

# ANIMAL MODELS OF SEPSIS: SETTING THE STAGE

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**Abstract** | Sepsis is a state of disrupted inflammatory homeostasis that is often initiated by infection. The development and progression of sepsis is multi-factorial, and affects the cardiovascular, immunological and endocrine systems of the body. The complexity of sepsis makes the clinical study of sepsis and sepsis therapeutics difficult. Animal models have been developed in an effort to create reproducible systems for studying sepsis pathogenesis and preliminary testing of potential therapeutic agents. However, demonstrated benefit from a therapeutic agent in animal models has rarely been translated into success in human clinical trials. This review summarizes the common animal sepsis models and highlights how results of recent human clinical trials might affect their use.

## MODEL ORGANISMS

Success, according to Sir Winston Churchill, “is the ability to go from one failure to another with no loss of enthusiasm.” From this perspective, the identification of novel sepsis therapeutics has been an unqualified success. However, for most scientists and clinicians, sepsis remains a long-unsolved medical mystery. Sepsis is a common disease with rising incidence and a mortality ranging from 28–47%<sup>1,2</sup>. Parallel research in humans and animal models has demonstrated the complex nature of sepsis development. The pathogenesis of sepsis was often described as an overzealous inflammatory response, with the logical implication that anti-inflammatory agents would represent the most successful class of sepsis therapy<sup>3</sup>. However, more than 20 years of clinical trials have demonstrated that this approach was not overtly successful<sup>4–6</sup>.

The failure of clinical therapeutics has prompted a rethinking of both the pathophysiological mechanisms of sepsis and the use and interpretation of animal models of sepsis. It is evident that the careful choice of sepsis models is crucial for capitalizing on emerging opportunities provided by the recent identification of major biochemical pathways that control inflammatory processes<sup>6–12</sup>. Manipulation of pathways that attenuate inflammation by appropriate targeting

of early and late inflammatory mediators could offer a previously unavailable chance to dramatically improve our understanding of sepsis mechanisms and the efficacy of clinical therapies<sup>6</sup>.

## Sepsis definitions and studies

Sepsis is currently viewed as a complex dysregulation of inflammation arising when the host is unable to successfully contain an infection (FIG. 1). Inflammatory dysregulation ultimately affects multiple organs through effects on endothelial, epithelial and immune cell types that leads to irreversible damage (FIG. 1). The sepsis inflammatory response can involve two distinct but not mutually exclusive phases: the systemic inflammatory response (SIRS), and the compensatory anti-inflammatory response (CARS) (FIG. 2)<sup>13</sup>. The development of SIRS, a functionally effective inflammatory response to infection or injury, is countered by a mechanism to terminate inflammation following elimination of the inciting agent. Achieving the correct balance of SIRS and CARS, as well as the intensity of these responses, greatly influences host survival. Imbalance and exaggeration of either response can result in host damage caused directly by excessive inflammation, or indirectly through immune dysfunction. An inappropriate response of SIRS or CARS, or both, might directly harm the host during the regulation of the inflammatory response, suggesting that both

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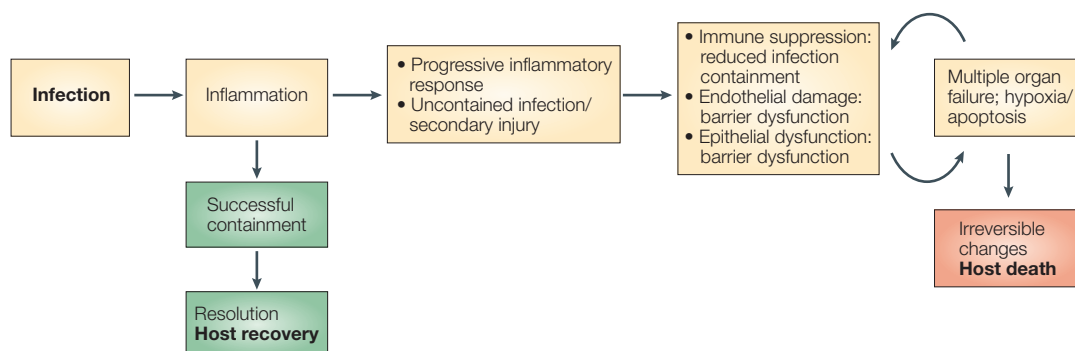


Figure 1 | **Sepsis pathogenesis.** Sepsis typically develops following infection or an inflammatory insult that is not contained and cleared by the host. The dysregulation of the inflammatory response leads to disruption and damage to the host immune system and several cell types. Endothelial and epithelial cells constitute an important barrier in the containment of infection and inflammation. Disruption of the endothelial and epithelial barrier could allow further dissemination of infection. Widespread cellular and immune dysfunction could then propagate resulting in organ failure, eventually resulting in an irrecoverable state

anti-inflammatory and pro-inflammatory agents might have a place in the treatment of sepsis<sup>13</sup>. The successful application of such opposite therapies requires that the agent (anti-inflammatory or pro-inflammatory) is delivered at the appropriate stage of sepsis to be beneficial and not harmful to the patient. The ideal treatment for sepsis should therefore be individually tailored to the known intensity and bias of the inflammatory status of each patient. However, such an individualized approach and sepsis staging remains an elusive goal, and requires more detailed study of biomarkers during the progressive stages of sepsis<sup>14</sup>.

The definition of sepsis has always been based on clinical features of the disease, and attempts are evolving to improve patient classification for research and therapeutic interventions<sup>15,16</sup>. At present, no panel of biomarkers is used to define sepsis in humans. Until 1992 sepsis patients were undifferentiated, as the prevailing definition of the disease was loosely associated with clinical symptoms and the presence of haemodynamic decompensation. In 1992 a standardized definition of sepsis was established in an attempt to improve clinical studies<sup>15</sup>. The definition of several clinical states during the development and progression of sepsis represented the first step towards the identification of distinct patient groups that could allow for more selective therapy administration. However, the initial recommendations have been actively revised to refine patient identification and improve the design of clinical trials<sup>16–18</sup>. Further opinion suggests that biomarker panels should be used with greater emphasis to define populations for sepsis staging and drug therapy<sup>14</sup>.

The goals of sepsis research — defining mechanisms of sepsis pathophysiology and developing therapeutic interventions — are intimately intertwined. Improved understanding of sepsis mechanisms has only recently generated clinically proven human therapies: recombinant human activated protein C (drotrecogin alfa (Xigris; Eli Lilly))<sup>19</sup> and an anti-tumour-necrosis factor- $\alpha$  (TNF $\alpha$ ) monoclonal antibody (afelimomab (Segard; Abbott))<sup>2</sup>. Prior to the success of drotrecogin alfa, many therapeutic agents that were effective in

animal studies failed to demonstrate a similar benefit in human clinical trials<sup>4</sup>. Several reasons could account for these failures: the lack of a defined staging system to enrol a homogeneous patient study sample; the heterogeneity of the study sample with respect to age, genetic background and co-morbidities; and the application of therapy based on incorrect or incomplete observations in animal sepsis models<sup>4</sup>.

Given the variability and difficulties of staging human sepsis, animal models of sepsis and septic shock have been established for the more reproducible study of sepsis pathophysiology<sup>20–22</sup>. These models of sepsis have often served as a preliminary testing ground for therapeutic agents prior to human clinical trials<sup>4,22</sup>. In-depth descriptions of animal models of sepsis as related to mechanisms and detailed procedures have been expertly reviewed elsewhere<sup>20–23</sup>. The purpose of this review is to re-examine and critically evaluate the most commonly used animal sepsis models in the context of drug therapy development. This review attempts to explain the failed translation of experimental therapeutics into clinical successes by the absence of sepsis-stage-specific treatments. Future studies should combine better staging of sepsis with advances in our understanding of the biochemical and pathophysiological changes that are hallmarks of different stages of sepsis.

#### Animal models of sepsis: general considerations

On the basis of the initiating agent, sepsis models can be divided into three categories: exogenous administration of a toxin (such as lipopolysaccharide (LPS), endotoxins or zymosan); exogenous administration of a viable pathogen (such as bacteria); or alteration of the animal's endogenous protective barrier (inducing colonic permeability, allowing bacterial translocation) (FIG. 3). All models have contributed significantly to our understanding of host defence mechanisms during infection. However, when considering the application of animal models to the development of sepsis therapeutics, there are many examples of controversy. Although several models are used to study mechanisms of sepsis

pathophysiology, which is the correct model to use in the development of drug therapy? The best answer would be the model that most closely mimics the course of human disease. Unfortunately, there are significant differences between each of the current animal sepsis models that prevent any single one from emerging as the perfect vehicle for sepsis drug discovery.

A central goal of all sepsis models is to faithfully reproduce clinically relevant pathogenesis that is similar to the disease observed in human beings. Several clinical features are characteristic of human sepsis and are used to validate the relevance of animal models (BOX 1)<sup>20</sup>. These features divide the clinical sequence of sepsis into two distinct early and late phases of disease. Sepsis development in humans is physiologically defined by two distinct haemodynamic phases. The first (early) phase is referred to as the hyperdynamic phase. This phase of sepsis is characterized by low systemic vascular resistance (SVR) and increased cardiac output (CO)<sup>24–28</sup>. During the progression of sepsis, CO declines without change in SVR, resulting in haemodynamic shock. The combination of low CO and SVR is the hallmark of septic shock and defines the second (late) hypodynamic phase of sepsis<sup>25</sup>. Haemodynamic measurements of CO and SVR in the various sepsis animal models have been used to critique their clinical relevance and applicability to the study of human disease. Models that do not closely mimic the haemodynamic changes of human disease are often regarded as less clinically relevant<sup>21,22</sup>. More recent studies suggest that some animal models mimic the human septic response with respect to immunoinflammatory characteristics and apoptosis (BOX 1)<sup>29–31</sup>. These similar responses have not been used for validation of clinical relevance among the various models of sepsis, although it seems reasonable that the greater number of similarities would predict a more accurate physiological model.

#### Caveats of sepsis animal models

The major advantages and drawbacks of the existing sepsis models have been discussed in earlier reviews (BOX 2)<sup>20–23</sup>. Two drawbacks common to all sepsis models with respect to clinical relevance are the timing of disease development and lack of supportive therapeutic interventions. Animal sepsis models are constructed to develop reproducible and rapid disease as compared with human sepsis. The onset and progression of sepsis to multi-organ failure occurs in hours to days in most animal models, whereas in human patients this progression occurs over days to weeks. Furthermore, human patients are promptly treated with various standard therapies such as source control (identification of infection with physical removal by invasive procedures), oxygen therapy, intubation and mechanical ventilation; fluid, antibiotic and vasopressor therapy; and nutritional support). Given the lack of supportive interventions in animal models, caution must be exercised before extrapolating efficacy results from animal to human disease. Despite these drawbacks, animal sepsis models have each contributed significantly to

our understanding of the host response to infection and the role of both pro- and anti-inflammatory mediators in the pathogenesis of sepsis.

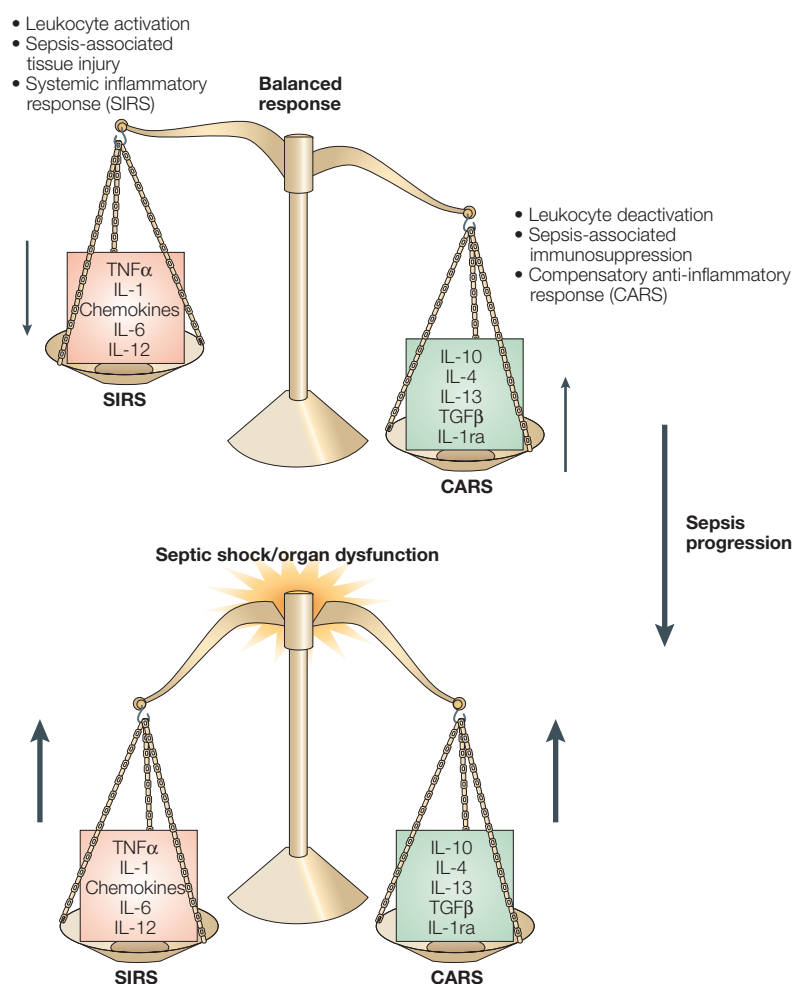
#### Toxaemia models

**Similarities with human disease.** The ability to tightly control an experimental system makes the use of specific chemical mediators that induce similar physiological changes to sepsis very attractive. Unfortunately, this also represents a major drawback of these models, as the isolated use of chemical mediators might not reproduce the complex physiological responses observed during sepsis progression caused by viable pathogens. A shock state can be induced by bolus injection of TOLL-LIKE RECEPTOR (TLR) agonists into laboratory animals. Endotoxemia models are widely used because they are simple to perform and human volunteer studies have shown that injection of low doses of endotoxin can induce pathophysiological alterations similar to those reported in sepsis patients<sup>21</sup>. Although activation of TLR4 through bolus injection of LPS is mostly applied in these models, a similar shock state can also be triggered by injection of immunostimulatory CpG DNA, which acts through TLR9<sup>32,33</sup> or by stimulation of TLR2 with synthetic lipopeptide agonists in sensitized mice<sup>34</sup>. Most laboratory animals, including mice, are relatively resistant to endotoxin and require administration of high LPS doses to induce the shock state. Mice are rendered considerably more sensitive to LPS by co-injection of D-galactosamine, which might increase sensitivity of hepatocytes to TNF-mediated apoptosis<sup>35</sup>.

**Characteristics of endotoxemia models and possible caveats.** Immune pathology of endotoxemia models using bolus injection is characterized by an overwhelming innate immune response, with inflammatory cytokines such as TNF representing crucial mediators<sup>4,36,37</sup>. It should be noted, however, that various endotoxemia models can show marked differences in immune pathology. TNF receptor p55-deficient mice are resistant to septic shock induced by LPS and D-galactosamine, but readily succumb to high-dose LPS<sup>38,39</sup>, whereas mice lacking macrophage migratory inhibition factor (MIF) or intercellular cell adhesion molecule-1 (ICAM1) survive a challenge with high-dose LPS but are as sensitive to LPS and D-galactosamine as wild-type mice<sup>40,41</sup>.

Bolus injection of LPS commonly induces a hypodynamic cardiovascular state immediately and unfortunately does not reproduce the haemodynamic changes observed in human sepsis and some infection models<sup>21,22</sup>. To circumvent the disadvantages associated with bolus injection of TLR agonists, models applying continuous infusion of LPS into mice have been developed. The infusion models were able to recreate the hyperdynamic phase of sepsis<sup>21,22</sup>. However, it was shown that sepsis induced by caecal ligation and puncture (CLP) altered the capacity of splenocytes to produce T<sub>H</sub>1 and T<sub>H</sub>2 cytokines upon stimulation with concanavalin A, whereas continuous infusion

TOLL-LIKE RECEPTORS (TLR). A class of cell surface proteins that recognize components of pathogenic organisms and regulate early activation of the immunoinflammatory response.



**Figure 2 | SIRS and CARS in sepsis.** The host inflammatory response can be viewed as a balanced response between pro-inflammatory mediators (referred to as the systemic inflammatory response (SIRS)) and anti-inflammatory mediators (referred to as the compensatory anti-inflammatory response (CARS)). SIRS mediators such as tumour-necrosis factor and interleukin-1 (IL-1), IL-6 and IL-12 activate the host immuno-inflammatory system, which can then be de-activated through the expression of CARS mediators, including IL-1 receptor antagonist (IL-1ra), IL-4, IL-10 and IL-13. During the development of septic shock, regulated expression of SIRS and CARS mediators is lost, resulting in an exaggerated and dysfunctional inflammatory response.  $TGF\beta$ , transforming growth factor- $\beta$ .

of LPS at levels comparable to those observed during CLP-induced septic peritonitis had no such effects<sup>42</sup>. Moreover, continuous LPS infusion and CLP resulted in greatly different survival rates<sup>43</sup>. Although this type of endotoxemia model has not been studied extensively, it seems that prolonged low-dose infusion of LPS does not reproduce important aspects of the immune response to a septic challenge caused by infection.

**Differences from human disease.** Inhibition of inflammatory mediators proved beneficial in endotoxemia models; however, neutralization of inflammatory mediators has failed to demonstrate substantial benefit in clinical trials<sup>45</sup>. Potential reasons for this failure could include overt differences between models of chemically induced septic shock and clinical disease. For example, bolus injection of LPS results in a very rapid and

transient increase in systemic cytokine levels. It is important to note that clinical sepsis differs from this response by showing a prolonged elevation of systemic cytokines and by exhibiting serum cytokine concentrations that are several orders of magnitude lower than in endotoxemia models<sup>21,22</sup>. Notably, the systemic cytokine profile observed in infection models such as CLP and colon ascends stent peritonitis (CASP) is more reminiscent of that observed in sepsis patients, demonstrating both continuous and sustained increase<sup>44,45</sup>. Furthermore, comparison of the response to endotoxin in humans and mice has demonstrated that mice are relatively more resistant to endotoxin, further limiting the extrapolation of studies between species<sup>46</sup>.

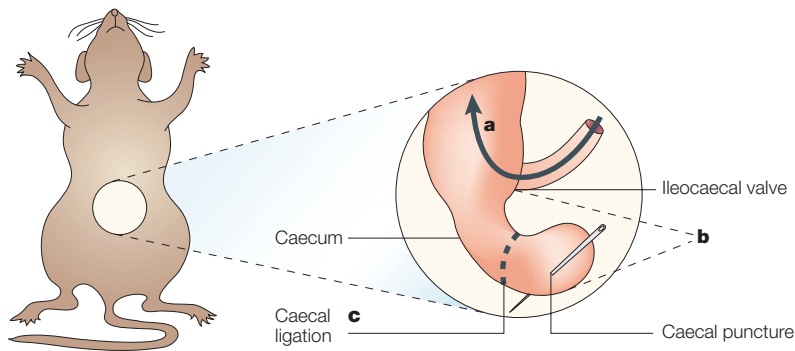
### Bacterial infection models

**Differences from human disease.** Although bacterial infection models do not recapitulate many important features of human sepsis, they can provide important insights into mechanisms of the host response to pathogens. Inoculation of animals with pure or mixed bacterial flora has been a common tool for studying sepsis mechanisms<sup>20–22</sup>. However, high doses of bacteria commonly administered do not typically colonize and replicate within the host, often due to rapid lysis by complement<sup>47</sup>. This leads to a potential model of intoxication with endotoxins rather than a true model of infection<sup>47</sup>.

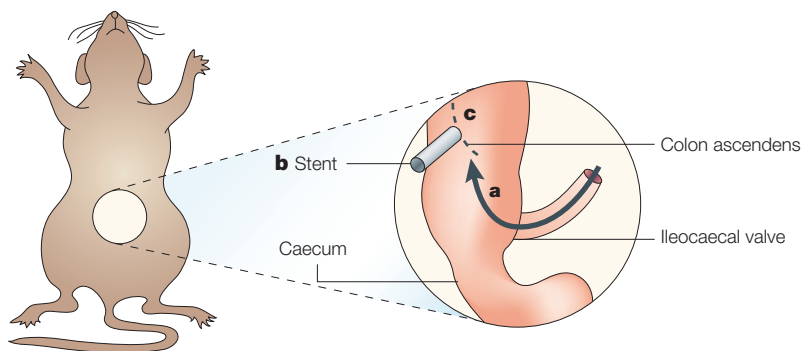
**Possible caveats: confounding variables in bacterial infection models.** Several factors confound the use of exogenous bacterial infection in the study of sepsis, including the infecting bacterial load, strain specificity of the infecting bacteria, strain specificity of the host response and the compartment of infection. These confounding factors can affect the clinical relevance of exogenous bacterial infection models. Bacterial strain is an important variable in sepsis models of exogenous infection. Studies on mechanisms of host defence suggest that interferon- $\gamma$  (IFN $\gamma$ ) has opposite effects on host survival following infection with different strains of bacteria, promoting host survival from *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* infection, while enhancing host demise during infection with *Staphylococcus aureus* and *Escherichia coli*<sup>48–52</sup>.

The route of infection can significantly affect the host response. An immediate and overwhelming introduction of bacteria into the peritoneum or blood compartment initiates rapid and distinct responses. A peritoneal or lung infection will primarily induce inflammatory and immune-cell migration into the infected compartment, whereas a blood-borne model of infection will have the greatest immediate effect on the endothelium and vascular system, with subsequent seeding of organs. The compartment-specific cytokine host response highlights this point. In the case of blood inoculation with *E. coli*, rapid changes are noted in the serum cytokine levels of interleukin-1 (IL-1), IL-6, and  $TNF\beta$ <sup>53</sup>. However, inoculation of the peritoneal cavity with the same dose of *E. coli* does not generate a robust serum cytokine response<sup>53,54</sup>. IL-10 also demonstrates a different role in the host

**Caecal ligation and puncture (CLP)**



**Colon ascendens stent peritonitis (CASP)**



**Figure 3 | Experimental models of sepsis: caecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP).** **a** | The CLP model of sepsis is induced by laparotomy and exposure of the junction between the large and small intestines. The small intestine joins the large intestine at the ileocaecal valve (blue arrow). The caecum extends inferiorly as a blind pouch, and normally faecal material passes distally into the large intestine (blue arrow). Caecal ligation takes place below the ileocaecal valve in a non-obstructing manner. The percentage of the caecum ligated is shown as 50% in the figure. The ligated caecum is punctured through-and-through allowing entrapped faecal material to leak into the normally sterile peritoneal cavity. **b** | The CASP model of sepsis is induced by laparotomy and exposure of the colon ascendens portion of the large intestine just distal to the ileocaecal valve. A stent is placed by incision (shown as a dotted line) of the colon ascendens allowing flow of faecal material from the colon into the peritoneal cavity to establish peritoneal infection.

response to infection in a compartment-dependent manner. In lung, IL-10 can impair host response and worsen mortality outcome following infection with *Klebsiella pneumoniae* or *Streptococcus pneumoniae*<sup>55,56</sup>. However, in peritoneal compartment infection, IL-10 activity protects the host from mortality<sup>57</sup>. Finally, IFN $\gamma$  elicits different effects during infection with *K. pneumoniae* depending on whether the inoculum is delivered into the lung compartment (IFN $\gamma$  host protective) or intravenously (IFN $\gamma$  independent)<sup>58</sup>.

Intravenous and intraperitoneal compartments represent the most common sites used for the exogenous bacterial sepsis models. However, pulmonary infection models deserve consideration, because lung infection represents a highly significant source of sepsis in human patients<sup>18</sup>. Pneumosepsis models are not typically characterized on the basis of the host haemodynamic response, but rather focus on the development of lung pathology. However, pneumosepsis models can produce detectable bacteraemia, distant organ damage following

initial infection of the lung and delayed mortality similar to other sepsis models<sup>58,59</sup>. These characteristics suggest that pneumosepsis models might deserve inclusion in the category of sepsis models if fulfilling haemodynamic and other immunological characteristics of human disease. One could argue that the lack of intervention with mechanical ventilation in pneumosepsis models makes this model less clinically relevant. However, the lack of ventilatory support is similar to the typical lack of surgical intervention in the CLP peritonitis sepsis model. With regard to the latter model, the inciting cause of sepsis would dictate surgical excision in the case of human disease. The use of pulmonary compartment sepsis models should be considered an important possible addition to the complement of current models if they fulfil similar characteristics used for sepsis model validation (BOX 1).

**Host-barrier disruption models**

Host-barrier disruption models of sepsis involve breaching the normal protective barriers that shield normally sterile compartments from pathogens. CLP and CASP represent examples of this category (FIG. 3). The predominant species used in the CLP and CASP system are rodents because of the relatively lower cost of performing experiments with large numbers of animals and the availability of genetically deficient mouse strains, which enables the study of specific genes during the sepsis response.

**The CLP model**

The CLP model is considered the gold standard for sepsis research<sup>20,60</sup>. The CLP model mimics the human diseases of ruptured appendicitis or perforated diverticulitis. The technique involves MIDLINE LAPAROTOMY, exteriorization of the caecum, ligation of the caecum distal to the ileocaecal valve and puncture of the ligated caecum. This process creates a bowel perforation with leakage of faecal contents into the peritoneum, which establishes an infection with mixed bacterial flora and provides an inflammatory source of necrotic tissue<sup>20,43</sup>. The severity of disease, as assessed by mortality, can be adjusted by increasing the needle puncture size or the number of punctures<sup>20</sup>. The severity of CLP can be adjusted such that mortality evolves rapidly over hours to days, or more slowly over 28 days<sup>61</sup>. A further advantage of the model is that it can identify an irreversible stage of sepsis, such that excision of the necrotic tissue is unable to improve survival<sup>62,63</sup>. The CLP technique has achieved its popularity because of its ease, general reproducibility and similarity to human disease progression<sup>20</sup>. Most notably, the CLP model recreates the haemodynamic and metabolic phases of human sepsis<sup>20</sup>. Subsequently, apoptosis of selected cell types and host immune responses seem to mimic the course of human disease, adding further clinical validity to this model<sup>30,64</sup>.

*Possible caveats: confounding variables of the CLP model.* Despite the multiple advantages of the CLP model, the mechanism of host response to infection could present a confounding variable. The host

MIDLINE LAPAROTOMY  
Surgical opening of the abdominal cavity in the midline.

Box 1 | **Mimicking the septic human response****Criteria for validation of animal models of sepsis:**

## Early-phase sepsis:

- Hyperdynamic cardiovascular state (low systemic resistance and elevated cardiac output = normotension)
- Hypermetabolic state (increased gluconeogenesis; hyperinsulinaemia)

## Late-phase sepsis:

- Hypodynamic cardiovascular state (low systemic resistance and low cardiac output = hypotension)
- Hypometabolic state (hypoglycaemia; hypoinsulinaemia)

## Features common to human sepsis and animal models not currently used to validate animal models:

- Apoptosis of specific cell populations (dendritic cell, endothelial cell and epithelial cell)
- Immune response (late-phase sepsis immunosuppression)

response to CLP is an attempt to wall off the infected/inflamed area through the creation of an abscess<sup>20,65</sup>. A certain percentage of animals successfully contain the infection, do not progress to septic shock and recover fully<sup>20</sup>. The ability of the host to survive by containing the infection suggests that therapies promoting abscess formation would improve outcome following CLP. It is therefore possible that an agent primarily enhancing abscess formation could be confused with a therapy for septic shock.

Variability within the CLP model itself might be underappreciated. Although the trends in sepsis outcomes might be similar, it is somewhat more difficult to compare experimental results between laboratory groups. A number of factors contribute to the variability within the CLP model, including age, sex and strain (BOX 3). Variability is highlighted by comparison of control mortality rates and time of death between laboratories reporting the same experimental techniques. Such a comparison might reveal different outcomes despite use of the same experimental system. For example, two studies evaluating the role of IL-10 in sepsis used female C57BL/6 mice and double-puncture CLP with a 20- and 22-gauge needle<sup>57,66</sup>. Survival was significantly greater in the 20-gauge needle puncture group (>90%) compared with the 22-gauge group (~25%). These results would not be predicted given the well-documented positive correlation of needle size with mortality<sup>67</sup>. This example highlights the importance of evaluating control group mortality when comparing experimental results. The percentage of caecum ligated during CLP can induce a subtle variability into the technique, as a greater percentage of caecum ligated correlates with increased mortality<sup>68</sup>. As this factor is not routinely described, it represents an important source of potential variability in CLP studies. Also, the load of faecal material that leaks from the ligated caecum is difficult to control for between different studies and could account for variability in the severity of disease.

*Staging of sepsis in the CLP model.* The difference in mortality rates and severity of sepsis in animal models could correlate to the human staging of sepsis as described by SIRS, sepsis, severe sepsis and septic shock. Often following CLP only a subset of animals progress to septic shock and death. It is more difficult to stage animal models with respect to severity of illness because of the lack of physiological monitoring commonly used for human patients. Severity of the individual CLP model is assessed through the mortality rate of the control group. This is an important exercise given the possible variability in disease severity, as mentioned above. The assessment via mortality increases the required number of study animals. Improved understanding of the animal model staging of sepsis could potentially allow for more focused studies that require fewer animal subjects. There is evidence suggesting that biomarkers could allow some staging of sepsis in the CLP model. Plasma IL-6 levels have been identified as a possible marker of disease severity and mortality predictor<sup>62</sup>. Elevation of IL-6 levels was predictive of mortality despite the administration of antibiotics<sup>69</sup>. The predictive power of IL-6 on survival has been demonstrated in the BALB/c and C57BL/6 mouse strains; however, IL-6 levels could not discriminate between early and late mortality in a fluid-resuscitated Sprague–Dawley rat model<sup>61,62,70</sup>. In the latter study, macrophage inflammatory protein-2 (MIP2), the chemokine KC, protein C and **C-reactive protein** were the most specific discriminators of early and late death following CLP<sup>70</sup>. Further studies in animal biomarkers could result in more focused preclinical testing and a reduction in animal usage.

**CASP model**

In the CASP model, a stent of defined diameter is implanted into the colon ascendens of mice, leading to persistent leakage of faecal content into the peritoneal cavity and infection with intestinal bacteria (FIG. 3)<sup>45,65</sup>. The procedure involves laparotomy, insertion and fixation of the stent, and fluid resuscitation of animals. As determined by survival rates, the severity of sepsis in the CASP model can be varied reproducibly by using stents of different diameters ranging from 14 to 22 gauge<sup>45</sup>. Peak lethality occurs 1–2 days after stent implantation. In addition, the stent can be removed after defined time periods, thereby mimicking surgical interventions in patients with the aim of eliminating the infectious focus. It was shown that mice can be rescued by early (3 hour) but not by late (9 hour) focus repair<sup>45</sup>. Lethality in the CASP model is accompanied by multiple organ failure including lung, liver and kidney<sup>71</sup>. Outcome in the CASP model is dependent on both the extent of inflammatory tissue damage and the efficiency of pathogen elimination.

CASP represents a murine model for acute polymicrobial septic peritonitis. Bacteraemia is detected as early as 12 hours after implantation of the stent<sup>65</sup>, whereas circulating LPS levels are elevated within 2 hours<sup>45</sup>. As determined by serum cytokine levels, both SIRS and CARS develop rapidly during septic peritonitis in the CASP model. Serum levels of pro-inflammatory

Box 2 | **Caveats of sepsis animal models****Endotoxaemia**

- Single toxin may not mimic responses in human sepsis
- Variable haemodynamic responses with different doses and infusion rates
- Intra- and inter-species variability in response to toxin

**Bacterial infection**

- Requires growth and quantification of bacteria prior to administration
- Significant inter-laboratory variability
- Large quantity of bacteria used may elicit confounding toxicosis response
- Host response is dependent on infecting bacterial strain
- Different host responses with infection of different compartments
- Variable host response dependent on bacterial load and infusion time
- Genetic background affects host responses to specific pathogens
- Human therapy potentially withheld could detract from validity of therapeutic agent

**Caecal ligation and puncture**

- Multiple bacterial flora
- Inter-laboratory variability
- Human therapy potentially withheld could detract from validity of therapeutic agent
- Age variability
- Strain variability
- Potential of ascribing sepsis therapy success to enhanced abscess formation mechanism

**Colon ascendens stent peritonitis**

- Multiple bacterial flora
- Human therapy potentially withheld could detract from validity of therapeutic agent
- Less characterized haemodynamic response
- Less experience to identify possible confounding variables

cytokines such as TNF, IL-1, IL-12 and IL-18, as well as anti-inflammatory IL-10, rise concomitantly rather than in a two-phase fashion, and high levels of these cytokines are detected as early as 3 hours after stent implantation<sup>45,65,72</sup>. Hepatic Kupffer cells are primarily responsible for increases in systemic levels of IL-10<sup>72</sup>. Systemic cytokine levels in the CASP model vary with the diameter of the implanted stent, but are typically greater than those observed in the CLP model<sup>65</sup>. In human patients with septic peritonitis, serum levels of TNF are comparable to those observed in the CASP model, whereas IL-6 and IL-10 levels were found to be substantially greater in the CASP model than in patients<sup>73,74</sup>. Taken together these data suggest that SIRS- and CARS-type responses can occur in a compartmentalized fashion. Consistent with this interpretation, lung extracts demonstrate strong induction of TNF but little expression of IL-10<sup>45</sup>, whereas liver extracts demonstrate high IL-10 and low TNF levels<sup>73</sup>.

Inflammatory gene expression in liver and lung during septic peritonitis in the CASP model is largely dependent on the presence of **MyD88**, a common signalling adaptor of TLRs<sup>73</sup>. In contrast, MyD88-deficiency

had only minor effects on gene expression in spleen<sup>73</sup>. Genetic ablation of MyD88 prevents the generation of SIRS in the CASP model without impairing antibacterial defence, resulting in markedly enhanced survival<sup>73</sup>. Mice deficient for IL-12p40 demonstrate greater mortality in the CASP model<sup>75</sup>. Notably, analysis of surgical patients has shown that impaired monocyte IL-12 production prior to surgery is associated with a high risk of lethality if post-operative sepsis develops<sup>76,77</sup>. These findings support the notion that immune functions associated with sepsis lethality in patients also influence outcome in the CASP model.

**Comparison of CASP and CLP models**

The CLP and CASP models of septic peritonitis were compared directly in a recent study<sup>65</sup>. Induction of systemic cytokines and bacterial counts in blood and other compartments were found to be considerably stronger in the CASP than in the CLP model. In the CLP model, the ligated caecum was rapidly covered by adhesive small-bowel loops, whereas the intestinal leakage in CASP mice was present for an extended time period. It therefore seems that diffuse peritonitis with persistent systemic infection predominates pathogenesis in the CASP model, whereas CLP could represent a model of intra-abdominal abscess formation. The CASP and CLP models could therefore reflect different kinds of diseases.

*Differential role of inflammatory mediators in CLP versus CASP.* A distinction should be made between peritonitis and intra-abdominal abscess when designing experiments and testing drugs in sepsis models. The presence of either diffuse peritonitis or intra-abdominal abscesses distinctly influences the outcome of sepsis in human patients<sup>78</sup>. These differences in the pathogenesis of infection could provide a possible explanation for the distinct effects of certain cytokines on outcomes in these models. Surprisingly, the pro-inflammatory mediator TNF was found to confer a protective role in CLP<sup>79,80</sup>, yet TNFRp55-deficient and wild-type mice showed comparable survival in the CASP model<sup>45</sup>. Conversely, IFN $\gamma$  receptor-deficient mice are highly susceptible in the CASP model, but show normal resistance to infection in CLP<sup>45,81</sup>. These results suggest that the IFN $\gamma$  pathway might be crucial to combat systemic infections, whereas TNF seems to be required for efficient intra-abdominal abscess formation in order to locally restrict the septic focus<sup>65,82</sup>. In contrast to pro-inflammatory pathways, neutralization of endogenous IL-10 at sepsis onset substantially increases mortality both in CLP and CASP<sup>57,72</sup>, indicating that an early anti-inflammatory response is essential, irrespective of the type of peritonitis. These data suggest that the CASP and CLP models might be complementary in the study of sepsis. It is attractive to speculate that comparison of results from each model could define disease mechanisms specific to both the inciting stimulus (abscess formation versus isolated peritoneal infection) and also to a final common pathway in the later stages of septic shock and organ failure.

Box 3 | **Variability factors****Toxicosis models**

- Type of toxin utilized (lipopolysaccharide subtype, use of sensitizing agent [d-Gal])
- Lethal or sub-lethal dose
- Route of administration
- Fluid resuscitation
- Host species and strain

**Bacterial infection models**

- Bacterial load
- Route of administration
- Timing of infusion
- Bacterial strain
- Host strain
- Antibiotics/fluid resuscitation

**Caecal ligation and puncture model**

- Needle size used for perforation
- Number of perforations
- Amount of caecum ligated/amount of necrosis induced
- Uncontrolled bacterial load (amount of stool milked into peritoneum)
- Antibiotic/fluid resuscitation
- Sex, age and strain

**Colon ascendens stent peritonitis model**

- Stent lumen diameter
- Load of stool transferred into peritoneum

**Possible caveats: confounding variables of the CASP model.** To date, the CASP model of sepsis seems to complement the CLP model as a model of ongoing peritoneal infection versus a model of abscess formation<sup>65</sup>. However, the CASP model represents a relatively new model system and issues of variability might not be fully realized given its limited use (BOXES 1,2). Although the CASP model does not involve the subtle variable of percentage of caecal ligation, it does represent a more challenging surgical procedure. One known technical issue is the requirement for careful surgical technique during placement of the stent to maintain patency and prevent abscess formation. As with CLP, operator consistency is the mainstay of reproducibility for CASP. Expanded use of the CASP model could generate a greater appreciation of its limitations and how it might best be used in the study of sepsis mechanisms.

**Lessons learned from human studies**

**Anti-TNF agents and activated protein C: a tale of two therapies.** Drug development typically begins with pre-clinical animal model studies and progresses to human clinical trials. This process has been remarkably unsuccessful with respect to the development of human sepsis

therapies<sup>4,5</sup>. In the past 4 years, two sepsis therapeutic agents have proven beneficial in large-scale clinical trials: recombinant human activated protein C (rhuAPC; drotrecogen alfa activated) and an anti-TNF $\alpha$  agent (afelimomab)<sup>2,19</sup>. However, the road to success was very different for each therapeutic approach with respect to the use of preclinical animal model testing. This raises the question whether animal studies of sepsis are sufficiently reliable to predict successful human therapy.

**Anti-TNF therapy.** The pro-inflammatory cytokine TNF has been a therapeutic target in multiple clinical trials<sup>4,5</sup>. Attention was given to TNF on the basis of observations that TNF levels became elevated in both human and animal subjects following administration of endotoxin<sup>47,83</sup>. Furthermore, exogenous TNF can recreate a shock state, supporting the notion that TNF is a central mediator of septic shock<sup>84</sup>. Finally, pre-treatment with an anti-TNF antibody was capable of preventing mortality in a baboon model of exogenous *E. coli* infection<sup>85</sup>. Given these preclinical results, significant hope and investment was directed towards anti-TNF therapy.

However, while clinical trials were underway, subsequent animal model studies raised doubts about the potential of anti-TNF therapy. Administration of a long-acting anti-TNF monoclonal antibody at the time of, or 8 hours after, CLP significantly enhanced mortality and these effects could be prevented with simultaneous administration of exogenous TNF<sup>79</sup>. Further studies demonstrated that anti-TNF antibody given at the time of CLP or endotoxin injection was unable to prevent mortality and that exogenous TNF could prevent CLP mortality<sup>86,87</sup>. These data suggest that TNF is required for the host response to CLP sepsis and that the timing of anti-TNF therapy administration has a central role in determining whether the experimental outcome is beneficial or harmful.

Subsequent to the preclinical studies, multiple clinical trials of anti-TNF therapy were performed without success<sup>5</sup>. One trial documented a detrimental effect of anti-TNF therapy on mortality<sup>88</sup>. A meta-analysis of all anti-TNF trials suggested that there could be a small benefit of anti-TNF therapy<sup>5</sup>.

**The prognosis-based choice of sepsis therapies.** Preclinical testing of a potential sepsis therapeutic agent might be facilitated by careful use of CLP and CASP models with predetermined levels of mortality in the experimental set-up. This suggestion arises from the hypothesis that the efficacy of a sepsis therapeutic agent depends on the severity of the underlying disease. Both animal and human data support this evolving concept<sup>89</sup>. For example, meta-analysis of human clinical trials has demonstrated that the degree of sepsis mortality influences the efficacy of anti-TNF therapy, with benefit observed only in those with high risk of mortality<sup>89</sup>. The CLP model was used to test this theory prospectively, and the beneficial response to anti-TNF therapy was found to be dependent on an increased mortality risk<sup>89</sup>.



The relationship of illness severity and response to TNF therapy was ultimately proven in the recent MONARCS (Monoclonal Anti-TNF: a Randomized Controlled Sepsis) trial, in which 2,634 patients were separated into two groups on the basis of IL-6 levels as an indicator of sepsis severity<sup>2</sup>. IL-6 was chosen as a discriminator of illness severity because elevated levels correlated with adverse outcomes<sup>2</sup>. These groups were then randomized to receive placebo or an anti-TNF F(ab')<sub>2</sub> monoclonal antibody fragment. With respect to mortality, anti-TNF therapy significantly benefited ( $p = 0.041$ ) only those subjects with elevated levels of IL-6. There was no benefit from TNF blockade in the overall population or those with low IL-6 levels.

The potential benefit of anti-TNF therapy is best realized after combined input from both preclinical and clinical trials. Had the decision to proceed with anti-TNF therapy been based solely on the initial CLP studies, it is possible that this therapy could have been abandoned. With hindsight, it is clear that testing of different levels of disease severity in combination with a clinically relevant animal model was required to demonstrate the efficacy of the therapy. This observation suggests that proper screening of pro- and anti-inflammatory therapeutic agents in sepsis should ideally be performed using carefully selected stages of sepsis within a relevant animal model.

**APC therapy.** The protein C (PC) pathway is important in the regulation of coagulation and inflammation during sepsis. PC circulates as an inactive precursor in the bloodstream and is converted to activated PC (APC) following proteolytic cleavage by the thrombin–thrombomodulin complex. APC, in conjunction with protein S, reduces the procoagulant activity of blood through inhibition of factors Va and VIIIa<sup>90</sup>. APC can also reduce the expression of tissue factor in endothelial cells and inhibit plasminogen activator inhibitor-1<sup>90,91</sup>. *In vitro* studies suggest that APC can exert an anti-inflammatory effect through inhibition of leukocyte-endothelial cell adhesion and inhibition of nuclear factor- $\kappa$ B activation in monocytes<sup>92–94</sup>. However, the *in vitro* anti-inflammatory effects of APC were generated at concentrations that exceed normal physiological conditions<sup>95</sup>. In all, the combined biological effects of APC in sepsis could improve survival through preserving microvascular flow and tissue perfusion while reducing local inflammation.

During human sepsis, PC is rapidly depleted over a prolonged period<sup>96–98</sup>. The reduction in PC and APC levels during sepsis blunts the multiple biological effects of APC and might correlate with greater sepsis severity in human sepsis patients<sup>99</sup>. The potential benefits of APC in sepsis and the observed reduction of APC levels in sepsis patients identify APC as an attractive sepsis therapeutic.

Remarkably, the first successful pharmaceutical sepsis therapy, APC, was tested in clinical trials after only one published study, in contrast to other potential sepsis therapies, which were informed by extensive animal studies<sup>100</sup>. Furthermore, the animal models selected

for preclinical study did not use the 'gold standard' CLP model<sup>60</sup>. The few published preclinical trials utilized LPS and live *E. coli* sepsis models in rats and baboons, respectively<sup>93,100,101</sup>. The first animal model demonstrating the efficacy of APC was based on a baboon model of intravenous infection with a LD<sub>100</sub> *E. coli*<sup>102</sup>. The baboon model was used to demonstrate the coagulopathic effects of *E. coli* bacteraemia<sup>100</sup>. This study demonstrated the severe depletion of PC in response to *E. coli*, and also that infusion of APC into baboons prevented mortality at 7 days (placebo mortality was 100% (5 baboons), versus 0% (4 baboons) in the APC-treated group)<sup>100</sup>. A second animal model of intravenous LPS administration in rats was used to determine the effect of APC on lung injury<sup>93,101</sup>. These studies demonstrated that exogenous APC reduced LPS-induced lung injury following 6 hours of study through inhibition of cytokine production and leukocyte activation<sup>93,101</sup>.

The positive effects of APC on survival echoed prior baboon studies using the same baboon model to test the efficacy of anti-TNF and corticosteroid therapies<sup>85,103</sup>. These latter therapies also improved survival in the baboon model; however, the large-scale clinical trials did not support their indiscriminate use in sepsis patients<sup>5</sup>. The success of anti-TNF therapy and corticosteroids in the baboon model and subsequent failure in clinical trials helped cast a disparaging shadow on the clinical relevance of this sepsis model. Such failures supported the claim that the baboon model represented an intoxication model rather than one of infection, as the bacterial load in these models ( $10^{11}$  and  $2.5 \times 10^{10}$  *E. coli* per kg) was considered to be within the intoxicating range<sup>22,47</sup>. On the basis of these data, one review of animal models concluded that "The moral of the TNF story is similar to that of the steroid story, which is that clinical trials based on inappropriate pre-clinical studies have little chance of clinical success"<sup>92</sup>. Given the dearth of preclinical data available, it is fortuitous that APC demonstrated success as the first pharmacological agent to improve mortality in a large-scale clinical trial<sup>19</sup>.

The effects of protein C in the CLP model were recently addressed, well after publication of the PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) trial. In a study of biomarkers during rat CLP sepsis, protein C levels rapidly declined similar to findings in the baboon model<sup>70</sup>. Furthermore, reduction of protein C served as a predictor of mortality<sup>70</sup>. Subsequent studies used genetically engineered mice that possessed heterozygous deficiency (PC<sup>+/-</sup>) in the gene encoding protein C<sup>104,105</sup>. The PC<sup>+/-</sup> mice were tested in a high-dose intravenous LPS-infusion sepsis model<sup>104</sup>. Heterozygosity of the PC gene resulted in worsened coagulopathy and lower survival rates at 12 and 24 hours as compared with the wild-type strain<sup>104</sup>. Using the widely accepted CLP sepsis model, 8-week old PC<sup>+/-</sup> mice did not demonstrate a significantly lower survival rate<sup>105</sup>. However, 24-week old PC<sup>+/-</sup> mice demonstrated a significant reduction in survival after CLP<sup>105</sup>. The 24-week old PC<sup>+/-</sup> mice also developed a more severe coagulopathy and haemodynamic shock state following the induction

LD<sub>100</sub>  
The lethal dose of a substance (for example, a toxin or infective agent) that causes 100% mortality in the study animals at a given time.

of CLP sepsis<sup>105</sup>. In the context of drug development, results from the CLP model alone (which typically uses mice 8–12 weeks of age) could have dampened the enthusiasm for APC therapy had older mice not specifically been tested. These data highlight the importance of variability within the CLP model itself, and the consideration of testing a potential therapy in selected populations (for example, aged and increased severity of illness).

With respect to the hypothesis raised by Eichacker, there are no data in animal models that evaluate the relationship between rhuAPC efficacy and the severity of illness<sup>89</sup>. Again, subgroup analysis of the human PROWESS trial data suggest that benefit of rhuAPC is greatest in those with severe sepsis<sup>19</sup>. The ADDRESS trial, which evaluated the efficacy of rhuAPC on less severe sepsis patients with a lower mortality risk, was stopped early due to futility<sup>106</sup>. These data seem to indicate that a key mechanistic threshold is crossed during the progression of sepsis. If so, animal models might contribute to defining this stage of sepsis mechanistically with respect to haemodynamics, coagulation and immuno-inflammatory parameters. Results from such studies could improve our ability to define human populations for successful therapeutic intervention.

#### Novel sepsis therapeutics

Several new inflammatory mediators of the SIRS/CARS axis have been identified and hold promise as potential sepsis therapeutic targets, including the complement C5/C5a protein, high-mobility group box-1 (HMGB1), the adenosine A<sub>2</sub> receptor, pro-apoptotic molecules and TLRs<sup>7,30,73,107</sup>. Preclinical evaluation of such pro- and anti-inflammatory drugs will ideally be performed in complementary animal models of sepsis. On the basis of previous experience, it will be important that models used for preclinical testing include different degrees of disease severity and several compartments of infection. Evaluation of potential therapies in better-defined animal models represents a method to reduce the overall costs of drug development. Although this

could represent a greater preclinical cost, hopefully the investment will translate into a reduction in the overall clinical trial sample size required to demonstrate efficacy. Reducing costs and improving the odds of success represent significant hurdles for re-engaging industry in sepsis therapy development.

#### Conclusions

Animal models of sepsis do not completely recreate the human disease or involve identical care delivered to human sepsis patients. However, it might still be possible to improve upon the animal models by better staging of sepsis. As more data in both human and animal studies support the theory proposed by Eichacker and colleagues<sup>14,16,89</sup>, it seems prudent to develop a better means of staging sepsis in each animal model and to routinely apply this categorization. This information could improve the comparability of animal studies and could potentially clarify some of the conflicting results reported using a particular animal model.

Sepsis has been viewed as potentially too complex for monotherapy. Although a multi-target approach would be ideal, it is also possible that the current problem is the inability to identify the sepsis stage most appropriate for a given monotherapy. Better definition of groups for targeted therapy is warranted, as these populations might respond differently to pro-inflammatory or anti-inflammatory agents. Indiscriminate use of either class of agent could result in lack of observed efficacy or in potential harm<sup>2,88,106</sup>. The failure of clinical trials has often been blamed on flawed animal models of sepsis<sup>4,22,60</sup>. However, it could be that the flaw lies less with the model, and more with our current understanding of how to use each model in the study of sepsis therapeutics. The criticism of animal models can be tempered by a better understanding of how to apply them in the study of sepsis therapeutic agents. Improvement in sepsis animal model staging would probably advance our ability to define appropriate human target populations for more focused and less costly sepsis drug therapy clinical trials.

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#### Competing interest statement

The authors declare no competing financial interests.

#### Online links

#### DATABASES

The following terms in this article are linked online to:

Entrez Gene:

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>  
C-reactive protein | HMGB1 | ICAM1 | IL1 | IL6 | IL10 | MIF | MyD88 | TLR2 | TLR4 | TLR9

#### FURTHER INFORMATION

Surviving Sepsis: <http://www.survivingsepsis.org/>

Society of Critical Care Medicine:

[http://www.sccm.org/professional\\_resources/guidelines/table\\_of\\_contents/index.asp](http://www.sccm.org/professional_resources/guidelines/table_of_contents/index.asp)

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