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MINIREVIEW

Can the Cecal Ligation and Puncture Model Be Repurposed To Better Inform Therapy in Human Sepsis?

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ABSTRACT A recent report by the National Institutes of Health on sepsis research has implied there is a trend to move away from mouse models of sepsis. The most commonly used animal model to study the pathogenesis of human sepsis is cecal ligation and puncture (CLP) in mice. The model has been the mainstay of sepsis research for decades and continues to be considered the gold standard to inform novel pathways of sepsis physiology and its therapeutic direction. As there have been many criticisms of the model, particularly regarding its relevance to human disease, how this model might be repurposed to be more reflective of the human condition begs discussion. In this piece, we compare and contrast the mouse microbiome of the CLP model to the emerging science of the microbiome of human sepsis and discuss the relevance for mice to harbor the specific pathogens present in the human microbiome during sepsis, as well as an underlying disease process to mimic the characteristics of those patients with undesirable outcomes. How to repurpose this model to incorporate these "human factors" is discussed in detail and suggestions offered.

KEYWORDS cecal ligation, intraabdominal infection, sepsis

The declarative statement "cecal ligation and puncture is the animal model most representative of human sepsis" is regularly present in the introduction of widely cited articles and books (1, 2). Although there is little doubt that CLP is most often used to model the course and outcome of human sepsis, that the model is representative of the human condition belies the complexity and protean manifestations of human sepsis and its variable causes of mortality. Today, of all patients that present with the diagnosis of sepsis, mortality rates are estimated to be between 10 and 15% (3, 4). This figure includes elderly patients from nursing homes, immunocompromised patients such as those undergoing liver transplantation, premature neonates, and the terminally ill. In a recent study sampling six United States academic medical centers, hospice-qualifying conditions, such as end-stage cancer, were present in 121 of 300 sepsis-associated deaths (40.3%) (5). Although suboptimal care (mostly delays in antibiotic administration) was identified in 68 of the 300 sepsis-associated deaths (22.7%) in this study, only 11 sepsis-associated deaths (3.7%) were judged to be definitely or moderately likely to be preventable. These sobering statistics indicate that most sepsis (i.e., >85%) is successfully treated in the short term (30-day mortality) when patients are not in an end-of-life situation. Yet despite early success in treating human sepsis with antibiotics, fluids, and source control, many patients, especially those with underlying comorbid conditions, go on to develop organ failure late in the course of their illness. Therefore, correlating findings in mouse models of CLP, in which most mortality develops within 48 h, to the human condition, remains challenging.

The term "late-onset sepsis" has now emerged to indicate that most deaths in ICU patients occur late in the course of their disease and are characterized by the presence

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of multidrug resistant health care-associated pathogens (4) and immunosuppression (6). These patients are frequently on multiple life support measures and, as mentioned above, are in an end-of-life situation. As such, it is not unexpected that they may die either of or with a secondary infection. Yet when death occurs in such cases, it is most often attributed not to the underlying morbid condition or the infection *per se*, but rather to the sepsis, which has been recently defined as "life-threatening organ dysfunction caused by a dysregulated host response to a known or suspected infection" (7). This assumes that patients are dying of the response itself and may explain, in part, why much research in the field focuses on elucidating the acute host response using mouse models such as CLP. Thus, the rationale for using the CLP model is to elucidate the immune and inflammatory pathways that are "dysregulated" among those mice that go on to die, as a mechanism to inform novel therapeutics that can be tested in human trials.

There is little doubt that much about pathways of acute inflammation has been elucidated by the CLP model, as the model allows incompletely treated infection to proceed unabated (*vide infra*). A significant amount of information has been generated by the CLP model that has contributed to our understanding of the intersection between immunology and inflammation. Yet, here it is argued that the time has come to repurpose the CLP model so it more appropriately represents those patients most at risk for organ failure and death, and in whom novel therapies may appropriately intervene. The rationale behind considering a redesign of the CLP is to inform strategies that will effectively intervene in those specific at-risk patients with sepsis, meaning not those that survive because of appropriate use of antibiotics, fluids, and source control, but those that die despite these treatments.

What is it about the CLP model that is so disconnected from human sepsis? The CLP model uses immunocompetent mice that undergo surgery to suture ligate the cecum, causing localized ischemia and necrosis, followed by puncture of the cecum to induce spillage of cecal contents into the peritoneum, localized infection, and peritonitis. Without the components of either ischemia/necrosis or peritoneal contamination with microbes, animals do not develop organ failure and mortality (8–10). The idea here is to create a life-threatening infection characterized by physiologic disturbance (organ failure) and ultimately mortality. Unfortunately, the precise composition of cecal material that drives the infectious process, including that which remains within the necrotic cecum as well as that material pushed into the peritoneum as part of the model, is rarely accounted for or sufficiently evaluated (i.e., species identification, phenotype, metagenomics, etc.) in the context of the organ failure or mortality. For example, a review of the top 10 most frequently cited publications over the last 5 years using SCOPUS using the keywords "Cecal Ligation and Puncture" demonstrates that among the top 10 publications, only 5 reported any culture data and only 4 reported peritoneal culture data (11–22). Furthermore, among these publications, reported peritoneal cultures were expressed as total numbers of bacteria without identification of the bacterial species present or any other level of key microbial detail (i.e., virulence phenotype, metagenomics, and/or metabolomics).

To compensate for this lack of detail, some investigators have attempted to control the composition of infectious inoculum in the CLP model by using a defined and characterized "cecal slurry" placed directly into the peritoneum in lieu of performing the CLP procedure itself (23). Yet, in the most heavily cited reports of this model (23), only bacteremia was reported and again, the value was expressed only as CFU/ml of blood without defining the species present, their community structure, or phenotype. Although the CLP model in this study yielded a 70% mortality rate, whether bacterial parameters in the peritoneum (bacterial density, species, phenotype) discriminated between those mice that lived (i.e., 30%) versus those that died (70%) was not determined, as no peritoneal bacterial assessment was made. Finally, no antibiotics were used in this study as would be administered to patients. Yet in this study and others, the inflammatory and immune response elements that characterize the organ

failure and mortality were studied under the assumption that, while microbes certainly initiate the process, their importance fades into the background as organ failure and mortality develop in consequence of "runaway" inflammation (24). Under this conceptual framework, the host response becomes pathoadaptive to recovery and therefore the therapeutic approach should focus on the host response rather than the inciting microbial agent(s) (25).

Another inconsistency in aligning the CLP model to human sepsis is that humans rarely if ever die of the primary event or lesion (i.e., the intestinal perforation, abscess formation, ischemia, etc.) (6). Rapid response teams that apply imaging, antibiotics, and source control (drainage/surgery) effectively and promptly contain the initial sepsis trigger (26–30). The incongruence of this clinical practice to the CLP model has much to do with the fact that within the first 24 to 48 h, most patients with severe sepsis survive the initial insult, whereas most CLP mice die. Furthermore, the patients that then go on to develop organ failure, prolonged hospitalization, colonization with health care-acquired pathogens, etc., i.e., the most at-risk for mortality, are the very target population for therapies informed by the CLP model. In many reported studies, the CLP model does not involve the use of antibiotics, source control obtained by surgical means (31), or goal-directed fluid administration, but rather follows untreated animals until they develop organ failure and/or death. In studies in which only fluids and antibiotics are applied, animals still remain with a frankly ischemic and necrotic portion of intestine and an undrained abscess or local peritonitis (32). Yet proper care of a CLP-treated mouse (i.e., antibiotics, fluid, surgical removal of the necrotic cecum and abscess) results in 100% survival with no visible sequelae (33). Therefore, the rationale behind how CLP might inform a therapeutic strategy to a patient with late-onset sepsis following a major event characterized by organ failure, life-support (ventilator, dialysis, total parenteral nutrition), immunosuppression, and colonization by health care-acquired pathogens remains difficult to reconcile. Those that espouse the use of the CLP model will argue that truncating the early inflammatory events informed by this model will prevent the occurrence, course, and outcome of late-onset sepsis, organ failure, and other sequelae, while accepting these limitations (34). Unfortunately, this idea ignores the fact that the great majority of patients to whom the CLP model might clinically mimic are, actually, successfully treated with antibiotics, fluids, and source control (35). Using a model of incomplete treatment of an ongoing undrained infection and ongoing intestinal ischemia/necrosis to inform how to interdict in the immune/inflammatory response to prevent the sequalae of human sepsis treatment in a modern hospital seems incongruent with our understanding of the natural history of human sepsis and its modern treatment.

The CLP model was originally developed to understand the natural history of untreated infection, with the idea being to mimic the pathophysiology of patients who present late in the course of a surgical infection, such as patients with perforated appendicitis, diverticulitis, or a perforated peptic ulcer (8, 36, 37). However, even today, most patients that present with such problems invariably present after several hours or days after the acute event and, in the overwhelming majority of cases, are successfully treated (5, 35, 38). Currently most sepsis-related deaths are not directly attributed to a delay in recognition or treatment of infection (although no doubt some are, it is just a small number), but rather from the cumulative effects of serious underlying conditions (i.e., cancer, frailty, advanced age, diabetes, chronic disease, ruptured aneurysm with profound shock) complicated by a serious infection (5). So this begs the question: which group of patients at risk of developing organ failure and dying of sepsis is the CLP model trying to represent?

Since there is little doubt that eliminating the offending infectious agent with antibiotics and source control is highly effective in rescuing the overwhelming majority of patients with sepsis (30), the real problem with human sepsis is the progression to sequential organ failure that leads to futile care and ultimately the withdrawal of care. So if virtually every drug trial performed over the last several decades informed by the

CLP model or LPS model has failed in clinical trials, why do investigators still use this model?

While animal models do not recapitulate human sepsis, they are useful to elucidate the pathobiology of inflammation. To be fair to our colleagues who have heavily invested in the CLP model or the use of a single bolus of endotoxin to mimic the pathobiology of sepsis, it is important to recognize that much about the pathophysiology of inflammation and immune function has been elucidated by these models (2). One line of reasoning, commonly used to justify the use of LPS or CLP, is that these models allow for the elucidation of specific immune/inflammatory pathways to be defined in the sepsis process using knockouts and other methods agnostic to the infectious inoculum phenotype. If common motifs and pathways can be identified, then therapy can emerge from these findings. No doubt much has been learned from these models and, to a certain degree, this argument remains valid. Yet here we assert that the failure of the LPS/CLP approach to inform effective therapy against human sepsis lies in the very assumption that all infectious inocula must somehow converge on immune/inflammatory elements common to them all (24). We argue that pathogen-pathogen interactions, differing not only at the species level, but also at the strain level, and pathogen-host interactions are a "matchless web of dense dynamic interactions" and the host response in each case is an emergent property (39). Perhaps it is time to consider that communities of pathogens that inhabit colonization surfaces of patients along the sepsis continuum have, over billions of years, evolved mechanisms to subvert and manipulate the host response even when selective host pathways are blocked (40). In an individual patient, the bacteria now present in the setting of critical illness have evolved multiple "work-arounds" to any immune/inflammatory blockade strategy informed by our current methods (41). It may be for this reason that anti-LPS, IL-RA, anti-TNF, and anti-TLR4, to name a few, have failed when applied to the most at-risk patients with sepsis and organ failure (42).

Can the CLP model be repurposed to inform the pathobiology of human sepsis? Invariably with the CLP model, antibiotics are under dosed, cultures are rarely performed, and complete source control is rarely, if ever, attempted or achieved. In essence, the two most critical and efficacious therapies that cure most cases of sepsis, early and appropriate antibiotic administration and adequate source control (30), are not routinely incorporated into the model. Even when antibiotics are administered, there is little evidence they actually target the organisms that drive the sepsis response (43). Investigators will argue that it is not possible to reoperate on mice following CLP as they are fragile and will not survive and that cultures cannot be performed without multiple blood draws or multiple sampling of peritoneal fluid. Yet studies demonstrate that adequate antibiotics and source control can be achieved with reoperation, is technically feasible, and results in nearly 100% survival following CLP (32, 33). Others might argue that enough patients present with a delay in diagnosis and treatment that even when properly treated (i.e., fluids, antibiotics, and source control) still develop organ failure and mortality and, therefore, understanding the tipping point at which organ failure develops in such cases will inform early treatment strategies. Perhaps in some way the CLP model can be repurposed to formally test these lines of reasoning in a way that will be more informative of therapies against human sepsis. However, it is first important to review the evidence that attempts to explain the mechanisms by which mice subjected to CLP develop organ failure and go on to die.

What are the mechanisms by which the CLP model leads to organ failure and mortality? In the CLP model, two complementary yet often competing mechanisms that lead to organ failure and mortality have centered around the cecal contents versus the immune/inflammatory response. To invoke causality of the cecal contents to the outcome of the model, investigators have taken the approach of directly procuring the cecal contents, molding them into a "cecal slurry," and then directly injecting this into the peritoneal cavity (44), as discussed above. This approach, in many cases, shows a similar inflammatory and mortality response to the original model itself. While the immunocentric view will concede the role of the cecal contents as causative to

mortality in the model, they will argue that the observation that targeted knockout of immune/inflammatory elements and pathways attenuates mortality invokes a major engagement and role for the immune/inflammatory system (45). They will further argue that, given that clinicians treat patients who have already developed the primary injury (appendicitis, pneumonia, necrotizing infection, etc.) when they present with life-threatening sepsis, aside from the administration of antibiotics, controlling the "dysregulated host response" should be the focus of novel strategies (45). They might also argue that although every patient might harbor a unique microbiome or pathobiome that drives the sepsis response, understanding how all driving agents converge on common elements of the immune/inflammatory system can lead to therapies that can be universally applied to all septic patients. Of course, this conceptual framework belies the complexity with which the host-pathogen interactome operates in a given patient with sepsis, but, nonetheless, in the context of this review we will attempt to unpack this logic and expose its incongruences.

First, it is important to recognize that mouse microbiota are not the same as the human pathobiota that predominate in patients who are critically ill with organ failure (46, 47). Second, most patients that develop organ failure and die from sepsis do not die from the primary injury (trauma, pancreatitis, burn injury, pneumonia, etc.), but rather a secondary insult or "hit," to use the common label of experimentalists (6). It is for this reason that many investigators use the CLP model and subject mice to a subsequent hit, such as exposure to a relevant human pathogen such as *Pseudomonas aeruginosa* (48). However, the results and implications of a series of studies performed by Murphey illustrate an important observation in the CLP model complicated by a second hit (49). Murphey performed CLP in mice and 5 days later mice were challenged with an intravenous dose of *P. aeruginosa* at 1×10^8 CFU, a substantial and near-lethal inoculum. Clearance of the *P. aeruginosa* and immune function were grossly impaired in the mice subjected to CLP. In order to determine which components of the CLP model lead to the impaired clearance of *P. aeruginosa* and the immunosuppressive response, Murphey isolated the individual components of the CLP model (cecal contents, ischemic/necrotic tissue, etc.) and tested their effect on the immunosuppression and *P. aeruginosa* clearance. Results demonstrated that mice subjected to either trauma alone or cecal ischemia/necrosis alone did not develop impaired clearance of intravenous *Pseudomonas*. In contrast to normobiotic mice, neither CLP performed in germfree mice nor abdominal contamination of mice with cecal contents from germfree mice adversely affected clearance of a subsequent *Pseudomonas* challenge. These data suggest that suppressed immune function after CLP is due to exposure to, and processing of, microbial ligands within the cecal lumen (i.e., the microbiota) rather than the tissue trauma, ischemia, or necrosis that is intrinsic to the model. Importantly, suppression of immune function did not appear to be due to exposure to LPS, as TLR4-deficient mice subject to abdominal contamination with cecal contents had diminished clearance of a *Pseudomonas* challenge similar to that seen in wild-type mice.

As discussed above, using the CLP model coupled with a secondary "hit" of an infectious inoculum can be useful to elucidate the mechanisms by which CLP alters immune function. The lung is a frequent site of pathogen inoculation following CLP and *Pseudomonas aeruginosa* is a commonly used pathogen (48). Local inoculation (of lung or gut) with health care-associated pathogens is based on the observation that intravenous administration of *P. aeruginosa*, at significantly high doses, does not cause immune activation (TNF-α, etc.) or mortality in animals as it does when introduced into the cecum or lungs (50, 51), suggesting the local activation of immune/inflammatory cells is more harmful than when bacteria enter the bloodstream. For example, when methicillin-resistant *Staphylococcus aureus* (MRSA) is introduced into the lungs of mice following CLP, immune/inflammatory responses are altered compared to CLP or MRSA exposure alone (52). Most importantly, MRSA lung infection in the background of CLP causes mortality, whereas its introduction alone causes no mortality when mice are medically treated with fluids and antibiotics. These studies indicate that much can be

learned using the CLP model when health care-associated pathogens are introduced into the model. At the same time, it may be important to consider the role of the microbiome in the immune response and outcome from CLP. For example, the microbiome has been recently shown to have a major impact on the outcome of the CLP model when *Listeria* is the pathogen introduced (53). Similarly, lung bacterial clearance mechanisms are impaired and mortality from pneumonia is increased when the gut microbiome becomes depleted (54). Production of key immunomodulatory metabolites produced by the microbiome may play a role in these observed effects. For example, butyrate, a short-chain fatty acid that has multiple immunomodulatory effects, is produced by gut anaerobes that use insoluble fiber as a substrate. Use of fiber-free diets and exposure to antibiotics, as often occurs during sepsis treatment, can impair both local and distant immune function (55–57). To define the key role of butyrate in CLP mortality, investigators supplemented CLP-treated mice with butyrate, which significantly improved survival, although the effect size was quite small and animals did not receive antibiotics (58). Yet, when taken together, these studies indicate the CLP model can potentially be rendered more relevant to the human condition by exposing the model to health care-associated pathogens, by feeding diets more representative of those fed to septic patients (i.e., fiber free), by exposing them to antibiotics, and by accounting for the effect of the gut microbiome on both mortality and immune function (59–61).

Incorporating the right microbes into the CLP model to mimic the human disease. In a general way, when microbiologists study sepsis they tend to vary the pathogen of interest while keeping the host response constant. In this manner, the precise virulence factors (adhesin, toxin, secretion system) that are necessary and sufficient to produce the clinical disease can be elucidated along the lines of the molecular Kock's postulates as proposed by Stanley Falkow (62, 63). Immunologists, on the other hand, often choose to keep the pathogen constant and vary elements within the immune systems in order to determine which immune elements are necessary and sufficient to produce the clinical phenotype in their animal model (64). Technological advances resulting in the ability to manipulate and study pathogens (microbial reporter strains, mutant bacteria, improved high-throughput sequencing), along with the improved ability to genetically modify rodents, have resulted in the ability for immunologists and microbiologists to vary both sides of the infection equation. The explosion in microbiome sciences, primarily advanced by sequencing technology and bioinformatics, adds yet another dimension to the complex biologic context of the "interactome" that is playing out in real time along the sepsis continuum from initial tissue insult (trauma, burn, pancreatitis) to changes in the microbiome, to immune alterations, and finally to pathogen exposure (65). For example, applying dual transcriptome sequencing (RNA-seq) to a given sample can create a heat map of not only all of the host genes that are expressed/repressed, but also all of the microbial genes across all species present in the sample (metatranscriptomics) (66, 67). Yet even with the possibility of gathering all such information, causality to organ failure and mortality along the sepsis continuum at the individual patient level remains elusive. Consider the following excerpt from Steven Hawking and Leonard Mlodinow's book *The Grand Design*: "According to quantum physics, no matter how much information we obtain or how powerful our computing abilities, the outcomes of physical processes cannot be predicted with certainty because they are not determined with certainty. Instead, given the initial state of a system, nature determines its future state through a process that is fundamentally uncertain" (68).

The above statement begs the question: can we repurpose the CLP model so it more aptly reflects the uncertainty of the course and outcome of human sepsis? This statement surely is antithetical to the scientific axiom "if you cannot order it, you cannot study it." It is for this reason that use of a single dose of purified LPS that consistently produces a predictable mortality rate (i.e., LD_{50}, the dose at which a 50% mortality occurs) is a highly desirable model to study the acute inflammatory response that characterizes the acute phase of sepsis. Perhaps the objection then should only be in

the use of the term sepsis, which implies that organ failure and death in the human condition are simply due to "dysregulated" inflammation. Here, we do not assert that LPS-mediated inflammation in mice has no scientific merit, as has been discussed (69). Rather, implying that this model reflects the human condition and therefore can somehow inform therapy for those patients that do not recover and go on to develop organ failure, prolonged critical illness, and mortality, is not appropriate. So how can we do better? Surely there must be a way to incorporate the host as a holobiont to model the human condition of sepsis.

Here, we propose five critical features of human sepsis to be incorporated into mouse models to enhance their relevance to the human condition. These features include many of the factors most recently demonstrated to dramatically affect the human microbiome and include diet, cancer, advanced age, antibiotic exposure, and carriage of health care-associated pathogens.

i. Feeding mice their standard chow diet does not reflect how patients eat prior to or during the septic insult; sepsis should be modeled in mice fed a western-type diet (70, 71) or a diet that resembles that which is fed to critically ill septic patients.
ii. The routine use of initial fluid resuscitation and daily antibiotics should be incorporated to all models of sepsis; the overwhelming majority of critical ill patients are continually exposed to multiple broad-spectrum antibiotics (43, 72).
iii. Mice should harbor an underlying condition such as a tumor or advanced age, as both conditions result in increased mortality and organ failure following CLP and represent those patients most at risk for organ failure and mortality (73–75).
iv. The mouse microbiome should be "humanized" in some way to be more reflective of the microbiota and pathobiota that critically ill patients harbor over the course of intensive care unit confinement (76). This can be easily accomplished by transferring feces from critically ill septic patients with organ failure to mice prior to CLP compared to feces from appropriate controls.
v. Animal models should undergo complete "source control" as part of the experimental setup at varying time points from the index CLP procedure to mimic the standard treatment applied to septic patients who harbor a clear focus of infection.

Finally, it is imperative that within-group differences in organ failure, cytokine profiles, microbiome composition/function, and mortality rate be accounted for in all experiments, as others have suggested (77). Invariably, between-group comparisons of mean values of a measured parameter belie their role in the mechanisms of death and organ failure at the individual mouse level. For example, if in the control group a 50% mortality rate is observed, it is critical that we understand why the other 50% survived, not simply that a given treatment reduced this to 25% in the experimental group with a P value of <0.05. This approach should be applied to all measured parameters and outcomes across treatment groups so that mechanisms can be elucidated at the individual mouse level. The biologic variability in the within-group analysis is likely to be most revealing into mechanisms of death and organ failure compared to knockout approaches which do not recapitulate the biologic variability in patients.

In closing, the CLP model has been useful as all well-defined models are useful, in this case to elucidate the host response when rodent microbiota are displaced into the peritoneum following cecal ligation and puncture and animals remain incompletely treated. However, the time has come to consider that this model belies the complexity of the human form of the sepsis when an underlying disease is complicated by infection and prolonged treatment (9). While the human condition might be characterized as simply a serious life-threatening infection with systemic signs of illness, it is important to accept that, once the infection is controlled and/or eradicated, the great majority of patients improve and do not go on to develop the life-threatening consequences of untreated infection, such as organ failure and mortality (5, 35). However, the current crisis today is to understand, at the most fundamental molecular level, the

determinants that drive one host phenotype toward recovery versus the other toward organ failure and mortality. The approach may not necessarily involve comparisons between groups of heterogeneously responding hosts only. While those determinants are likely to be a reflection of the genetics of the host, more important may be the life history of the host (i.e., diet, lifestyle, age, etc.) and the life history of its microbiota (i.e., diet, vaccination status, global travel, prior antibiotic exposure, etc.). Incorporating these elements into the CLP model may offer a mechanism to more closely align this important model to the human condition, where life-threatening infections with multiple organ failure occur in patients with complex underlying disorders whose care requires exposure to sterile, fiber-free, chemically defined diets, health care-associated pathogens, multiple antibiotics, and a markedly altered microbiome.

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