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REFINEMENT OF ANIMAL MODELS OF SEPSIS AND SEPTIC SHOCK

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ABSTRACT—This report aims to facilitate the implementation of the Three Rs (replacement, reduction, and refinement) in the use of animal models or procedures involving sepsis and septic shock, an area where there is the potential of high levels of suffering for animals. The emphasis is on refinement because this has the greatest potential for immediate implementation. Specific welfare issues are identified and discussed, and practical measures are proposed to reduce animal use and suffering as well as reducing experimental variability and increasing translatability. The report is based on discussions and submissions from a nonregulatory expert working group consisting of veterinarians, animal technologists, and scientists with expert knowledge relevant to the field.

KEYWORDS—Animal models, animal welfare, refinement, Three Rs, sepsis, septic shock

RECOMMENDATIONS

The report includes a number of recommendations, many of which are highlighted in Table 1. These recommendations are not intended by the authors to change current regulatory practice, rather to guide the reader to an appreciation of the animal welfare issues in sepsis research. They represent some key points that were raised and discussed during meetings of the expert working group (EWG) and during the preparation of this article.

INTRODUCTION

Sepsis research represents an area where many of the models used have the potential to cause high levels of suffering for animals. Therefore, there is an ethical imperative to address the issue of the validity of animal models for sepsis research, and the implementation of the Three Rs of replacement, reduction, and refinement is a priority. Because higher welfare standards go hand in hand with better science (1–3), this nonregulatory EWG was voluntarily established to (i) identify welfare issues associated with the study of sepsis in animals and (ii) set out practical refinements that can be used to reduce suffering so as to improve both welfare and research quality by minimizing experimental variability. Although sepsis has been studied using a range of species, this report will concentrate on rats and mice because these two species are used most commonly. Although the members of the EWG are all based in the United Kingdom and are most familiar with UK and European legislation (4, 5), we intend this nonregulatory guidance to be applicable internationally.

Sepsis is a complex syndrome that commences with a systemic immune response to an infection that can progress to severe sepsis and septic shock resulting in multiple organ failure and death (6). Current therapeutic approaches are based on a combination of fluid resuscitation (to maintain target hemodynamics and oxygen saturation) and antimicrobial therapy (to address the infection) and, as the syndrome progresses, vasopressor, cardiac ionotropes, and corticosteroids may be administered (7, 8). However, in the United States, sepsis is responsible for more than 200,000 deaths a year (9) and about 40,000 in the United Kingdom (10), so there is still a need for new approaches for diagnosis and treatment of sepsis. Although the identification of clinical biomarkers in patients that facilitate earlier diagnosis and rapid therapeutic intervention is likely to have the greatest impact on survival rates (11–13), understanding disease mechanisms and the therapeutic efficacy of novel pharmacological/genetic interventions is of critical importance, and it is for this purpose that animal studies are currently conducted.

There is considerable current debate as to the predictive validity and translational value of many animal models used in medical research in a range of disease areas and research disciplines including sepsis (14–19). It is essential to acknowledge that all experimental models have limitations and that an animal model can never fully replicate all of the features of human disease. Where predictive validity is poor, any benefit that may result from an animal study is limited and such work is hard to justify on ethical grounds.

For the purposes of this article, we will use the term *sepsis model* to cover all interventions that lead to a sepsis-like syndrome in animals.

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This article represents the outcome of discussions from an expert working group (EWG) on behalf of the RSPCA as part of its work toward ending severe suffering for animals in research. Elliot Lilley, Rachel Armstrong, Nicole Clark, Peter Gray, Penny Hawkins, Simon Jackson, Karen Mason, Manasi Nandi, Noelia López-Salesansky, Anne-Katrien Stark, and Christoph Thiemermann were all members of the EWG. The manuscript was written by Elliot Lilley and Manasi Nandi and was edited and revised by the EWG.

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Table 1. **EWG recommendations**

The EWG makes the following recommendations:
1) Researchers should use the least severe model feasible to answer their research question, ensuring that the model is standardized and fully characterized to minimize interlaboratory variability.
2) Appropriately validated quantitative biomarkers and use of humane end points should replace death as an end point in sepsis studies whenever possible.
3) Animals should be allowed to make a complete recovery from any surgical instrumentation (for biomarker measurement) before induction of sepsis.
4) Provision of analgesia should always be the default position; exclusion should be supported by specific evidence that such use would negatively impact experimental validity.
5) Comorbidity studies should only be performed when their translational value is high and the potential for animal suffering can be minimized.
6) Fluid resuscitation should ideally be via the intravenous route and, wherever possible, using a vascular access port.
7) Where relevant, the dosage of sepsis-inducing agents should be stated/calculated as units of activity per kilogram rather than milligrams per kilogram to account for batch variance or quantified appropriately.
8) The bacterial load given should be appropriately quantified, where feasible.
9) Biomarker data are needed to define the most refined and consistent humane end points and to assist in building better *in silico* models to reduce reliance on animal studies. To facilitate this, experimental data should be shared, positive and negative results and any adverse effects should be reported, and all studies should be published according to reporting standard guidelines.
10) Social animals should be group-housed rather than singly housed, unless there is a compelling scientific or welfare reason for not doing so.
11) Objective animal welfare assessment is of paramount importance, and a collaborative working arrangement with researchers, animal technologists, and veterinarians is essential. Clinical end points need to be well defined, and every effort is made to minimize suffering.
12) Where potential welfare issues are unknown or poorly characterized (e.g., when using a new genetically altered line), pilot studies should be performed.

General considerations regarding the use of animals in sepsis research

As with any area of medical research, different *in silico*, *in vitro*, *in vivo*, and clinical approaches can be used to address scientific questions. The decision to use animals in sepsis research must only be taken after all *in vitro* and *in silico* approaches and human clinical studies have been ruled out based on scientific or ethical grounds. Knowledge of the strengths and weaknesses of, and alternatives to, *in vivo* sepsis models is essential to choose the most appropriate model system to use. In this context, it is important to stay up-to-date with current best practice in sepsis model choice, design, refinement, and general animal welfare science through the use of published literature and from scientific meetings where new methods and refinements can be discussed.

The choice of animal model should be guided by animal welfare considerations, with the aim of minimizing suffering. The EWG views this as an integral part of the process for selecting the best scientific approach. It should also be noted that *in vitro* work also raises ethical and welfare issues, for example, where primary cell lines must be generated or animal serum is required for cell or tissue culture.

Potential for replacement

The complete replacement of animal models of sepsis with cell culture systems is currently difficult because of the complexities of the immune and multitissue responses associated with sepsis. However, *in vitro* cell culture models may be useful to analyze specific aspects of the biological response and may be used in the initial steps toward understanding mechanisms.

Considerable advances have been made in detailing the molecular mechanisms underlying the acute inflammatory response, and the complexity of this process makes inflammation a prototypical case study for the application of systems and computational biology (20, 21). Combining data from mouse models and *in vitro* cell models of lipopolysaccharide (LPS)-induced inflammation allowed a mechanistic computational model that simulates whole-animal inflammation to be developed (22).

Macrophages form part of the first line of antimicrobial defense, and macrophage differentiation and activation are important to the type and magnitude of the responses they elicit. Therefore, better understanding of the factors that govern macrophage differentiation and activation is an area of current sepsis and infection research. However, primary tissue macrophages of sufficient number and quality are difficult to obtain and, because of a limited life span, often require large numbers of animals (23) and are therefore not in themselves without welfare and ethical issues. Recently, a simple method yielding self-renewing, nontransformed, GM-CSF/STAT5–dependent macrophages (MPI cells) from mouse fetal liver was reported (24). This technique is important because it has the potential to dramatically reduce the numbers of animals needed to study primary tissue macrophages.

In addition to conventional two-dimensional monolayers, there is growing use of organotypic three-dimensional (3D) cell culture models to explore infectious disease mechanisms. Three-dimensional cell cultures more closely mimic the morphological and functional features of their *in vivo* parental tissues (25–28). These 3D systems have enormous potential for bridging the gap between cell-based research and animal models for studying both host–pathogen interactions and human disease progression, as well as for the development of novel drugs and therapeutics (29–31). Infection mechanisms explored using 3D culture models include *Pseudomonas aeruginosa* lung infections (32), *Salmonella typhimurium* in the small intestine (26), hepatitis C virus in the liver (33), and LPS in lung and liver models (28).

In vitro models can be used to investigate specific aspects of sepsis pathophysiology, allowing a more focused approach to be used in animal models that might better translate into the human condition.

New approaches could also be explored to improve current models both in terms of application of the Three Rs and improving translational validity. These could include genomic descriptions from patient studies to better define the human disease and using identified disease-modified pathways as a guide to develop the most appropriate animal model. The translational value of the animal model could then be determined by how well it reproduces the human disease on a molecular basis rather than simply phenotype (17). In addition, *in vitro* reconstitution of disease-related cell types or tissues could be used in the development of synthetic human models that would also improve current disease models (34). Moreover, new genomic information, such as the availability of personal genomes (35) or exomes (36), to capture the disease heterogeneity directly from patients or systematic interpretation of genome-wide signatures in human diseases (37, 38) will complement or even replace the need for mouse models in disease discovery and drug development.

The use of living animals in sepsis research

When, following consideration of ethical and welfare issues, including a harm–benefit analysis, the choice is taken to use animals to model human disease, the data that are produced should allow for translation of relevant information from animal models to the human condition. In an ideal world, the model should be robust, reproducible, and cause no distress to the animals involved. Unfortunately, based on these criteria, the ideal model of sepsis does not currently exist (39).

The following sections will describe issues relating to animal models of sepsis, including points to consider and recommendations for refinement by the EWG. More detailed refinements related to how experimental sepsis procedures are conducted are presented in the section titled "Potential Adverse Effects in Experimentsl Sepsis and How These can be Refined," as well as in Table 3.

Species used

Rodents are the most commonly used species in preclinical research. This is because of the ease of introducing genetic modification, large litter sizes, short generation time, and relative ease of housing and care. Large mammals such as sheep and pigs have also been used in sepsis research to enable interventions that more closely align with the clinical intensive care setting and therefore are considered useful for proof-of-concept studies before human studies. These include high-volume fluid resuscitation to mimic the hyperdynamic state of the cardiovascular system and invasive measurement of multiple physiological parameters, which may be technically challenging in smaller species (40). However, it is beyond the scope of this article to critique the different species choice for sepsis studies, and species selection must be guided by the research question (39, 41).

Genetically altered animals—Genetically altered animals (induced or naturally occurring), including animals that are immunocompromised, may also be used for mechanistic sepsis studies. The nature of the mutation may make animals more susceptible to sepsis, resulting in more severe clinical signs (in terms of intensity or duration) than those that occur in wild-type animals.

Where data do not currently exist, the EWG recommends the use of carefully designed pilot studies (see section titled "Pilot Studies") or a staged approach to characterize the impact of sepsis models in such animals. This can facilitate the identification of humane end points and indicate other potential refinements.

Nature of sepsis models

To identify and define refinements of animal models of sepsis, it is important to understand the different types of model that are currently used. The EWG has highlighted specific considerations for these models that can reduce the impact on animal welfare and/or reduce experimental variability to facilitate the choice of sepsis model.

Animal models of sepsis broadly fall into one of three categories: exogenous administration of a bacterial toxin (toxemia models); exogenous administration of a viable purified and/or fecal-derived pathogen (bacterial infection models); or alteration of the animals endogenous protective barrier (host–barrier disruption models). For reviews of animal models of sepsis, see (14, 41–44):

Toxemia models—These are often used to study the basic biology of septic shock, for proof of concept, and, in particular, in mechanistic studies into the role of Toll-like receptor signaling. Injectable chemical agents such as LPS, peptidoglycan, lipoteichoic acid, CpG DNA, zymosan, and synthetic lipopeptides are typically used. Lipopolysaccharide causes a severe systemic inflammatory response in the absence of an ongoing infection, bypassing opsonization, and does not create a model of sepsis per se. Experiments (especially those investigating the effects of anti-inflammatory interventions) may need to be repeated in a model with a microbial source of infection because such interventions may modify the host defense mechanisms.

From a refinement point of view, the advantage of toxemia models is that there is a rapid onset of pathological changes and they can therefore be performed in terminally anesthetized animals (45–47). In addition, toxemia models can have relatively low interanimal variability because the exact dose and route of administration can be standardized. However, the EWG recommends that the dosage should be calculated based on the units of activity per unit body weight rather than as milligrams per kilogram to account for batch variance.

Live bacterial infection models—These models involve administration of either pure or mixed bacterial flora into the animal via an appropriate route to mimic different clinical scenarios. There is a wide range of published models including intravenous bolus injection of a pure strain of bacteria (e.g., *Escherichia coli*) (48, 49), inhalation of *Streptococcus pneumoniae* (50, 51), or intraperitoneal administration of filtered fecal slurry (52).

There are a number of potential confounding factors with these types of sepsis model, including the choice of bacterial strain, the bacterial load, and the susceptibility of the host animal and the compartment of infection. It should be noted that the usually high bacterial load administered may not, in all cases, go on to colonize and replicate in the host—often caused by rapid lysis by complement, resulting in the release of endotoxins (53). The likelihood of colonization will depend on

the species and strain of animal and serotype of bacteria being studied as well as the site of infection (50, 54).

Pneumosepsis models (pulmonary infection of respiratory pathogens) deserve specific mention because previous respiratory tract infection is a common cause of sepsis in patients. These models not only produce lung pathology but also can go on to produce bacteremia and distant organ damage (50). A distinction must be made between experiments where lung pathology is the sole focus of the study and experimental models where sepsis, secondary to lung pathology, is a key component. Clearly, the latter introduces additional welfare considerations to the former, and this should be taken into account during harm–benefit evaluation of studies of this type. The intraperitoneal administration of fecal slurry derived from a "donor" animal to a "recipient" animal is another method used to induce sepsis.

These models do not require surgery to induce sepsis (see host-barrier disruption models below) that reduces the impact on the animal. However, care should be taken to avoid unintended organ damage or gut perforation when performing an intraperitoneal injection. In addition, efforts should be made to standardize the microbial composition and load administered wherever possible. To reduce interanimal variability and better enable comparison of the published literature, the EWG recommends appropriate quantification of the bacterial load given, where feasible.

Host-barrier disruption live bacterial models—These models involve compromise of the protective barrier separating the normally sterile internal compartments of the body from bacteria and other pathogens and give rise to a polymicrobial insult reflective of the flora of the individual animal. Typically, sepsis models of this type involve damage to the intestine that causes leakage of fecal material into the normally sterile peritoneal cavity. Examples include cecal ligation and puncture (CLP) (55, 56), cecal ligation and incision (CLI) (57), and colon ascendens stent peritonitis (CASP) (58). Of these, CLP is the most widely used and is viewed by many as the gold standard for sepsis research (16, 59) because it is considered to mimic the human clinical profile and time course of abdominal peritonitis, appendicitis, and perforated diverticulitis, all of which can lead to sepsis and septic shock.

These models require full surgical anesthesia (with recovery for CLP and CASP), midline laparotomy, and exteriorization of the cecum. In addition, in CLP and CLI, the cecum is ligated below the ileocecal valve. In all three models, the cecum is perforated with either single or multiple punctures with a syringe needle (CLP), a blade incision (CLI), or introduction of a stent into the ascending colon distal to the ileocecal valve (CASP).

For CLP, the proportion of cecum that is ligated, the gauge of needle used, and the number of punctures given can all introduce variability in the severity of sepsis for the animal (60). Researchers should standardize and fully characterize this model to minimize severity and interlaboratory variability.

CASP was developed to attempt to deal with some of this variability by removing the need to ligate the cecum and by using a stent rather than puncture of the bowel. However, this requires more complicated surgery and may involve a second surgical procedure to remove the stent if the model is being used to evaluate the effect of surgical intervention on disease progression (61). Stent size is not standardized; larger stents will produce more severe disease (58). As with CLP, researchers should standardize and fully characterize this model to minimize both severity and interlaboratory variability.

CLI was developed recently and is proposed as an acute-onset model of severe sepsis (62, 63). The advantage of this model is that the whole procedure is performed under nonrecovery anesthesia and, therefore, minimal suffering should be experienced by the animals. Given that this model is likely to cause less suffering that either CLP or CASP its use should be given careful consideration. However, this technique has not been used widely, and its clinical relevance has not yet been fully evaluated.

Risk factors and comorbidities

In the clinical setting, sepsis mortality is highest in very young and elderly patients who often present with comorbidities, representing highly heterogeneous population. By definition, this means that there is no single animal model that fully recapitulates the clinical sepsis syndrome (15, 64). A number of studies have indicated that gender and age both influence sepsis progression in animal models in a manner consistent with patient data (65–68). However, most preclinical sepsis studies use young adult male rodents with no comorbidities, and this may represent an issue with respect to translational validity (64, 69).

From a welfare perspective, although the use of older animals or neonates may produce more clinically translatable results, the severity of the septic insult is likely to be higher. This raises an important ethical dilemma requiring careful assessment of both harms and benefits associated with such studies. It is the view of the EWG that minimizing the potential for suffering should be a priority and, therefore, studies in aged or neonatal animals should only be undertaken to obtain mechanistic data specific to these age groups. Suffering should also be minimized through refinement and use of humane end points.

Given the highly comorbid clinical population, it has been argued that incorporation of comorbid injury/insult into animal models of sepsis may increase their clinical relevance (44, 64). However, the addition of comorbidity to a sepsis model will clearly increase the cumulative severity of the procedure, raising further ethical issues and increasing the experimental variability without sufficiently recapitulating the clinical pathology. It can be argued that disease model systems should be kept simple and unconfounded, so that specific mechanisms can be studied and data can be compared across different laboratories.

The EWG members debated the clinical relevance of comorbidity models in sepsis research, and not all were convinced of the translational value of current comorbidity approaches. In our view, there is currently insufficient evidence to support use of these models. However, given the clinical prevalence of comorbidity in sepsis, research in this field is needed. The scientific community (both preclinical and clinical) needs to reach a consensus on the translational value of comorbidity models based on existing and new data as they emerge. We recommend that careful pilot studies are performed to evaluate the animal welfare implications and scientific value of any comorbidity study, and that every effort should be made to minimize animal suffering.

Biomarker measurement

Biomarker data are highly valuable for both translation of animal model data into clinical trial design and to identify and validate early humane end points for use in future studies.

General considerations

Provided that it will not cause additional suffering or confound data quality, measuring multiple biomarkers in the same animal can provide a global overview of syndrome progression. Suffering can be reduced by conducting procedures on anesthetized animals and/or using the most refined approach available. However, it is important to recognize the harms associated with repeated capture, handling, and multiple exposures to anesthetic agents and to weigh them against the benefits of the additional experimental data.

The surgical implantation of devices required for biomarker measurements has the potential to cause postsurgical pain, infection, and wound breakdown. Appropriate aseptic technique and perioperative analgesia must be used and adequate postsurgical care given to prevent and control such complications and their potentially confounding effects. Importantly, animals should be allowed to recover fully from any surgery before the induction of the septic insult to minimize the potential adverse welfare impact, stress artifacts, and variability in subsequent results. Recovery should be assessed through monitoring body weight, feeding and behavioral patterns, and other standard clinical observations (70).

Researchers should ensure that they are aware of any relevant advances in technology that permit reproducible measurements to be obtained from animals with a lower potential for causing welfare harms. Up-to-date equipment should be used, and outdated equipment should be replaced at the earliest opportunity wherever more refined alternatives become available.

Blood pressure and heart rate measurements—Blood pressure and heart rate can be monitored in both anesthetized and conscious animals. The latter raises greater potential welfare concerns (71) but, despite this, tail cuff, tethering, and radiotelemetry are commonly used techniques. Both tail cuff and tethering approaches necessitate restraint known to introduce a stress response/artifact that can impact significantly on cardiovascular function, introducing experimental variability in both naive and septic animals (72–74).

Advances in monitoring technology during the last 15 years have introduced significant refinement to animal models. Radiotelemetry represents the current gold standard in remote physiological monitoring. In contrast to tethered systems, signal loss and dropout are rare (75) and systems can provide simultaneous readings such as electrocardiography, locomotor activity, and temperature. However, use of telemetry is not free from adverse animal welfare consequences (76) and is, in itself, a technique that needs refining (77). As with all procedures that involve the use of animals, use of telemetry should be subject to a harm-benefit analysis, taking into account the broader lifetime experience of the animal.

Animals should be allowed to fully recover from telemetry surgery to minimize any negative welfare impact and to enable accurate baseline measures to be obtained before giving a septic insult. Surgical recovery after arterial catheterization for implantation of a radiotelemetry device has been reported to take up to 7 days. After this period, a robust circadian core body temperature is likely to have been reestablished and locomotor activity patterns and body weight normalized (75, 78).

Multichannel digital biotelemetry devices are becoming available, allowing animals to be group housed, rather than singly housed, and this should be the default for social animals, unless there is a robust scientific argument against it.

The use of single-frequency transmitters does not justify single housing in itself. The telemetered animal can be cohoused with a nontelemetered cage mate or, alternatively, animals with devices can be group housed and separated for recording or implanted with devices that can be switched on and off and used one at a time (77).

Temperature—Core temperature can also be used as a biomarker of overall health status in sepsis and may be useful in defining humane end points (79).

However, it should be noted that hypothermia may be an adaptive and protective response to sepsis. This has been suggested in human patients (80), and some of the EWG believe that the same may also be true in rodents (unpublished observation). Mammals and birds are endotherms and can regulate their body temperature via their metabolism (81). Indeed, it has recently been suggested that hypometabolism may prevent hypoxia in a rat model of endotoxic shock (82). It should therefore not be assumed that hypothermia is always an indicator of poor prognosis in sepsis.

There is a variety of methods available including use of microchip transponders (83), telemetry devices (84), or noncontact thermography (85, 86). Careful consideration needs to be given as to the most appropriate method for measuring temperature to ensure that there is minimal adverse welfare impact on the animal, taking into account the level of invasiveness and the type and frequency of handling.

Echocardiography—Echocardiography can be used to measure clinically relevant cardiovascular parameters, such as cardiac output, ventricular dilatation, and ejection fraction (87). The overall welfare benefit of this method is that it is noninvasive and is usually performed under anesthesia. Multiple paired measures can be obtained during syndrome progression, although repeated use of anesthetics raises welfare issues (see below). Importantly, studies have shown that cardiac contractility can accurately predict survival outcome and therefore should be used as a surrogate biomarker to facilitate the use of humane end points where possible (87).

Microcirculatory monitoring—Sepsis is recognized as a disease of the microcirculation, and poor microvascular blood flow in sepsis patients correlates with multiple organ failure and poor prognosis (6, 88–90). Microcirculatory monitoring can be performed by sidestream dark field imaging, laser Doppler, or laser speckle contrast-based techniques and is typically performed under anesthesia in a variety of vascular beds. As technology improves and data become available to better understand the relationship between microvascular flow and disease progression and prognosis, this approach may be useful for identification and evaluation of both novel therapeutic targets and humane end points in animal models of sepsis.

The EWG is of the opinion that as long as it is feasible and would not increase the welfare burden, monitoring of the microcirculation in anesthetized animals may be a valuable addition to the biomarker repertoire.

Blood biomarkers—Blood biomarkers of sepsis are highly important for early and accurate diagnosis in the clinic and can similarly predict survival in preclinical studies (91–93). Such measurements, incorporating biochemical markers of organ function and metabolic acidosis and cytokine profiling, improve the translational impact of these studies and facilitate the implementation of early humane end points.

The method used for blood sampling must be carefully selected, with consideration for the welfare impact of the sampling method, frequency, and volume of blood that will be removed (for review of good practice, see [70]). It is now possible to analyze very small blood sample volumes for certain biomarkers, and repeat low-volume blood sampling can allow for close monitoring of changes in biomarker levels, in individual animals, across time (94, 95). Where repeat sampling is not required, arterial and venous bleeds should be performed at the end of the study under terminal anesthesia.

Postmortem histopathology—Although blood biochemistry markers are useful indices of organ function, postmortem histology should be conducted on freshly isolated tissues to establish the extent of inflammation, organ damage, and necrosis and to inform subsequent experiments. Consideration must be given to maximizing the use of postmortem analyses especially where this may have the potential to reduce or replace *in vivo* procedures or to inform the development or use of humane end points.

Other considerations for use of living animals in sepsis research

Anesthesia

In some cases, it may be necessary for animals to undergo repeated short-term anesthesia (e.g., for repeat echocardiography). Although inhaled anesthetics are generally preferred to injectable agents (because of ease of regulation), repeated exposure to inhaled general anesthetics can lead to welfare issues, including possible aversion stress in rodents, and repeated anesthetic use in general may influence disease progression (96, 97). In addition, animals with septicemia may be more difficult to safely anesthetize—as in the human clinical setting (98, 99). Anesthetic agents should be selected (in consultation with a veterinarian) on the basis of their suitability for the study both in terms of minimizing any welfare concerns for the species being studied and minimizing any adverse impact on data quality.

Fluid resuscitation

Fluid resuscitation is a key component in sepsis models, but provision of clinically relevant resuscitation volumes to mimic hyperdynamic sepsis is a challenge in rodents because of their size, although hyperdynamic examples exist (100–102).

The EWG recommends that any resuscitation regimen should use the least invasive approach, consistent with the research question being addressed. The intravenous route is likely to be the most effective and clinically relevant. If large-volume frequent resuscitation is required, we recommend the use of a preimplanted venous access port because this negates the need for chronic tethering.

Mechanical ventilation

Sepsis patients are given respiratory support and mechanical ventilation can be successfully used in rodent models of sepsis (101, 102). Use of mechanical ventilation should be considered as long as appropriate anesthetic depth is maintained and the mode of ventilation does not cause any additional welfare problems or confound the experimental outcome.

Analgesia

Analgesics such as morphine-based derivatives or nonsteroidal anti-inflammatory drugs should be administered perioperatively in relation to the surgical implantation of devices for biomarker measurements, and appropriate analgesia should be maintained until animals are fully recovered. However, the use of analgesia after induction of sepsis is more contentious and provision of analgesia during or after surgery for CLP or CASP or to alleviate any pain during sepsis itself is rarely reported (14). The primary reason expressed for the reluctance to use analgesia is the concern that such use may interfere with the inflammatory processes that are involved in the development of sepsis. This may be true for nonsteroidal anti-inflammatory drugs and morphine but not for other opioid analgesics that are classified as having less immunosuppressive activity (e.g., buprenorphine, tramadol [103). Recent studies (101, 102, 104) suggest that it may be possible to use buprenorphine in CLP studies in rodents.

Given that pain is not only undesirable on ethical grounds but also may affect disease outcome and experimental variability (105), the EWG recommends that analgesia should always be used where required, unless there is clear, specific, scientific evidence to preclude its use.

Antimicrobial agents

In the clinic, patients receive broad-spectrum antibiotics as part of the treatment regimen. It has been suggested that animals should also receive these in preclinical studies especially when the efficacy of a nonantibiotic treatment regimen is being assessed (106). However, the use of antibiotics should be carefully considered because they could cause a variety of side effects and potentially interfere with the pharmacokinetics of therapeutic agents under investigation (107).

Septic animals with impaired renal and hepatic functions could experience antibiotic-induced toxicity at normal doses because of a reduced ability to metabolize and clear them (108). Other adverse reactions that should be taken into account and monitored accordingly are antibiotic-associated diarrhea and colitis (107) and changes in blood biochemical and hematological parameters (109).

Finally, the long-term use of broad-spectrum antibiotics in large numbers of animals could lead to the development of antibiotic-resistant bacteria that would have broader implications and may impact on all housed animals within research facilities (110).

Pilot studies

Pilot studies are a useful way to evaluate the welfare impact of a particular procedure or intervention where prior knowledge is lacking. In particular, pilot studies can be used to evaluate a refinement of an existing procedure or model, for example, the use of analgesia. However, although animals used in a full study will benefit, pilot studies demonstrating that a particular refinement is effective also have the potential to cause suffering and will require additional animal use. They should therefore be subject to a harm-benefit assessment.

Humane end points

Many current sepsis models can cause high levels of morbidity and mortality. In the clinic, the success of treatment for sepsis is often measured by the survival rate of patients at 28 days. As a consequence, when evaluating the efficacy of a new therapeutic approach in experiments using animals, researchers often wish to use survival as the principal study readout. However, death as an end point raises significant ethical concerns, especially because it is likely to be preceded by severe suffering (111). As for every other field of research where animals are used, humane end points are needed in sepsis research (39, 112–114). A *humane end point* can be defined as a set of criteria that enables a study to be ended earlier or to alleviate pain or distress so that the suffering of the animals can be reduced or ideally eliminated (115).

The identification and use of quantitative biomarkers will facilitate the development of humane end points that truly predict outcome in animal models of sepsis. Indeed, some progress has already been made in this area (6, 87, 93, 94).

The EWG is of the opinion that death as an end point should not be used. Prudent use of quantitative biomarkers, which empirically describe the extent of sepsis progression and the underlying pathophysiological processes, is more scientifically valuable than using mortality measures alone, where the exact cause of death may be difficult to establish and where valuable biomarker data may be lost.

Experimental design and the potential for reduction

As in most areas of animal research, it is rare for power calculations to be reported in sepsis publications (although in some EU countries, it is mandatory to perform an appropriate statistical evaluation to obtain ethical approval). This can make it impossible to judge whether experiments were underpowered (using too few animals, wasting animals, and producing unreliable data) or overpowered (using too many animals and causing avoidable suffering). In addition, a recent study into the reporting standards of animal research in critical care journals indicated that *ethical quality* (as judged by reporting of Three Rs–related information) was poor (116).

There are a number of reporting guidelines (117–119) for studies that involve animals, and all require power calculations as part of the minimum information that should be included in

TABLE 2. **Sources of variation in experimental sepsis**

Source of variation	Recommendation
Stress/pain/discomfort	Avoid restraint and minimize direct investigator intervention wherever possible. Provide analgesia (using a standardized protocol) when appropriate by default, unless there is specific evidence that this will invalidate the particular study in question.
Lack of quantification of sepsis insult	1) Calculate/administer doses of sepsis-inducing agents as units of activity per kilogram.
	2) Standardize and characterize bacterial load for bacteremia models and report this clearly.
	3) Ensure that administration methods are standardized and reported (and published) clearly (e.g., injecting into a "tent" when performing i.p. injections).
	4) In CLP studies, ensure needle size, number of punctures and percentage of the cecum ligated are consistent, and report this clearly.
	5) In CASP studies, ensure that stent size, location, and placement duration are consistent and reported in publications
Blood pressure signal loss/dampening because of lack of catheter patency in tethered animals	Flush with fluid to maintain patency or consider alternative methods to monitor blood pressure
Insufficient recovery time after instrumentation surgery for biomarker measurements	Ensure animals are fully recovered before induction of sepsis. Where appropriate, use a pilot study to determine the appropriate recovery period.
Experimental bias	Exclusion criteria must be preestablished and clearly reported. Studies and analyses should be blinded.
Variations in and quality of husbandry and care	Within a study, ensure that the experimental and housing environments are consistent and that the nature and frequency of human intervention (e.g., cage changes, room cleaning) are standardized.
Statistical power—underpowered studies will be misleading	Conduct power calculations, use appropriate numbers, and define appropriate statistical analysis at the project planning stage. Use pilot experiments, where appropriate, to evaluate welfare issues and statistical power.
Variations in health status, for example, pinworm infection	Apply good health care and colony management led by animal technologists and the attending veterinarian.

a scientific publication (and therefore included in the design of the study).

This EWG recommends that specialist statistical advice is sought when designing experiments to ensure that they are adequately powered, that the minimum number of animals required to generate satisfactory data is used, and that appropriate statistical analyses are applied to data.

A staged approach, where the overall experiment is divided into a series of smaller experiments with smaller group sizes, may be useful when assessing the effect of a novel intervention. A well-designed pilot study may form part of this staged approach, and interim analysis at the end of each stage can highlight issues that lead to early termination of the study (e.g., for welfare reasons). However, care must be taken not to introduce bias into the study design.

Experimental systems, by definition, must have sufficiently low control variability to allow scientifically valid conclusions to be drawn. Minimizing experimental variability also increases the power of the study, thereby reducing the number of animals required. It has been argued that animal models should reflect the heterogeneity present within the patient population that has the clinical condition being modeled (64). However, this approach raises important ethical and scientific challenges. Increasing variability may make it impossible to reproducibly and reliably test hypotheses and may require an ethically challenging increase in group sizes. A balance must be sought where the translational value of the model is as high as possible while minimizing extraneous variability and using the minimum number of animals.

Extraneous variability can be minimized through:

i) Careful selection of the study cohort, incorporating scientifically valid species, age, and sex, with appropriate controls (e.g., littermates or use of paired controls).
ii) Standardization of protocols used to induce experimental sepsis.
iii) Use of monitoring techniques/technology with a high signal-to-noise ratio that permits the acquisition of reproducible measures of physiological biomarkers.

The EWG has developed recommendations that aim to reduce experimental variability and has highlighted recommendations to minimize these in Table 2.

Potential adverse effects in experimental sepsis and how these can be refined

A useful approach to achieving refinement is to set out the whole life experience of the animal and consider how each potentially painful or distressing event could be refined, such that the overall impact is a significant reduction in severity. The overarching principle of this approach is the *accumulation of marginal gains*, in which each individual refinement may not make a significant difference in itself but, when implemented all together, the effect may be that a procedure is significantly less severe to an individual animal (120).

Some refinements have already been described in preceding sections. Table 3 sets out potential adverse effects that may be experienced by animals used in sepsis studies, with suggested ways of ameliorating pain or distress in line with the accumulation of marginal gains principle. The EWG understands that not all of the refinements will be possible, or applicable, within every project, and additional text to explain some of the entries and complement the Table is set out below.

Housing and care refinements

Animals with sepsis are likely to have special husbandry needs. Highly debilitated animals will have limited ability to move around the holding cage and will have difficulty in feeding, drinking, and maintaining body temperature. For these animals, additional provision will be required in addition to standard good practice (as defined by local legislation and/or guidance). The use of soft/soaked food or fluid blocks can reduce inappetance or the impact of the consequential weight loss associated with the condition.

Sick animals are more likely to lose body heat and will benefit if housed with untreated animals because they can group associate with other animals to aid thermoregulation. An alternative approach is to place the cage on a heated mat; this can be placed under part of the cage to give the animals a choice as to whether they require supplementary heating. However, septic animals can *shut down* and become very cold, almost hibernating (state of torpor), which may be an adaptive/protective response; a similar effect has been reported in human sepsis patients (80). The adaptive/protective hypothermic response in animals requires additional study, and the decision whether to provide thermoregulatory aid should be informed by up-to-date scientific understanding and veterinary advice.

Animals should be weighed regularly, but this will involve capture, handling, and restraint, which may be stressful to the individual (121). As increased stress may exacerbate sepsis symptoms, it may be important to minimize handling and to remove stressors from the environment as far as possible. However, it is necessary to balance this with the need to accurately observe and examine animals to ensure that adverse effects are identified early, so that appropriate action can be taken and humane end points can be applied.

Assessing animal well-being, pain, suffering, or distress in sepsis studies

To assess the welfare status of animals used in any scientific study, it is essential to understand what *normal* behavior and baseline physiological parameters are for the species strain and sex of animal being studied. It is important to be able to rapidly recognize indicators of problems associated with the model and techniques (e.g., kinking of tether lines, hypothermia, weight loss, immobility).

An effective day-to-day welfare assessment system, tailored to the species, strain, and protocol, should be developed. This will require a team approach, input from the researcher(s), animal technologists, and attending veterinarian (122, 123). Indicators of suffering can be obvious (e.g., weight loss, lack of voluntary movement, diarrhea) or more subtle (e.g., facial expression [124, 125] or nest building/latrine location [126]). All those responsible for assessing animals should receive adequate training in recognizing indicators of suffering associated with each project and in using the recording systems in place.

TABLE 3. **Procedures that can lead to adverse effects and how these can be refined**

Procedure	Adverse effect	How this may be refined
Instrumentation before induction of sepsis		
Capture and restraint	• Aversion stress and anxiety	• Catch mice by cupping in the hands, or in their home cage tunnel, instead of by the tail. These methods of capture are less aversive and induce less anxiety (121).
Surgery	• Aversion toward anesthetic	• Ensure that the most effective and least aversive anesthetic agent that is compatible with the scientific objectives has been selected.
	• Postoperative pain	• Provide appropriate perioperative analgesia.
	• Infection	• Use aseptic techniques.
	• Organ/tissue damage	• Ensure optimal surgical approach and handle tissues gently during surgery so as to minimize tissue damage.
	• Suffering because of poor postoperative care	• Ensure that the surgeon is adequately trained and competent; record postoperative outcomes including behavioral observations and analgesia requirements.
		• Ensure that postoperative husbandry and care are appropriately refined, including soft diet, heat pads (see text).
		• Monitor weight and food/water intake during recovery and introduce a clinical scoring system.
		• Regularly review postoperative monitoring protocols, including use of score sheets.
Tethering	• Surgical issues	• See surgery information above.
	• Discomfort caused by tether	• Use most refined tether system suitable for the species, which maximizes the range of movement for the animals and minimizes the impact of the harness worn by the animal.
Radiotelemetry	• Surgical issues	• See surgery information above.
	• Discomfort caused by telemetry device	• Use the smallest lightest telemetry system available and site the device to minimize the impact on the animal. Systems that record multiple parameters simultaneously should be considered provided that the size of the implanted device does not add to the welfare burden on the animal.
	• Stress caused by single housing (in normally social animals)	• Use a multichannel system that allows social animals to be group housed (where this is suitable for the species, strain, and sex of animals used) or use another solution such as cohousing telemetered and nontelemetered animals.
Baseline blood biomarker measurements	• Aversion toward anesthetic	• Take into account current knowledge of the effect of repeated short-term use of anesthetic on animal welfare and use the least aversive agentpossible for the species and strain being used.
	• Pain or discomfort caused by blood sampling technique	• When taking either single or repeated blood samples, techniques must be refined to minimize suffering. Regularly research new approachesto blood sampling; for example, facial vein sampling of low volumes of blood has recently been reported (95). Retro-orbital sampling cancause tissue damage and should not be used.
Induction of sepsis		
Capture and restraint	• Aversion stress and anxiety	• See above
Surgery	• Surgical issues	• See above
	• Postoperative pain	• Provide appropriate perioperative analgesia, unless there is compellingevidence that it would invalidate the experiment.
	• Sepsis severity with potential for high morbidity/mortality	• For CLP/CASP, the least severe model (number of punctures, proportion of cecum that is ligated, stent size, etc) that enablesthe research question to be answered.
		• Consider the CLI method performed under nonrecovery anesthetic.
Administration of agents	• Pain/discomfort caused by injection	• Refine techniques to minimize suffering, that is, use sharp sterile needles of the smallest gauge possible for administration of sepsis-inducing agents.
	• Tissue/organ damage	• Carefully select the site and technique of injection to minimize the risk of tissue damage or accidental injection of material directly into organs (e.g., injecting into a “tent” when conducting i.p. injections).
	• Aversion toward anesthetic	• If anesthetic is used to sedate animals for administration of agents, use the most effective and least aversive anesthetic agent that iscompatible with the scientific objectives.
	• Pain/discomfort caused by injection of fluid. Issues caused by tethering (see above)	• Use an indwelling intravenous cannula to administer fluid resuscitation or pharmacological agents (for both bolus injections and for transient infusions via tether; see above for information regarding tether use).
Sepsis		
Suffering caused by sepsis	• Postsepsis discomfort, hypothermia	• Provide additional nesting material and litter, refuge, and environmental enrichment.
	• Inappetance, dehydration, weight loss	• Provide supplementary nutrition (e.g., wet mash, liquid nutrition) and facilitate easy access to food and water (e.g., lower feeding/drinking nozzles, provide food in a tray on the cage floor). Provide any novel foods before the study to ensure that animals are habituated and prepared to eat them.
	• Morbidity and potential for mortality	• Use a monitoring and recording system (e.g., clinical sign scoring sheet) specifically tailored for the species and strain of animals and the nature of sepsis model being used (see below).
		• Maintain animals for as short a time on the study as is consistent with acquiring satisfactory data.
		• Tailor monitoring regimen to ensure that animals are observed sufficiently frequently during the most severe phase of the syndrome to ensure that adverse effects are identified early and appropriate action is tobe taken promptly to minimize suffering.
		• Use biomarker data to establish early humane end points wherever possible.
Fluid resuscitation	• Pain or discomfort	• Use a venous access port when performing multiple frequent resuscitation procedures (see surgery above).
	• Inadequate resuscitation	• Provide fluid via the i.v. or i.p. routes; avoid the s.c. route.
	• Pulmonary edema	• Select the nature (colloidal or crystalloid) and volume of resuscitation fluid based on scientific and welfare considerations.
		• Monitor hematocrit to ensure that the resuscitation fluid is entering the vasculature.
		• Check for signs of pulmonary edema when optimizing the fluid resuscitation regimen and alter the rate or volume of resuscitation where necessary.
Humane killing	• Pain or discomfort	• Use the most humane method possible—refer to current literature

TABLE 4. **M-CASS scoring sheet**

Clinical criteria				
Fur aspect	Actively grooming	Dulling of hair coat	Rough hair coat	Piloerection
Activity	Normal activity	Reduced activity disturbed	No activity disturbed, reduced activity stimulated	Nil activity disturbed or stimulated
Posture	Normal	Slightly hunched, moving freely	Hunched with stiff movement/posture	Hunched with no movement stimulated
Behavior	Normal	Slow normal when disturbed	Abnormal disturbed, relocates only when stimulated	Abnormal when disturbed or stimulated, no relocation
Chest movements	Normal	Mildly dyspneic	Moderately dyspneic	Severely dyspneic with thoracic abdominal respiration
Chest sounds	No	Occasional chirping	Frequent chirping	Wet chirping increased when stimulated
Eye lids	Normally opened spontaneously	Normally opened disturbed	Near closed when stimulated and disturbed	Closed disturbed, near closed stimulated
Body weight loss	0%–5%	5%–10%	>15%	>20%
Score	1	2	3	4
Monitoring frequency	12 hourly	6 hourly	4 hourly	Hourly

During the day, animals will be humanely killed if they reach a score of 4 in all eight parameters.
During the night, animals will be humanely killed if they reach a score of 4 in two parameters.
Regardless of total score, any animal found to exhibit a complete lack of activity or eyes closed when stimulated, gasping, or collapsed with head down will be humanely killed immediately.

One approach that can be used to facilitate objective and refined humane end points is the use of welfare/clinical sign scoring systems (Table 4). These require careful design and staff training and familiarization. Attentive monitoring should be used at a frequency that reflects the time course and severity of the model so that suffering can be minimized and humane end points can be effectively implemented.

Welfare/clinical sign scoring systems

Huet and colleagues (114) published a scoring system that they developed for mice used in a pneumonia model of sepsis. The M-CASS (mouse clinical assessment scoring system) represents a scoring system for murine sepsis (reproduced in Table 4). Of particular note is the graded severity scale for each of the clinical signs and the inclusion of a change in monitoring frequency that correlates with sepsis symptom severity. Although this *approach* is useful, the humane end points described in the M-CASS article, in the opinion of a number of the EWG members, are not sufficiently early or humane.

The EWG believes that the use of early humane end points is an ethical obligation when using animals in research and recommends that scoring systems are used to help achieve this.

A one-size-fits-all approach to welfare assessment is not recommended, and researchers should develop their own systems in collaboration with their animal care staff, veterinarian, and ethics or animal care and use committee (127). Scoring systems and monitoring regimens should be specifically tailored to reflect the study design and scientific question being asked. Frequency of monitoring needs to take into account the stage of disease, severity, and the likely speed at which an animal's condition might deteriorate. For examples of how to develop a welfare scoring system based on physiological and behavioral signs, see (123).

A list of potential physiological and behavioral signs that can be used in scoring systems is presented in Table 5.

TABLE 5. **Indicators of suffering in experimental sepsis studies**

Indicators of suffering in experimental sepsis studies	
Piloerection	Gait
Facial expression ("pain face")	Posture
Voluntary movement	Aversion to touch
Ocular discharge	Chest movements
Chest sounds	Nest building/latrine location
Body weight loss	Persistent tremors
Low body temperature (cold to touch)	Bloating
Alertness	Fluid intake
Diarrhea	Bladder control
Appetite	Grooming

CONCLUSIONS

Applying the Three Rs in sepsis research is a serious challenge. There are many factors to consider, including the translational validity of current models, availability of humane alternatives to living animals, balancing the need to minimize animal suffering while generating meaningful data, and high interlaboratory variability in the way studies are conducted. Applying the R of refinement of animal studies can be a highly effective way to reduce suffering and improve scientific quality. This report gives some guidance to help researchers apply the Three Rs to sepsis research with a focus on practical refinement approaches that can be used to reduce animal suffering. The authors hope that these refinements will be used and further developed by researchers working in this field.

REFERENCES

1. Poole P: Happy animals make good science. *Lab Anim* 31:116–124, 1997.
2. Baumans V: Science-based assessment of animal welfare: laboratory animals. *Rev Sci Tech* 25(2):503–514, 2005.
3. Lloyd M, Foden B, Wolfensohn S: Refinement: promoting the Three Rs in practice. *Lab Anim* 42:284–293, 2008.
4. *Home Office: Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*. London, UK: The Stationary Office, 2014.
5. European Commission: Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* L276: 33–79, 2010.
6. Iskander K, Osuchowski M, Stearns-Kurosawa D, Kurosawa S, Stepien D, Valentine C, Remick D: Sepsis: multiple abnormalities, heterogeneous responses, and evolving understanding. *Physiol Rev* 93:1247–1288, 2013.
7. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M: Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med* 345(19):1368–1377, 2001.
8. Dellinger R, Levy M, Rhodes A, Annane D, Gerlach H, Opal S, Sevransky J, Sprung C, Douglas I, Jaeschke R, et al.: Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock. *Crit Care Med* 41(2):580–637, 2010.
9. Gaieski D, Edwards J, Kallan M, Carr B: Benchmarking the incidence and mortality of severe sepsis in the United States. *Crit Care Med* 41(5):1167–1174, 2013.
10. McPherson D, Griffiths C, Williams M, Baker A, Klodawski E, Jacobson B, Donaldson L: Sepsis-associated mortality in England: an analysis of multiple cause of death data from 2001 to 2010. *BMJ Open* 3:e002586, 2013.
11. Ma Y, Vilanova D, Atalar K, Delfour O, Edgeworth J, Ostermann M, Hernandez-Fuentes M, Razafimahatratra S, Michot B, Persing D, et al.: Genome-wide sequencing of cellular microRNAs identifies a combinatorial expression signature diagnostic of sepsis. *PLoS ONE* 8(10):e75918, 2013.
12. Fairchild K: Predictive monitoring for early detection of sepsis in neonatal ICU patients. *Curr Opin Pediatr* 25(2):172–179, 2013.
13. Moyer M: New biomarkers sought for improving sepsis management and care. *Nat Med* 18(7):999, 2012.
14. Marshall J, Deitch E, Moldawer L, Opal H, van der Poll T: Preclinical models of shock and sepsis: what can they tell us? *Shock* 24(s1):1–6, 2005.
15. Rittersch D, Hoesel L, Ward P: The disconnect between animal models of sepsis and human sepsis. *J Leukoc Biol* 81:137–143, 2007.
16. Dejager L, Pinheiro I, Dejonckheere E, Libert C: Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends Microbiol* 19(4): 198–208, 2011.
17. Seok J, Warren H, Cuenca A, Mindrinos M, Baker H, Xu W, Richards D, McDonald-Smith G, Gao H, Hennessy L, et al.: Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 110(9):3507–3512, 2013.
18. McGonigle P, Ruggeri B: Animal models of human disease: challenges in enabling translation. *Biochem Pharmacol* 87(1):162–171, 2014.
19. Osuchowski M, Remick D, Lederer J, Lang C, Aasen A, Aibiki M, Azevedo L, Bahrami S, Boros M, Cooney R, et al.: Abandon the mouse research ship? Not just yet! *Shock* 41(6):463–475, 2014.
20. Zak D, Aderem A: Systems biology of innate immunity. *Immunol Rev* 227: 264–282, 2009.
21. Vodovotz Y, Csete M, Bartels J, Chang S, An G: Translational systems biology of inflammation. *PLoS Comput Biol* 4:e1000014, 2008.
22. Namas R, Bartels J, Hoffman R, Barclay D, Billiar T, Zamora R, Vodovotz Y: Combined *in silico*, *in vivo*, and *in vitro* studies shed insights into the acute inflammatory response in middle-aged mice. *PLoS One* 8:e67419, 2013.
23. Verschoor C, Puchta A, Bowdish D: The macrophage. In: *Leucocytes: Methods and Protocols*. New York, NY: Springer, 2012.
24. Fejer G, Wegner M, Györy I, Cohen I, Engelhard P, Voronov E, Manke T, Ruzsics Z, Dölken L, Prazeres da Costa O, et al.: Nontransformed, GM-CSF–dependent macrophage lines are a unique model to study tissue macrophage functions. *Proc Natl Acad Sci USA* 110:E2191–E2198, 2013.
25. Nickerson C, Honer zu Bentrup K, Ott C: Three-dimensional cell culture models for drug discovery and infectious disease. *Bioforum Eur* 6:34–36, 2005.
26. Nickerson C, Richter E, Ott C: Studying host–pathogen interactions in 3-D: organotypic models for infectious disease and drug development. *J Neuroimmune Pharmacol* 2:26–31, 2007.
27. Barrila J, Radtke A, Crabbé A, Sarker S, Herbst-Kralovetz M, Ott C, Nickerson C: Organotypic 3D cell culture models: using the rotating wall vessel to study host–pathogen interactions. *Nat Rev Microbiol* 8:791–801, 2010.
28. Liu J, Abate W, Xu J, Corry D, Kaul B, Jackson S: Three-dimensional spheroid cultures of A549 and HepG2 cells exhibit different LPS receptor expression and LPS-induced cytokine response compared with monolayer cultures. *Innate Immun* 17:245–255, 2010.
29. Schmeichel K, Bissell M: Modeling tissuespecific signaling and organ function in three dimensions. *J Cell Sci* 116:2377–2388, 2003.
30. Lin R, Chang H: Recent advances in threedimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 3:1172–1184, 2008.
31. Xu J, Patton D, Jackson S, Purcell W: *In vitro* maintenance and functionality of primary renal tubules and their application in the study of relative renal toxicity of nephrotoxic drugs. *J Pharmacol Toxicol Methods* 19:210–214, 2013.
32. Carterson A, Höner zu Bentrup K, Ott C, Clarke M, Pierson D, Vanderburg C, Buchanan K, Nickerson C, Schurr M: A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* 73(2):1129–1140, 2005.
33. Sainz B, TenCate V, Uprichard S: Three-dimensional Huh7 cell culture system for the study of hepatitis C virus infection. *Virol J* 6:103, 2009.
34. Huh D, Mathews B, Mammoto A, Montoya-Zavala M, Yuan Hsin H, Ingber D: Reconstituting organ-level lung functions on a chip. *Science* 328:1662–1668, 2010.
35. 1000 Genomes Project Consortium: A map of human genome variation. *Nature* 467:1061–1073, 2010.
36. Ng S, Turner E, Robertson P, Flygare S, Bigham A, Lee C, Shaffer T, Wong M, Bhattacharjee A, Eichler E, et al.: Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* 461:272–276, 2009.
37. Dudley J, Sirota M, Shenoy M, Pai R, Roedder S, Chiang A, Morgan A, Sarwal M, Pasricha P, Butte A: Computational repositioning of the anticonvulsant topiramate for inflammatory bowel disease. *Sci Transl Med* 3(96):96ra76, 2011.
38. Sirota M, Dudley J, Kim J, Chiang A, Morgan A, Sweet-Cordero A, Sage J, Butte A: Discovery and preclinical validation of drug indications using compendia of public gene expression data. *Sci Transl Med* 3(96):96ra77, 2011.
39. Nemzek J, Hugunin K, Opp M: Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comp Med* 58(2):120–128, 2008.
40. Doi K, Leelahavanichkul A, Yuen P, Star R: Animal models of sepsis and sepsis-induced kidney injury. *J Clin Invest* 119:2868–2878, 2014.
41. Fink M, Heard S: Laboratory models of sepsis and septic shock. *J Surg Res* 49:186–196, 1990.
42. Buras J, Holzmann B, Sitkovsky M: Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* 4:854–865, 2005.
43. Zonotti-Cavazzoni S, Goldfarb R: Animal models of sepsis. *Crit Care Clin* 25:703–719, 2009.
44. Mai S, Khan M, Liaw P, Fox-Robichaud A: Experimental sepsis models, sepsis—an ongoing and significant challenge. ISBN: 978-953-51-0780-4, InTech, DOI: 10.5772/52876, 2012. Available at: http://www.intechopen.com/books/sepsis-an-ongoing-and-significant-challenge/experimental-sepsis-models, Accessed January 16, 2015.
45. Leiper J, Nandi M, Torondel B, Rust J, Malaki M, O'Hara B, Rossiter S, Anthony S, Madhani M, Selwood D, et al.: Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat Med* 13(2):198–203, 2007.
46. Chase J, Lambermont B, Starfinger C, Hann C, Shaw G, Ghuysen A, Kolh P, Dauby P, Desaive T: Subject-specific cardiovascular system model-based identification and diagnosis of septic shock with a minimally invasive data set: animal experiments and proof of concept. *Physiol Meas* 32:65–82, 2011.
47. Geloen A, Chapelier K, Cividjian A, Dantony E, Rabilloud M, May C, Quintin L: Clonidine and dexmedetomidine increase the pressor response to norepinephrine in experimental sepsis: a pilot study. *Crit Care Med* 41(12):e431–e438, 2013.
48. Van Schaik S, Abbas A: Role of T-cells in a murine model of *Escherichia coli* sepsis. *Eur J Immunol* 37:3101–3110, 2007.
49. Maddens B, Vandendriessche B, Demon D, Vanholder R, Cheirs K, Cauwels A, Meyer E: Severity of sepsis-induced kidney injury in a novel mouse model is age dependent. *Crit Care Med* 40(9):2638–2646, 2012.
50. Chaivolini D, Pozzi G, Ricci S: Animal models of *Streptococcus pneumoniae* disease. *Clin Microbiol Rev* 21(4):666–685, 2008.
51. Aslami H, Haitsma J, Hofstra J, Florquin S, Dos Santos D, Streutker C, Zhang H, Levi M, Slutsky A, Schultz M: Plasma-derived human antithrombin attenuates ventilator-induced coagulopathy but not inflammation in a *Streptococcus pneumoniae* pneumonia model in rats. *J Thromb Haemost* 10:399–410, 2012.
52. Brealey D, Karyampudi S, Jacques T, Novelli M, Stidwill R, Taylor V, Smolenski R, Singer M: Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. *Am J Physiol Regul Integr Comp Physiol* 286(3):R491–R497, 2004.
53. Cross A, Opal S, Sadoff J, Gemski P: Choice of bacteria in animal models of sepsis. *Infect Immun* 61(7):2741–2747, 2013.
54. Sandgren A, Albiger B, Orihuela C, Tuomanen E, Normark S, Henriques-Normark B: Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans. *J Infect Dis* 192:791–800, 2005.
55. Wichterman K, Baue A, Chaudry I: Sepsis and septic shock—a review of laboratory models and a proposal. *J Surg Res* 29:189–201, 1980.
56. Rittirsch D, Huber-Lang M, Flierl M, Ward P: Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* 4(1):31–36, 2009.

57. Scheiermann P, Hoegl S, Revermann M, Ahluwalia D, Zander J, Boost K, Nguyen T, Zwissler D, Muhl H, Hofstetter C: Cecal ligation and incision: an acute onset model of severe sepsis in rats. *J Surg Res* 151:132–137, 2009.
58. Zantl N, Uebe A, Neumann B, Wagner H, Siewert J-R, Holzmann B, Heidecke C-D, Pfeffer K: Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis. *Infect Immun* 66(5):2300–2309, 1998.
59. Schabbauer G: Polymicrobial sepsis models: CLP versus CASP. *Drug Discov Today Dis Models* 9(1):e17–e21, 2012.
60. Ebong S, Call D, Nemzek J, Bolgos G, Newcomb D, Remick D: Immunopathologic alterations in murine models of sepsis of increasing severity. *Infect Immun* 67(12):6603–6610, 1999.
61. Traeger T, Koerner P, Kessler W, Cziupka K, Diedrich S, Busemann A, Heidecke C-D, Maier S: Colon ascendens stent peritonitis (CASP)—a standardized model for polymicrobial abdominal sepsis. *J Vis Exp* 46:e2299, 2010.
62. Fink T, Heymann P, Taha-Melitz S, Taha A, Wolf B, Rensing H, Volk T, Mathes A: Dobutamine pretreatment improves survival, liver function, and hepatic microcirculation after polymicrobial sepsis in rats. *Shock* 40(2):129–135, 2013.
63. Fink T, Glas M, Wolf A, Kleber A, Reus E, Wolff M, Keifer D, Wolf B, Rensing H, Volk T, Mathes A: Melatonin receptors mediate improvements of survival in a model of polymicrobial sepsis. *Crit Care Med* 42(2):1–10, 2014.
64. Esmon C: Why do animal models (sometimes) fail to mimic human sepsis? *Crit Care Med* 32(5):S219–S222, 2004.
65. Drechsler S, Weixelbaumer K, Raeven P, Jafarmadar M, Khadem A, van Griensven M, Bahrami S, Osuchowski M: Relationship between age/gender-induced survival changes and the magnitude of inflammatory activation and organ dysfunction in post-traumatic sepsis. *PLoS One* 7(12):e51457, 2010.
66. Coldewey S, Khan A, Kapoor A, Collino M, Rogazzo M, Brines M, Cerami A, Hall P, Sheaff M, Kieswich J, et al.: Erythropoietin attenuates acute kidney dysfunction in murine experimental sepsis by activation of the β-common receptor. *Kidney Int* 84(3):482–490, 2013.
67. Coldewey S, Rogazzo M, Collino M, Pate N, Thiemermann C: Inhibition of IκB kinase reduces the multiple organ dysfunction caused by sepsis in the mouse. *Dis Model Mech* 6(4):1031–1042, 2013.
68. Khan A, Coldewey S, Patel N, Rogazzo M, Collino M, Yaqoob M, Radermacher P, Kapoor A, Thiemermann C: Erythropoietin attenuates cardiac dysfunction in experimental sepsis in mice via activation of the β-common receptor. *Dis Model Mech* 6(4):1021–1030, 2013.
69. Raven K: Rodent models of sepsis found shockingly lacking. *Nat Med* 18(7): 998, 2012.
70. Wolfensohn S, Lloyd M: *Handbook of Laboratory Animal Management and Welfare*. 4th ed. Oxford, United Kingdom: Wiley-Blackwell, 2013.
71. Zhao X, Ho D, Gao S, Hong C, Vatner D, Vatner S: Arterial pressure monitoring in mice. *Curr Protoc Mouse Biol* 1:105–122, 2011.
72. Schumacher S, Morris M, Riddick E: Effects of restraint by tether jackets on behaviour in spontaneously hypertensive rats. *Clin Exp Hypertens A* A13(5):875–884, 1991.
73. Fortaleza E, Scopinho A, Corrêa F: Adrenoceptors in the medial amygdaloid nucleus modulate the tachycardiac response to restraint stress in rats. *Neuroscience* 227:170–179, 2012.
74. Matsuura T, Takimura R, Yamaguchi M, Ichinose M: Estimation of restraint stress in rats using salivary amylase activity. *J Physiol Sci* 62:421–427, 2012.
75. Nandi M, Kelly P, Torondel B, Wang Z, Starr A, Ma Y, Cunningham P, Stidwill R, Leiper J: Genetic and pharmacological inhibition of dimethylarginine dimethylaminohydrolase 1 is protective in endotoxic shock. *Arterioscler Thromb Vasc Biol* 32:2589–2597, 2012.
76. Morton D, Hawkins P, Bevan R, Heath K, Kirkwood J, Pearce P, Scott L, Whelan G, Webb A: Refinements in telemetry procedures. *Lab Anim* 37: 261–299, 2003.
77. Hawkins P: Refining housing husbandry and care for animals used in studies involving biotelemetry. *Animals* 4:361–373, 2014.
78. Helwig B, Ward J, Blaha M, Leon L: Effect of intraperitoneal radiotelemetry instrumentation on voluntary wheel running and surgical recovery in mice. *J Am Assoc Lab Anim Sci* 51(5):600–608, 2012.
79. Miao P, Kong Y, Ma Y, Zeng H, Yu Z: Hypothermia predicts the prognosis in colon ascendens stent peritonitis mice. *J Surg Res* 181(1):129–135, 2013.
80. Wendon J: Critical care "normality": individualized verses protocolized care. *Crit Care Med* 38(10):S590–S599, 2010.
81. Cannon B, Nedergaard J: Nonshivering thermogenesis and its adequate measurement in metabolic studies. *J Exp Biol* 214:242–253, 2011.
82. Corrigan J, Fonseca M, Flatow E, Lewis K, Steiner A: Hypometabolism and hypothermia in the rat model of endotoxic shock: independence of circulatory hypoxia. *J Physiol* 592(17):3901–3916, 2014.
83. Kort W, Hekking-Weijima J, TenKate M, Sorm V, VanStrik R: A microchip implant system as a method to determine body temperature of terminally ill rats and mice. *Lab Anim* 32:260–269, 1997.
84. Clement J, Mills P, Brockway B: Use of telemetry to record body temperature and activity in mice. *J Pharmacol Methods* 21(2):129–140, 1989.
85. Ludwig N, Gargano M, Luzi F, Carenzi C, Verga M: Technical note: applicability of infrared thermography as a noninvasive measurement of stress in rabbit. *World Rabbit Sci* 15:199–206, 2007.
86. Szenttkuti A, Kavanagh H, Grazio S: Infrared thermography and image analysis for biomedical use. *Periodicum Biologorum* 113(4):385–392, 2011.
87. Rudiger A, Singer M: Mechanisms of sepsis-induced cardiac dysfunction. *Crit Care Med* 35(6):1599–1608, 2007.
88. De Backer D, Ortiz J, Salgado D: Coupling microcirculation to systemic hemodynamics. *Curr Opin Crit Care* 16(3):250–254, 2010.
89. Sakr Y, Dubois M, De Backer D, Creteur J, Vincent J: Persistent microcirculatory alterations are associated with organ failure and death in patients with septic shock. *Crit Care Med* 32(9):1825–1831, 2004.
90. De Backer D, Creteur J, Preiser J, Dubois M, Vincent J: Microvascular blood flow is altered in patients with sepsis. *Am J Respir Crit Care Med* 166:98–104, 2002.
91. Iskander K, Craciun F, Stepien D, Duffy E, Kim J, Moitra R, Vaickus L, Osuchowski M, Remick D: Cecal ligation and puncture–induced murine sepsis does not cause lung injury. *Crit Care Med* 41(1):159–170, 2013.
92. Chan T, Gu F: Early diagnosis of sepsis using serum biomarkers. *Expert Rev Mol Diag* 11(5):487–496, 2011.
93. Osuchowski M, Welch K, Siddiqui J, Remick D: Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. *J Immunol* 177:1967–1974, 2006.
94. Osuchowski M, Connett J, Welch K, Granger J, Remick D: Stratification is the key: inflammatory biomarkers accurately direct immunomodulatory therapy in experimental sepsis. *Crit Care Med* 37(5):1567–1573, 2009.
95. Weixelbaumer K, Raeven P, van Griensven M, Bahrami S, Osuchowski M: Repetitive low-volume blood sampling method as a feasible monitoring tool in a mouse model of sepsis. *Shock* 34(4):420–426, 2010.
96. Tremoleda J, Kerton A, Gsell W: Anaesthesia and physiological monitoring during *in vivo* imaging of laboratory rodents: considerations on experimental outcomes and animal welfare. *EJNMMI Res* 2(44):1–23, 2012.
97. Wong I, Makowska J, Weary D: Rat aversion to isoflurane verses carbon dioxide. *Biol Lett* 9(1):20121000, 2013.
98. Eissa D, Carton E, Buggy D: Anaesthetic management of patients with severe sepsis. *Br J Anaesth* 105(6):734–743, 2010.
99. Yoon S: Concerns of the anesthesiologist: anesthetic induction in severe sepsis or septic shock patients. *Korean J Anesthesiol* 63(1):3–10, 2012.
100. Hollenberg S, Dumasius A, Easington C, Colilla S, Neumann A, Parrillo J: Characterization of a hyperdynamic murine model of resuscitated sepsis using echocardiography. *Am J Respir Crit Care Med* 32(11):2589–2597, 2001.
101. Albuszies G, Radermacher P, Vogt J, Wachter U, Weber S, Schoaff M, Georgieff M, Barth E: Effect of increased cardiac output on hepatic and intestinal microcirculatory blood flow, oxygenation, and metabolism in hyperdynamic murine septic shock. *Crit Care Med* 33(10):2332–2338, 2005.
102. Barth E, Radermacher P, Thiemermann C, Weber S, Georgieff M, Albuszies G: Role of inducible nitric oxide synthase in the reduced responsiveness of the myocardium to catecholamines in a hyperdynamic, murine model of septic shock. *Crit Care Med* 34(2):307–313, 2006.
103. Sacerdote P: Opioids and the immune system. *Palliat Med* 20:s9–s15, 2006.
104. Hugunin K, Fry C, Shuster K, Nemzek J: Effects of tramadol and buprenorphine on select immunologic factors in a cecal ligation and puncture model. *Shock* 34(3):250–260, 2010.
105. National Research Council: *Recognition and Alleviation of Pain in Laboratory Animals*. Washington, DC: The National Academies Press, 2009.
106. Deitch E: Animal models of sepsis and shock: a review and lessons learned. *Shock* 9(1):1–11, 1998.
107. Morris T: Antibiotic therapeutics in laboratory animals. *Lab Anim* 29(1): 13–36, 1995.
108. Granowitz E, Brown R: Antibiotic adverse reactions and drug interactions. *Crit Care Clin* 24(2):241–242, 2008.
109. Dinev T, Zapryanova D, Lubomir L: Changes in some blood biochemical and haematological parameters in goats after aminoglycoside and aminocyclitol treatment at therapeutic doses. *Turk J Vet Anim Sci*, 31(3):179–188, 2007.
110. van den Bogaard A, Stobberingh E: Epidemiology of resistance to antibiotics. Links between animals and humans. *Int J Antimicrob Agents* 14(4):327–335, 2000.
111. Festing M, Altman D: Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR J* 43:244–258, 2002.
112. Nemzek J, Remick D: Humane endpoints in shock research. *Shock* 21(2): 189–190, 2004.
113. Radermacher P, van Griensven M, Thiemermann C: Humane end points in experimental models of septic shock: a must, not a superfluous nightmare for researchers! *Shock* 37(6):541–542, 2013.
114. Huet O, Ramsey D, Miljavec S, Jenney A, Aubron C, Aprico A, Stefanovic N, Balkau B, Head G, de Haan J, et al.: Ensuring animal welfare while meeting

scientific aims using a murine pneumonis model of septic shock. *Shock* 39(6):488–494, 2013.
115. Stokes W: Reducing unrelieved pain and distress in laboratory animals using humane endpoints. *ILAR J* 41:59–61, 2000.
116. Bara M, Joffe A: The ethical dimension in published animal research in critical care: the public face of science. *Crit Care* 18:R15, 2014.
117. Hooijmans C, Leenaars M, Ritskes-Hoitinga M: A gold standard publication checklist to improve the quality of animal studies, to fully integrate the Three Rs and to make systematic reviews more feasible. *Altern Lab Anim* 38:167–182, 2010.
118. Kilkenny C, Browne W, Cuthill I, Emerson M, Altman D: Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8(6):e1000412, 2010.
119. ILAR: *Guidance for the Description of Animal Research in Scientific Publications*. Washington, DC: National Academy of Science, National Academy Press, 2011.
120. Lilley E, Jennings M: Refinement: lessons from the 2012 Olympics. *Altern Lab Anim* 41:P28–P29, 2013.
121. Hurst J, West R: Taming anxiety in laboratory mice. *Nat Methods* 7:825–826, 2010.
122. Hawkins P, Morton D, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan J, Wells S, et al.: A guide to defining and implementing protocols for the welfare assessment of laboratory animals. *Lab Anim* 45:1–13, 2011.
123. European Commission: Examples to illustrate the process of severity classification, day-to-day assessment and actual severity assessment. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/examples.pdf, 2013. Accessed January 16, 2015.
124. Langford D, Bailey A, Chanda M, Clarke S, Drummond T, Echols S, Glick S, Ingrao J, Klassen-Ross T, et al.: Coding of facial expressions of pain in the laboratory mouse. *Nat Methods* 7:447–449, 2010.
125. Sotocinal S, Sorge R, Zaloum A, Tuttle A, Martin L, Wieskopf J, Mapplebeck J, Wei P, Zhan S, Zhang S, et al.: The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. *Mol Pain* 7(1):55–65, 2011.
126. Jirkof P, Fleischmann T, Cesarovic N, Rettich A, Vogel J, Arras M: Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring. *Lab Anim* 47(3):153–161, 2013.
127. European Commission: Working document on a severity assessment framework. Available at: http://ec.europa.eu/environment/chemicals/lab_animals/pdf/Endorsed_Severity_Assessment.pdf, 2012. Accessed January 16, 2015.