Attenuation of Responses to Endotoxin by the Triggering Receptor Expressed on Myeloid Cells-1 Inhibitor LR12 in Nonhuman Primate

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ABSTRACT

Background: The triggering receptor expressed on myeloid cells-1 is an immunoreceptor that amplifies the inflammatory response mediated by toll-like receptors engagement. Triggering receptor expressed on myeloid cells-1 inhibitory peptides such LR12 have been shown to prevent hyperresponsiveness and death in several experimental models of septic shock.

Methods: Twelve adult male Cynomolgus (*Macaca fascicularis*) monkeys exposed to an intravenous bolus of endotoxin (10 μg/kg) were randomized to receive LR12 or placebo (n = 6 per group) as an initial intravenous bolus followed by an 8-h continuous intravenous infusion. An additional group of four only received vehicle infusion. Vital signs were monitored for 8 h. Blood was sampled at H0, 1, 2, 4, and 8 for analysis of clinical chemistries, leukocyte count, coagulation parameters, and cytokine plasma concentration.

Results: LR12 showed no effect on heart rate and body temperature. By contrast to the placebo group, which experienced a 25 to 40% blood pressure decrease after endotoxin administration, LR12-treated monkeys remained normotensive. Endotoxin induced leukopenia at 2 h (mean leukocyte count, 7.62 g/l *vs.* 21.1 at H0), which was attenuated by LR12. LR12 also attenuated cytokine production.

Conclusions: The triggering receptor expressed on myeloid cells-1 inhibitor LR12 is able to mitigate endotoxin-associated clinical and biological alterations, with no obvious side effects. This study paves the way for future phases Ia and Ib trials in humans. **(Anesthesiology 2014; XXX:XX-XX)**

T HE "genomic storm" that occurs during sepsis or trauma is the result of an excessive and overamplified host response to aggression.[1] Among the potential candidates acting as amplifiers of the innate immune response, the triggering receptor expressed on myeloid cells (TREM)-1 appears to play a central role.[2] TREM-1 is expressed by neutrophils, macrophages, and mature monocytes (CD14^{high}), as well as by hypoxic dendritic cells.[2–4] The engagement of TREM-1 with agonist monoclonal antibodies has been shown to stimulate the production of proinflammatory cytokines and chemokines such as interleukin (IL)-8, monocyte chimoattractant protein (MCP)-1, MCP-3, and macrophage inflammatory protein (MIP)-1α, along with rapid neutrophil degranulation and oxidative burst. The activation of TREM-1 in presence of toll-like receptor-2, -4, or nod-like receptors ligands amplifies the production of proinflammatory cytokines (tumor necrosis factor-α, IL-1β, granulocyte-macrophage colony–stimulating factor), as well as the inhibition of IL-10 release.[5–7] Of note, the TREM-1 pathway was among the most up-regulated ones in the Xiao *et al.*'s study.[1] The TREM-1 blockade by the use of a fusion protein or LP17, a short inhibitory peptide that mimics a portion of the extracellular domain of TREM-1, was associated with a survival improvement in animal models of experimental sepsis.[8–10]

What We Already Know about This Topic

- The triggering receptor expressed on myeloid cells-1 is an immunoreceptor that amplifies the inflammatory response mediated by toll-like receptors engagement

What This Article Tells Us That Is New

- The triggering receptor expressed on myeloid cells-1 inhibitor LR12 mitigated endotoxin-associated clinical and biological alterations, with no obvious side effects in experimental primates

These protective effects are also evident in other models of acute or chronic inflammatory disorders.[11–17]

In addition to TREM-1, the TREM gene cluster includes TREM-like transcript-1 (TLT-1). TLT-1 is abundant and specific to the platelet and megakaryocyte lineage. Upon platelet activation with thrombin or lipopolysaccharide, TLT-1 is translocated to the platelet surface and may act as a coactivating receptor.[18,19] We have shown that a soluble fragment of TLT-1 is present in human plasma, the level of which is highly correlated to disseminated intravascular coagulation scores during sepsis.[20] Soluble TLT-1 binds to

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fibrinogen and augments platelet aggregation *in vitro*. Interestingly, crystallographic studies reveal structural similarities between TLT-1 and TREM-1, which suggest the existence of interactions between TLT-1 and TREM-1.[21]

Indeed, we recently showed that TLT-1 and a 12–amino acid TLT-1 derived peptide (LR12) exhibit antiinflammatory properties by dampening TREM-1 signalling and thus behave like a naturally occurring TREM-1 inhibitor. The mechanism by which LR12 inhibits TREM-1 signalling derives from its ability to bind to the TREM-1 ligand.[22] We further demonstrated that this same peptide also modulates *in vivo* the inflammatory cascade triggered by infection, thus inhibiting hyperresponsiveness, organ damage, and death during sepsis in mice and minipigs.[22,23]

As mouse models of septic shock are far from recapitulating the human physiology,[24] we prospectively investigated the effects of LR12 during endotoxemia in the nonhuman primate. Here we show that LR12 infusion was able to attenuate endotoxin-induced inflammatory and clinical responses.

Materials and Methods

Animals

Male cynomolgus monkeys (*Macaca fascicularis*) (2.8 to 3.5 kg, 24 months old; Le Tamarinier, La Route Royale, Tamarin, Mauritius) were used. Animals were fasted the day before lipopolysaccharide challenge but had full access to water. CIToxLAB France Ethical Committee (CEC, Evreux, France) reviewed and approved the study plans (Nr CEC: 02221). This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Helsinki Declaration. Thus, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher.

Endotoxin Preparation

Purified endotoxin (lipopolysaccharide 0127:B8; Sigma-Aldrich, Saint-Quentin Fallavier, France; source strain ATCC12740, 500,000 endotoxin unit per milligram [100 mg] per vial) was diluted in sterile water and administered as a 10 min intravenous bolus at a dose of 10 μg/kg of body weight.

Reconstitution of LR12 and Placebo

Placebo and LR12 were labelled identically and appeared identical in a lyophilized form. Active drug was reconstituted with sterile water and diluted in 0.9% saline to a 7.5 mg/ml concentration. Corresponding placebo infusions were prepared in an identical manner.

LR12 is a 12-amino acid peptide derived from TLT-1 (LQEEDAGEYGCM), and placebo is the corresponding scrambled peptide (YQMGELCAGEED). These peptides have been described elsewhere.[23] The scrambled peptide has no biological nor clinical effect *per se* and thus constituted the control of choice.

Drug Administration and In Vivo *Lipopolysaccharide Challenge*

Vital signs and weight were recorded the day before the lipopolysaccharide challenge. The next morning, baseline clinical laboratory samples were collected, and vital signs were recorded. The drug was administered into the cephalic or saphenous vein *via* a teflon catheter. Contralateral vein was used for lipopolysaccharide administration.

Monkeys were randomized to receive LR12 or placebo (n = 6 per group). An additional group of four was constituted to only receive vehicle (NaCl, 0.9%) infusion and served as the control group.

At time point 0, a 15 min intravenous infusion of LR12 or placebo solution was begun, at the rate of 12 ml/h (5 mg/kg, 10 min, 2 ml), delivered by a calibrated syringe pump (Harvard Apparatus, Les Ullis, France). A continuous infusion was then administered for further 8 h at the rate of 2 ml/h (1 mg kg^{-1} h^{-1}, 8 h, 16 ml). Just before treatment infusion, an intravenous bolus of lipopolysaccharide (10 μg/kg) was administered over the course of a 10-min period into the contralateral catheter. Animals remained awake in an upright position in restraint chairs and continued to fast without food for the whole study duration.

Monitoring of Vital Signs and Blood Sampling

Oral temperature, pulse rate, and blood pressure were monitored every 15 min for 1 h, then every 30 min for 7 h. Blood was sampled into 5 ml lithium-heparinized tubes at H0, 1, 2, 4, and 8 for analysis of clinical chemistries, leukocytes count, coagulation parameters, LR12 plasma concentration, and cytokine plasma concentration.

Biological Measurements

Clinical chemistries were analyzed on a Siemens (Châtillon, France) ADVIA 1650 chemistry analyzer, leukocyte counts on a Siemens ADVIA 120 hematology system, and coagulation parameters on an ACL ELITE PRO (Beckman Coulter, Villepinte, France).

Plasma concentrations of cytokines were determined using a multiplex method (Luminex, Oosterhout, The Netherlands) for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, interferon-γ, MCP-1, MIP-1α, MIP-1β, and tumor necrosis factor-α.

Statistics

After testing for their normal distribution (Kolmogorov–Smirnov test) data were presented as means (SD). Between-group differences were tested by two-way ANOVA with Bonferroni correction. Analyses were performed using Graphpad Prism Software (La Jolla, CA).

Results

Pharmacokinetics

LR12 was administered as a 5 mg/kg bolus over 15 min followed by a continuous 1 mg kg^{-1} h^{-1} infusion. A peak concentration at 159.3 ± 22.8 ng/ml was achieved after the
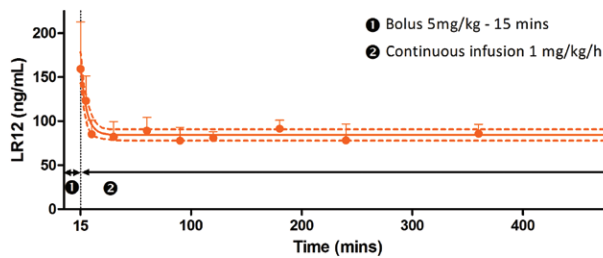
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Fig. 1. Pharmacokinetics of LR12. After an initial bolus followed by a continuous infusion, steady LR12 plasma concentration was reached and maintained throughout the 8-h study period.

bolus, then the LR12 concentration decreased to a steady state at 91.4 ± 5.1 ng/ml and the interindividual variability was very small (fig. 1). LR12 half-life was 2.25 min *in vivo*.

Vital Signs

Heart rate transiently increased after lipopolysaccharide injection, with no effects of LR12 infusion (fig. 2A). Lipopolysaccharide challenge induced a slight increase of body temperature, especially between H1 and H2, although LR12 contained the body temperature increase it was mild and the difference between placebo (LR12scr) and LR12-treated animals was not significant (fig. 2B).

Although the doses of endotoxin used were small, transient hypotension developed in the placebo-treated group: systolic arterial pressure decreased up to 25% at 180 min and diastolic arterial pressure up to 40% ($P < 0.001$ *vs.* LR12 or control groups). By sharp contrast, LR12-treated monkeys never experienced a hypotensive state and their arterial pressure did not differ from that of control animals (fig. 3).

Leukocyte Counts

Animals that received placebo (LR12scr) developed a leukopenia (mean leukocyte count, 7.62 g/l *vs.* 21.1 at H0) at 2h after challenge ($P < 0.001$ *vs.* control and LR12 groups). This leukopenia was the result of neutropenia, lymphopenia, and monocytopenia (fig. 4). Although LR12 had no effect on lymphocyte counts, its administration totally blunted neutropenia

during most of the observation period. Nevertheless, a marked leukocytosis (interesting mainly neutrophils) occurred at the end of the 8-h study period: leukocyte count was three times higher at H8 than at H0 ($P < 0.001$). Of note, platelet count remained unchanged between groups (not shown).

Blood Chemistry

Endotoxin challenge had almost no effect on clinical chemistries except on liver function. Indeed, total bilirubin and aspartate aminotransferase progressively increased and were higher at H8 than at baseline. LR12 completely abrogated this phenomenon. Of note, a brief hypoglycemia occurring at H2 was present in placebo animals whereas it was absent in the LR12 group (table 1). There was no effect of lipopolysaccharide or treatment on prothrombin, activated partial thromboplastin times or fibrinogen concentration.

Plasma Cytokine Concentrations

Endotoxin induced increase of IL-6, IL-8, interferon-γ, MCP-1, MIP-1α, MIP-1β, and tumor necrosis factor-α, which reached a maximum concentration between H1 and H2. LR12 attenuated the rise of IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and tumor necrosis factor-α plasma concentrations by 20 to 50% (fig. 5).

Side Effects

The animals were regularly examined up to 1 month after lipopolysaccharide/LR12 administration. There were no treatment side effects: in particular, no infection, electrocardiographic modification, or visual impairment.

Discussion

The use of mouse models still constitutes the cornerstone of medical research to evaluate new therapeutic approaches to various conditions, sepsis being one of the most studied. Unfortunately, Seok *et al.*[24] recently demonstrated that this approach carries many limitations. Indeed, genomic responses in mouse correlate very poorly with the human conditions: among genes that were significantly regulated
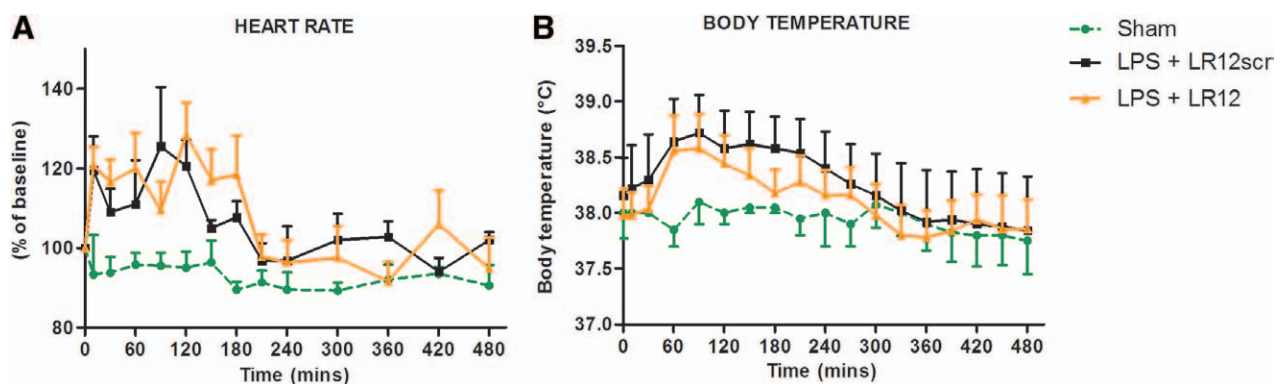

Fig. 2. Effects of LR12 on endotoxin-induced heart rate and body temperature changes. LR12 has no effect on endotoxin-induced heart rate (*A*) and body temperature (*B*) increases. LPS = lipopolysaccharide.
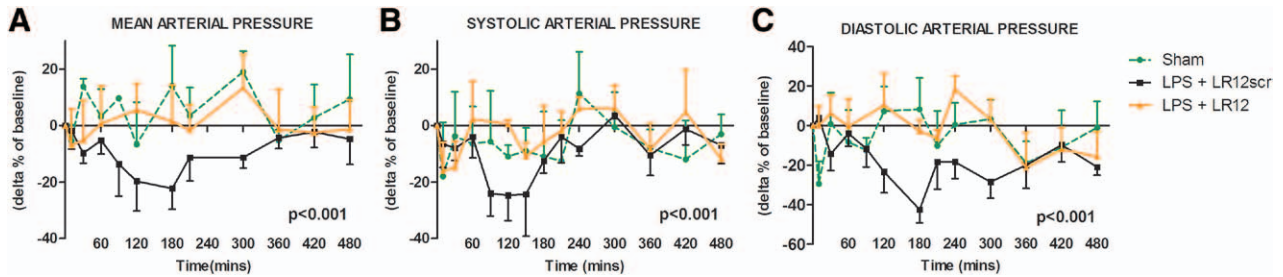
Fig. 3. LR12 protects from endotoxin-induced hypotension. (*A–C*) Endotoxin induced a transient drop in blood pressure (mean, systolic, and diastolic) that was completely prevented by LR12 (group effect: *P* < 0.001 placebo *vs.* LR12 or control groups). LPS = lipopolysaccharide.

in humans, the murine orthologs are close to random. This seminal article even generated a commentary in the *New York Times*, urging for a change of our experimental models in sepsis.[25] To attempt to overcome such pitfalls, we have chosen to focus on nonhuman primates in order to test the hypothesis that the modulation of TREM-1 by the inhibitory peptide LR12 protects from endotoxin-induced inflammatory and clinical responses.

Xiao *et al.*[1] recently characterized the circulating leukocyte transcriptome after severe trauma, burns, or during endotoxemia in healthy volunteers. They observed that as early as 4 h after injury, more than 80% of gene pathways were altered. This phenomenon, coined genomic storm, consisted of an increased expression of genes involved in innate immunity, systemic inflammatory and antiinflammatory responses, concomitant with a decreased expression of genes regulating adaptive immunity. They also observed that complications such as nosocomial infections arose independently of the existence of a second hit injury but were under the dependence of the magnitude and the duration of the initial leukocytes reprogramming. This new paradigm thus clearly suggests that a targeted therapy aimed at limiting this initial leukocyte genomic storm may be a valuable approach to improve patients' outcome.
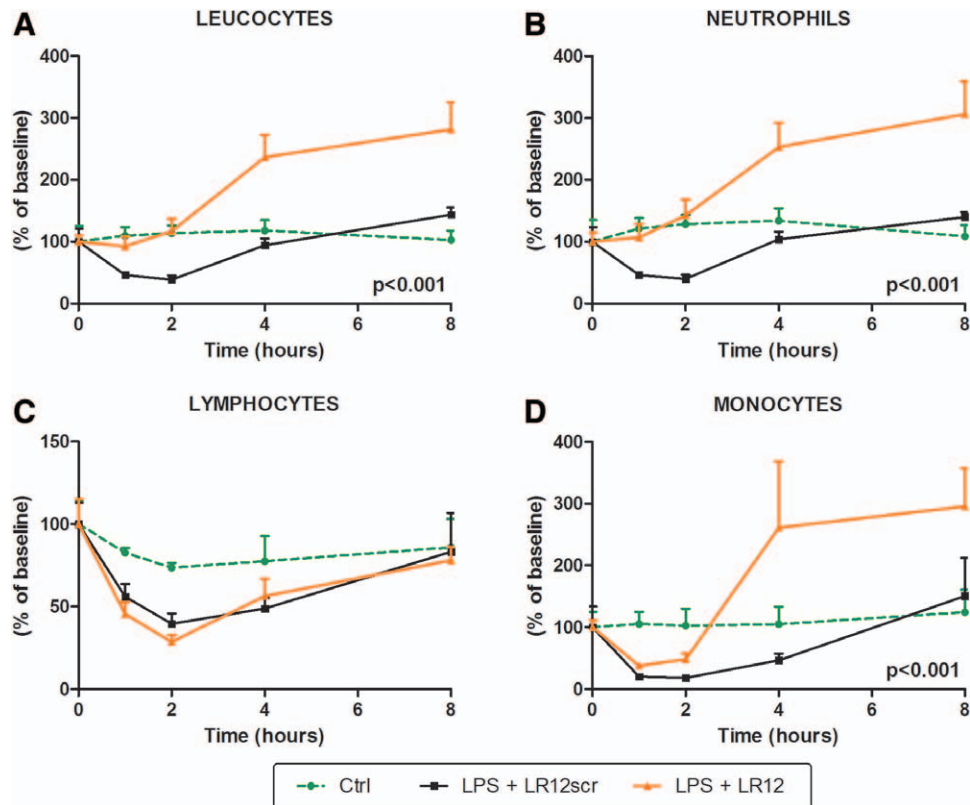

Fig. 4. (*A–D*) Effects of LR12 on blood leukocyte count. Animals that received placebo demonstrated a leukopenia (mean leukocytes count, 7.6 g/l *vs.* 21.1 g/l at H0) at 2 h after challenge (group effect: *P* < 0.001 *vs.* control and LR12 groups) resulting from neutropenia, lymphopenia, and monocytopenia. LR12 administration totally blunted neutropenia. A marked leukocytosis, mainly interesting neutrophils, occurred at the end of the 8-h study period (*P* < 0.001). Ctrl = control group; LPS = lipopolysaccharide.

Table 1. Blood Chemistry and Coagulation Parameters

	H0	H1	H2	H4	H8
Sodium (mM)					
Placebo	151.8±0.9	151.5±0.8	151.8±1.4	150.5±2.9	153.3±2.1
LR12	149.0±0.7	149.8±0.7	150.8±0.5	148.7±1.2	152.0±0.8
Potassium (mM)					
Placebo	4.2±0.2*	4.2±0.3	4.2±0.4*	4.3±0.1*	4.6±0.3
LR12	3.6±0.1	3.9±0.2	3.5±0.1	3.6±0.1	4.4±0.1
Chloride (mM)					
Placebo	108.2±2.2	110.5±3.0	110.2±2.9	107.7±3.9	109.3±1.6
LR12	107.8±1.7	108.2±1.6	110.5±1.5	108.5±1.4	110.8±2.3
Calcium (mM)					
Placebo	2.40±0.03	2.39±0.05	2.30±0.03	2.29±0.02	2.15±0.07
LR12	2.49±0.05	2.48±0.07	2.37±0.08	2.35±0.06	2.26±0.04
Glucose (mM)					
Placebo	4.1±0.2	3.3±0.5	2.6±0.5*†	3.6±1.0	3.8±1.2
LR12	3.6±0.3	3.4±0.3	3.7±0.3	4.1±0.4	4.0±0.4
Urea (mM)					
Placebo	5.9±0.6	6.4±0.6	6.4±0.6	6.7±0.9	7.3±1.0
LR12	5.6±0.5	5.4±0.5	5.7±0.2	6.1±0.9	5.8±1.0
Creatinine (μM)					
Placebo	82.6±3.1	83.7±5.2	81.2±3.2	79.5±1.7	80.5±1.2
LR12	86.0±5.7	82.8±4.1	83.1±5.5	83.3±0.3	80.0±0.9
Total bilirubin (μM)					
Placebo	3.0±0.6	4.2±0.7	5.0±1.0*	4.5±0.5	6.7±1.2*†
LR12	2.7±0.9	3.3±1.2	3.0±0.5	3.5±0.8	5.0±0.8
Alkaline phosphatase (U/l)					
Placebo	1,645±443	1,641±334	1,657±366	1,648±331	1,661±314
LR12	1,791±358	1,780±362	1,735±332	1,640±303	1,709±351
Aspartate amino transferase (U/l)					
Placebo	82±114	156±110	151±105	172±115	192±82*†
LR12	42±4	46±8	48±11	63±25	60±12
Total protein (g/l)					
Placebo	75±1	74±1	68±1	66±1	70±3
LR12	81±2	79±3	73±2	69±1	76±1
Albumin (g/l)					
Placebo	43±2	42±2	39±2	38±1	40±2
LR12	46±1	43±2	40±1	40±1	43±2
Fibrinogen (g/l)					
Placebo	3.94±0.4	3.42±0.4	3.53±0.2	3.69±0.3	3.58±0.2
LR12	3.64±0.3	3.31±0.4	3.17±0.3	3.15±0.3	3.40±0.3
Prothrombin time (s)					
Placebo	12.3±0.5	12.3±0.8	13.3±0.4	13.4±0.9	13.2±0.6
LR12	11.9±0.3	12.7±0.3	13.3±0.2	13.3±0.2	13.5±0.5
Activated partial thromboplastin time (s)					
Placebo	18.8±1.0	19.7±1.1	19.5±0.8	19.8±1.0	19.4±1.0
LR12	17.7±0.8	19.1±1.2	17.7±0.8	18.3±1.0	17.7±0.7

* $P < 0.05$ placebo *vs.* LR12-treated animals. † $P < 0.05$ *vs.* baseline.

Several proteins are known to amplify the initial inflammatory response, acting as amplification loops. Among them, TREM-1 seems to have an important role as its modulation demonstrated encouraging results in experimental models of sepsis, ischemia–reperfusion, pancreatitis, inflammatory bowel diseases, or chronic arthritis.[26]

Although the natural TREM-1 ligand remains unknown, we recently observed that another member of the TREM-1 family, TLT-1, was able to bind this ligand, therefore dampening TREM-1 engagement.[22] TLT-1 is one of the most abundant proteins released by activated platelets[27] whose role is to promote platelet aggregation through binding to fibrinogen. Large amounts of a soluble form of TLT-1 (sTLT-1) are released during sepsis[20] and we proposed that TLT-1 might prevent sustained and prolonged inflammation.[22]

In order to identify which portion of sTLT-1 was involved in this protective effect, we designed several
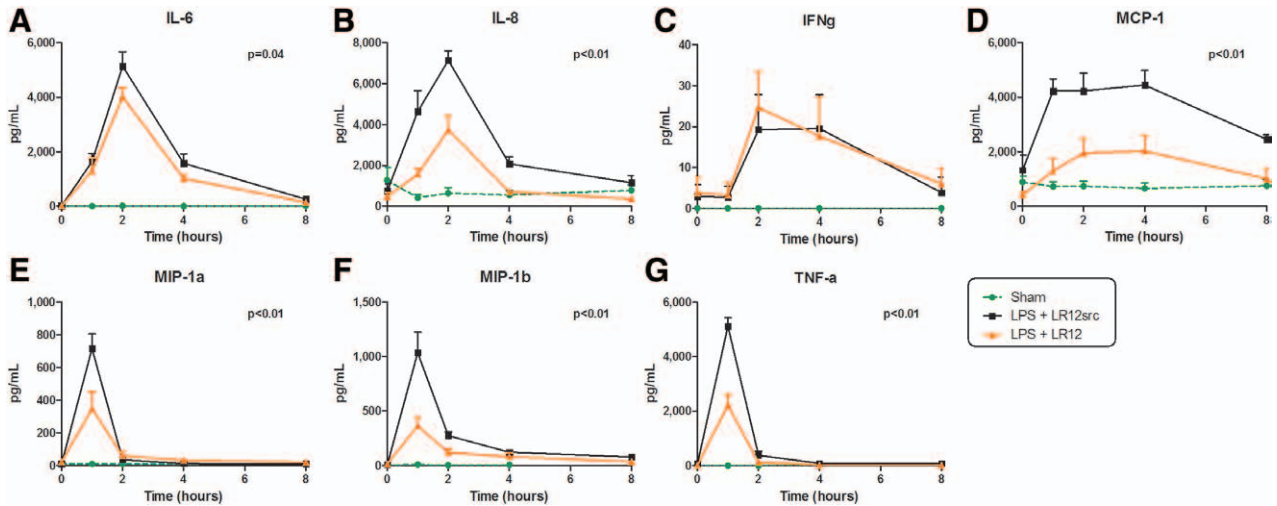
Fig. 5. LR12 administration reduced cytokine plasma concentrations. (*A–G*) Lipopolysaccharide (LPS) induced an increase of interleukin (IL)-6, IL-8, interferon (IFN)-γ, monocyte chimoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and tumor necrosis factor (TNF)-α peaking between H1 and H2. LR12 decreased IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α plasma concentrations by 20 to 50%. Indicated *P* values are for LR12 *versus* control group effect.

TLT-1 peptides representative of various potential ligand-binding regions.[22] Among these, a 12-amino acid sequence representative of residues 94 to 105, named LR12, was shown to be responsible for the antiinflammatory effect of sTLT-1. LR12 administration was associated with protective effects during sepsis both in murine and minipigs sepsis models.[23]

We designed the current study in primates in order to mimic a future phase Ib study in healthy volunteers and to accumulate robust information on LR12 safety and pharmacology.

We observed that LR12 administration was able to reduce endotoxin-induced systolic as well as diastolic hypotension, even though the blood pressure decrease subsequent to lipopolysaccharide administration was transient. This hemodynamic improvement is an effect we previously observed during experimental peritonitis in minipigs.[23] Indeed, recent unpublished data from our group (March 2013) suggest that LR12 protects from sepsis or endotoxin-induced endothelial dysfunction (and thus from vascular hyporeactivity), as well as from cardiac failure.

As expected, endotoxin administration induced a rapid and brief leukopenia involving neutrophils, monocytes, and lymphocytes. LR12 prevented this leukopenia essentially by blunting neutropenia. The effect on monocytes was modest and even null on lymphocytes. Interestingly, on LR12 treatment, both neutrophilia and monocytosis occurred. Although not completely elucidated, this observation suggests that TREM-1 engagement plays an important role in cellular recruitment and mobilization.[28]

We have repeatedly showed that TREM-1 pathway modulation was associated with a decrease of cytokine plasma concentrations: the same findings were observed here with a 20 to 50% reduction of most studied cytokines.

Regarding clinical chemistry or coagulation parameters, we only noticed a slight liver dysfunction upon endotoxin administration: this disorder was prevented by LR12. There were no deleterious effects of LR12 on any studied parameters.

The model of endotoxin administration, even in nonhuman primates, is a helpful but definitely not an absolute predictor to assess the complex physiopathology of human sepsis: whether LR12 will prove beneficial in humans remains to be proved, especially when considering the numerous and recent failures to translate promising experimental data to the clinical setting.[29,30]

Nevertheless, this work adds important data to those previously accumulated in polymicrobial models of sepsis in rodents and minipigs and therefore suggests that LR12 is able to mitigate endotoxin-associated clinical and biological alterations, with no obvious deleterious side effects in the nonhuman primate.

Although endotoxin does not mimic the complex physiopathology of sepsis, this study in nonhuman primates constitutes an important stepping-stone to help designing and conducting future phase Ia and Ib trials in humans.

Acknowledgments

Competing Interests

Drs. Derive and Gibot are cofounders of INOTREM, a society developing triggering receptor expressed on myeloid cells-1 inhibitors.

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