TLR2 activation causes no morbidity or cardiovascular failure, despite excessive systemic nitric oxide production

Anje Cauwels1,2†*, Benjamin Vandendriessche1,2†, Jennyfer Bultinck1,2, Benedicte Descamps3, Elke Rogge1,2, Tom Van Nieuwenhuysen1,2, Magdalena Sips1,2, Christian Vanhove3, and Peter Brouckaert1,2

1Department for Molecular Biomedical Research, VIB, Technologiepark 927, Ghent B-9052, Belgium; 2Department of Biomedical Molecular Biology, Ghent University, Ghent B-9052, Belgium; and 3Infinity Lab, MEDISIP, iMinds, Ghent University, Ghent B-9000, Belgium

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Aims
Septic shock is the leading cause of death in intensive care units worldwide, resulting from a progressive systemic inflammatory reaction causing cardiovascular and organ failure. Nitric oxide (NO) is a potent vasodilator and inhibition of NO synthases (NOS) can increase blood pressure in septic shock. However, NOS inhibition does not improve outcome, on the contrary, and certain NO donors may even provide protection. In addition, NOS produce superoxide in case of substrate or cofactor deficiency or oxidation. We hypothesized that excessive systemic iNOS-derived NO production is insufficient to trigger cardiovascular failure and shock.

Methods and results
We found that the systemic injection with various synthetic Toll-like receptor-2 (TLR2), TLR3, or TLR9 agonists triggered systemic NO production identical to that of lipopolysaccharide (LPS) or tumour necrosis factor. In contrast to the latter, however, these agonists did not cause hypothermia or any other signs of discomfort or morbidity, and inflammatory cytokine production was low. TLR2 stimulation with the triacylated lipopeptide Pam3CSK4 not only caused identical NO levels in circulation, but also identical iNOS expression patterns as LPS. Nevertheless, Pam3CSK4 did not cause hypotension, bradycardia, reduced blood flow, or inadequate tissue perfusion in the kidney or the liver.

Conclusion
We demonstrate that excessive iNOS-derived NO in circulation is not necessarily linked to concomitant cardiovascular collapse, morbidity, or mortality. As such, our data indicate that the central role of iNOS-derived NO in inflammation-associated cardiovascular failure may be overestimated.

Keywords
LPS • Shock • NOS • TLR2 • Hypotension

1. Introduction
Sepsis and septic shock are systemic inflammatory response syndromes resulting from and complicating an infection. They are the leading cause of death in intensive care units, and the incidence continues to increase. When micro-organisms invade a host, (immune) cells are stimulated to produce powerful pro-inflammatory mediators, such as tumour necrosis factor (TNF), IL-1, and IL-6. The host responds by producing nitric oxide (NO) and reactive oxygen species (ROS), and by releasing enzymes in order to kill the invading pathogen and clear the infection. However, when this inflammatory reaction becomes systemic and uncontrolled, sepsis ensues. Septic shock is defined as severe sepsis complicated by refractory hypotension. Historically, NO was considered the central mediator of inflammatory hypotension, since NO production is enhanced in experimental and clinical sepsis, and inhibition of NOS may increase blood pressure (BP). However, several studies also indicated serious adverse effects of NO inhibition, and some actually hinted towards the existence of other shock-inducing factors that are...
Excessive iNOS-derived NO does not cause hypotension.
2. Methods

2.1 Laboratory animals

Female C57BL/6J mice were purchased from Janvier (France). We also used animals on a C57BL/6J background, deficient in TLR2 (Jackson laboratories), MyD88,11 TRIF,12 eNOS,13 and iNOS.14 Age-matched control mice of the corresponding genetic and SPF background were used in all experiments. All mice were housed in temperature-controlled, air-conditioned facilities with 14/10 h light/dark cycles, food and water ad libitum, and used at 7–12 weeks. All the experiments were approved by, and performed according to the guidelines of the Animal Ethics Committee from Ghent University, Belgium, and the investigation conforms to the Directive 2010/63/EU of the European Parliament. For each experiment, mice were monitored several times daily for 5 days. Moribund or surviving animals were euthanized by CO2 asphyxiation followed by cervical dislocation.

2.2 Reagents and injections

Phenol-extracted E. coli LPS (serotype 0111:B4) was purchased from Sigma (St Louis, MO); ultrapure E. coli LPS (serotype 0111:B4, extracted by successive enzymatic hydrolysis steps and purified by phenol-TEA-DOC extraction15) was purchased from Invivogen (Toulouse, France). LPS, Pam3CSK4 (Pam, EMC Collections), FSL-1, poly I:C (pI:C), ODN1826 (ODN), and its negative control without CpGs (ODNc) (Invivogen) and L-NAME (Novabiochem, 100 mg/kg) were injected i.v. Cyclophosphamide (Sigma) was injected intraperitoneally (i.p.).

2.3 Blood collection, NOx−, and cytokine measurements

Blood was collected from the tail vein or via cardiac puncture. Determination of NO2− + NO3− (NOx−) was done as described.16 For TNF, IL-1, and IL-6 determination after LPS challenge, bioassays were used.17 For detailed analysis of the cytokine induction profiles of Pam and FSL-1 compared with LPS, negative control without CpGs (ODNc) (Invivogen) and L-NAME (Novabiochem, 100 mg/kg) were injected i.v. Cyclophosphamide (Sigma) was injected i.p. 4 days (5 mg) and 1 day (2 mg) before the experiment, inducing profound leucopenia for several days, as evaluated by differential white blood cell counting, FACS analysis and immunohistochemistry (data not shown).

2.4 Body temperature, BP, and HR measurements

Rectal body temperature was recorded with an electronic thermometer (model 2001; Comark Electronics). BP and HR were measured in conscious mice via radiotelemetry,18 PA-C10 probes (Data Sciences International) were surgically implanted. Mice received ibuprofen orally for 1 week, starting 1 day before surgery. For surgery, they were anaesthetized with 2% iso-flurane, and the cervical area skin cleaned with povidone-iodine and alcohol. At Day 10–14 after surgery, continuous, 24-h data collection began using the Dataquest ART Acquisition System (Data Sciences International, version 4.1).

2.5 Immunohistochemistry

Detection of iNOS was performed as described.19

2.6 MR acquisition and image analysis

MR images were acquired on a 7 T system (Bruker PharmaScan 70/16, Germany) with a mouse body volume coil. Mice were anaesthetized with iso-flurane (5% induction and 1.5% maintenance) and warmed with a water-based heating blanket. Anatomical information was obtained with a T1-weighted sequence (RARE) with the following parameters: TR 1464, TE 9.1 ms, 4 averages, echo train length 4, field of view 3 cm × 3 cm, matrix size 250 × 250, 120 µm in-plane spatial resolution, 30 contiguous slices with 600 µm thickness, and acquisition time 4′35″. Dynamic contrast-enhanced MR images were acquired for a single slice using a fast low angle shot sequence with the following parameters: TR 12 ms, TE 3.4 ms, field of view 3 cm × 3 cm, matrix 112 × 112, 268 µm in-plane resolution, 550 repetitions, temporal resolution 1.344 s, and acquisition time 12′19″. The oblique slice was rotated such that both kidneys and liver were covered. The gadolinium-based contrast agent (Dotarem, Guerbet, France, 2 mmol/kg, in 20 µL) was injected i.v. 3 min after start of acquisition. Total acquisition time per session was 25 min. Based on the anatomical MRI image, different regions of interest (ROI) of fixed size were determined for the abdominal artery (one ROI), kidney medullas (two ROIs) and cortex (four ROIs), liver (three ROIs), spleen (one ROI), and dorsal muscles (two ROIs). Care was taken to define the ROIs in similar locations for all mice (n = 14). For every ROI, the gadolinium (Gd)-enhanced intensity as a function of time was exported. Detection of time-to-peak (TTP) values was done in Matlab v7.13 [The MathWorks, Inc., Natick, MA (USA)]. Briefly, movement-induced outliers were removed by fitting a sixth-order polynomial to the data, followed by setting outlier boundaries arbitrarily at ± 15 000 (small-spread filter) or ± 30 000 intensity measures (wide-spread filter). Two filter settings were required due to differences in movement-induced data spread depending on the target tissue. Choice of filter for every ROI was done manually; all further data analysis was automated. Since all rapid Gd-based intensity changes were located in the first 60 s, a sixth-order polynomial was fit through the outlier corrected data in that time window. The roots of the first derivative of this fit were calculated and used to extrapolate the time-to-peak values.

2.7 Statistics

Statistics were performed using Graph-Pad Prism. Significant differences in systemic TNF, IL-1, IL-6, and NOx− were examined using a one-way ANOVA with the Bonferroni test (for comparison of all pairs). Data shown are always means ± SEM. Two-way ANOVA was used to compare temperature curves (repeated measures) and DCE-MRI time-to-peak values (standard), with Sidak’s correction for multiple comparisons. Survival curves were compared with a logrank test.

![Figure 2](image-url)
3. Results

3.1 Synthetic TLR2, TLR3, and TLR9 agonists induce iNOS and \( \text{NO}_x \) in mice, without causing morbidity or mortality

Pam3CSK4 (Pam) is a synthetic triacylated lipopeptide. It mimics the triacylated amino terminus of bacterial lipoproteins and signals via the interaction between TLR2 and TLR1. FSL-1 (Pam2CGDKP]HPSF) is a synthetic diacylated lipopeptide mimicking mycoplasmal lipoprotein, recognized by the TLR2/6 dimer. To analyse iNOS expression and \( \text{NO}_x \) production in vivo, we injected mice with Pam or FSL-1 and compared their serum \( \text{NO}_x \) induction with that induced by LPS. Identical doses of LPS and Pam caused identical levels of NOS-derived systemic \( \text{NO}_x \) (Figure 1A). The TLR2/6 agonist FSL-1 induced NOS-derived systemic \( \text{NO}_x \) as well, albeit at lower levels (Figure 1A). Also systemic challenge with the TLR3 agonist poly i:C (pI:C, a synthetic dsRNA) or the TLR9 agonist ODN1826 (ODN, a synthetic oligonucleotide containing

Figure 3  Comparison of Pam- and LPS-induced morbidity, cardiovascular failure, and perfusion. (A and B) Hypothermia and mortality. Mice were injected i.v. with PBS, 200 or 400 µg Pam, or 200 µg LPS. (C and D) MAP and HR were monitored in radiotelemetered conscious mice injected with PBS or 200 µg Pam. (E and F) DCE-MRI, see Methods for details. WT mice were injected i.v. with PBS (n = 5), Pam (n = 4) or LPS (n = 5) at t = 0, 7 h later Gd was injected i.v. and Gd intensity changes were recorded. Thirteen ROIs were defined per animal. For every ROI, the Gd intensity as a function of time was exported and TTP values were determined as an index of perfusion. (E) Representative example for the model fits used to derive TTP values for the kidney medullas of all individual mice. (F) TTP values for the abdominal aorta, kidney, liver, and spleen; there was no significant perfusion detectable in the dorsal muscle. LPS results were compared with PBS (*) and Pam (#) using two-way ANOVA. ***/###P < 0.001, ****/####P < 0.0001.
unmethylated CpG motifs) induced large amounts of NO\textsubscript{\textsuperscript{2}} in circulation (Figure 1B). Nevertheless, none of these NO-inducing TLR agonists caused any signs of morbidity, in contrast to LPS, evaluated by monitoring body temperature (Figure 1C). In addition, they did not cause diarrhea, and mice were as active and grooming as those that had been injected with PBS.

### 3.2 Excessive systemic NO\textsubscript{\textsuperscript{2}} induced by Pam does not cause any cardiovascular problems

Since immunohistochemical analysis showed that Pam-induced iNOS expression was situated in exactly the same cells as iNOS induced by LPS, viz. Paneth cells, epithelial cells in both small and large intestine, and hepatocytes (Figure 2), we decided to evaluate the effects of Pam in vivo in more detail. Pam-induced systemic NO\textsubscript{\textsuperscript{2}} originated entirely from iNOS and not from eNOS (Figure 1D), was induced via TLR2 and MyD88 signaling (Figure 1E), and was not produced by leucocytes (Figure 1F). Also systemic NO\textsubscript{\textsuperscript{2}} induced by LPS, TNF, or bacterial infections does not originate from leucocytes.\textsuperscript{19} Nevertheless, despite the identical iNOS expression pattern and the identical endogenous NO\textsubscript{\textsuperscript{2}} levels in circulation, Pam did not cause any hypothermia or mortality, not even when given in a higher dose (Figure 3A and B). In addition, Pam did not cause any hypotension or bradycardia (Figure 3C and D), and did not affect organ perfusion, in contrast to LPS, evaluated using dynamic contrast-enhanced MRI analysis (Figure 3E and F). Representative examples for the model fits used to determine TTP values for the kidney medullas for all individual mice are shown in Figure 3E. In the abdominal aorta, kidney medulla and cortex, and liver, TTP values were significantly increased after LPS treatment, compared with PBS or Pam treatment (Figure 3F).

### 3.3 TLR2 stimulation induces little systemic inflammation as such, but is highly synergistic with LPS or TNF

Synthetic TRL2 stimulation by i.v. Pam or FSL-1 did not induce any detectable TNF, IL-1\textbeta or IFN\gamma levels (Figure 4A–C). Other pro-
Inflammatory cytokines such as IL-1α, IL-6, and IL-12 were slightly elevated early after challenge (2 h), but returned to baseline only a couple of hours later, in contrast to LPS (Figure 4D–F). The combination of Pam with a sublethal dose of ultrapure LPS synergistically induced hypothermia and mortality (Figure 5A and B), and the production of pro-inflammatory cytokines such as TNF, IL-1β, and IL-6 (Figure 5C–E). Also with a sublethal challenge of TNF, Pam was highly synergistic (Figure 5F–H).
4. Discussion

Our results show that TLR2 triggering by Pam, a synthetic triacylated lipopeptide mimicking bacterial lipoprotein, induces systemic iNOS-derived NO in similar quantities as observed after triggering of TLR4 by LPS, without resulting in hypotension, bradycardia, reduced blood flow rate, or inadequate tissue perfusion. Since the iNOS expression patterns and NO\textsubscript{x} levels were identical for innocuous Pam and shock-inducing LPS or TNF, our results thus suggest that high systemic levels of iNOS-derived NO are not sufficient to cause cardiovascular collapse, as is generally assumed.

In this paper, we show that increased levels of systemic NO\textsubscript{x} may be induced by synthetic agonists of TLR2/1 and TLR2/6, namely Pam and FSL-1, synthesized on the basis of bacterial and mycoplasmal lipoproteins, respectively, as well as by the synthetic dsRNA and TLR3 agonist poly I:C, and by the TLR9 agonist ODN1826. However, none of the TLR2, TLR3 or TLR9 agonists caused any discomfort, hypothermia or mortality, in contrast to LPS. Since injection of identical amounts of Pam and LPS induced identical NO\textsubscript{x} levels in circulation, as well as identical iNOS expression patterns in parenchymal cells of the liver and gut, we decided to evaluate the cardiovascular effects of Pam in more detail. In contrast to LPS, Pam did not induce hypotension or bradycardia, which we measured in conscious non-restrained mice using telemetry. In addition, LPS significantly affected the blood flow rate and perfusion in the abdominal aorta, kidney, and liver, while these parameters were not altered after treatment with Pam. Hence, our findings suggest that endogenous induction of iNOS, resulting in excessive systemic NO\textsubscript{x} production, is not sufficient to cause cardiovascular collapse or shock, and that factor(s) different from NO might be more centrally involved in cardiovascular failure. Identifying these may provide new opportunities for future shock therapeutics.

We have previously demonstrated that haematopoietic cells do not contribute to systemic NO production in bacteraemic, endotoxaemic, or TNF-induced shock in mice. We also demonstrated that the parenchymal cellular sources of iNOS in these models are predominantly intestinal enterocytes, Paneth cells, and hepatocytes. In the present study, immunohistochemical analysis and experiments in leucopenic mice again demonstrate that parenchymal cells are the principal source of iNOS-derived NO in vivo, both after TLR4 or TLR2 stimulation.

In the late 1980s, NO, synthesized from L-arginine by NOS, was recognized as the endogenous endothelium-derived relaxing factor. Not long thereafter, L-arginine analogues such as L-NMMA and L-NAME were identified as convenient means to block NO enzymes and study the involvement of NO. Soon, NO (or rather, in retrospect, NOS, as the evidence was obtained using general NOS inhibitors) was put forward as the principal endogenous vasodilator and BP regulator and NOS/NO was considered the major mediator, and therefore a promising therapeutic target, of refractory hypotension associated with septic shock. More recently, however, it has become clear that NOS inhibition may indeed increase SVR and BP in septic shock patients (as it does in healthy subjects), but does not provide protection from cardiac failure and mortality at all, rather contrary, implying that NO also exerts extremely important protective effects. These may include anti-oxidant effects, hypoxic vasodilation necessary to maintain microcirculation and organ function, as well as cardioprotection. In addition, NO was recently shown to protect against endotoxaemia by suppressing inflammasome activation and the production of pro-inflammatory cytokines IL-1β and IL-18.

After the large phase III clinical trial had to be prematurely discontinued because of increased lethality, the existence of certain beneficial effects of NO in shock was finally generally accepted. Nevertheless, the paradigm that NO was the principal mediator of hypotension and shock kept dominating the field, and selective inhibition of the inflammatory iNOS isozyme was suggested. Meanwhile, pre-clinical studies with iNOS inhibitors have been conducted, with variable results. Nevertheless, iNOS-deficiency cannot provide survival benefit in endotoxaemic or septic shock and certain NO donor compounds can even provide protection. Some of the early pioneer studies using NO donors and NO donors already suggested that other shock-inducing factors may be more crucial than NO, and that NO may even be required to antagonize these unknown detrimental factors. Unfortunately, these reports have not been given much attention since, not even in the wake of the negative NOS inhibition results in the phase III trial. Moreover, when drawing conclusions from NOS inhibition studies, we should not forget that uncoupled NOS produces ROS, rather than NO. Uncoupling occurs in case of L-arginine or BH4 deficiency or oxidation, characteristics of systemic inflammation and sepsis.

It was recently suggested that mouse models would poorly mimic human inflammatory diseases, including endotoxaemia. This hypothesis was concluded after comparing LPS doses that cause a similar IL-6 response early after challenge in mice and men. Nevertheless, the LPS doses chosen induce a febrile, cardiovascular, and haematological response in humans but not in mice. In addition, the genomic study was conducted on total blood leucocytes, whereas leucocytes are not critically involved in the onset of endotoxic shock, or in the release of systemic NO. Hence, we believe that the results described in this paper, indicating that the presence of excessive systemic NO is not necessarily associated with cardiovascular or perfusion failure, are clinically relevant. To our knowledge, TLR2 agonists have not been used in humans yet, and no literature data could be found linking TLR2 stimulation with hypotension.

In conclusion, our observations show that TLR2 stimulation causes endogenous iNOS and systemic NO induction, identical in location and amount as if induced by shock-inducing LPS or TNF, without having any effect on peripheral body temperature, BP, HR, blood flow rate, organ perfusion, or morbidity. Therefore, our data imply that it is not only so that NO has protective effects next to its presumed deleterious ones, but they also question the magnitude of NO’s presumed deleterious effects. This has important therapeutic consequences. Indeed, several pre-clinical experiments have indicated that the administration of agonists (instead of antagonists) of the NO\textsubscript{(GOA). For this research, A.C. and J.B. received a postdoctoral and PhD fel-
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