

One-hit, two-hit . . . is there really any benefit?

J. D. Lang and J. M. Hickman-Davis
Department of Anaesthesiology, Center for Free Radical Biology, The University of Alabama at Birmingham, Birmingham, Alabama, USA

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Correspondence: John D. Lang MD, Center for Free Radical Biology, Department of Anaesthesiology, Center for Free Radical Biology, The University of Alabama at Birmingham, Birmingham, Alabama, 35205–3703, USA.

E-mail: jlang@uab.edu

Lung injury resulting from the systemic inflammatory response syndrome (SIRS), be it infectious (sepsis) or not, remains a significant contributor to patient morbidity and mortality worldwide [1]. While the mortality from acute respiratory distress syndrome (ARDS) has decreased to between 30 and 40%, approximately 20% will die of refractory hypoxaemia and the rest from the multiple organ dysfunction syndrome (MODS) [2]. Thus, with the prevalence of lung injury remaining substantial and the acquisition of lung injury not being inconsequential, investigators continue to search for mechanisms, management strategies and therapeutic treatments that will benefit those patients in need.

Models of lung injury used by investigators internationally are numerous in their insults of initiation, maintenance and host organisms utilized. Despite their diversity, it is extremely difficult to mimic lung injury encountered at the bedside. Examples of inherent differences just among species are numerous and do not allow replication of human disease, but rather provide to some degree *in vivo* biological proof of principle (Fig. 1) [3,4].

Patients afflicted with lung injury more commonly than not encounter more than 'one-hit' modulating the immunological response to injury by increasing duration and amplitude of the inflammatory response. In fact, many second 'hits' occur after proinflammatory responses (SIRS) have waned and patients manifest a compensatory anti-inflammatory responses (CARS) with suppressed immunity and diminished resistance to infection. This scenario seemingly places the patient at risk for manifesting clinically significant lung injury and MODS [5].

While human studies lack clarity in this area, recently published animal studies have been designed with 'two-hits' to better mimic and allow for insight into the 'yin and yang' of immuno-inflammatory responses due to injury. Recently, Murphy *et al.* tested whether changes in innate immune cell reactivity following two-hit injury contributes to the development of heightened inflammation and organ dysfunction

[6]. Using a rodent model of thermal injury followed by intraperitoneal administration of endotoxin at either 1 or 7 days, the investigators demonstrated significantly elevated Toll-like receptor 4 (TLR4), a family of pattern recognition receptors that respond to endotoxin, mediated increases in interleukin (IL)-1 β , tumour necrosis factor (TNF)- α and interleukin (IL)-6 in spleen cells stimulated *ex vivo* by endotoxin. While increases in cytokines concentrations were significant at day 1, the response was twofold greater in magnitude at day 7. In support of enhanced TLR-4 responses was the observation that 75% of the animals died within 48 h when endotoxin was administered to animals on day 7, but not on day 1. To determine cause of death, vital organs were assessed for severity of injury after 7 days. Lung and liver from the endotoxin-thermally injured animals demonstrated the most significant injury. In an attempt to relate mortality and pathology, selected serum and organ-derived cytokines and chemokines were measured serially in endotoxin-treated animals. Interestingly, no significant increases were observed in the injured animals or sham animals at day 1. However, in the day 7 group, cytokine (TNF- α , IL-6, IL-10) levels were significantly higher and remained there for much longer intervals as compared to sham animals. At day 7, both chemokines [macrophage inflammatory protein (MIP)-1 α], keratinocyte-derived chemokine, monocyte chemoattractant protein-1] and cytokines (TNF- α , IL-1 β , IL-6, IL-10) measured in lung tissue extracts of endotoxin-challenged animals were significantly elevated as compared to their sham cohorts. Blocking TNF- α with soluble TNF-R55-Ig infusion 2 h prior to endotoxin challenge at day 7 post-injury resulted in a dramatic reduction in mortality. In conclusion, the authors provided evidence that a second-hit could enhance injury, and that the elapsed time from which the second-hit occurred was pivotal in evoking innate immunological responses necessary for instigating injury. To strengthen the two-hit model argument, Rizoli *et al.* sought to assess the influence of shock followed by endotoxin instil-

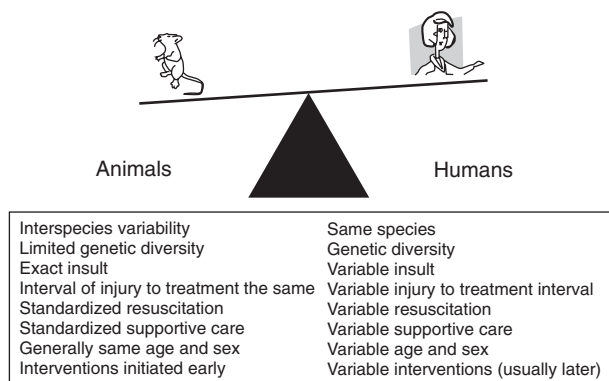


Fig. 1. Imbalance between animal models and humans [3,4]. Inherent differences between animals and humans makes modelling lung injury challenging. Thus, the efforts to mimic the bedside situation by utilizing animal models must be conducted carefully and the conclusions interpreted with caution.

lation on C-X-C chemokine responses [7]. These families of chemokines are potent neutrophil chemoattractants implicated in neutrophil influx to acutely inflamed lungs. Rodents were subjected to haemorrhagic shock by blood withdrawal subsequently reducing mean arterial pressure (MAP) to 50 mmHg within 15 min. The MAP was maintained between 35 and 40 mmHg for 60 min, after which time the animals were resuscitated over a 2 h interval. At 1 h post-resuscitation the animals underwent tracheostomy for mechanical ventilation, and either endotoxin or normal saline was administered intratracheally. Animals exposed to both hits demonstrated significantly greater lung injury reflected by increased transpulmonary albumin flux, increased neutrophil counts and cytokine-induced neutrophil chemoattractant (CINC) concentrations compared to sham animals and those animals given only normal saline. Additionally, anti-CINC antibodies administered 10 min prior to endotoxin instillation, significantly reduced lung neutrophil sequestration. The authors conclude that lung injury was more significant with a second hit, and that augmented release of CINC was responsible for the observed injury.

In this issue of *Clinical and Experimental Immunology*, Vuichard *et al.* utilize another variation of the two-hit model in an attempt to better characterize endotoxin-mediated lung injury, in hopes of better reflecting the 'clinical situation'. Endotoxin was instilled intratracheally into rats, followed by placing the animals in a chamber with either an oxygen concentration of 21% (normoxia) or 10% (hypoxia) within 60 min of endotoxin instillation. While an additional animals receiving hypoxia without endotoxin were tested, these data were de-emphasized due to insignificant findings when compared to the endotoxin–normoxia and endotoxin–hypoxia animals. Pulmonary inflammatory responses were measured at 2, 4, 6 and 8 h. In animals exposed to endotoxin–hypoxia, lung myeloperoxidase (MPO), neutrophil

counts, TNF- α , MIP-1 β and surfactant protein-B were measured in bronchoalveolar lavage fluid (BALF) and all were significantly increased as compared to animals exposed to endotoxin–normoxia. It was interesting that when macrophages were depleted using clodronate liposomes, lung MPO, neutrophil counts, TNF- α and MIP-1 β measured at 6 h were significantly reduced, thus implicating the alveolar macrophage as the major effector cell. In addition to providing mechanistic insights into the complex disease that is lung injury, this experimental design better mimics the clinical condition. Insults occurring via an inflammatory nidus followed by a secondary insult such as hypoxia or severe and protracted hypotension is commonly encountered in the critically ill patient. Also, the observation of compartmentalization (alveolar *versus* interstitial) was fascinating. Measurements derived from BALF were significantly altered (except for MPO) by this two-hit model compared to measurements derived from lung homogenates. This may have arisen from the mode of inducing injury, time interval between the first and second hit (short at 60 min) and the total experimental time (8 h). Nevertheless, the study by Vuichard *et al.*, as well as the previously cited studies, epitomizes legitimate efforts to more effectively model experimentation to enhance patient care. With that said, is the two-hit model really necessary?

There is a diverse body of literature aimed at determining the ideal 'clinically relevant' animal models for the study of ALI (acute lung injury) and ARDS. The difficulty in determination of the single best choice for these studies lies in a lack of uniformity in regards to methodology and clinical endpoints. Direct comparison of one- *versus* two-hit models of lung injury is compounded further by the lack of definition for lung injury that appropriately mimics ARDS in human patients; i.e. is it enough to study specific inflammatory and histological changes that correspond to ARDS or must the development, progression and resolution of the syndrome also mirror the human clinical scenario? Vuichard and colleagues accurately point out that, based on the complexity of the illness, two-hit models of ALI should more accurately reflect comorbidities and risk factors found in patients. These authors therefore utilized intratracheal endotoxin and hypoxia to enhance acute lung injury. While the use of hypoxia in combination with endotoxin represents a relatively new (if not unique) two-hit model for ARDS [8,9], the use of endotoxin alone or in combination with a second hit has been well utilized as a model for lung injury (Table 1).

Indeed, within the parameters of inflammation as characterized by Vuichard *et al.* there are a number of single-hit endotoxin models that closely parallel their findings. Increasing doses of endotoxin (5–5000 $\mu\text{g}/\text{ml}/\text{kg}$) instilled intratracheally in the rat has been shown to increase airway hyperinflation and oedema, induce the production of TNF- α , MCP-1, IL-6 and IL-1 β , and trigger neutrophilia [10]. Intraperitoneal injection of endotoxin into mice has also

Table 1. Examples of endotoxin models of ARDS and ALI.

Animal model	Endotoxin type ^a	Route	Dose (mg/kg)	Second-hit	Ref.
Rat	NR	i.t.	0.75	Hypoxia ^b	Vuichard 2005
Rat	0111:B4	i.t.	0.03	Haemorrhage ^b	[7]
Rat	ST	i.p.	15.0	–	[20]
Rat	0111:B4	i.v.	0.20/h ^d	–	[21]
Rat	026:B6	i.t.	0.005–0.5 ^e	–	[10]
Rat	0111:B4	i.t.	5.0	–	[13]
Mouse	FP	i.p.	–	Acid aspiration ^c	[14]
Mouse	0111:B4	i.p.	15.0	–	[11]
Mouse	0128:B12	Aerosol	0.1–1.0 ^e	–	[12]
Mouse	0111:B6	i.p.	1.0	–	[22]
Pig	FP	i.p.	–	Ischaemia/reperfusion ^b	[18]
Pig	FP	i.p.	–	Haemorrhage ^c	[15]
Pig	0111:B4	i.v.	2500/h ^d	–	[23]
Pig	0111:B4	i.v.	0.20	–	[24]

^a*Escherichia coli*. ^bIntroduction of a second-hit increased inflammation of model. ^cIntroduction of second-hit had no effect on inflammation of model. ^dConstant infusion; ^edose–response; NR = not reported; FP = fecal peritonitis; i.t. = intratracheal; i.p. = intraperitoneal; i.v. = intravenous; ST = *Salmonella typhimurium*; ARDS = acute respiratory distress syndrome; ALI = acute lung injury.

been reported to display the key features of ARDS including increased pulmonary oedema, neutrophil infiltration, inflammatory cytokine and chemokine expression and mortality [11]. Interestingly the use of aerosolized endotoxin in mice has demonstrated that low dose exposure (100 µg/ml) causes a rapid and transient activation of macrophages with a concomitant spike in neutrophilic inflammation within the airspace [12], and in a rat model a single high dose (5 mg/kg) intratracheal injection of endotoxin identified the macrophage as the ‘pivotal’ cell type responsible for both inflammation and repair during lung injury [13]. These latter data correlate well with the clodronate-mediated macrophage depletion data presented by Vuichard *et al.* The arguments for a single-hit endotoxin model of lung injury are clear: reproducibility, rapid onset of clinical signs and lack of expense make these uncomplicated models extremely attractive. Similarly, several articles have reported that the introduction of a second-hit had no impact on inflammation or increased lung injury [14,15].

However, despite the appeal of the single-hit model, the reasoning for the use of a two-hit model is still extremely logical. The development of ALI and ARDS in human patients is complex and rarely occurs as the result of a single instigating factor. Therefore the concept of priming the immune response to react exuberantly to a second stimulus makes eminent sense for a model system [16]. A number of two-hit models have been proposed with apparent success. The use of haemorrhagic shock prior to the introduction of low dose (0.03 mg/kg) intratracheal endotoxin in rats produced significant increases in neutrophil infiltration as well as protein leakage across the lung epithelial barrier as compared to endotoxin alone [7]. Similarly, this same model was utilized to determine that an antioxidative or anti-inflammatory agent applied after haemorrhagic shock might ameliorate

effectively the development of serious lung injury [17]. Development of two-hit models has become extremely sophisticated. Recently Steinberg and colleagues [18], created a two-hit model utilizing ischaemia reperfusion injury and fecal peritonitis to initiate ARDS in pigs. These authors have argued against the use of endotoxin in favour of bacterial sepsis, on the grounds that endotoxin speeds up the development of lung disease in such a way as to make it irrelevant to the human condition. They point out that a number of human clinical trials using anti-inflammatory treatment strategies proven efficacious in acute animal models of ARDS have failed to improve lung function and mortality [18]. The failure of these interventions to provide any significant improvement to patients with ALI and ARDS underscores the importance for the development of a relevant animal model (either single-hit or two-hit) that mirror the clinical and physiological development of ARDS in humans.

At the end of the day, stating definitively that two-hit models reign superior to one-hit models may be premature. Vuichard and colleagues should be complimented for their work, but significant controversy still remains and will require further investigation. The one-hit model’s simplicity, combined with reduced resources expended in a ‘do more with less’ environment, will continue to be preferred by many laboratories. While bodies such as the National Heart, Lung and Blood Institute are recommending two-hit models, prospective investigations designed to directly compare one- *versus* two-hit models head-to-head must be highly encouraged [19].

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