further independent validation of our findings, which is a major limitation.

In summary, we conclude that MBL deficiency is not a risk factor for very severe influenza infection in children and adolescents. Children predicted to have MBL deficiency were not at higher risk of more severe critical illness or development of influenza-associated complications such as ALI or bacterial co-infection. We did confirm a previously reported association of higher carriage of the B allele in children that died from influenza-MRSA co-infection, but our confidence in this finding is low due to the small number of patients. It must be noted, that the majority of critical illness from influenza virus at PICFLU sites is preventable by vaccination (58), which in addition to supportive care and influenza antivirals is currently the most effective way to decrease influenza-related mortality.

PRELIMINARY VERSIONS OF THIS WORK HAS BEEN PRESENTED AT THE FOLLOWING MEETINGS

PAS 2016: "Mannose-Binding Lectin and Susceptibility to Pediatric Influenza-Related Critical Illness." Pediatric Academic Societies Annual Meeting, Baltimore, MD. April 2016. Abstract #750580.

SCCM 2018: "Association of Mannose-Binding Lectin with Influenza Critical Illness in Children." Society of Critical Care Medicine Annual Meeting, San Antonio, TX. February 2018. Abstract #41.

ETHICS STATEMENT

This study was carried out with approval of the Boston Children's Hospital and all participating site Institutional Review Boards with written informed consent from at least one parent or guardian on behalf of the child, and parents consented for their own participation, in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Boards at each enrolling hospital listed in the acknowledgments.

AUTHOR CONTRIBUTIONS

EL, W-KY, MS, YZ, PALISI PICFLU Investigators, and AR made substantial contributions to the conception or design of the study. EL, W-KY, MS, JF, AM, MN, YZ, HS, GM, AnS, LL, SW, MH, NC, AdS, KT, PALISI PICFLU Investigators, and AR made contributions to the acquisition, analysis, and interpretation of the data for this work. EL, W-KY, MS, and AR drafted the work and JF, YZ, HS, GM, SW, MH, and KT revised it critically for important content. EL, W-KY, MS, JF, AM, YZ, HS, GM, AnS, LL, SW, MH, NC, AdS, KT, PM, PALISI PICFLU Investigators, and AR gave final approval of the version to be published. This work represents the findings and conclusions of the authors and does not necessarily represent the official position of the Centers for Disease Control and Prevention or the National Institutes of Health.

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REVIEW
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Epigenetics in Sepsis: Understanding Its Role in Endothelial Dysfunction, Immunosuppression, and Potential Therapeutics

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Sepsis has a complex pathophysiology in which both excessive and refractory inflammatory responses are hallmark features. Pro-inflammatory cytokine responses during the early stages are responsible for significant endothelial dysfunction, loss of endothelial integrity, and organ failure. In addition, it is now well-established that a substantial number of sepsis survivors experience ongoing immunological derangement and immunosuppression following a septic episode. The underpinning mechanisms of these phenomena are incompletely understood yet they contribute to a significant proportion of sepsis-associated mortality. Epigenetic mechanisms including DNA methylation, histone modifications, and non-coding RNAs, have an increasingly clear role in modulating inflammatory and other immunological processes. Recent evidence suggests epigenetic mechanisms are extensively perturbed as sepsis progresses, and particularly play a role in endothelial dysfunction and immunosuppression. Whilst therapeutic modulation of the epigenome is still in its infancy, there is substantial evidence from animal models that this approach could reap benefits. In this review, we summarize research elucidating the role of these mechanisms in several aspects of sepsis pathophysiology including tissue injury and immunosuppression. We also evaluate pre-clinical evidence for the use of "epi-therapies" in the treatment of poly-microbial sepsis.

Keywords: sepsis, epigenetics, immunosuppression, endothelial dysfunction, histone deacetylase inhibitors

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INTRODUCTION

An Overview of Sepsis Pathophysiology

Sepsis is a syndrome with a broad clinical manifestation, defined by The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) as "a life-threatening organ dysfunction caused by dysregulated host responses to infection" (1). Due to the numerous possible presentations, sepsis can be a difficult clinical condition to recognize, especially during the early stages if patients exhibit non-specific symptoms of being unwell (1–4) or if archetypal signs of infection are absent, e.g., in young infants, the elderly, and the immunocompromised (5–8). Signs which are highly suggestive of sepsis include (but are not limited to) acute confusion, hypotension, tachycardia, and tachypnoea, hypoxia, reduced urine production, a high blood lactate level and a non-blanching rash. Only one of these signs may be present, and none are unique to sepsis (9).

Screening tools have been developed to aid identification of patients who are seriously ill with suspected sepsis (10–12). An example of one such tool, the Sequential Organ Failure Assessment (SOFA) score, codifies the progression of sepsis-related organ failure (13). However, despite these efforts to improve diagnostics, sepsis still can be missed, leading to delays in treatment which can dramatically worsen outcomes. Rapid administration of antibiotics is critical; for every hour of delayed treatment, mortality risk increases by 7.6% (14). The development of tests that accurately predict the onset of sepsis before organ failure occurs would be useful for improving outcomes. The broad manifestation and rapid onset of sepsis make this very challenging, but it nevertheless continues to be an active area of research (15–22).

Infection-driven inflammation causes substantial tissue injury and organ dysfunction during acute sepsis and represents a major cause of mortality (22, 23). The binding of commonly expressed, conserved pathogen antigens (pathogen-associated molecular patterns, PAMPs) to pattern recognition receptors activates NF-κB signaling and promotes transcription of a wide range of pro-inflammatory factors (24–26). The endothelium becomes activated, increasing its permeability as well as the adherence and migration of leucocytes (27). Loss of endothelial integrity drives intravascular leak, hypotension, and widespread oedema (27, 28). The production of damage-associated molecular patterns (DAMPs) from host cells feeds the inflammatory response, resulting in more tissue injury, and thereby, creating a vicious circle. Concurrent to this, cytokines with anti-inflammatory properties are produced in efforts to promote resolution of inflammation and tissue repair; antigen presenting cells become less responsive to lipopolysaccharide (LPS) and other PAMPs, widespread apoptosis of leucocytes is observed, and myeloid-derived suppressor cells (MDSCs) are substantially increased (9, 29). It was once thought that acute hyperinflammatory responses preceded an immunosuppressive phase, however, it is now believed that there two phases can exist simultaneously (30).

Individuals who clear infection can still exhibit protracted, deranged immune responses following a septic episode. Persistent inflammation, immunosuppression, and catabolism syndrome (PICS), whilst not a universal phenomenon in sepsis, describes a clinical syndrome that patients with longer ICU stays can exhibit (31, 32). One characteristic of PICS is increased susceptibility to opportunistic infections and reactivation of latent viruses, which contributes to morbidity and mortality after the initial infective insult has resolved (33, 34). The factors which contribute to PICS are multi-factorial but the significant risk of rehospitalization with infection may suggest an ongoing perturbation of the immune response (35). Indeed, a study by Arens et al. demonstrated persistent immunoparalysis weeks to years after sepsis (36). There is a paucity of studies which investigate the persistence of PICS after hospital discharge, however, it is notable that sepsis survivors have a significantly reduced survival rate over the years following acute infection vs. age matched individuals, occurring independently of health status preceding the septic episode (37–39). The increased death rate may result from persisting sequalae of sepsis; e.g., increased frailty, irreversible impairment in organ function and/or from sepsis-associated, sustained changes in immune function e.g., immunosuppression. Work is underway to elucidate the mechanisms behind these modifications, with some studies suggesting they arise from changes to the epigenome of leucocytes.

Epigenetics: Definition and Mechanisms

Epigenetics refers to the regulation of gene expression not caused by underlying changes in DNA sequence (40). In eukaryotes, DNA forms a stable structure with octomers of histone proteins; this stable structure is known as chromatin [**Figure 1**, (41)]. The "openness" of chromatin structure affects the accessibility of DNA to transcription factors and RNA polymerase II, and is therefore a key factor in determining the rate of mRNA expression (42). Three major epigenetic mechanisms are described, two of which exert their effect by influencing chromatin compaction (see **Figure 1**). DNA methylation is a modification of cytosine residues mainly in the context of cytosine-guanine (CpG) motifs. The majority of the mammalian genome is CpG poor, with enriched regions occurring at transcriptional regulatory loci such as promotors and enhancers (termed CpG islands). Around 60–70% of promotors contain CpG islands (43). The second mechanism is histone modification; post-translational modifications of the amino acids in the tail region of histone proteins which include acetylation, phosphorylation, ubiquitylation, and methylation. Modifications of amino acids at specific locations in the protruding tails either strengthen or weaken the interaction between DNA and histones. The final mechanism involves non-coding RNAs (ncRNAs), which can modulate gene expression by binding to either sites in the genome to prevent gene transcription or mRNA transcripts to prevent translation (44, 45).

There is growing evidence that modifications of the epigenome impacts the phenotype of immune cells in such a way as to affect responses to infection, and are involved in propagating inflammatory disorders (46, 47). Whilst most epigenetic marks are generally stable over time, those at certain loci show high plasticity in response to environmental factors such as smoking, diet, and disease, making them of interest in the context of various pathologies (48–50). The rewritable nature of epigenetic modifications and the responsiveness of epigenetic enzymes to inhibitor therapy creates great potential for this avenue of treatment in patients both with chronic and acute inflammatory diseases such as sepsis.

In this review, the epigenetic modifications associated with various stages of sepsis will be discussed. Specifically, we cover mechanisms involved in endothelial dysfunction during the hyperinflammatory response and those underpinning aspects of immunosuppression in PICS. The pre-clinical evidence for use of epi-therapies will also be described.

SEARCH STRATEGY

References were identified through Ovid using search terms ("sepsis" OR "septic shock" OR "endotoxin tolerance (ET)") AND ("epigenomics" OR "epigenetic" OR "DNA methylation"

FIGURE 1 | (A) Histone modifications: The negative charge of DNA allows it to bind tightly to positively charged histone proteins. DNA wraps around octomers of histone proteins and forms discrete units known as nucleosomes, the basis of chromatin. The overall structure and openness of chromatin is dictated by chemical modifications of the N terminal amino acid tails of the histone proteins. Chemical modifications include acetylation, methylation, phosphorylation, SUMOylation, citrullination, and ADP-ribosylation. **(B)** CpG methylation: The majority of cytosines found in cytosine-guanine dinucleotides (gray circles) are methylated. CpG-rich sections of the genome (CpG islands) occurs in areas requiring transcriptional control e.g., retrotransposons and gene promotors. Here, methylation status is more dynamic, with some hypomethylated CpGs (white circles) facilitating promotor accessibility and gene transcription. **(C)** Small non-coding RNAs interact with complementary sequences in DNA and on mRNA to interfere with gene transcription and translation respectively. A well-known species of small RNAs are microRNAs. Mature single stranded microRNAs molecules (21–24 nt long) are incorporated into the RNA induced silencing complex (RISC) and then bind to a complementary sequence in the 3'UTRs of mRNA molecules. This binding inhibits mRNA translation and results in either mRNA degradation or storage.

OR "Histone modifications" OR "histone" OR "non-coding RNA" OR "micro RNA"). Bibliographies of papers of interest were searched by hand to identify additional studies. Relevant papers identified in the database were included.

EPIGENETIC CHANGES ASSOCIATED WITH SEPSIS PATHOPHYSIOLOGY

Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs) as Regulators of Inflammation

Histone acetylation is a key process involved in regulating inflammatory response genes (51, 52). Addition or removal of acetyl groups is mediated by two families of antagonistic enzymes, histone acetyltransferases (HATs), and histone deacetylases (HDACs). There are numerous studies that associate levels of histone acetylation with expression of pro-inflammatory cytokines and other anti-microbial products (52). Therefore, understanding the relative activities of these two enzymatic groups has great relevance to sepsis. To date, five families of HATs enzymes have been discovered. Using acetyl-CoA as a substrate, these enzymes target primarily lysine residues on histones 3 and 4 (53). In humans, 18 HDACs have been discovered, grouped into four classes based on sequence homology with their yeast counterparts. Classes I, II, and IV represent the "classical" HDACs and are the most extensively studied. Class III HDACs, otherwise known as sirtuins, utilize a distinct mechanism for lysine deacetylation requiring NAD+ as a substrate, in contrast with classical HDACs which are Zn^{2+}-dependent metalloproteases (54).

Whilst acetylation is generally considered a pro-transcriptional modification, increasing evidence suggests this is an over-simplistic view and that the effect of acetylation on chromatin structure is in fact site-specific (55, 56). Therefore, the roles of these enzymes in transcriptional regulation is likely to be highly complex and requires detailed elucidation as they are pursued as targets of therapeutics. In addition to histones, HATs, and HDACs have multiple non-histone targets that are critical for a range of cellular processes including metabolism and cell cycle (54).

Epigenetic Changes Associated With Endothelial Dysfunction, Tissue Injury, and Organ Failure in Sepsis

Endothelial damage, as a result of an excessive cytokine response, is one of the initiating steps that ultimately leads to sepsis-associated organ dysfunction. Other than provision of fluids and use of inotropic drugs, there are no interventions available to restore loss in arterial partial pressure and organ perfusion (57). During sepsis, the endothelium is activated and adhesion molecules including ICAM, VCAM, and E-selectin are upregulated (58, 59). These adhesion molecules are critical for leucocyte infiltration into tissues. Entry of neutrophils into the endothelium in particular has been paradoxically associated with both containment of infection and exacerbation of tissue injury (60). Besides adhesion molecule upregulation, endothelial cell junctions become "loose," leading to an increase in permeability and a loss of fluid from the vascular system into the surrounding tissues. Whilst neither of these processes are pathological in themselves, the extent to which they occur in sepsis is a major driver of organ failure. Therefore, stabilizing endothelial disruption could be an effective avenue of therapeutic intervention in sepsis.

Loss of histone acetylation during acute lung injury may partially drive the over-expression of adhesion molecules and regulate endothelial permeability. Acetylation loss at the

promotors of *Angp1*, *Tek,* and *Kdr*–genes with critical roles in both Tie2/Angiopoietin and vascular endothelial growth factor (VEGF/VEGFR) signaling cascades–was observed in lung and extra-pulmonary organs in a mouse sepsis model (61). Loss of acetylation was suggested to be responsible for a significant reduction in gene expression 6 h post-induction of sepsis and for increased albumin leak. Despite the limitations of this study [as discussed in detail by Bataille et al. (62)], these findings highlight a potential mechanism by which inflammatory factors can influence epigenetic regulation and drive maladaptive changes in endothelium. Indeed, ICAM-1, and E-selectin expression are markedly reduced in the lungs of mice with poly-microbial sepsis if they are pretreated with histone deacetylase inhibitors (HDACi) (63, 64). Neutrophil infiltration and albumin leak were both minimized and associated with improved survival.

Other epigenetic mechanisms are also implicated in mediating tissue injury [extensively reviewed in (44)]. Perturbation of several micro RNAs (miRNAs) during sepsis has been described in plasma and the endothelium (65). MiR-181b expression in endothelial cells minimizes leucocyte invasion of tissues by reducing expression of adhesion molecules, mediated by suppression of NF-κB signaling (66). Injection of miR-181b mimics in a mouse model of endotoxemia downregulated VCAM-1 in the lung and reduced leucocyte adhesion and lung histopathology scores (66). Interestingly, 24 intensive care patients with sepsis have lower circulating levels of miR-181b than those with other inflammatory conditions, suggesting this mechanism is particularly pertinent in driving sepsis-associated overexpression of adhesion molecules (66). Suppression of NF-κB signaling by miRNAs may also confer protective effects in other organs. Animal models of sepsis-associated cardiac dysfunction have shown that miR-146a expression can attenuate NF-κB activation and inflammatory responses in both the myocardium and peripheral blood, changes associated with improved survival (67–69). Further mechanistic studies would be helpful to fully elucidate the functions of these miRNAs, and beyond this should explore whether their therapeutic modulation would be of benefit in sepsis.

Persistent Inflammation, Immunosuppression, and Catabolism Syndrome (PICS)

Immunosuppression in critically ill patients was first noted in 1970's when it was discovered that these patients did not develop delayed hypersensitivity responses to common antigens (70). It is now recognized that ongoing immunological disturbance following sepsis occurs in a subset of patients, keeping them in intensive care with a milieu of symptoms despite clearance of initiating infection. These symptoms are collectively referred to as PICS. Individuals may exhibit inappropriately elevated protein catabolism (leading to loss of lean body mass and thus increased frailty), poor wound healing, an increased susceptibility to infection, and prolonged immunosuppression. Current prognosis is poor with many requiring extensive stays in intensive care and high rates of mortality (71). Understanding of the mechanistic features that drive immunosuppression is essential and likely to involve epigenetic elements.

Expansion of Myeloid-Derived Suppressor Cells (MDSCs)

Several studies have highlighted the extensive apoptosis of immune cells during acute sepsis as a prominent driver of subsequent immune dysfunction. Besides a depletion in sheer numbers of cells, several studies have noted functional abnormalities in the remaining subsets of the immune system. The proportional number of regulatory T-cells (Tregs) and other immunosuppressor subsets is significantly increased in patients with PICS. MDSCs are a subset of immature myeloid cells with highly immunosuppressive properties [reviewed extensively by Schrijver et al. (72)]. Specifically, their production of arginase-1, reactive oxygen species, TGF-β, and IL-10 critically suppress T-cell and NK cell function (73). These cells are largely absent in healthy individuals but form a major component of tumor micro-environments in cancer and are detectable in blood following sepsis (74). MDSCs associate with deleterious outcomes and are highly elevated in patients with PICS. Their considerable expansion following sepsis and function as immunosuppressors make understanding of their development an important area of research.

Epigenetic modulation of myeloid progenitors may explain the disproportional expansion in MDSCs. Transcriptional regulators in these cells are precisely controlled by numerous epigenetic mechanisms. Regulation of nuclear factor 1A (NFI-A) has been shown to be critical for myeloid cell differentiation (75). MiR-181b and miR-21, through a synergistic mechanism, negatively modulate NFI-A expression in mice subjected to cecal ligation and puncture (76). In this study, miR-181b and miR-21 were both upregulated in bone marrow, and blockade of these miRNAs substantially impeded MDSC expansion, improved the capacity of these mice to clear peritoneal infection, and increased survival. Other transcription factors, including C/EBP-β and Runx1, are also epigenetically regulated and drive MDSC expansion. HDAC11 is recruited to the C/EBP- β promoter and negatively controls its expression; in knockout models, loss of HDAC11 significantly increases MDSC populations (77). Whether HDAC11 is upregulated during sepsis is unclear. MDSC differentiation and suppressive capacity can also be altered by regulation of miR-9 which in turn exerts its effect by regulation of Runx1 (78). Elevation of miR-9 expression during sepsis is not confirmed, however it should be noted that it is inducible by LPS and several pro-inflammatory cytokines (79), making a strong case for activity during sepsis. Whether targeting the mechanisms that drive MDSC development could be of therapeutic benefit in sepsis is an unanswered question. The miRNAs and acetylation enzymes highlighted here have multiple targets and their inhibition may have other undesirable effects. Furthermore, specific targeting of regulatory mechanisms in these cells alone may prove a challenge. However, further exploration of this area is undoubtedly warranted.

Endotoxin Tolerance as a Mechanism Mediating Immunosuppression

The second notable aspect of immunosuppression is the hypo-responsiveness, particularly of innate immune cells, to subsequent challenge. ET is a well-described clinical phenomenon whereby pro-inflammatory responses to LPS are repressed during secondary encounter. Not all refractory responses are necessarily harmful–acute modulation of pro-inflammatory responses may in fact be beneficial during early sepsis. However, protracted suppression has detrimental consequences, potentially making patients more vulnerable to Gram-negative infections. Several epigenetic mechanisms have been linked to the persistence of ET (80, 81). Elevated miR-221 and miR-222 following prolonged LPS exposure were recently found to have a role in regulating Brahma-related gene 1, which in turn mediated transcriptional silencing of several pro-inflammatory products (80). In addition, failure to induce pro-transcriptional histone modifications–namely, acetylation and tri-methylation of histone 3– was shown by Foster et al. to repress pro-inflammatory gene expression on secondary LPS encounter, whilst leaving anti-microbial, and metabolic gene expression intact (81). Other studies have also demonstrated alterations in promotor histone profiles during sepsis–including loss of marks of active transcription–downregulating genes involved in pro-inflammatory responses and antigen presentation (82).

Some of the epigenetic machinery responsible for modifying histones has been demonstrated to be directly regulated by LPS [reviewed in (9)], potentially providing a mechanistic link between epigenetics and immune regulation. Expression of histone demethylase enzyme, JMJD3, was shown to be induced by LPS stimulation via NF-κB signaling in macrophages (83, 84). Furthermore, the activity of HAT and HDAC enzymes can also be modulated by LPS, although the extent to which their activity contributes to ET remains unclear. CREB-binding protein (CBP), a transcriptional co-activator with HAT activity, is critically involved in NF-κB signaling and regulation of the inflammatory responses (85). LPS exposure increases CBP stability by stimulating the removal of ubiquitin and blocking proteosomal degradation (86). This in turn correlates with increased histone acetylation and cytokine release. Stabilization of histone acetyltransferase HBO1 via a similar mechanism has also been reported (87). Conversely, sirtuins (class III HDAC) have demonstrable suppressive roles in cytokine regulation. Sirtuin 1 (SIRT1) rapidly accumulates at the proximal promotors of TNFα and IL1B following LPS stimulation and induces facultative heterochromatin formation thus silencing gene expression (88). In the same study, SIRT1 was additionally shown to deacetylate (and deactivate) the transcription factor NF-κB p65, a critical inducer of inflammatory signaling, preventing further transcription of pro-inflammatory genes. Another prominent sirtuin family member, SIRT6, can also act as an inflammatory repressor by deacetylating histone 3 at lysine 9 (H3K9) and inducing heterochromatin formation at NF-κB target gene promoters (89). In addition to acetylation changes, methylation and the enzymes which regulate methylation state are observed to negatively regulate expression of some of pro-inflammatory gene loci such as TNFα during sepsis immunosuppression (90).

Cytokines, TNF-α and type I interferons, have also been shown to modulate monocyte responsiveness to LPS through changes in the epigenome. Pre-treatment of monocytes *in vitro* with TNF-α prior to LPS stimulation was shown to block accumulation of euchromatin-associated H4ac and H3K4me3 at promotor regions of NF- κB target genes (90). When stimulated with LPS pre-treated monocytes had significantly lower pro-inflammatory mRNA expression than those without prior TNF-α exposure. Conversely, type I interferons propagated LPS responses by priming chromatin to respond, heightening sensitivity to weak upstream signaling. The ability of the immune response to self-modulate may represent a beneficial protective mechanism in the short-term. It is the timing and extent of the immunosuppression, specifically an inappropriate continuation once infection has cleared, which generates harm. It is notable that in most of these studies a very small selection of cytokines have been investigated (typically TNF-α and IL-6). Furthermore, no studies have characterized the persistence of epigenetic modifications, for example, in re-hospitalized patients after sepsis. Therefore, the ability of these described changes to potentiate long term suppression is difficult to assess. Repression of pro-inflammatory cytokines represents only one element of immunosuppression. Therefore, the contribution of epigenetic modulation of immune function to overall patient outcome remains to be fully elucidated.

Additional Epigenetic States of Potential Relevance to Sepsis

Other epigenetic states have been associated with modulation of immune function and may be pertinent to inflammatory disorders such as sepsis. Trained immunity in innate cells was reported in 2011 by Netea et al. (91), defined as a heightened immune response to secondary challenge following sub-lethal exposure to an initial stimulus. This phenomenon was subsequently linked to deposition of permissive histone modifications, H3K4me3, at promotors of *tnfα*, *il6,* and *tlr4* in monocytes following antigen exposure (92). Primed monocytes were found to mount a stronger pro-inflammatory cytokine response during secondary challenge. Priming with other antigens such as fungal β-glucan has also been shown to increase H3K4me3 occupancy at pro-inflammatory gene promotors and correlate with increased cytokine release (93).

The induction of ET or trained immunity appears to be dependent on the microbial stimulus itself and antigen concentration (94). Stimulation of monocytes via Nod Like Receptor (NLR) or Toll Like Receptor (TLR) pathways resulted in unique effector functions, epigenetic and metabolic profiles (95, 96). Whilst TLR stimulation via LPS induced strongly immunosuppressive effects, NLR engagement had the opposite effect, enhancing effector function in a dose-dependent manner. Interestingly, tolerized monocytes regain responsiveness when

stimulated with β-glucan (97). These findings underline the complexity of proposed innate immunological "memory." A plethora of factors including the host cytokine milieu, the antigen in question and antigen concentration all influence the development of either refractory or enhanced effector function. In a complex immune response such as during sepsis, it is likely that a combination of these features occurs simultaneously. Which factors, epigenetic or otherwise, contribute to persistence of ET still require complete elucidation. That immune states such as trained immunity have been shown to propagate via progenitor cells suggests that alteration of host epigenetic regulation can persist extensively (98, 99). Therefore, characterization of histone alterations in sepsis survivors over a prolonged period of time would provide useful information on the longevity of sepsis-induced changes.

EPIGENETIC THERAPEUTICS: POTENTIAL AND LIMITATIONS IN TREATMENT OF SEPSIS-ASSOCIATED TISSUE INJURY

Histone Deacetylase Inhibitors (HDACi)

A significant amount of research has examined the effect of modulating epigenetic enzymes upon sepsis-associated organ dysfunction and outcome. Numerous histone deacetylase inhibitor studies in pre-clinical models of sepsis have been conducted (summarized in **Table 1**), discussed in detail in this section. Characterizing the effect of HDACi at the tissue level is difficult in humans. Animal models circumvent this limitation and have brought valuable insights.

Histone deacetylases inhibitors targeting classical HDACs are currently used in a number of clinical contexts including cancer. HDACi administration has been shown to attenuate tumor growth and cause apoptosis in tumorigenic cells though the exact mechanism of action is unknown. Synergistic beneficial effects of combinational HDACi use have been demonstrated, although how synergy is achieved is unclear. Vorinostat (or suberoylanilide hydroxamic acid, SAHA) was licensed in 2006 for the treatment of relapsed and/or refractory cutaneous T-cell lymphoma (120). Two other pan-HDACi, for peripheral T-cell lymphoma and multiple myeloma, are also in use (121, 122). Outside of oncology, valproic acid (VPA) is used as an anticonvulsant which acts on class I and II HDACs, and trichostatin A (TSA) is an antifungal which also acts on class I and II HDACs. These examples demonstrate that HDACi treatment, in principle, has an acceptable safety profile, therefore, their use in sepsis is a realistic option should they prove effective.

Pre-clinical Evidence of HDACi Efficacy in Sepsis

In addition to the positive effects of HDACi on the endothelium during sepsis in mice (discussed above), HDACi treatment has been shown to curb other pro-inflammatory and innate immune responses in pre-clinical models of sepsis. Leoni et al. were the first to report anti-inflammatory properties of Vorinostat both *in vitro* and *in vivo* (123). Prophylactic administration of Vorinostat in mice reduced pro-inflammatory cytokine production upon challenge with LPS. Other reports reveal the impact of VPA and TSA on macrophage activity. Host anti-bacterial responses are inhibited via multiple mechanisms: phagocytic receptors are downregulated and release of reactive oxygen species and nitric oxide is reduced. Bacterial killing, demonstrated in mice with *E. coli* and *S. aureus,* is significantly impeded (123).

Roger et al. demonstrated a significant reduction in mortality in mouse models of toxic shock induced by Pam_3CSK_4 and cecal ligation and puncture when HDACi were given (64). TSA negatively regulated the expression of several pattern recognition receptors involved in microbial antigen detection. In addition, they observed that treatment with TSA, Vorinostat, and VPA all repressed cytokine release following TLR stimulation. A recent study exploring the effects of the HDAC6 inhibitor Tubastatin A in a cecal ligation and puncture model of sepsis demonstrated strong therapeutic efficacy. Survival was greater in Tubastatin A-treated mice vs. controls, and pro-inflammatory TNF-α and IL-6 were significantly reduced in peritoneal fluid and plasma of treated animals (106). In addition, a significant reduction in lung injury and bacterial load in the spleen 24 h after cecal ligation and puncture was observed (23 h after HDAC6 inhibitor treatment) (106).

Histone deacetylase inhibitors have been used synergistically with other epigenetic modifiers to ameliorate endothelial integrity and prevent lung injury. Prophylactic inhibition of histone deacetylation alone or combined with inhibition of histone methylation reduced capillary leak and pulmonary oedema in endothelium *in vivo* and substantially minimized lung histopathology (109).

Evidence from the clinic suggests that HDACi could be useful in attenuating the deleterious pro-inflammatory responses seen in sepsis as there is already a precedent for using HDACi in inflammatory disorders. Vorinostat and another HDACi, Givinostat, are licensed therapies for autoimmune inflammatory disorders graft-vs.-host disease and systemic onset juvenile idiopathic arthritis (SOJIA) (juvenile onset Still's disease), respectively (124, 125).

Considerations and Limitations of HDACi Use

A potential caveat of HDACi treatment is the associated increased risk of subsequent infection that accompanies a reduction in pro-inflammatory responses. Some phase I and II trials of HDACi as cancer treatments noted an increase in severe infections (126, 127) although trials of Vorinostat in graft-vs.-host disease and Givinostat in systemic onset juvenile idiopathic arthritis (SOJIA) have not reported such findings.

It should be noted that the effects of HDACi on the inflammatory response may not be restricted to alterations to the epigenome. The exact effect of HDACi on histone and non-histone acetylation is difficult to characterize, particularly for pan-inhibitors where alterations are likely to be widespread. This impairs our understanding of the exact mechanism driving potentially beneficial effects, in turn hampering the improvement of therapeutic specificity. HDACs have a degree of functional redundancy, therefore knockdown of a given enzyme

TABLE 1 | Summary of pre-clinical studies investigating the therapeutic potential of various HDACi inhibitors.

Inhibitor	Target	Study	HDACi dose	Sepsis model	Animal model	Effect
Valproic acid (VPA)	HDAC1 HDAC2	(100)	50 mg/kg	LPS	Beagles	Significant reduction in TNF-α and IL-6 mRNA in PBMCs 3 and 6 h post-treatment. No difference in clinical symptoms between treated and untreated groups.
		(101)	300 mg/kg	CLP	C57Bl/6J mice	Reduced TNF-α, IL-1β, and IL-6 in peripheral blood, reduced histopathological events, and oxidative damage in renal tissue. No comment on survival.
		(102)	Prophylactic and therapeutic doses given.	CLP	Sprague-Dawley rats	Improves survival of treated mice, inhibits transcription of TNF-α and IL-6, reduced oxidative burst. Reduced acute lung injury All animals were exposed to haemorrhagic shock 24 h prior to CLP.
		(103)	Prophylactic and therapeutic doses given.	CLP	BALB/c mice	Anti-apoptotic effect in lung and spleen tissue. No effect on serum cytokine levels or inflammation in lungs. No controls used.
		(104)	100 mg/kg	CLP	C57BL/6 mice	No significant difference in survival between treated and untreated mice. Hippocampal IL-1β levels were reduced in VPA group, Spatial learning ameliorated in treated mice.
Tubastatin A (Tub-A)	HDAC6	(105)	70 mg/kg	CLP	C57Bl/6J mice	Improved survival, inhibits transcription of TNF-α and IL-6, reduced oxidative burst. All animals were exposed to haemorrhagic shock 24 h prior to CLP.
		(106)	70 mg/kg	CLP	C57BL/6J mice	Improved survival, reduced TNF-α and IL-6 in peritoneal fluid and plasma, reduced lung injury, and macrophage apoptosis.
Trichostatin A (TSA)	HDAC1 HDAC2	(107)	1 mg/g (co-administered with DNA methyltransferase inhibitor, Aza)	LPS	C57BL/6J mice	Reduced apoptosis in lung tissue, reduced pro-apoptotic gene expression in lung.
		(108)	1 μg/g (administered alone or DNA methyltransferase inhibitor, Aza)	LPS	C57BL/6J mice	Treatment with both epigenetic modifiers had synergistic effect. Increased M2 macrophages in lungs, reduced pro-inflammatory cytokines in plasma, increased acetylation of STAT3 promotor in BMDMs, increased STAT3–Bcl2 signaling, reduced p38MAPK activation.
		(109)	3.3 μmol/L/kg (administered alone or DNA methyltransferase inhibitor, Aza) Co-administered with 4.4 μmol/L/kg Aza	LPS	C57BL/6 mice	Treatment with both epigenetic modifiers had synergistic effect. Reduced inflammation in lung Reduced pulmonary microvascular permeability Reduced apoptosis of lung cells
		(110)	10 mg/kg	CLP	Male Sprague–Dawley rats	Daily treatment for 7 days reduced neuronal cell death and improved spatial learning and memory defects induced by sepsis.
		(111)	10 mg/kg	CLP	Sprague-Dawley rats	HDAC inhibition increased skeletal muscle catabolism 4 h after sepsis induction, atrogin-1 expression is upregulated.
Suberoylanilide hydroxamic acid (SAHA or Vorinostat)	Pan-inhibitor	(112)	50 mg/kg	CLP	C57BL/6J mice	Improved survival, ameliorated coagulation disturbances at 48 h post-sepsis induction.
		(113)	50 mg/kg	CLP	C57BL/6J mice	Improved survival, reduced cytokine levels in peritoneal fluid and blood, reduced acute liver injury.
		(114)	Prophylactic and therapeutic doses given.	LPS	C57B1/6J mice	Reduced phosphorylation of MAP kinase proteins at 3 h post-induction, reduced neutrophil, and macrophage activity in the liver, reduced pro-inflammatory cytokine levels in liver tissue.

(Continued)

TABLE 1 | Continued

Inhibitor	Target	Study	HDACi dose	Sepsis model	Animal model	Effect
		(115)	50 mg/kg	LPS	C57BL/6J mice	Improved survival, reduced MyD88 gene expression, reduced TNF-α and IL-6 production.
		(116)	Prophylactic and therapeutic doses given.	LPS	C57BL/6J mice	Improved survival, reduced inflammatory infiltration into lungs and spleen, increased histone acetylation, reduced TNF-α in blood, reduced pro-inflammatory gene expression in lung.
Sodium butyrate	HDAC1 HDAC2	(117)	500 mg/kg (2 doses)	CLP	Wister rat	Improved survival 6 days post-sepsis, protective effect on liver, kidney, and lung
Cambinol	SIRT1 SIRT2	(118)	Prophylactic and therapeutic doses given.	LPS	BALB/c mice	Improved survival, lowered TNFα levels and bacteraemia, blocked phosphorylation of MAPKs.
EX-527	SIRT1	(119)	47 mg/kg	CLP	C57BL/6J mice	Improved survival, reduced TNF-α and IL-6 levels, attenuated bone marrow atrophy.

is frequently compensated for by another of the same class (128). In addition, several HDAC enzymes are known to form multiple complexes, each of which targets a different histone substrate (128).

In several studies of the effects of HDACi on sepsis outcomes treatment was given either prophylactically or very soon following sepsis induction (within an hour), well ahead of the development of symptoms (in CLP models the first symptoms generally appear around 6–12 h post-induction) (**Table 1**). Therefore, the impact of HDACi treatment in a clinical setting when administered during symptomatic disease is currently unclear.

Histone deacetylase inhibitors may not be appropriate for use in individuals with latent infections. Vorinostat has been proven to reactivate transcription of the HIV reservoir in infected CD4+ T-cells (129). To this end, it has been heavily investigated as part of the "shock and kill" strategy for HIV reservoir eradication (130). Several latent infections such as Epson-Barr virus (EBV) and other herpesviruses also enter lytic replication following HDACi treatment (131). Given the ubiquity of herpesviruses and the seriousness of HIV, reactivation of latent infections during or after a septic episode could be highly detrimental. Whilst HDACi treatment in the context of sepsis is unlikely to be administered long-term, more detailed understanding of HDACi effects on viral latency and reactivation is critical for safe usage.

The Role of HATs Inhibitors in Inflammation

Given the role of acetylation in sepsis, there is a surprising paucity of data examining the role of HATs activity and inhibition. Whilst less well-characterized than that of HDACs, several studies suggest that HATs inhibition could also elicit anti-inflammatory effects. Several HATs inhibitors including delphinidin, gallic acid, epigallocatechin-3-gallate, diferuloylmethane, and cerulenin have all been shown to reduce pro-inflammatory cytokine release by regulating NF-κB acetylation (132–136). In animal models of acute respiratory distress syndrome and renal injury, elevated HATs activity associated with worsened tissue injury suggesting these inhibitors could have therapeutic benefits in cases of sepsis (137, 138). However, contradictory findings have been reported with some suggesting HATs inhibition has either no effect on pro-inflammatory responses or could in fact exaggerate cytokine release (139, 140). This discordance demonstrates the highly context-specific effect of these drugs. Further, exploration of their role *in vivo* and in sepsis pathophysiology would be welcome.

CONCLUDING REMARKS

Sepsis has a worldwide clinical burden with significant associated morbidity and mortality. Whilst our understanding of the underlying immunopathology has improved over the last 30 years, this has yet to inform effective therapeutic strategies. In this review, we have collated evidence from a large number of studies that highlight the epigenetic mechanisms underlying some of the major aspects of sepsis pathology. Together these reveal the importance of epigenetic changes at transcriptional promotors or enhancers in driving many pathological adaptions. It is key to note that the cell-specific context and stage of sepsis in which these changes occur is important for determining phenotypic effect. The potential use of HDACi as therapeutics in inflammatory disorders has garnered interest over the past decade. These drugs have proven tolerability and are already used in the treatment of a number of cancers. Their mechanism of action is incompletely understood and there are legitimate concerns about off-target effects. Histone deacetylase enzymes are involved in modulating thousands of genes and there are likely to be numerous non-histone targets within the cell that are also affected by their activity. Therefore, detailed exploration of enzyme selectivity and development of more targeted inhibitors are vital next steps in the clinical development of HDACi for use in inflammatory disorders.

We are only just beginning to understand the full scale of epigenetic influence on immune function (141). A critical question to address is the longevity of these adaptions. A limitation of many of the above studies is the relatively short time frame in which epigenetic changes are reported. Results from Mitroulis et al. which demonstrate sustained epigenetic modulation in myeloid progenitors now need to be expanded and built upon (98). A more comprehensive description of both the nature of epigenetic changes and the retention of them is needed

to fully understand epigenetic contribution to sepsis pathology and outcome.

AUTHOR CONTRIBUTIONS

DC wrote the first draft on the manuscript. RD contributed significantly to the discussion of the clinical presentation of sepsis. RD and DC produced the figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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