Novel therapeutic approaches to modulate inflammation

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I

Introduction and outline of the thesis

Inflammation can be caused by various insults such as microbial infection and tissue injury, and is a protective response of the body to ensure removal of detrimental stimuli and to stimulate the repair of damaged tissues [1]. Inflammation is, however, also a major pathophysiological factor in several illnesses such as sepsis, renal ischemia-reperfusion (I/R) injury, and cancer. It is generally thought that a controlled inflammatory response is beneficial (for example, in providing protection against infection), but that it becomes detrimental if dysregulated (for example, in septic shock). Therefore, restoring proper control of inflammatory reactions may be of benefit in pathological inflammatory conditions. Although many experimental anti-inflammatory approaches have been developed and tested, most of these have not led to successful clinical application. Therefore, there is still an urgent need for novel approaches to better control inflammation in severe clinical conditions. This thesis describes the immunomodulatory effects of human chorionic gonadotropin (hCG)-related oligopeptides, dexamethasone, and dietary restriction in animal models of sepsis, renal I/R injury, and cancer metastases, in which inflammation is a central pathophysiological component.

1.1 SEPSIS

Sepsis is characterized by the presence of bacteria in blood, and can provoke a complex syndrome resulting from a dysregulated immune response of the host to the invasive pathogen. Sepsis is clinically defined as an infection with evidence of systemic inflammation, as reflected by increased or decreased body temperature (>38ºC or <36ºC), abnormal leukocyte count (white blood cell count >12.000 cells/µl or <4.000 cells/µl), tachycardia (heart rate >90/min), and tachypneu (respiratory rate >20/min) or hyperventilation ($PaCO_2$ <32 mmHg) [2, 3]. In the United States approximately 750.000 cases of sepsis occur annually. During the last decades the incidence of sepsis, sepsis-related hospitalization, and sepsis-associated mortality increased substantially [4-6]. This emphasizes the need for new effective immunomodulatory therapeutics.

Initially the septic response results in excessive release of pro-inflammatory mediators relative to anti-inflammatory mediators, resulting in a hyperinflammatory phase defined as the systemic inflammatory response syndrome (SIRS) [3, 7, 8]. SIRS is associated with the development of hypoperfusion, tissue hypoxemia, tissue damage, and multiple organ dysfunction syndrome. Upon persistence, the septic response shifts towards a progressive anti-inflammatory response, the so-called compensatory anti-inflammatory response syndrome (CARS) [8, 9]. CARS is characterized by increased production of anti-inflammatory mediators as well as immune cell apoptosis, and may develop into immunoparalysis, failure to clear the primary infection, and increased susceptibility to new infections.

Although septic patients may die during the uncontrolled pro-inflammatory response, most sepsis-related deaths occur during the immunosuppressive phase [7, 10, 11]. These findings have contributed to the consensus that the balance between the pro-inflammatory and anti-inflammatory responses is vital in order to survive sepsis treatment. Numerous experimental therapeutic approaches to modulate the inflammatory response were successful in animal models of sepsis or septic shock. However, with the exception of recombinant activated protein C [12, 13], most of these therapeutical approaches were unsuccessful in clinical sepsis treatment. Therefore, detailed knowledge on the biology of sepsis-related immune activation and specific approaches to modulate this necessary. A more detailed description on the pathobiology of the septic response and treatment strategies is provided in the general discussion.

1.2 ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion (I/R) injury is initiated by a lack of blood flow (ischemia) which results in a state of tissue oxygen and nutrient deprivation that is characterized by adenosine triphosphate (ATP) depletion and buildup of toxic byproducts. Subsequent restoration of blood flow (reperfusion) causes further damage, at first by inappropriate activation of cellular oxidases and subsequently by the release of inflammatory mediators in response to tissue damage [14]. Successful renal transplantation, which is considered to be the treatment of choice for people with end-stage renal disease, is negatively influenced by I/R injury [15, 16]. I/R injury is the major cause of ischemic acute renal failure (ARF) in transplanted kidneys [14], and is responsible for detrimental consequences such as delayed graft function and subsequent loss of kidney grafts [17, 18]. Currently, there is no specific therapy for ischemia induced ARF.

Renal I/R injury induces an inflammatory reaction that is characterized by the production of cytokines (e.g. IL-1, TNF-α), chemokines (e.g. CXCL1, MIP-2), and reactive oxygen species (ROS) [19]. These factors activate endothelial cells and enhance the expression of adhesion molecules such as E-selectin, P-selectin, and ICAM-1 [19]. Activated leukocytes, such as monocytes and neutrophils, subsequently adhere to endothelial cells with resultant infiltration into the renal tissue, inflammation (e.g. cytokine release), and extension of cellular injury [19]. Neutrophil activation and infiltration may be controlled by other leukocytes, such as natural killer (NK)-T cells, as well. NKT cells play an important role in renal I/R injury and they have the capacity to rapidly (within 2 hours) produce large amounts of cytokines (IFN-γ, IL-4, TNF-α) [20, 21]. This rapid and strong cytokine response subsequently regulates the function of other immune cells such as dendritic cells (DC), regulatory T cells, NK cells, and B cells. Blockade of NKT cells with an anti-CD1d monoclonal antibody prevents renal accumulation of IFN-γ-

producing neutrophils and protects against renal I/R injury in mice [20]. The importance of inflammatory mediators in the pathogenesis of ischemic acute renal injury is evident from blocking studies (see below).

Tubular epithelial cells also play a pro-inflammatory role in renal I/R injury. Complement activation is required to stimulate the production of the chemokines CXCL1 and MIP-2 by renal tubular epithelial cells [22]. These chemokines attract neutrophils and macrophages to the injured kidney. In addition, TLR-4 expression is upregulated by tubular epithelial cells after renal I/R injury, while TLR-4 deficiency blunts the production of pro-inflammatory cytokines and chemokines induced by I/R injury [23].

Several immunomodulatory approaches for renal I/R injury have been investigated in experimental animal models. Treatment with an anti-ICAM-1 or CX3CL1 antibody, or an oral inhibitor of platelet activating factor protected animals from renal I/R injury [24-26]. In addition, overexpression of the antioxidant glutathione peroxidase reduced renal chemokine expression, renal neutrophil infiltration, and improved renal function following renal I/R [27]. Treatment with the synthetic glucocorticosteroid dexamethasone reduced apoptosis and necrosis of proximal tubular cells, and reduced renal ICAM-1 expression as well as neutrophil influx following renal I/R [28]. Also apoptosis inhibiting agents have proven to be effective against renal I/R injury in animal models. For instance, caspase-1 inhibition reduced necrotic and apoptotic cell death, improved renal function, and reduced inflammation in a murine I/R model [29]. However, as with septic shock, these experimental therapeutical approaches failed in a clinical setting [17].

1.3 SURGERY-RELATED INFLAMMATORY RESPONSE AND CANCER

Cancer is one of the leading causes of mortality and morbidity worldwide [30]. Although the immune system clearly exerts anti-tumor effects, there is growing evidence that inflammation plays a major role in the development and progression of many types of cancer [31].

Colorectal cancer is the third most common cancer [32] and surgical resection of the primary tumor remains the treatment of choice. Unfortunately, 30%-50% of all patients undergoing curative resections develop local recurrence or distant metastases, predominantly in the liver [33]. Surgical trauma and intra-operative blood loss promote tumor growth in animal models and patients undergoing surgery for colorectal cancer [34-36]. It was found that induction of surgical trauma to healthy tissue promotes adhesion and/or growth of circulating tumor cells (CTC) [37], and a recent meta-analysis demonstrated an increased hepatic metastases rate in CTC positive patients undergoing surgery for colorectal cancer [38]. In addition, a randomized study demonstrated that the

probability of survival of colon cancer patients was significantly higher when operated using laparoscopy as compared with an open colectomy procedure [39], demonstrating that less extensive surgical tissue damage reduces the chance of metastatic tumor development.

The surgery-induced inflammatory response has many parallels with a septic response and is characterized by increased levels of pro-inflammatory mediators (IL-1β, IL-6, TNF-α, MCP-1, MIP-1α) and adhesion molecules (E-selectin, ICAM-1, VCAM-1), leukocyte activation, and the occurrence of a postoperative immunosuppression that may be severe and long-lasting [40, 41]. The invasive capacity of malignant cells can increase in the presence of inflammatory cytokines such as IL-1β, IL-6, and TNF-α [42] and upregulation of adhesion molecules (e.g. E-selectin) on endothelial cells. This further contributes to metastases by facilitating adhesion of tumor cells to endothelial cells and subsequent outgrowth. E-selectin and TNF-α have been identified as key mediators of hepatic metastases in a murine hepatic metastases model [43, 44]. This suggests that downregulation of these molecules may reduce the occurrence of hepatic metastases following surgery. Surgery also induces a shift from cellular to humoral immunity, which may impair NK cell and Kupffer cell function. These cell types have an important role in eradication of tumor cells that are retained within the liver vasculature. Thus, impairment of their activity enhances the risk of hepatic metastases [45-47].

Overall, surgically-induced immune changes may create an environment that potentiates the metastatic potential of CTC, which are already present prior to surgery or originate as a result of hematogenic tumor spill due to tumor handling [45]. Therefore, the perioperative period may provide a window of opportunity for immunomodulatory intervention to reduce adhesion and outgrowth of CTC in the liver. Up to now few perioperative anti-inflammatory approaches have been investigated to reduce the occurrence of metastases after cancer surgery. Postoperative dexamethasone treatment significantly reduced tumor recurrence and hepatic metastases in mice upon inoculation of human pancreatic cancer cells followed by subtotal pancreatectomy [48]. Considering this, research into immunomodulatory approaches that prevent metastases upon surgical intervention is warranted.

1.4 NOVEL THERAPEUTIC APPROACHES TO MODULATE INFLAMMATION

Human chorionic gonadotropin-related oligopeptides

The fact that clinical manifestations of auto-immune diseases, such as rheumatoid arthritis and multiple sclerosis, can improve during pregnancy drew the attention to the pregnancy-associated hormonal milieu as potential regulator of the maternal immune

system. The human pregnancy hormone chorionic gonadotropin (hCG) has already long been recognized to exert immune inhibitory effects [49]. During pregnancy degradation products of the β-chain of hCG are present in serum and urine [50] and a peptide fraction of 400-2000 Dalton, that was present in urinary hCG preparations, reduced diabetes in NOD-mice [51] and protected BALB/c mice from lipopolysaccharide (LPS)-induced shock [52]. More recently, in our department the concept emerged that small oligopeptides of 3 - 7 amino acids that are related to the primary sequence of the hCG-β-chain exert immunomodulatory activities [53]. For instance, LQGV and VLPALP, sequences which are both present within the primary sequence of loop 2 of β-hCG, protected BALB/c mice from LPS-induced septic shock [52, 54]. A mixture of VLPALP, LQGV and its alanine replacement variant AQGV reduced the signs of septic shock in rhesus monkeys after *Escherichia coli* infusion [54]. Furthermore, *in vivo* administration of LQGV reduced the capacity of splenocytes to proliferate and to produce IL-6 and TNF-α upon LPS stimulation *in vitro*, indicating that LQGV reduces Toll-like receptor-driven immune activation [54, 55]. Before these hCG-related anti-inflammatory oligopeptides can be tested in human clinical trials a better insight is required into the mechanism(s) by which these agents exert their immunomodulatory activity. In addition, the immunomodulatory effect of hCG-related oligopeptides in clinically more relevant animal models of sepsis, such as the cecal ligation and puncture (CLP)-induced polymicrobial sepsis model, needs to be investigated.

Glucocorticosteroids

Stressors such as infection, surgery, and trauma are accompanied by activation of the hypothalamic-pituitary-adrenal axis, and the release of corticotropin and glucocorticosteroids like cortisol [56, 57]. Endogenously produced glucocorticosteroids play a pivotal role in the control and resolution of inflammation [58-60]. Specific activation of the adrenal glands to stimulate glucocorticosteroid production has been postulated as a way to control inflammation [61]. Furthermore, synthetic glucocorticosteroids are effective anti-inflammatory drugs that are commonly used for the treatment of many chronic inflammatory and immune mediated diseases [58].

During the last decades studies on the therapeutic value of general anti-inflammatory agents, such as glucocorticosteroids, in severe sepsis and septic shock have yielded contradictory results. Some studies demonstrated survival improvement after high-dose glucocorticosteroid treatment [62] while others did not [63, 64]. Recently, an extensive literature review suggested that low-dose glucocorticosteroid treatment has a beneficial effect on short-term mortality in patients with vasopressor-dependent shock, in contrast to high-dose glucocorticosteroid treatment [65]. This suggests that these immunosuppressive glucocorticosteroids, which are applied for a long time already, may

be of benefit to treat sepsis but that their application should be changed. Furthermore, the mechanism(s) by which low-dose glucocorticosteroid treatment might protect against sepsis-induced mortality remains elusive, and warrants further studies.

Dietary restriction

Dietary restriction (DR) may be achieved by various regimens such as caloric restriction (CR) and fasting. CR refers to a reduced energy intake without causing malnutrition, while fasting is abstinence of food, with an *ad libitum* access to water. Life-long daily CR is associated with extended longevity in different species such as fish, rodents, and non-human primates [66-68]. Randomized controlled trials in humans demonstrated that prolonged CR reduces the risk of cardiovascular events [69] and improves insulin sensitivity [70]. Besides the long-term effects of DR on lifespan, short-term DR has been shown to improve stress resistance. Preoperative fasting (1 to 4 days) improved liver function and survival following orthotopic liver transplantation [71] and protected other organ systems such as brain, heart, and retina against I/R injury in animal models [72]. CR has also been shown to reduce angiogenesis and tumor growth in a brain tumor model in mice [73].

DR modulates immune activity, which may contribute to the observed effects of DR in clinical settings. DR reduces the expression of pro-inflammatory cytokines and adhesion molecules in mouse models of renal and hepatic I/R injury [74]. In addition, DR improves survival and kidney function following renal I/R injury in mice, which is associated with reduced oxidative injury and cell death [74]. In line with this, reduced serum cytokine levels (e.g. IL-6, IL-1β, and TNF-α) have been described in case of DR prior to myocardial ischemia-reperfusion in mice [75] and in a murine Sjögren's syndrome model [76]. The effect of short-term DR in humans is largely unknown, but reduced TNF-α serum levels and improved well-being were found in asthma patients after short-term DR [77]. More recently, a clinical trial demonstrated that a 4 day DR regimen prior to surgery increased postoperative IL-8 plasma levels [78]. In addition, the capacity of postoperatively obtained blood cells to produce TNF-α upon LPS stimulation was decreased by DR [78], suggesting that DR interferes with the inflammatory response.

Overall, these studies demonstrate that short-term DR increases stress resistance both in animals and in humans. The proposed protective mechanisms of DR include upregulation of cytoprotective molecules such as heat shock proteins and hemoxygenase-1 [72], and decreased production of pro-inflammatory molecules. The mechanism by which DR exerts its immunomodulatory effects remains elusive, which warrants further investigations to facilitate translation to the clinical setting.

1.5 AIM OF THESIS

Inflammation is crucially involved in the pathogenesis of sepsis, I/R injury, and in tumor recurrence following cancer surgery. Therapeutic approaches to modulate inflammation may therefore be of benefit in the treatment of these conditions. The overall aim of this thesis was to examine the immunomodulatory effects of hCG-related oligopeptides, dexamethasone, and dietary restriction in animal models of sepsis, renal I/R injury, and surgery-enhanced tumor metastases growth. **Chapter 2** describes the effect of the β-hCG related oligopeptide LQGV on inflammation and mortality in a CLP-induced polymicrobial sepsis model in mice. In **Chapter 3** the immunomodulatory mechanism of LQGV was explored using an *in vivo* LPS-induced shock model in mice and *ex vivo* murine adrenal gland cultures. **Chapter 4** describes the effect of low and high doses of dexamethasone on CLP-induced inflammation and mortality in mice. In **Chapter 5** the potential anti-inflammatory properties of several hCG-related oligopeptides in a murine model of renal I/R-induced inflammation and injury were examined**.** In **Chapter 6** the protective effect of dietary restriction through fasting on renal I/R injury, and the role of corticosterone release and glucocorticosteroid receptor signaling herein, was explored. In **Chapter 7** the effect of caloric restriction on the adhesion of tumor cells to endothelial cells and hepatic tumor growth was investigated in a murine model of hepatic colorectal cancer. **Chapter 8** gives a literature overview of the immunobiology of sepsis with regard to regulation and potential novel therapeutic targets, as well as recommendations to facilitate successful implementation of (novel) immunomodulatory therapies such as LQGV, dexamethasone, and dietary restriction into the human setting. In addition, the studies performed in this thesis, and the implications of these studies, are summarized and discussed in the context of available literature. Finally, suggestions for future research in this field are given.

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II

The β-human chorionic gonadotropin-related peptide LQGV reduces mortality and inflammation in a murine polymicrobial sepsis model

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ABSTRACT

Objective: Mortality in sepsis remains high and efforts to modulate the inflammatory response so far mostly failed to improve survival. The human chorionic gonadotropin-related tetrapeptide LQGV was recently shown to exert anti-inflammatory activity. The aim of this study was to assess the effect of LQGV on cecal ligation and puncture-induced mortality and inflammation.

Design: Animal study.

Setting: University research laboratory.

Subjects: Male C57BL/6 mice.

Interventions: To examine the effect of LQGV by itself on cecal ligation and puncture-induced mortality and inflammation C57BL/6 mice were exposed to a moderate cecal ligation and puncture procedure (40% ligation and double puncture) with a mortality rate of approximately 80% within 5 days in control mice. In addition, to examine whether LQGV was of additive value to standard sepsis care (fluid resuscitation and antibiotics) a more severe cecal ligation and puncture procedure was used (80% ligation and double puncture), yielding approximately 100% mortality within 12 days in control mice. LQGV (5 mg/kg body weight), phosphate-buffered saline (as control), or dexamethasone (2.5 mg/kg body weight) was administered perioperatively. Survival was monitored for 21 days and inflammatory markers were determined in plasma, peritoneal cavity, and lungs.

Measurements and Main results: LQGV significantly improved survival from 20% to 50% during the first 5 days after moderate cecal ligation and puncture. This was associated with reduced cytokine and E-selectin levels in peritoneal lavage fluid, lungs, and, to a lesser extent, in plasma. LQGV treatment also reduced pulmonary nuclear factor-κB activation and pulmonary damage. In the severe cecal ligation and puncture model, LQGV combined with fluid resuscitation and antibiotics resulted in significantly better survival (70%) than that observed with fluid resuscitation and antibiotics alone (30%).

Conclusions: LQGV improves survival after cecal ligation and puncture. This is likely established by a modest reduction of the acute inflammatory response through a nuclear factor-κB-dependent mechanism. Furthermore, LQGV may be a valuable additive next to the standard care in polymicrobial sepsis.

INTRODUCTION

Sepsis and septic shock resulting in multiple organ dysfunction syndrome are leading causes of morbidity and mortality [1-3]. Sepsis is characterized by an early Toll-like receptor (TLR)-driven hyperinflammatory response, defined as systemic inflammatory response syndrome (SIRS). SIRS, induced by pathogen recognition through TLR, is characterized by leukocyte extravasation and release of cytokines (eg, interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α)) and chemokines (eg, chemokine (C-C motif) ligand 2 (CCL2) and macrophage inflammatory protein-1α (MIP-1α)) by inflammatory cells and endothelial cells [4]. This proinflammatory environment causes the release of secondary mediators such as reactive oxygen species and nitric oxide that further augment the inflammatory reaction and subsequent organ damage [5]. The early hyperinflammatory response is followed by a state of immunosuppression characterized by abundant presence of anti-inflammatory cytokines (eg, IL-10 and transforming growth factor-β (TGF-β)) as well as anergy and apoptosis of immune effector cells (eg, B cells, T cells, and dendritic cells) [5-8]. Septic patients may die during the early hyperinflammatory phase of sepsis but mostly die at the late immunosuppressive phase [7]. The fact that sepsis-related morbidity and mortality still increases emphasizes the need for new therapeutics with immune regulatory properties [3].

During pregnancy, the maternal immune system is regulated by a shift away from a type 1 (cellular) immune response toward a type 2 (humoral) immune response [9], a process in which downregulation of nuclear factor-κB (NF-κB) activity in type 1 cells seems to be involved [10]. Consequently, clinical manifestations of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis improve during pregnancy, whereas symptoms of systemic lupus erythematosus, in which the principal pathology is autoantibody-driven, tend to flare up [11]. The pregnancy-associated hormonal milieu is thought to contribute to maternal immune modulation. Human chorionic gonadotropin (hCG) is one of the first hormones formed during pregnancy and exerts immunomodulatory actions [12, 13]. Degradation products of β-hCG are also present during pregnancy [14], and previously we have demonstrated that the peptides LQGV and VLPALP, which are present within the primary structure of loop 2 of β-hCG, have anti-inflammatory activities in models of autoimmune diabetes, hemorrhagic shock, and lipopolysaccharide (LPS)-induced shock [15-17]. Furthermore, *in vivo* administration of LQGV reduced the capacity of splenocytes to produce IL-6 and TNF-α upon lipopolysaccharide and heat-killed *Listeria monocytogenes* stimulation *in vitro*, indicating that LQGV reduces TLR-driven cytokine production [18].

Several potent anti-inflammatory therapeutics such as anti-TNF-α monoclonal antibodies have been tested successfully in LPS models [19-21]. However, they appeared to be noneffective in both cecal ligation and puncture (CLP)-induced polymicrobial

sepsis in mice and clinical trials involving septic patients [21-24]. Recently, we described that LQGV effectively prevents LPS-induced shock and mortality in mice [17]. The CLP-model, however, more adequately resembles the human immune reaction during sepsis than the acute and sterile LPS-induced shock model. Also, CLP more closely mimics the clinical setting, because fluid resuscitation and antibiotics can be administered to evaluate the potential of new anti-inflammatory agents [25, 26].

To examine the potential of LQGV to reduce mortality and to modulate the inflammatory response during sepsis, we first determined the effect of LQGV by itself in a polymicrobial murine sepsis model. Thereafter, to examine a potential additive value of LQGV to standard sepsis care, we determined the effect of LQGV in combination with fluid resuscitation and antibiotics. We demonstrate that LQGV improves survival after CLP, which is associated with reduced inflammation, especially in the peritoneal cavity and the lungs. Furthermore, we demonstrate that combination treatment of LQGV with fluid resuscitation and antibiotics significantly improves survival as compared with fluid resuscitation and antibiotics alone. Our results demonstrate that the β-hCG-related tetrapeptide LQGV acts as an anti-inflammatory agent and is a useful addition to the current standard treatment for sepsis.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice with an average weight of 25 g were purchased from Harlan (Horst, The Netherlands). The experimental protocol was approved by the local animal care and use committees.

Moderate CLP-induced polymicrobial sepsis

Moderate polymicrobial sepsis, defined as approximately 70 - 80% mortality during the acute phase of sepsis (first 5 days), was used to test potential effects of LQGV in sepsis. Hereto, mice underwent low-grade CLP as described [27, 28]. Briefly, the cecum was located, exteriorized, and ligated 1 cm from its distal end without causing intestinal obstruction. Subsequently, the cecum was punctured twice with an 18-gauge needle and manipulated to ensure extrusion of feces into the abdominal cavity. Postoperatively mice received a single subcutaneous (s.c.) injection of 0.5 mL of saline. Mice were monitored every 12 hrs during the first 5 days (the acute hyperinflammatory phase of sepsis), followed by daily monitoring up to 21 days (the chronic immunosuppressive phase of sepsis).

Severe CLP-induced polymicrobial sepsis

Severe CLP-induced polymicrobial sepsis (defined as approximately 100% mortality when mice were postoperatively treated with fluid resuscitation only, and approximately 50% mortality when mice were postoperatively treated with fluid resuscitation and antibiotics) was used to test whether LQGV is of additive value to standard sepsis treatment. Hereto, the cecum was ligated just below the ileocecal valve without causing intestinal obstruction followed by double puncture with an 18-gauge needle and manipulation to ensure extrusion of feces [27]. To reach a mortality of approximately 50% during the chronic phase of sepsis, mice received subcutaneous injections of Tienam (25 mg/kg body weight (BW); Merck Sharp & Dohme, Haarlem, The Netherlands,) dissolved in 1 mL 0.9% NaCl starting 2 hrs after surgery followed by subsequent injections every 12 hrs during the first 5 days. Control mice postoperatively received 1 mL 0.9% NaCl at the same time points. In survival experiments, mice were monitored up to 21 days.

LQGV and dexamethasone treatment

LQGV (GL Biochem, Shanghai, China) was dissolved in phosphate-buffered saline (PBS). In both CLP models, mice received an intravenous (i.v.) injection of LQGV (5 mg/kg BW) or PBS (as control) 5 mins before and 20 mins after the CLP procedure. The time-points of administration and dosage were based on previous studies [16, 29]. Furthermore, in the moderate CLP-model, the effect of i.v. injection of dexamethasone (25 mg/kg BW; Sigma Aldrich, Zwijndrecht, The Netherlands, dissolved in PBS) [30] administered 20 mins after the CLP procedure was examined.

Blood

Blood was obtained at various time-points after CLP by cardiac puncture in tubes containing EDTA (Greiner, Bio-one, Alphen aan den Rijn, The Netherlands). Blood was centrifuged (1500 rpm; 5 mins), and plasma was stored at -80ºC until assayed.

Peritoneal lavage

The peritoneal cavity was washed with 2 mL ice-cold PBS, followed by a second wash with 5 mL ice-cold PBS. Total cell counts were determined using a cell counter (Beckman Coulter BV, Woerden, The Netherlands). The first 2 mL peritoneal wash was centrifuged (1500 rpm; 10 mins), and supernatant was stored at -80ºC until assayed. Peritoneal cells from both washes were combined and resuspended (10^6 cells/mL). Cytospin preparations were stained (Diff-Quick, Medion Diagnostics, Dudingen, Switzerland) and cell differentials were determined by counting 300 cells per cytospin.

Bacterial culture of peritoneal lavage fluid and blood

Bacterial counts were determined in blood and the first 2 mL peritoneal wash by plating serial dilutions onto blood agar plates (Columbia blood agar, BD Pharmingen, Breda, The Netherlands). Plates were incubated for 24 hrs at 37ºC. Colony-forming units (CFU) numbers were determined and expressed as $\log_{10}$ CFU per ml peritoneal fluid or blood.

***In vitro* culture of peritoneal cells**

Peritoneal cells (10^6 cells/mL in RPMI 1640 containing 5% fetal calf serum and antibiotics) were cultured overnight and supernatants were collected for cytokine measurements.

Cytokine determination

Cytokines in plasma were determined using a cytometric bead array (BD Biosciences, San Diego, CA, USA) [29]. Supernatant from the first 2 mL peritoneal wash and peritoneal cell-culture supernatants were analyzed for IL-6, TNF-α, chemokine (C-X-C motif) ligand 1 (CXCL1) **,** and IL-10 by ELISA (R&D Systems Europe, Abingdon, UK). In experiments with dexamethasone, CCL2 and IL-6 levels were determined by ELISA (R&D Systems Europe).

Histologic analysis

Hematoxylin and eosin-stained lung sections, from 6 and 24 hrs after CLP, were analyzed under a light microscope (Axioskop 2 plus, Carl Zeiss, Sliedrecht, the Netherlands).

RNA isolation and real-time quantitative PCR

RNA was isolated (RNeasy Micro Kit; Qiagen, Hilden, Germany) from lung tissue obtained 6 hrs after CLP and reverse-transcribed into cDNA [29]. IL-6, TNF-α, CXCL1, IL-10, and E-selectin mRNA levels were determined by real-time quantitative PCR (RQ-PCR) using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA) and quantified by normalization against ABL [29].

Electrophoretic mobility shift assay

NF-κB activity was evaluated in nuclear extracts from lung tissue of mice sacrificed 6 hrs after CLP. Lung tissue was ground and resuspended in ice-cold lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and protease inhibitor cocktail (mini protease inhibitor, ethylenediaminetetraacetic acid (EDTA) free, Roche, Basel, Switzerland). After 20 mins incubation on ice followed by centrifugation, nuclei were lysed in ice-cold lysis buffer containing 20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and a protease inhibitor cocktail (mini protease inhibitor,

EDTA free, Roche). Electrophoretic mobility shift assay was performed with 10 μg of nuclear extracts as described previously using double-stranded γ-adenosine triphosphate (ATP)-^{32}P labeled oligonucleotide probes with specific recognition sequence for NF-κB or organic cation transporter-1 (OCT-1) [31].

Statistical analysis

Data are presented as the mean values ± standard error of mean. Statistical analysis was performed using SPSS version 15 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by log-rank survival analyses. Log-rank survival analyses was performed during both the acute phase of sepsis (until day 5) and the chronic phase of sepsis (until day 21) and corrected for population stratification of different experiments. All cytokine and mRNA levels were log-transformed to get normal distribution. The *t* test was used to compare the mean cytokine and mRNA levels for the two subgroups. When data were evaluated over time, a two-way analysis of variance was performed. Data shown in figures are geometric means with standard error of mean, or indicated otherwise. A p-value <0.05 was considered statistically significant.

RESULTS

LQGV treatment improves survival following moderate CLP-induced sepsis

In PBS-treated mice, CLP resulted in 20% survival during the acute phase of sepsis, after which no further mortality was observed during the chronic phase (Fig. 1). LQGV treatment significantly ($p<0.05$) improved survival up to 50% during the acute phase of sepsis (Fig. 1). In the chronic phase, mortality increased in the LQGV-treated group to a comparable level as in the PBS-treated group due to deaths occurring during the last week of follow-up (Fig. 1).

LQGV treatment moderately reduces the systemic inflammatory response following moderate CLP-induced sepsis

We next examined whether LQGV treatment affected the acute inflammatory response. Both in PBS and LQGV-treated mice, IL-6, TNF-α, CCL2, and IL-10 plasma levels rapidly increased after CLP, peaking at 24 hrs (Fig. 2). In the LQGV-treated mice, plasma levels were consistently lower, although this never reached significance, because analysis of variance showed that the profiles of the mean cytokine levels did not significantly differ between treatment groups (Fig. 2). Collectively, these data suggest that LQGV treatment reduces CLP-induced systemic cytokine levels and enhances the resolution of CLP-induced systemic inflammation, which is consistent with better survival.

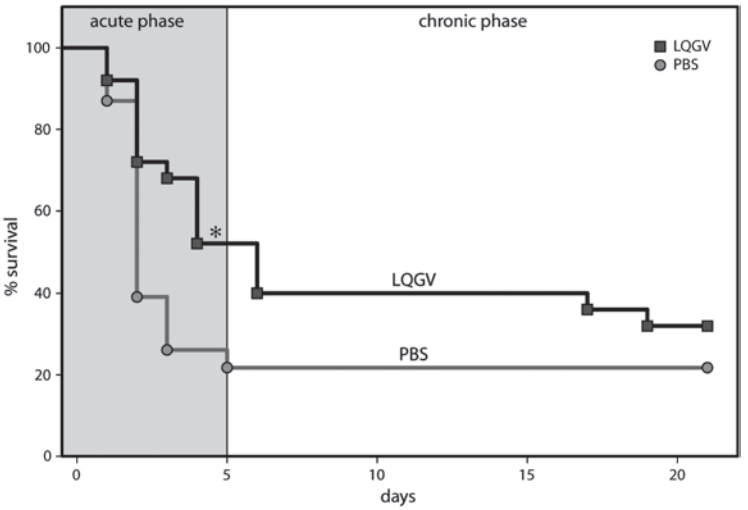

Figure 1. LQGV treatment improved survival.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP). Survival was monitored for 21 days. LQGV treatment improved survival during the acute phase of sepsis (≤ day 5). Presented results were obtained in three identical independent experiments. Log-rank survival analyses were performed as described in "Materials and methods". PBS, n = 25; LQGV, n = 23. *p<0.05.

LQGV treatment is associated with reduced peritoneal inflammation following moderate CLP-induced sepsis

To examine the effect of LQGV treatment on CLP-induced local inflammation, peritoneal lavage fluid from 6 and 24 hrs post CLP was analyzed for IL-6, TNF-α, CXCL1, and IL-10. This revealed a significant ($p<0.05$) increase of these cytokines, peaking at 6 hrs after CLP in both groups (Fig. 3). However, at 6 hrs after CLP, IL-6 and IL-10 levels were significantly ($p<0.05$) lower in LQGV-treated mice (Fig. 3), whereas TNF-α levels were not affected (Fig. 3*B*).

Next, we evaluated the effect of LQGV treatment on the production of IL-6, TNF-α, CXCL1, and IL-10 by cells obtained by peritoneal lavage at 6 hrs and 24 hrs following CLP. In both groups, peritoneal cell numbers were significantly ($p<0.05$) higher at 24 hrs post CLP than 6 hrs post CLP (Fig. 4*A*). LQGV treatment did not affect the total number of peritoneal cells (Fig. 4*A*), or the cellular composition, which mainly consisted of neutrophils at 6 and 24 hrs post CLP (Table 1). In general, peritoneal cells from LQGV-treated mice produced less cytokines, being significant ($p<0.05$) for IL-10 at 24 hrs after CLP (Fig. 4*E*). In PBS-treated mice, peritoneal cells obtained 24 hrs after CLP produced significantly ($p<0.05$) more IL-10 than cells obtained 6 hrs post CLP (Fig. 4*E*). No such

increase in IL-10 production was observed for peritoneal cells from LQGV-treated mice. In LQGV-treated mice, peritoneal cells obtained 24 hrs after CLP produced significantly ($p<0.05$) less CXCL1 than peritoneal cells obtained 6 hrs after CLP. This decrease did not occur in PBS-treated mice (Fig. 4*D*).

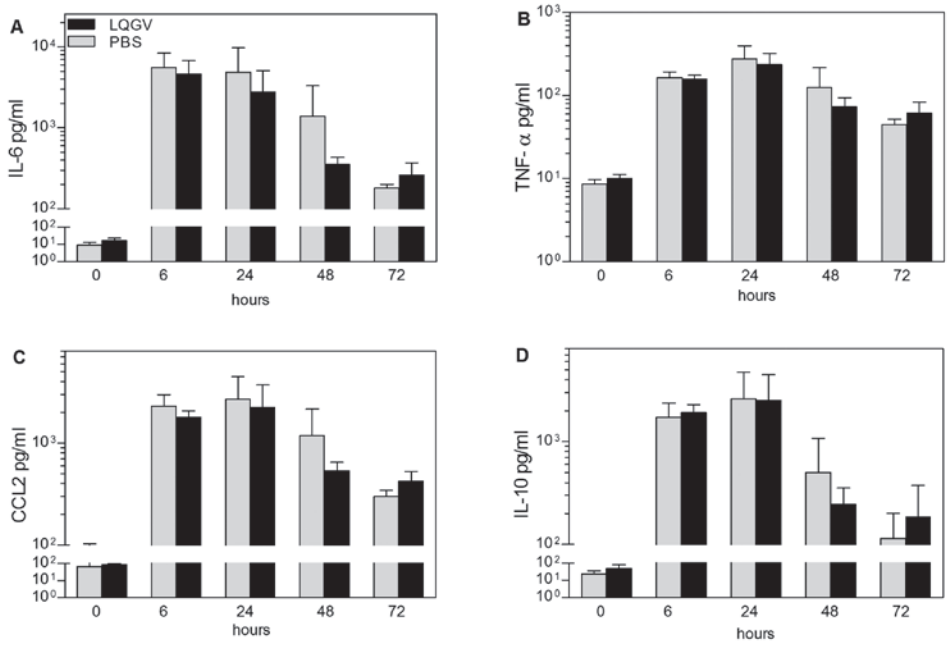

Figure 2. LQGV treatment moderately reduced plasma cytokine levels.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP). CLP induced an increase of plasma IL-6 (A), TNF-α (B), CCL2 (C), and IL-10 (D) in both PBS- and LQGV-treated mice with maximum levels for all cytokines at 24 hrs after CLP. LQGV treatment was associated with lower IL-6, TNF-α, CCL2, and IL-10 levels at most time points. n = 5 mice/group at 0 hrs post CLP. n = 17 mice/group at 6 hrs; n = 9 mice/group at 24 hrs. PBS, n = 7; LQGV, n = 10 at 48 hrs. PBS, n = 7; LQGV, n = 8 at 72 hrs.

Table 1. Effect of LQGV on peritoneal cell population

Time	6 hours post CLP		24 hours post CLP	
Treatment	PBS	LQGV	PBS	LQGV
Peritoneal lavage				
Granulocytes (%)[a]	83.2 [79.3 - 88.1]	84.0 [80.1 - 88.5]	75.7 [74.3 - 76.8]	74.3 [72.0 - 76.6]
Macrophages (%)[a]	16.8 [11.9 - 20.7]	16.0 [11.5 - 19.9]	24.3 [23.2 - 25.7]	25.7 [23.4 - 28.0]

[a] Mean [range]. n = 4 mice/ group.

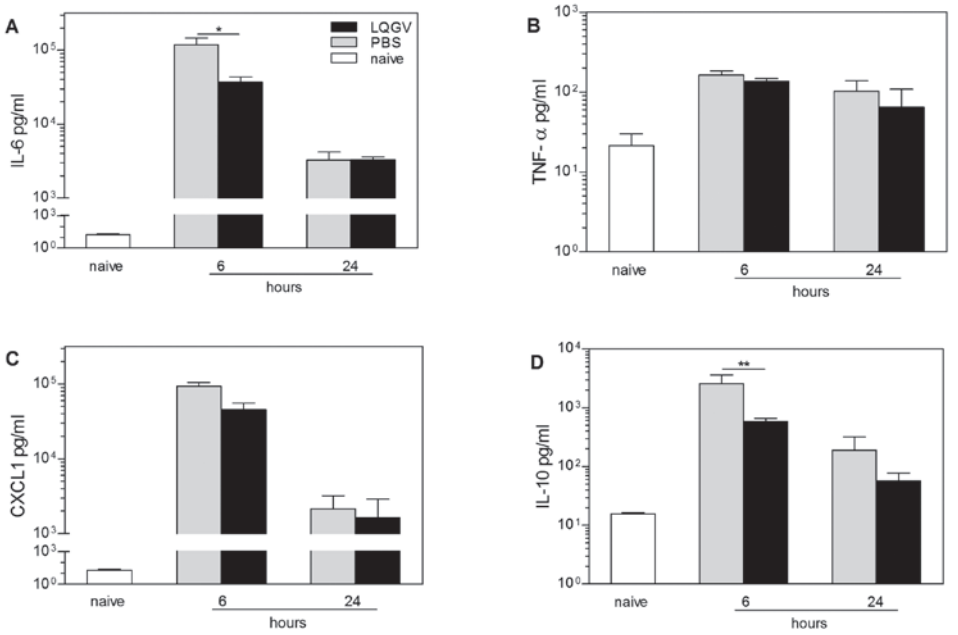

Figure 3. LQGV treatment reduced peritoneal cytokine levels.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP). Peritoneal washes were performed at 6 hrs and 24 hrs after CLP and cytokine levels were determined. CLP induced an increase of IL-6 (A), TNF-α (B), CXCL1 (C), and IL-10 (D) levels. LQGV treatment was associated with reduced cytokine levels as compared with PBS-treated mice. n = 4 mice/group. *p<0.05, **p<0.01.

LQGV treatment is associated with reduced pulmonary inflammation following moderate CLP-induced sepsis

Lung involvement is frequently observed during sepsis. Therefore, we examined whether LQGV treatment influenced pulmonary IL-6, TNF-α, CXCL1, IL-10, and E-selectin mRNA levels at 6 hrs after CLP. CLP resulted in a significant ($p<0.05$) increase in mRNA expression of all examined cytokines and the adhesion molecule E-selectin in both experimental groups (Fig. 5*A*). LQGV treatment significantly ($p<0.05$) reduced IL-6, CXCL1, and E-selectin mRNA levels in the lungs (Fig. 5*A*). Also, IL-10 mRNA levels were reduced upon LQGV treatment, although not statistically significant (Fig. 5*A*). LQGV treatment did not affect the increase of pulmonary TNF-α mRNA (Fig. 5*A*). The transcription factor NF-κB regulates the production of many cytokines and initial experiments demonstrated clear pulmonary NF-κB activity at 6 hrs following CLP (data not shown). Therefore, we determined whether LQGV treatment affected the pulmonary NF-κB activity at 6 hrs after CLP. Pulmonary NF-κB activation was lower in 75% of the LQGV-treated mice as compared with PBS-treated mice (Fig. 5*B*). Because LQGV

reduced pulmonary inflammation and NF-ϰB activation we next evaluated the effect of LQGV treatment on lung histology. Lungs obtained from PBS-treated mice at 6 hrs after CLP had thickened alveolar septa, mainly resulting from edema (Fig. 5*C*). LQGV treatment reduced these histopathologic alterations (Fig. 5*C*).

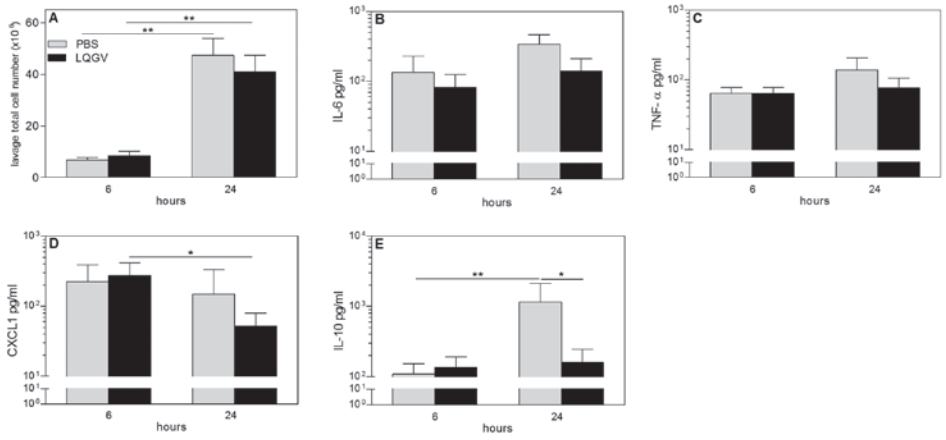

Figure 4. LQGV treatment reduced cytokine production by peritoneal cells.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP). Peritoneal wash were performed at 6 hrs and 24 hrs after CLP and cell numbers were determined. (A) CLP resulted in an increase of total cell numbers in both groups, which peaked at 24 hrs. LQGV treatment did not influence the number of peritoneal cells retrieved by peritoneal lavage as compared with PBS treatment. Peritoneal cells were cultured overnight and levels of IL-6 (B), TNF-α (C), CXCL1 (D), and IL-10 (E) were determined. LQGV treatment was associated with slightly reduced IL-6 (B), TNF-α (C), and CXCL1 (D) production levels at 6 or 24 hrs. CXCL1 production levels (D) decreased significantly between 6 and 24 hrs in the LQGV-treated mice, whereas this was not observed in the PBS-treated mice. IL-10 production (E) increased significantly between 6 and 24 hrs post CLP in PBS-treated mice, while this did not occur in the LQGV-treated mice. The IL-10 level (E) was significantly lower in the LQGV-treated mice at 24 hrs after CLP. n = 8 mice/group. *p<0.05, **p<0.01.

LQGV treatment does not affect bacterial load following moderate CLP-induced sepsis

To examine whether LQGV treatment interfered with bacterial dissemination in the peritoneal cavity and blood, we determined bacterial loads. During the first 72 hrs after CLP, the number of CFU in blood and peritoneal lavage fluid did not differ between both groups (Fig. 6).

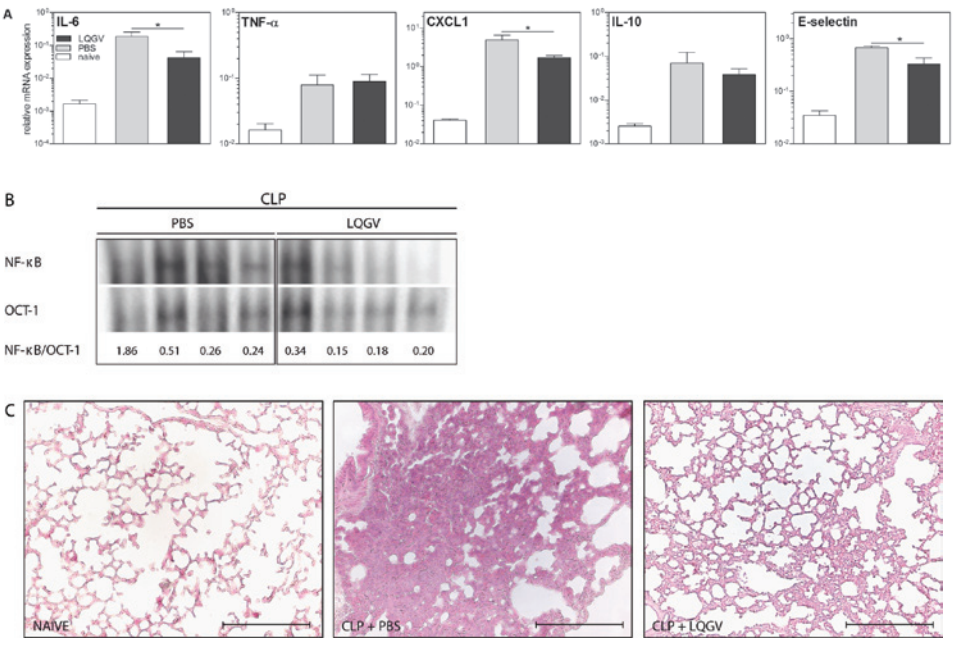

Figure 5. LQGV treatment reduced pulmonary inflammation.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP). Lung tissue was obtained at 6 hrs after CLP. (A) LQGV treatment was associated with reduced IL-6, CXCL1, and E-selectin mRNA expression levels. (B) Pulmonary NF-κB activity was reduced in nuclear extracts of LQGV treated mice. (C) Representative histological sections showed that LQGV treatment was associated with reduced alveolar septal thickening resulting from diminished edema. n = 4 mice/group, *p<0.05. Bars, 50 μm.

Dexamethasone treatment reduces systemic, peritoneal, and pulmonary inflammation without improvement of survival following moderate CLP-induced sepsis

To compare the effect of LQGV with a commonly used anti-inflammatory agent, we investigated the effect of dexamethasone on CLP-induced mortality and inflammation. Dexamethasone had no effect on survival in the moderate CLP-model when compared with PBS-treated mice (Fig. 7*A*). Dexamethasone treatment did however significantly ($p<0.05$) reduce plasma IL-6 and CCL2 levels as well as peritoneal IL-6 and CCL2 levels at 6 hrs after CLP (Fig. 7*B*). Dexamethasone treatment also significantly ($p<0.05$) reduced pulmonary IL-6 mRNA levels, while TNF-α remained unaffected (Fig. 7*B*).

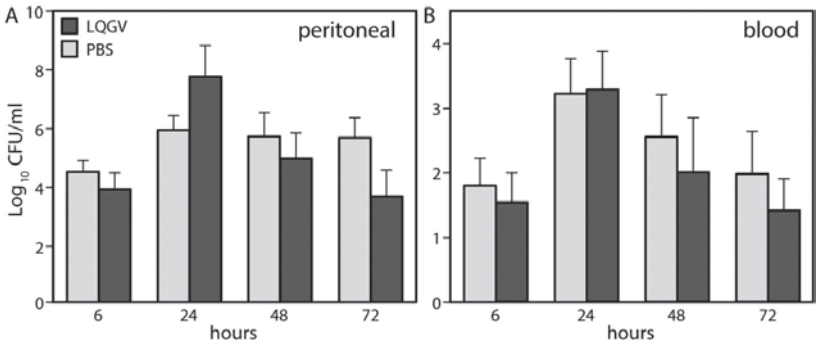

Figure 6. LQGV treatment did not alter bacterial load.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after low-grade cecal ligation and puncture (CLP) and bacterial load was determined in blood and peritoneal fluid obtained at different time points after CLP. LQGV treatment did not affect bacterial load in peritoneal fluid (A) and blood (B). Similar results were obtained in two identical independent experiments. n = 8 – 10 mice/group.

LQGV as addition to standard sepsis treatment improves survival following moderate CLP-induced sepsis

To determine a potential role of LQGV in the standard treatment of sepsis we evaluated the effect of LQGV in combination with fluid resuscitation and antibiotics, in a severe CLP-induced sepsis. In control mice that only received fluid resuscitation 100% mortality was observed (Fig. 8). In this severe CLP-model treatment with LQGV plus postoperative fluid resuscitation alone did not improve survival (Fig. 8). In control mice, treated with PBS followed by postoperative treatment with fluid resuscitation and antibiotics, survival increased to 30% ($p<0.01$) after 21 days (Fig. 8). However, when perioperative LQGV treatment was combined with postoperative fluid resuscitation and antibiotics, survival increased to 70% ($p<0.05$) compared with fluid resuscitation and antibiotics alone (Fig. 8).

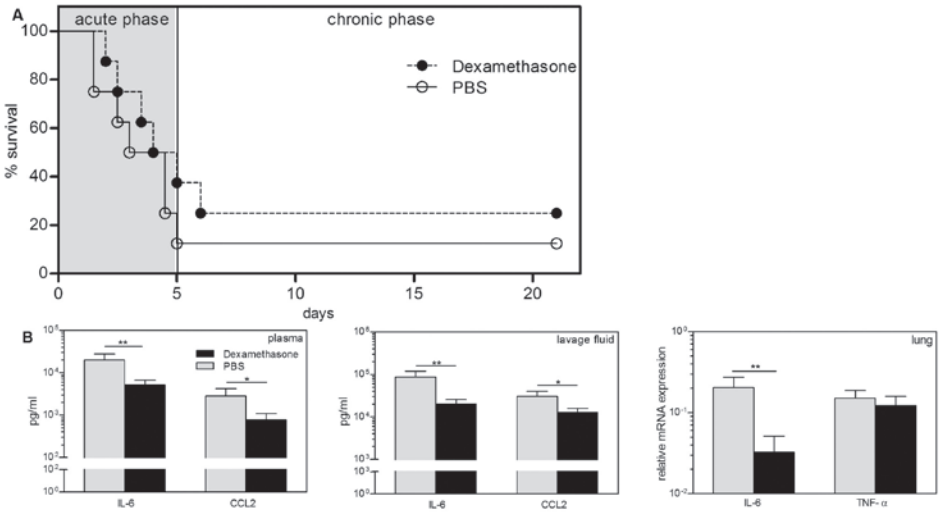

Figure 7. Dexamethasone treatment did not alter mortality and inflammation.
Dexamethasone was administered (2.5 mg/kg body weight) intravenously 20 mins after low-grade cecal ligation and puncture (CLP). (A) Survival was monitored for 21 days. Dexamethasone treatment did not alter mortality but did alter inflammation during the acute and chronic phases of sepsis. n = 8 mice/group. (B) IL-6 and CCL2 levels were determined in plasma and peritoneal lavage at 6 hrs after CLP. Relative IL-6 and TNF-α mRNA expression levels were determined in lung tissue obtained at 6 hrs post CLP. Dexamethasone reduced the IL-6 and CCL2 plasma levels as well as the peritoneal IL-6 and CCL2 levels and the IL-6 mRNA expression level in the lung. n = 6 mice/group. *p<0.05, **p<0.01.

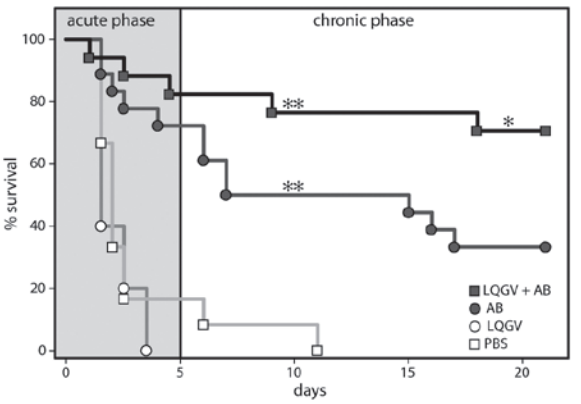

Figure 8. LQGV treatment in combination with standard care improved survival.
LQGV was administered (5 mg/kg body weight) intravenously 5 mins before and 20 mins after high-grade cecal ligation and puncture (CLP), followed by 5-day treatment with fluid resuscitation and antibiotics or fluid resuscitation alone. Survival was monitored for 21 days. LQGV treatment in combination with fluid resuscitation alone [LQGV] did not affect mortality. Phosphate-buffered saline (PBS) treatment followed by postoperative treatment with fluid resuscitation and antibiotics [AB] was associated with improved survival. LQGV treatment in combination with fluid resuscitation and antibiotics [LQGV + AB] was associated with improved survival as compared with PBS-treatment in combination with fluid resuscitation and antibiotics. Presented results were obtained in three identical independent experiments. Log-rank survival analyses were performed as described in "Materials and methods". PBS, n = 12; LQGV, n = 6; AB, n = 18, LQGV + AB, n = 17. *p<0.05, **p<0.01.

DISCUSSION

Sepsis-related hospitalization and mortality still increase [3]. Although inflammation is a well-recognized component contributing to the pathology of sepsis and septic shock, most anti-inflammatory treatment strategies applied in sepsis gained disappointing results. Therefore, novel therapeutic interventions are required. CLP induces a polymicrobial peritoneal infection in the presence of necrotic gut tissue in the abdominal cavity, and is characterized by bacteremia, SIRS, septic shock, multiple organ dysfunction syndrome, and eventually death and is considered to reflect the pathophysiology of human sepsis [25, 27]. In line with other studies [30, 32-34], we observed that most mice typically die from CLP-induced sepsis during the early acute hyperinflammatory septic phase (< 5 days after CLP). Here, we demonstrate for the first time that treatment with the β-hCG-related tetrapeptide LQGV significantly improves survival during the early hyperinflammatory phase of CLP-induced polymicrobial sepsis in mice. This was associated with a significant reduction of inflammation in the peritoneal cavity and lungs, whereas only a modest reduction of systemic inflammation was observed. Furthermore, we demonstrate that LQGV has additive value to standard sepsis care with fluid resuscitation and antibiotics.

In the present study, LQGV improved survival in a moderate CLP-induced polymicrobial sepsis model. This survival benefit was associated with only a modest reduction of IL-6, TNF-α, IL-10, and CCL2 plasma levels. However, LQGV treatment was associated with enhanced resolution of plasma cytokine levels, suggesting decreased cellular activation upon LQGV treatment. Contrary to the systemic inflammatory response, LQGV treatment downregulated inflammation in the peritoneal cavity as reflected by significantly reduced IL-6 and IL-10 levels. This appeared not to be related to differences in total cell numbers recruited to the peritoneal cavity nor the cellular composition of the infiltrate. A consistent trend towards less cytokine production by these cells from LQGV-treated animals was found, which was significant for IL-10. These data suggest that although these cells are recruited equally effective to the peritoneal cavity, their activity is altered by LQGV treatment. We cannot exclude that cells other than the recruited inflammatory cells contributed to peritoneal cytokine levels as well. Endothelial cells and mesothelial cells are well-recognized producers of cytokines upon activation with LPS [35-37] and may therefore have been targeted by LQGV as well. CLP-induced sepsis is associated with NF-κB-driven pulmonary inflammation and damage [38-42]. LQGV treatment resulted in reduced pulmonary NF-κB activation in combination with a significant reduction of IL-6, CXCL1, and E-selectin mRNA levels and reduced histological pulmonary damage. LQGV treatment exerted a long-term beneficial effect on survival. However, small compounds such as LQGV, which has a molecular weight of 415 Dalton, are rapidly removed as a result of renal clearance [43]. This suggests that the protective effect of LQGV must be due to reduction of the early SIRS-response,

which is supported by our observation that LQGV reduces early immune activation after *Listeria monocytogenes* infection [18]. This implies that early interference with the SIRS-response can result in long-term beneficial effects on survival.

TLR activation by bacterial antigens or molecules released upon tissue damage activates transcription factors such as NF-κB, which subsequently drive the production of cytokines. Therefore, TLR activation is considered a key event in the initiation of the inflammatory response during sepsis and tissue damage [44-47]. Our current study demonstrates that LQGV treatment reduces systemic and peritoneal cytokine responses as well as pulmonary NF-κB activation and cytokine and adhesion molecule production after CLP. Positive correlations between the intensity of the cytokine response and bacterial load have been described elsewhere [18, 48]. Here, we observed no effect of LQGV on the bacterial load in blood and peritoneal cavity after CLP. Therefore, we consider it unlikely that the anti-inflammatory effect of LQGV following CLP is related to bacterial load. Previously, we found that LQGV administration to mice impaired the capability of splenocytes to produce IL-6 and TNF-α in response to LPS or *Listeria monocytogenes* antigens, indicating that LQGV interferes with TLR-driven immune activation [18]. Recent data from our laboratory demonstrate that the effect of LQGV involves the induction of a secondary anti-inflammatory mediator that activates glucocorticoid receptor signaling [49]. Overall, these data suggest that LQGV, at least partly, exerts its effect through activation of the glucocorticoid receptor and subsequent inhibition of TLR driven gene activation.

Dexamethasone is a well known anti-inflammatory agent that efficiently ameliorates the systemic inflammatory response following LPS injection [50, 51]. Here, we found that dexamethasone significantly, and more effectively than LQGV, reduced the CLP-induced inflammatory response. However, dexamethasone treatment was not associated with survival improvement, which is in line with previous observations [50]. Therefore, our results suggest that extensive downregulation of the inflammatory response during polymicrobial sepsis (as established by dexamethasone) does not improve survival, whereas a relatively modest downregulation (as established by the LQGV treatment used in this study) does improve survival. This notion is supported by studies demonstrating that moderate IL-6 inhibition by neutralizing antibodies improves survival following CLP, whereas this survival benefit was not observed when a higher dosage of IL-6 neutralizing antibody was used [52] and point at an important physiological role of the inflammatory response following CLP. Overall, these data suggest that moderate downregulation of the inflammatory response, as observed after LQGV treatment, is a prerequisite to improve survival after CLP.

In the severe CLP-model, treatment with LQGV alone did not result in survival improvement. Bacterial dissemination can be expected to be higher in the severe model

as compared with the moderate model. The pathology and mortality in the severe CLP-model are likely the result of a higher extent of bacterial dissemination and growth as well as a more severe inflammatory reaction. Inhibition of the inflammatory reaction by LQGV alone is apparently not sufficient to improve survival in this model. This is probably the result of the inhibition of the antibacterial response as part of the inhibited inflammatory response [17]. However, at present, fluid resuscitation and antibiotics are the cornerstones of sepsis treatment, and in line with previous studies [8, 53], we found that this treatment combination improved long-term survival following CLP. Remarkably, the addition of LQGV to fluid resuscitation and antibiotics further improved long term survival as compared with fluid administration and antibiotics alone. This suggests that modest downregulation of the inflammatory response in combination with standard sepsis care might be a beneficial therapeutic approach.

CONCLUSIONS

Our data demonstrate that the β-hCG-related tetrapeptide LQGV is able to improve survival during the acute hyperinflammatory phase following CLP-induced polymicrobial sepsis in mice. This beneficial effect is likely the result of the moderate immunosuppressive effect of LQGV. Importantly, the immunosuppressive effect of LQGV is of additive value to antibiotics and fluid administration in improving survival following CLP-induced severe sepsis in mice. Therefore, LQGV might be a useful addition to the standard treatment of sepsis.

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III

The β-human chorionic gonadotropin-related peptide LQGV exerts anti-inflammatory effects through activation of the adrenal gland and glucocorticoid receptor in C57BL/6 mice

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ABSTRACT

The systemic inflammatory response syndrome (SIRS) is a complex host response to a variety of clinical insults, generally leading to severe pathology. The human chorionic gonadotrophin β-chain related tetrapeptide LQGV reduces hemorrhagic and lipopolysaccharide (LPS)-induced SIRS, but its mechanisms of action are not yet fully understood. Through the combination of *in vivo, in vitro* and *ex vivo* approaches we demonstrate that LQGV actively stimulates corticosterone production in mice and thereby suppresses *in vivo* TLR-4 directed inflammation upon LPS administration. Blocking *in vivo* glucocorticosteroid receptor signaling reduced the prosurvival effect of LQGV. Also, upon multiple TLR activation by heat-killed *Listeria monocytogenes*, splenocytes from LQGV-treated mice produced significantly less TNF-α and IL-6, which was absent after *in vivo* blockage of the glucocorticosteroid receptor. Using adrenal gland and adrenal cell line cultures, we show that LQGV stimulates corticosterone production. Moreover, by using specific pharmacological inhibitors of the adrenocorticotropic hormone (ACTH) receptor and cyclic AMP signaling, we demonstrate that LQGV stimulates the ACTH-receptor. These data show that the β-hCG-related tetrapeptide LQGV stimulates adrenal glucocorticosteroid production through activation of the ACTH receptor with consequent glucocorticoid receptor activation and immunosuppression in C57BL/6 mice.

INTRODUCTION

Pregnancy is characterized by specific control of the maternal immune system, which is necessary to prevent rejection of the fetal allograft [1]. Consequently during pregnancy, symptoms of autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, decline, whereas susceptibility to intracellular pathogens, such as *Listeria monocytogenes*, *Toxoplasma goundii, Leishmania major,* and *Plasmodium species,* increases [1-4]. The exact mechanisms that modulate the immune system during pregnancy are largely unknown, but most likely specific hormonal changes are involved [5].

Human chorionic gonadotrophin (hCG) is a human pregnancy hormone that, besides its endocrine functions, also influences the functionality of the immune system [6-9]. In addition to intact hCG, several other isoforms of hCG exist during pregnancy [10]. It is well recognized that loop 2 of β-hCG is nicked by leukocyte elastase-like proteases, generating hCG β-core and several nicked β-hCG forms [11, 12]. These molecules can be detected in serum and urine especially in the late second trimester and the third trimester of pregnancy [10]. Benner and Khan [9, 13, 14] postulated that, besides the generation of hCG β-core and nicked β-hCG, small breakdown products of three to seven amino acids long are generated from loop 2 of β-hCG and that such small peptides can exert immunomodulatory effects.

The systemic inflammatory response syndrome (SIRS) is a complex host response that may be inflicted by a variety of insults, such as severe trauma-hemorrhage, ischemia-reperfusion injury, pancreatitis, sepsis, and septic-shock [15]. SIRS is characterized by excessive production of proinflammatory mediators, such as IL-1, IL-6, TNF-α, CCL2, CXCL1, and CXCL2 [16, 17]. High levels of these proinflammatory mediators contribute to severe organ damage and multiple organ dysfunction syndrome [18]. Recently, we demonstrated that the synthetic β-hCG-related tetrapeptide LQGV reduces hemorrhagic shock-associated inflammation and liver damage, prevents LPS-induced mortality in mice, and ameliorates the early chemokine response following *Listeria monocytogenes* infection [19-21]. However, the regulatory mechanism by which LQGV exerts anti-inflammatory effects remains elusive.

Endogenous glucocorticosteroid production and function are crucial in the control and resolution of inflammation [22-24]. In line with this, administration of synthetic glucocorticosteroids is commonly used in clinical settings to control inflammation [22]. Also, specific activation of endogenous glucocorticosteroid production by the adrenal glands has been postulated as a way to control an inflammatory event [25]. An increase in circulating cortisol (of which the rodent analogue is corticosterone) is observed during the late second trimester and the third trimester of pregnancy, the phase wherein the highest degree of proteolytic cleavage of β-hCG occurs. Therefore, we examined whether the β-hCG-related tetrapeptide LQGV exerts anti-inflammatory effects through

the stimulation of glucocorticosteroid receptor (GR) signaling and glucocorticosteroid production.

By using combined *in vivo, in vitro,* and *ex vivo* approaches we demonstrate that LQGV exerts anti-inflammatory effects through GR activation via stimulation of corticosterone production by the adrenal glands. This LQGV-induced corticosterone production is mediated through adrenocorticotropic hormone (ACTH) receptor activation and subsequent cAMP-signaling.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 male mice (Harlan, Horst, The Netherlands), aged 8–12 weeks, were used in the experiments, which were approved by the local animal experiments committee.

LPS-induced shock

Mice were injected intraperitoneally (i.p.) either with 200 μl LQGV (50 mg/kg body weight (BW) dissolved in PBS, purity 99.2%; GL Biochem, Shanghai, China) or PBS (as control), directly followed by a second i.p. injection with 500 μl of the GR antagonist mifepristone (RU38486; 10 mg/kg BW, dissolved in dimethylsulfoxide; Sigma-Aldrich, Zwijndrecht, The Netherlands) or vehicle. The next day, mice were challenged with an lipopolysaccharide (LPS) injection (30 mg/kg BW of *Escherichia coli* strain 0111:B4; Sigma-Aldrich), and survival was scored every 12 h for 3 days.

Adrenalectomy

Adrenalectomy (ADX) was performed as described [26], after which mice acclimatized for 7 days before start of the experiment. All mice received postoperatively 0.9% NaCl solution as drinking water.

Ex vivo adrenal gland culture

Complete adrenal glands were isolated and cultured as described [27, 28]. Briefly, 300 μl RMPI 1460 medium containing antibiotics, 5% FBS, and LQGV *(*50, 5, or 0.5 μg/ml) or PBS was added to the adrenal gland culture for 6 h. The cAMP-blocker H-89 (10 μM; Enzo Life Sciences, Plymouth Meeting, PA) and the ACTH-receptor antagonist corticotropin-inhibiting peptide (CIP) (10^{-6} mM, 10^{-7} mM, 10^{-8} mM, or 10^{-9} mM; Phoenix Pharmaceutics, Phoenix, AZ) were added 5 mins before LQGV stimulation. Culture supernatants and adrenal glands were collected and stored at -80 ºC until assayed.

In vitro stimulation of LHR-Y1-cells

Murine adrenal cells (LHR-Y1; kindly provided by Dr. Bill Moyle, University of Medicine and Dentistry of New Jersey, Newark, NJ) were cultured as described [29]. The cAMP-blocker H-89 (10 μM) and the ACTH-receptor antagonist CIP (10^{-6} mM) were added 5 mins before addition of recombinant hCG (300 U/ml Sigma-Aldrich) or LQGV (5 or 50 μg/ml). Cells and supernatants were collected and stored at -80 ºC until assayed.

In vitro stimulation of splenocytes and cytokine analysis

ADX and non-ADX mice were i.p. injected with 200 µL LQGV (50 mg/kg BW) or PBS. Thirty mins later, 500 μl of the GR antagonist mifepristone or vehicle was administrated i.p. Mice were euthanized 18 hours later; blood was collected by cardiac puncture, and splenocytes were isolated and stimulated as described [21]. Plasma was obtained by centrifugation (3000 rpm, 10 min), immediately frozen, and stored at -80°C until assayed. In addition, splenocytes (10^6 cells/ml) from untreated mice (mice that received neither LQGV nor PBS) were isolated and cultured overnight in the presence of plasma from either LQGV- or PBS -treated mice or with culture media from either LQGV- or PBS-stimulated adrenal glands, with or without $7Log_{10}$ heat-killed Listeria monocytogenes (HKLM) [21], and with or without mifepristone (0.3 μg/ml). Culture supernatants were collected, and TNF-α and IL-6 levels were determined by ELISA (R&D Systems Europe, Abingdon, U.K.).

Corticosterone quantification

Corticosterone levels in plasma (obtained between 3:00 and 4:00 pm) and culture supernatant were determined by ELISA (IBL, Hamburg, Germany).

Evaluation of mRNA expression levels by real-time quantitative (RQ)-PCR

RNA was isolated using the GenElute RNA kit (Sigma-Aldrich). *CYP11B1* (encoding 11β-hydroxylase) gene expression levels were determined by RQ-PCR using an Applied Biosystems 7900 PCR machine (Applied Biosystems, Foster City, CA). The expression levels were quantified by normalization against the mRNA levels of the household gene *ABL* [30]. Primers and probes used are available upon request.

Statistical analysis

Statistical analysis was performed using SPSS version 15 software (SPSS Inc., Chicago, IL). Intergroup differences were analyzed using Mann-Whitney *U*-test, and $p<0.05$ was considered statistically significant. For survival analysis, a Kaplan-Meier analysis followed by a log-rank test was performed. Correlation coefficients were determined with Pearson's correlation analyses with significance set at $p<0.05$.

RESULTS

LQGV reduces LPS-induced mortality via GR activation

Previously, it was demonstrated that LQGV protected BALB/c mice against LPS-induced mortality [19]. In this study, we found that LQGV administration 24 h prior to LPS injection significantly ($p<0.05$) improved the 3-day survival from 20% to 60% in C57BL/6 mice (Fig. 1). This pro-survival effect of LQGV was completely reversed when mice received the GR antagonist mifepristone in combination with LQGV (Fig. 1). These data indicate that the pro-survival effect of LQGV in this model is dependent on GR activation.

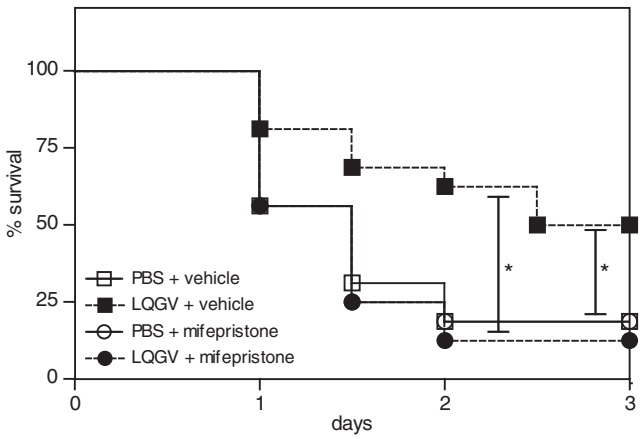

Figure 1. Mifepristone injection abolishes the LQGV prosurvival effect.
Mice were injected i.p. with either 200 μl LQGV (50 mg/kg BW) or PBS, directly followed by a second i.p. injection with 500 μl mifepristone (10 mg/kg BW) or vehicle. The next day, mice were challenged with an LPS injection. Survival was scored every 12 h. Data depicted are from nineteen mice per group. *, $p<0.05$ for LQGV + vehicle treatment compared with LQGV + mifepristone treatment, or PBS + vehicle.

In vivo LQGV treatment reduces the *in vitro* responsiveness of splenocytes to *L. monocytogenes* antigens

Next, we examined how LQGV treatment activated the GR in immune cells. Splenocytes from untreated mice were *in vitro*-stimulated with HKLM (which activates multiple pattern-recognition receptors and evokes a stronger inflammatory response than LPS [21]), in the presence of LQGV (50 μg/ml). HKLM stimulation induced TNF-α and IL-6 production by splenocytes, which was not affected by addition of LQGV to the cultures (Fig. 2*A*, *B*). In contrast, splenocytes obtained from mice 18 h after LQGV (50 mg/kg BW) administration produced significantly less TNF-α ($p<0.01$) and IL-6 ($p<0.05$) upon HKLM stimulation than that of splenocytes from PBS-treated mice (Fig. 2*C*, *D*). This suppressive effect of LQGV was completely reversed when LQGV-treated mice

also received mifepristone (Fig. 2*C*, *D*). These data demonstrate that LQGV does not directly stimulate GR activity in splenocytes and does not directly interfere with cytokine production and secretion but suggests that the immunosuppressive effect of LQGV is established through an *in vivo*-released secondary mediator that stimulates the GR.

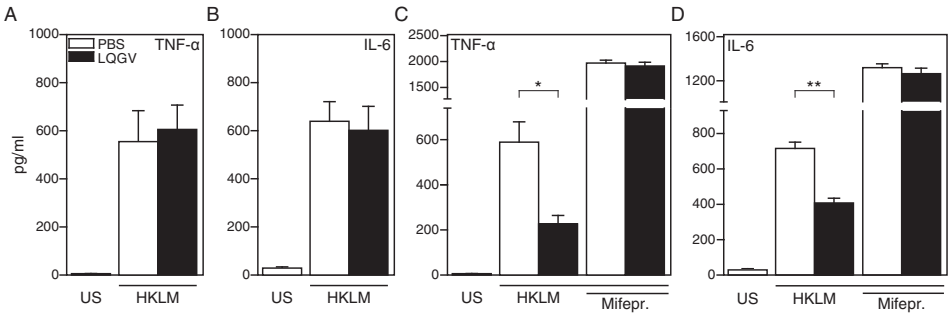

Figure 2. ***In vivo*** **LQGV administration reduces HKLM-induced TNF-α and IL-6 production by splenocytes.**

Splenocytes from untreated mice were stimulated overnight with $7\log_{10}$ HKLM in the presence of 50 μg/ml LQGV, after which TNF-α (*A*) and IL-6 (*B*) levels in culture supernatants were determined by ELISA. In other experiments, mice were treated with LQGV (50 mg/kg BW) or PBS, in combination with or without mifepristone, and 18 h later splenocytes were isolated and cultured overnight with $7\log_{10}$ HKLM, after which TNF-α (*C*) and IL-6 (*D*) levels in the culture supernatants were determined by ELISA. Data depicted are from five mice per group. *, $p<0.05$; **, $p<0.01$. US, unstimulated.

LQGV induces the systemic release of an immunosuppressive mediator

Next, we determined whether LQGV induced the systemic release of an immunosuppressive mediator that acts through the GR. Plasma was obtained from mice 18 h after PBS or LQGV injection. Plasma from both LQGV- and PBS-treated mice inhibited HKLM-induced TNF-α production by naïve splenocytes in a dose-dependent manner (Fig. 3*A*). When a total volume of 30% (v/v) plasma was added to the culture, plasma from LQGV-treated mice reduced HKLM-induced TNF-α ($p<0.01$) and IL-6 ($p<0.05$) production to significantly lower levels than observed with plasma from PBS-treated mice (Fig. 3*B*, *C*). Addition of mifepristone to the cultures completely reversed the immunosuppressive effect of plasma from LQGV-treated mice (Fig. 3*B*, *C*). Collectively, these data suggest that the reduced capacity of splenocytes to respond to HKLM antigens, as displayed after *in vivo* LQGV administration, is established through the systemic release of a GR activating factor.

The immunosuppressive effect of LQGV is abolished by adrenalectomy

To examine the role of the adrenal glands in LQGV-induced immunosuppression, splenocytes from both LQGV- and PBS-treated ADX mice were stimulated with HKLM. Removal of the adrenal glands completely abolished the immunosuppressive effect of LQGV (Fig. 4*A*), as reflected by the TNF-α and IL-6 levels detected in

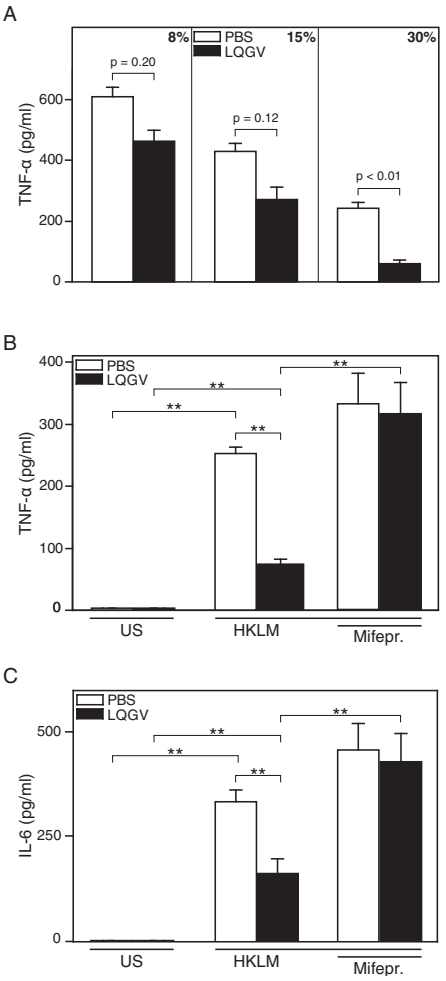

Figure 3. LQGV induces the release of an immunosuppressive mediator in plasma.
Mice were treated with LQGV (50 mg/kg BW) or PBS, and 18 h later plasma was obtained and used in increasing amounts (v/v) in overnight cultures of splenocytes from untreated mice stimulated with $7\log_{10}$ HKLM. Thereafter, TNF-α levels in culture supernatants were determined by ELISA. Data depicted are from 4 mice per group (*A*). In other experiments, mice were treated with LQGV or PBS, and 18 h later plasma was isolated, and 30% (v/v) was used in an overnight stimulation of splenocytes from untreated mice with $7\log_{10}$ HKLM in combination with or without mifepristone. TNF-α (*B*) and IL-6 (*C*) levels in culture supernatants were determined by ELISA. Data depicted are from seven mice per group. *, $p<0.05$; **, $p<0.01$. US, unstimulated.

culture supernatants. Also, plasma from LQGV-treated ADX mice did not reduce the HKLM-induced TNF-α and IL-6 production by splenocytes from untreated mice (Fig. 4*B*). These data demonstrate that the adrenal glands are involved in establishing the immunosuppressive effect of LQGV.

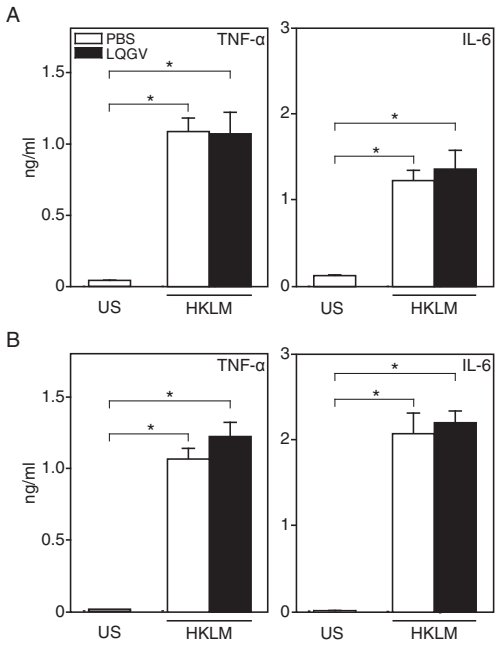

Figure 4. Adrenalectomy abolishes the immunosuppressive effect of LQGV.
ADX mice were treated with LQGV (50 mg/kg BW) or PBS and 18 h later splenocytes were obtained and cultured overnight with $7\log_{10}$ HKLM, and TNF-α and IL-6 levels in culture supernatants were determined by ELISA (*A*). In other experiments, ADX mice were treated with LQGV or PBS, and 18 h later plasma was isolated and 30% (v/v) was used in an overnight stimulation of splenocytes from untreated non-ADX mice with $7\log_{10}$ HKLM, and TNF-α and IL-6 levels in culture supernatants were determined by ELISA (*B*). Data depicted are from five mice per group. *, $p<0.05$. US, unstimulated.

LQGV stimulates adrenal corticosterone production

The previous experiments suggest that LQGV induces the release of an adrenal-derived immunosuppressive mediator that exerts its action through the GR. Therefore, we determined plasma corticosterone levels at different time points after LQGV administration. Corticosterone plasma levels were significantly higher at 1 h (0.265 μM/l vs. 0.074 μM/l; $p<0.05$), at 6 h (0.664 μM/l vs. 0.055 μM/l; $p<0.05$), and at 24 h (0.406 μM/l vs. 0.050 μM/l; $p<0.05$) in LQGV-treated mice than in PBS-treated mice (Fig. 5*A*). *Ex vivo* stimulation of complete adrenal glands revealed that LQGV dose-dependently and significantly ($p<0.05$) induced corticosterone secretion (Fig. 5*B*). Recombinant hCG and the irrelevant tetrapeptide EPPE did not stimulate adrenal corticosterone secretion (data not shown). Next, we examined whether the adrenal gland culture media affected HKLM-induced TNF-α and IL-6 production by splenocytes. Splenocytes were isolated from untreated mice and stimulated with HKLM in the presence of culture media from adrenal glands stimulated with either LQGV or PBS. Culture media obtained from

LQGV-stimulated adrenal glands inhibited HKLM-induced TNF-α production in a dose-dependent manner (Fig. 5*C*). When a total of 8% (v/v) adrenal gland culture medium was added to the culture, media from LQGV-stimulated adrenal glands reduced HKLM-induced TNF-α ($p<0.05$) (Fig. 5*D*) and IL-6 ($p<0.05$) (Fig. 5*E*) production to significantly lower levels than observed with media from PBS-stimulated adrenal glands. This effect was completely abolished when mifepristone was co-added to the cultures (Fig.5*D, E*). These data suggest that LQGV activates the adrenal glands to synthesize and secrete corticosterone, which reduces the *in vitro* responsiveness of splenocytes to Listeria antigens.

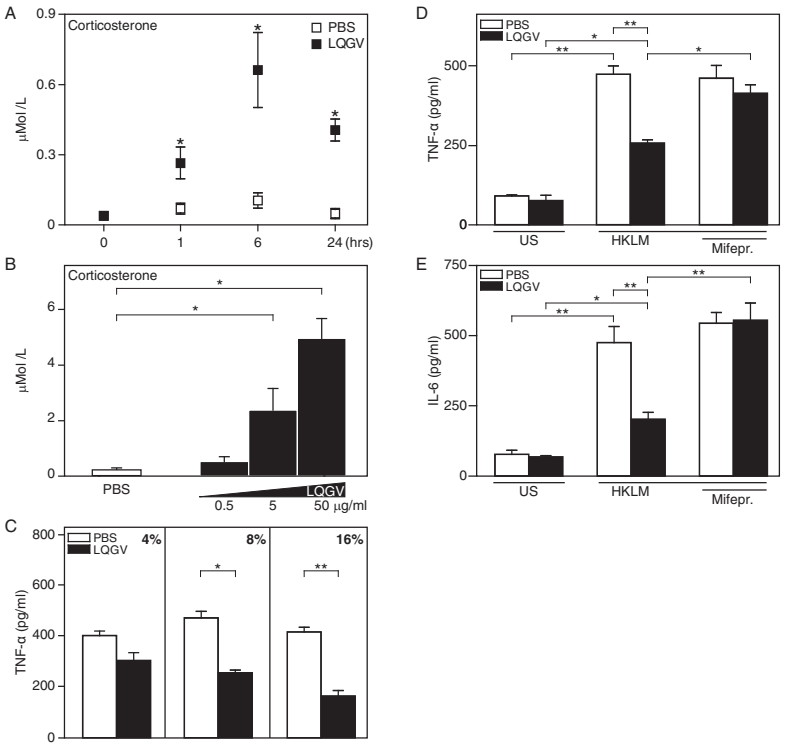

Figure 5. LQGV activates the adrenal glands to secrete corticosterone.
Mice were treated with LQGV (50 mg/kg BW) or PBS, and plasma corticosterone levels were determined at 0, 1, 6, and 24 hours after treatment. Plasma corticosterone levels in LQGV-treated mice increased and peaked at 6 h, after which the levels slowly declined (*A*). Data depicted are from 8 mice per time point. Adrenal glands from naïve mice were *ex vivo*-stimulated with either 0.5, 5, or 50 μg/ml LQGV, and corticosterone levels in supernatant were determined (*B*). Culture supernatant collected from *ex vivo* adrenal gland stimulation was used in increasing amounts (v/v) in overnight stimulation of splenocytes from untreated mice with $7\log_{10}$ HKLM. Thereafter, TNF-α levels in culture supernatants were determined by ELISA. Data depicted are from four mice per group (*C*). Culture supernatant (8%, v/v) was added to an overnight stimulation of splenocytes from untreated mice with $7\log_{10}$ HKLM with or without co-activation of mifepristone, and TNF-α (*D*) and IL-6 (*E*) concentrations in culture supernatants were measured by ELISA. Data depicted are from 7 mice per group. *, $p<0.05$; **, $p<0.01$. US, unstimulated.

LQGV activates the adrenal ACTH-receptor

Next, we determined the mRNA expression levels of 11β-hydroxylase, the enzyme that converts deoxycorticosterone to corticosterone. LQGV dose-dependently enhanced the mRNA expression levels of 11β-hydroxylase (Fig. 6*A*), which correlated positively and significantly (r = 0.426; p<0.05) with the corticosterone levels detected in the culture media (Fig. 6*B*). The cAMP-blocker H-89 and ACTH-receptor antagonist CIP completely abolished the LQGV-induced corticosterone release (Fig. 6*C*). Moreover, CIP inhibited the LQGV-induced increase of 11β-hydroxylase mRNA expression in a dose dependent manner (Fig. 6*D*). H-89 and CIP alone did not affect the basal adrenal 11β-hydroxylase mRNA expression levels (data not shown). These data suggest that LQGV activates the adrenal glands to actively synthesize and secrete corticosterone through an ACTH-receptor activated cAMP-signaling cascade.

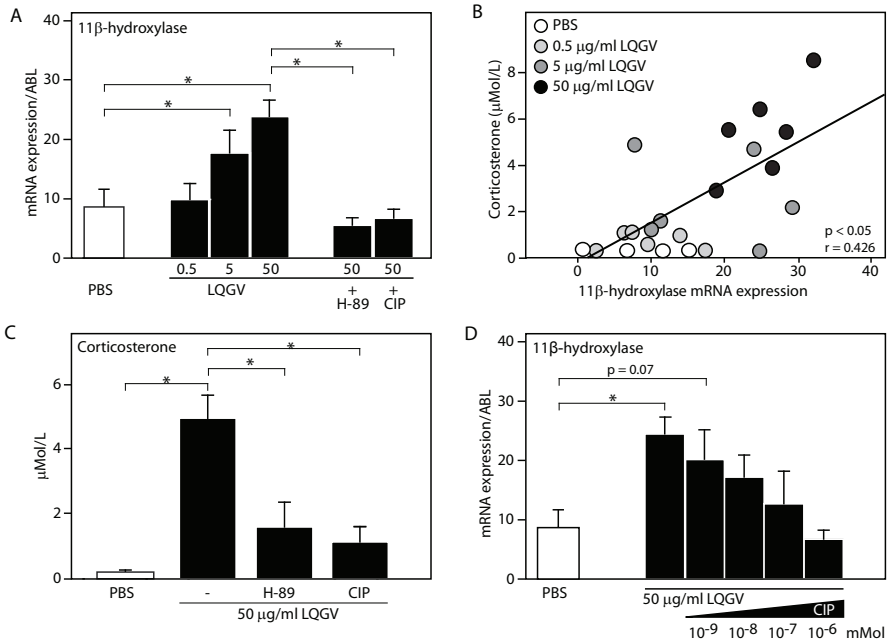

Figure 6. LQGV activates the adrenal ACTH-receptor.

Adrenal glands were *ex vivo*-stimulated with either 0.5, 5, or 50 μg/ml LQGV or with a combination of 50 μg/ml LQGV with either 10 μM of the cAMP-blocker H-89 or 10^{-6} mM ACTH-receptor antagonist CIP, and 11β-hydroxylase mRNA expression levels were determined (*A*). Correlation analysis between adrenal 11β-hydroxylase mRNA expression levels and corticosterone levels with different LQGV stimuli. Statistical significance was determined by Pearson's correlation analyses (*B*). Adrenal glands were *ex vivo*-stimulated with 50 μg/ml LQGV alone or in combination with either 10 μM cAMP-blocker H-89 or 10^{-6} mM ACTH-receptor antagonist CIP, and corticosterone levels in supernatant were determined (*C*). Adrenal glands were *ex vivo*-stimulated with PBS or 50 μg/ml LQGV in combination with either 10^{-9} mM, 10^{-8} mM, 10^{-7} mM, or 10^{-6} mM CIP and 11β-hydroxylase mRNA expression levels were determined (*D*). Data depicted are from five or six *ex vivo* stimulated adrenal glands from different naïve mice per group. *, p<0.05.

LQGV activates the ACTH-receptor on the murine adrenal cell line LHR-Y1

To further study the specificity of LQGV for the ACTH-receptor, we examined the effects of LQGV on ACTH-receptor by using the adrenal cell line LHR-Y1. These cells were stimulated with recombinant hCG or LQGV in the presence or absence of H-89 or CIP. In line with previous observations [31, 32],, recombinant hCG increased the corticosterone level and 11β-hydroxylase mRNA expression level. This increase was inhibited by H-89 (Fig. 7*A, B*). LQGV dose dependently increased 11β-hydroxylase mRNA expression levels and corticosterone levels, which were reduced by H-89 and CIP (Fig. 7*A, B*). In addition, mRNA expression levels of 11β-hydroxylase correlated positively and significantly with corticosterone levels detected in culture media (Fig. 7*C*). The irrelevant tetrapeptide EPPE did not increase the 11β-hydroxylase mRNA expression (data not shown). These results demonstrate that LQGV induces 11β-hydroxylase mRNA expression through ACTH-receptor induced cAMP signaling.

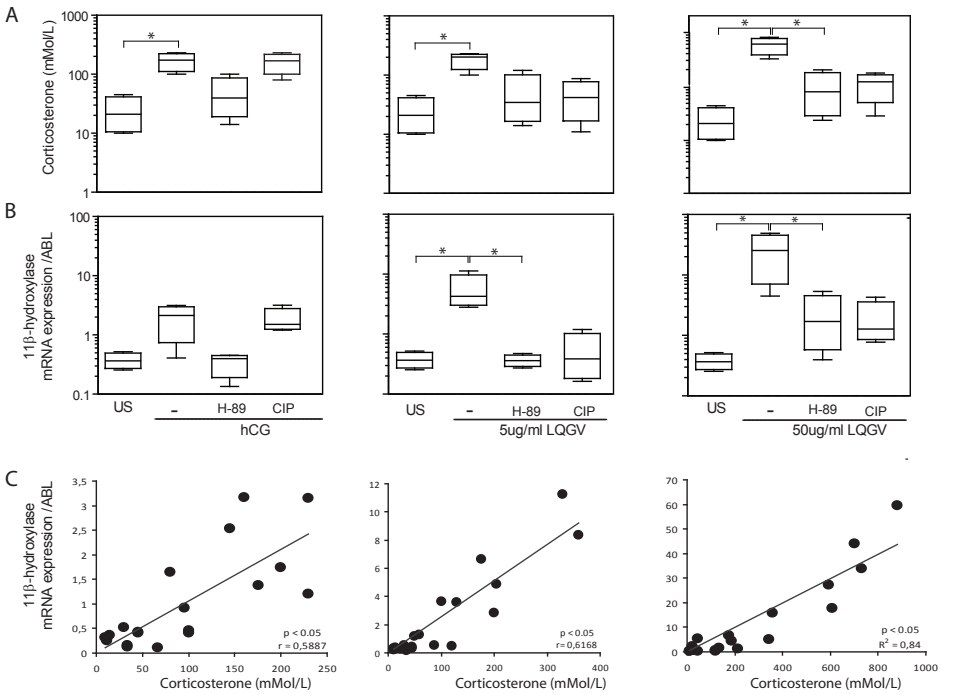

Figure 7. LQGV activates the ACTH-receptor on the murine adrenal cell line LHR-Y1.
LHR-Y1 cells were stimulated with either 300 U recombinant hCG, 5, or 50 μg/ml LQGV alone or in combination with either 10 μM the cAMP-blocker H-89 or 10^{-6} mM ACTH-receptor antagonist CIP. Subsequently, 11β-hydroxylase mRNA expression levels (*A*) and corticosterone levels in supernatant (*B*) were determined in the cultured cells. Correlation analysis between 11β-hydroxylase mRNA expression levels and corticosterone levels after 6 hours stimulation with either 300 U recombinant hCG, 5 μg/ml LQGV, or 50 μg/ml LQGV in combination with either the cAMP-blocker H-89 or ACTH-receptor antagonist CIP (*C*). Data depicted are from four single experiments. *, p<0.05

DISCUSSION

Endogenous glucocorticosteroid production and function are crucial in the control and resolution of inflammatory responses [22, 25, 33]. In this study, we demonstrate that the hCG-related tetrapeptide LQGV, which reduces immune activation in response to hemorrhagic shock and resuscitation, *L. monocytogenes* infection, and LPS injection [19-21], exerts anti-inflammatory effects through the induction of corticosterone production and secretion by the adrenal glands. In addition, we show that the LQGV-induced corticosterone production and secretion is mediated through ACTH-receptor activation and subsequent cAMP signaling.

Inflammation is a physiological reaction to infection and tissue injury [34]. However, an uncontrolled inflammatory response can culminate in SIRS and finally multiple organ dysfunction syndrome (MODS), which is a major cause of in-hospital deaths worldwide [35]. LPS challenge of mice is a TLR4 driven *in vivo* SIRS model that can result in MODS and eventually death [35]. In line with a previous study [19], we found that LQGV (50 mg/kg BW) can inhibit LPS-induced mortality in mice. This effect was completely reversed by mifepristone administration, demonstrating that LQGV exerts its *in vivo* prosurvival effect through GR activation. Our observations are in line with those of others who have demonstrated that GR-signaling blockage increases the vulnerability of organisms to tissue injury and LPS-induced inflammation [23, 36].

Glucocorticosteroids exert their immunosuppressive effect through binding to the cytoplasmic GR [22, 33]. Upon ligand binding, GR translocates into the nucleus, where it interacts with glucocorticoid responsive elements in the promoter region of target genes and regulates their expression [22]. The activated GR can also regulate gene expression through direct interaction with transcription factors, such as AP-1, NF-κB, and signal transducers of activation and transcription (STATs) [37]. We found that splenocytes from LQGV-treated mice produced significantly less of the AP-1 and NF-κB controlled cytokines IL-6 and TNF-α when stimulated with HKLM *in vitro*, which was reversed when the GR-blocker mifepristone was coadministrated with LQGV to the mice. Also, *in vitro* blockage of GR activity reversed the inhibitory activity of plasma from LQGV-treated mice with regard to HKLM-induced IL-6 and TNF-α production by splenocytes. Furthermore, LQGV administration to mice resulted in increased corticosterone levels in plasma. Our study also shows that *in vitro* administration of LQGV to splenocyte cultures did not inhibit HKLM-induced IL-6 and TNF-α production by itself. Together, these data indicate that LQGV renders cells less susceptible to multiple TLR activation through corticosterone-induced GR activity. We cannot exclude that other steroids, such as progesterone, are also produced and secreted upon LQGV-induced ACTH receptor activation. However, corticosterone has a 5 to 10 times higher affinity for the GR than that of progesterone [38, 39]. Therefore, we believe that corticosterone is the major

contributing anti-inflammatory glucocorticosteroid induced by LQGV treatment in our studies [21, 40].

Glucocorticosteroid production is regulated by 11β-hydroxylase within the zona fascilata of the adrenal cortex [41]. In rodents, 11β-hydroxylase converts deoxycorticosterone to corticosterone, and in humans it converts deoxycortisol to cortisol [41]. The expression of 11β-hydroxylase is dependent on ACTH-induced ACTH receptor activation and subsequent cAMP signaling [41-43]. LQGV increased 11β-hydroxylase mRNA expression levels and stimulated corticosterone production in *ex vivo* adrenal gland cultures and the murine adrenal cell line LHR-Y1. These effects were abolished when cAMP signaling or ACTH receptor activation were blocked. Thus LQGV actively stimulates the ACTH-receptor of adrenal cells, but more studies are needed to fully explain the mechanism by which LQGV activates the ACTH-receptor.

The *in vivo* corticosterone plasma levels were increased from at least 1 h up to 24 h after LQGV administration. These kinetics clearly differ from the *in vivo* stress-induced release of pre-existing corticosterone, which typically rises and declines again within 30-60 mins [44]. Also, the *ex vivo* LQGV-induced 11β-hydroxylase mRNA expression levels correlated positively with the corticosterone levels detected in adrenal gland culture supernatants. These data suggest that LQGV indeed stimulates corticosterone production and secretion by adrenal cells. Alternatively, the increase in plasma corticosterone level after LQGV administration could be due to an increased level of corticosteroid-binding globulin (CBG), which upon binding enhances the corticosterone half-life from 30 to 60 mins to approximately 5 days [45]. We regard this as unlikely because CBG is produced in the liver [46] and therefore could not have influenced the increase in corticosterone levels detected in our adrenal gland culture supernatants. Furthermore, glucocorticoids bound to CBG are biologically inactive [47], whereas we here show that LQGV stimulation results in the release of bioactive corticosterone, as reflected by the fact that mifepristone abolishes the LQGV effects. All together, these data indicate that LQGV can stimulate *de novo* corticosterone production and secretion by murine adrenal glands.

Previously, we demonstrated that LQGV reduced inflammation associated with hemorrhagic shock and resuscitation and reduced LPS-induced septic shock, but LQGV enhanced the susceptibility to *L. monocytogenes* infection [19-21]. GR blockage has been shown to increase disease severity during hemorrhagic shock and resuscitation as well as LPS-induced septic shock [25, 44, 48], whereas synthetic glucocorticosteroids are protective in these models [49, 50]. High corticosterone levels also render mice more susceptible to *L. monocytogenes* infection [44, 51-53]. Therefore, we suggest that LQGV-stimulated adrenal glucocorticosteroid production and subsequent GR activation contributed to the immunosuppressive effects that we found in our previous studies [14, 19-21]. However, we do not exclude the possibility that LQGV exerts other effects as well.

Synthetic glucocorticosteroids are the most frequently used drugs to treat autoimmune and inflammatory diseases. Long-term glucocorticosteroid treatment can lead to several severe side effects, such as atrophy of the skin and muscles, osteoporosis, and adrenal gland insufficiency. In the clinic, glucocorticosteroid treatment has to be tapered during a period of weeks to several months to prevent the patient from a state of hypocortisolism. Treatment with LQGV may have the advantage that the adrenal function remains intact without the risk of developing adrenal gland insufficiency and the potentially toxic long-term glucocorticosteroid tapering schemes.

CONCLUSIONS

In conclusion, the data presented indicates that the hCG-related tetrapeptide LQGV can stimulate adrenal corticosterone production through activation of the ACTH-receptor with consequent GR activation and immunosuppression in mice. This effect of LQGV may have therapeutic potential, for instance for treating severe inflammatory responses or in case of adrenal insufficiency as may occur after cranial irradiation.

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IV

Mild versus strong anti-inflammatory therapy during early sepsis in mice: a matter of life and death

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ABSTRACT

Objective: A recent literature-based study suggested that low-dose corticosteroid treatment has a beneficial effect on mortality in septic patients, whereas high-dose corticosteroid treatment has not. This suggests that mild downregulation of the inflammatory response during early sepsis may be beneficial while extensive reduction of the inflammatory response is not. To investigate this hypothesis, we examined the effect of dexamethasone in varying doses on cecal ligation and puncture-induced inflammation and mortality.

Design: Animal study.

Setting: University research laboratory.

Subjects: Male C57BL/6 mice.

Interventions: Mice were subjected to cecal ligation and puncture, and dexamethasone was administered intravenously at a dosage of 0.05 (L/DEX), 0.25 (M/DEX), or 2.5 (H/DEX) mg/kg body weight 20 mins postoperatively. Mice receiving phosphate-buffered saline served as controls. Survival was recorded up to 21 days and inflammatory markers were determined in plasma, lungs, liver, and kidneys at 6 hrs following CLP as well as bacterial load in blood and peritoneal fluid.

Measurements and main results: L/DEX treatment significantly improved survival compared with control mice, whereas treatment with higher concentrations of dexamethasone (M/DEX and H/DEX) did not. Treatment with either M/DEX or H/DEX was associated with significantly ($p<0.05$) reduced cytokine plasma levels as compared with controls at 6 hrs after cecal ligation and puncture. In addition, M/DEX or H/DEX powerfully reduced cytokine messenger RNA expression in the lungs, liver, and kidneys. In contrast, treatment with L/DEX was associated with a mild, but nonsignificant, reduction of cytokine plasma levels. In addition, L/DEX moderately reduced cytokine messenger RNA expression in lung, liver, and kidney tissue, and reduced the occurrence of bacteremia.

Conclusions: A modest down-regulation of the early sepsis associated inflammatory response improves survival in a murine cecal ligation and puncture model. We propose that the success of anti-inflammatory therapies in a septic setting fundamentally depends on finding a treatment balance that reduces the hyperinflammation-induced pathology but still allows adequate defense against pathogens.

INTRODUCTION

Sepsis is a complex clinical syndrome resulting from a harmful host response to infection, and the incidence is still increasing [1]. Sepsis is characterized by an early hyperinflammatory response, defined as the systemic inflammatory response syndrome (SIRS) [2]. SIRS is represented by an excessive production of pro-inflammatory cytokines relative to anti-inflammatory cytokines [3-5]. In time, SIRS will transit into a state of immunosuppression due to increased production of anti-inflammatory cytokines as well as anergy and apoptosis of immune cells [2, 4, 6-8]. The current treatment of septic patients mainly consists of administration of broad-spectrum antibiotics, fluid resuscitation, and ventilation and has essentially remained unchanged during the last decades. The sepsis-related inflammatory response may develop into septic shock and multiple organ dysfunction syndrome, which remain leading causes of morbidity and mortality in critically ill patients [9, 10]. This emphasizes the need for immunoregulatory therapeutic strategies.

During the last decades the use of general anti-inflammatory agents, such as corticosteroids, has been investigated in severe sepsis and septic shock. These studies have yielded contradictory results. Some studies demonstrated survival improvement after high-dose corticosteroid treatment [11] while others did not [12, 13]. Other therapeutic interventions which aimed at blocking a single pro-inflammatory mediator, for instance with tumor necrosis factor alpha (TNF-α)-specific monoclonal antibodies, have also been investigated. Although such studies showed a remarkable survival improvement in lipopolysaccharide-induced shock in animals [14, 15], no survival benefit was observed in the murine cecal ligation and puncture (CLP) polymicrobial sepsis model [16-18] nor in clinical trials involving septic patients [19-21]. Recently, an extensive literature review suggested that low-dose corticosteroid treatment, if administered as replacement therapy for adrenal insufficiency, has a beneficial effect on short-term mortality in patients with septic shock, in contrast to high-dose corticosteroid treatment [22]. These data illustrates the need for further studies to improve our understanding about the effectiveness of anti-inflammatory therapies on morbidity and mortality during sepsis and septic shock. The observations by Annane et al [22] led us to hypothesize that mild immunosuppression is beneficial during a septic event, whereas extensive immunosuppression is not.

To examine this hypothesis we investigated the effect of three different dexamethasone (DEX) dosages on CLP-induced mortality, acute inflammation, and bacteremia in mice. Our study demonstrates that low-dose DEX (L/DEX) treatment (0.05 mg/kg body weight (BW)) improves survival, while it only moderately downregulates inflammatory parameters such as cytokine levels and adhesion molecules in plasma, lungs, liver, and kidneys. In contrast, treatment with a medium-dose DEX (M/DEX; 0.25 mg/kg BW) or high-dose DEX (H/DEX; 2.5 mg/kg BW) did significantly reduce the inflammatory response but had no beneficial effect on survival. Low-dose DEX treatment

was also associated with significant inhibition of bacteremia, while M/DEX and H/DEX treatment were not. Monocytes of CLP mice that were treated with L/DEX produced higher levels of reactive oxygen species (ROS) when stimulated with phorbol 12-myristate 13-acetate (PMA) than monocytes from mice treated with the higher DEX dosages, indicating better preservation of antimicrobial defense mechanisms. Our data strongly suggest that the success of anti-inflammatory therapies in a septic setting fundamentally depends on finding a treatment balance that is able to reduce SIRS-induced pathology but still allows the immune system to raise an adequate host defense to the invading pathogens.

MATERIAL AND METHODS

Mice

Male C57BL/6 mice (8-12 weeks of age) with an average weight of 25 gram were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 hr light/dark cycle and were allowed food and water *ad libitum*. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Moderate CLP-induced polymicrobial sepsis model

Moderate polymicrobial sepsis, defined as ~60% mortality during the acute phase of sepsis (first 5 days), was used to test the effect of different DEX dosages in a septic setting. Hereto, mice underwent CLP, as described previously [23, 24]. Briefly, the cecum was ligated 1 cm from its distal end followed by double puncture with an 18-gauge needle, and manipulated to ensure extrusion of feces into the abdominal cavity. Postoperatively, all mice received a single subcutaneous injection of 0.5 mL of 0.9% NaCl solution. Mice were monitored every 12 hrs during the first 5 days (the acute hyperinflammatory phase of sepsis) followed by daily monitoring up to 21 days (the chronic immunosuppressive phase of sepsis). Sham mice underwent the same procedure but without ligation and puncture of the cecum.

Severe CLP-induced polymicrobial sepsis

Severe CLP-induced polymicrobial sepsis (defined as ~80-90% mortality when mice were postoperatively treated with fluid resuscitation only and ~50% mortality when mice were postoperatively treated with fluid resuscitation and antibiotics) was used to test the effect of different DEX dosages in combination with standard sepsis treatment. Hereto, the cecum was ligated just below the ileocecal valve followed by double puncture with an 18-gauge needle and manipulated to ensure extrusion of feces [23, 24]. To reach a mortality

of ~50% during the chronic phase of sepsis mice received subcutaneous injections of Tienam (25 mg/kg BW, Merck Sharp & Dohme, Haarlem, The Netherlands; dissolved in 1 mL 0.9% NaCl) starting 2 hrs after surgery followed by subsequent injections every 12 hrs during the first 5 days. Control mice postoperatively received 1 mL 0.9% NaCl at the same time points. Mice were monitored up to 21 days.

DEX treatment

Mice received a single intravenous injection of DEX (Sigma Aldrich, Zwijndrecht, The Netherlands; dissolved in phosphate-buffered saline (PBS)) 20 mins after the CLP procedure. This time point was based on previous studies [25]. Treatment groups were as follows: L/DEX (0.05 mg/kg BW), M/DEX (0.25 mg/kg BW), H/DEX (2.5 mg/kg BW). Control mice received an injection with PBS.

Cytokine measurement

Blood was obtained at indicated time-points following CLP and collected in ethylenediaminetetraacetic acid containing tubes (Greiner, Bio-one, Alphen aan den Rijn, The Netherlands). Plasma was obtained by centrifugation (1,500 rpm; 10 mins), immediately frozen, and stored at -80ºC until assayed. Proinflammatory cytokines interleukin (IL)-1β, IL-6, and TNF-α, the chemokines chemokine (C-C) motif ligand 2 (CCL2), chemokine (C-X-C motif) ligand (CXCL)-1, CXCL-2, and eotaxin, as well as the anti-inflammatory cytokines and soluble cytokine receptors IL-1 receptor antagonist (IL-1ra), IL-10, soluble TNF-receptor (sTNF-r)1, and sTNF-r2 were determined in plasma obtained at 6 hrs after CLP, using a sequential enzyme-linked immunosorbent assay method as described previously [26]. Antibody pairs and recombinant proteins were obtained from R&D Systems Europe (Abingdon, UK).

RNA isolation and real-time quantitative polymerase chain reaction (PCR)

RNA was extracted from lung, liver, and kidney tissue obtained 6 hrs after CLP using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA as described previously [27, 28]. CCL2, IL-6, TNF-α, E-selectin, and intercellular adhesion molecule (ICAM)-1 messenger RNA (mRNA) levels were determined by real-time quantitative polymerase chain reaction using an Applied Biosystems 7900 Polymerase Chain Reaction machine (Foster City, CA, USA) and quantified by normalization against the household gene ABL [28].

Bacterial culture of peritoneal lavage fluid and blood

Bacterial counts were determined in blood and peritoneal lavage fluid (the peritoneal cavity was washed with 2 mL ice-cold PBS) obtained at 24 hrs after CLP by plating

serial dilutions onto blood agar plates (Columbia blood agar, BD Pharmingen, Breda, The Netherlands) as described previously [23]. CFU numbers were determined and expressed as $\log_{10}$ CFU per mL peritoneal lavage fluid or blood.

Corticosterone measurement

Corticosterone levels in plasma obtained at 6 and 24 hrs after CLP were determined by enzyme-linked immunosorbent assay (IBL, Hamburg, Germany).

Reactive oxygen production by monocytes

Whole blood obtained at 24 hrs after CLP was stimulated for 10 mins with PMA (25 ng/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) after which a specific probe (H2DCF-DA (10 μm); Invitrogen, Breda, The Netherlands) that emits a fluorescent signal when activated by reactive oxygen species (ROS) was added. Samples were analyzed with a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) using Diva software (BD Biosciences). Monocytes were gated based on their scatter pattern and in every sample 10,000 events were obtained. Within this gate, H2DCF-DA-positive cells were evaluated with and without stimulation with PMA and the mean fluorescence intensity per cell was determined and considered as a measure for the cellular capacity to produce ROS. Data are presented as the difference in mean fluorescence intensity between PMA-stimulated and unstimulated cells.

Statistical analysis

Data are presented as the mean values ± standard error of mean. Statistical analysis was performed using SPSS version 15 (SPSS Inc., Chicago, IL). Log-rank survival analyses was performed during both the acute phase of sepsis (until day 5) and the chronic phase of sepsis (until day 21) and corrected for population stratification of different experiments. All cytokine and mRNA levels were log transformed in order to get normal distribution. The t-test was used to compare the mean cytokine and mRNA levels between the subgroups. The Mann-Whitney *U*-test was used to compare the number of positive blood cultures between the subgroups. Data shown in figures are geometric means with standard error of mean, or indicated otherwise. A p value <0.05 was considered as statistically significant.

RESULTS

The effect of DEX treatment on survival in a moderate CLP-induced sepsis model

In control mice, CLP resulted in an overall survival of 33% at day 21. Treatment with M/DEX or H/DEX had no effect on survival; 39% and 33%, respectively. L/DEX treatment significantly improved survival to 75% at day 21 (L/DEX vs. PBS $p<0.05$; L/DEX vs. H/DEX $p<0.05$) (Fig. 1).

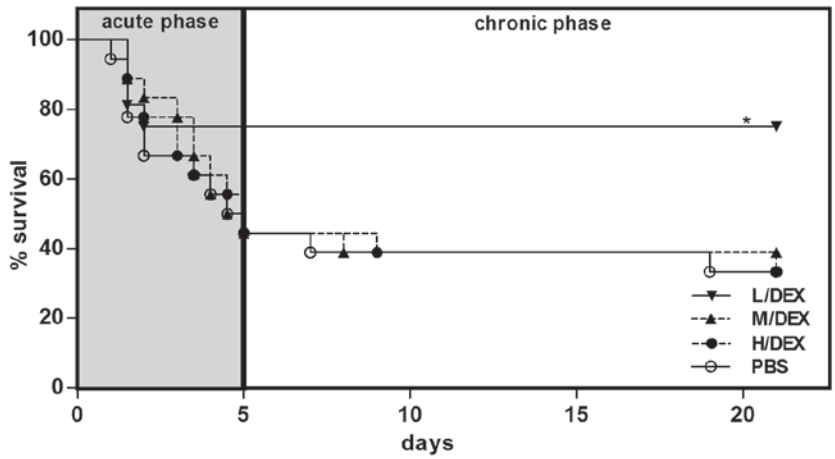

Figure 1. Effect of dexamethasone (DEX) treatment on survival.
Dexamethasone was administered intravenously 20 mins after moderate cecal ligation and puncture (CLP)-induced sepsis. Survival was monitored for 21 days after CLP. Low-dose DEX (L/DEX; 0.05 mg/kg body weight) treatment was associated with improved survival as compared with treatment with phosphate-buffered saline (PBS), medium-dose DEX (M/DEX; 0.25 mg/kg body weight), or high-dose DEX (H/DEX; 2.5 mg/kg body weight). Presented results were obtained in three identical independent experiments and corrected by stratification. n = 16 – 18 mice/group. * = $p<0.05$.

The effect of DEX treatment on plasma cytokine levels in a moderate CLP-induced sepsis model

We next examined the effect of the different DEX dosages on systemic cytokine levels. Plasma cytokine levels were determined 6 hrs after CLP. In all experimental groups, CLP induced an increase of plasma CCL2, CXCL1, CXCL2, eotaxin, IL-1β, IL-1ra, IL-6, IL-10, sTNF-r1, sTNF-r2, and TNF-α levels. In mice treated with M/DEX or H/DEX CCL2, CXCL1, CXCL2, eotaxin, IL-1ra, IL-6, sTNF-r1, and sTNF-r2 plasma levels were mostly significantly ($p<0.05$) reduced compared with control mice (Fig. 2). In the L/DEX-treated mice CCL2, CXCL1, CXCL2, eotaxin, IL-1ra, IL-6, IL-10, sTNF-r1, and sTNF-r2 plasma levels were consistently lower than in control mice, although this was never significant (Fig. 2). The tested DEX concentrations did not affect the CLP-induced increase of plasma IL-1β and TNF-α (Fig. 2).

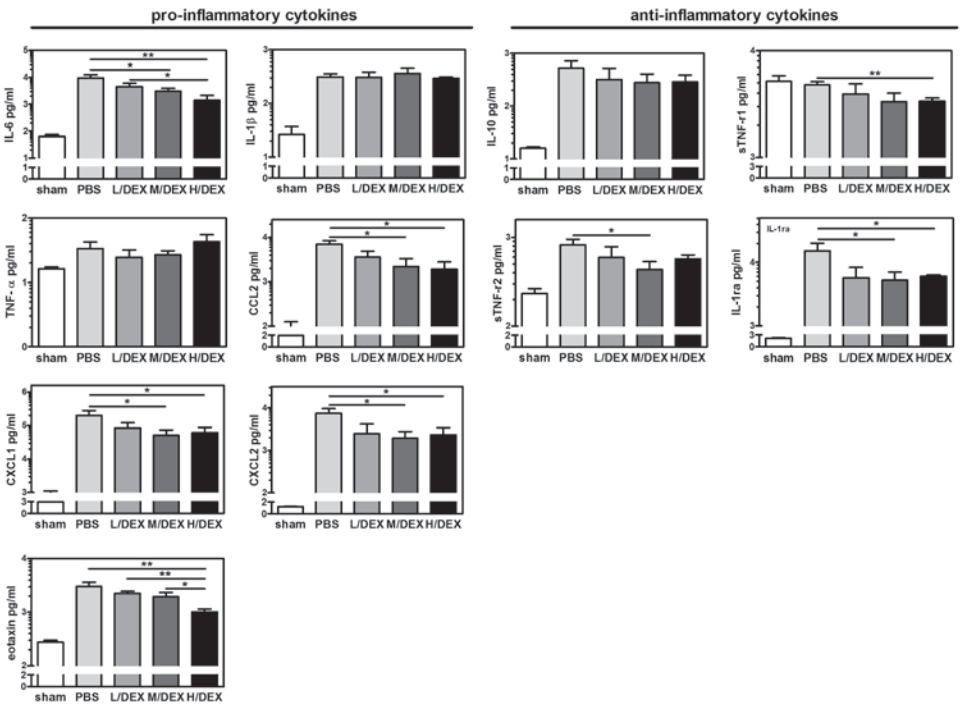

Figure 2. Effect of dexamethasone (DEX) treatment on systemic inflammatory response.
Dexamethasone was administered intravenously 20 mins after moderate cecal ligation and puncture (CLP)-induced sepsis. Plasma cytokine levels were determined at 6 hrs following CLP. Low-dose DEX (L/DEX; 0.05 mg/kg body weight) treatment was associated with reduced plasma levels of proinflammatory cytokines and anti-inflammatory cytokines, although never significant. Medium-dose DEX (M/DEX; 0.25 mg/kg body weight) and high-dose DEX (H/DEX; 2.5 mg/kg body weight) treatment was associated with significantly reduced interleukin (IL)-6, chemokine (C-C) motif ligand 2 (CCL2), chemokine (C-X-C motif) ligand (CXCL)-1, CXCL2, eotaxin, soluble tumor necrosis factor receptor (sTNF-r)1, sTNF-r2, and IL-1-receptor antagonist (IL-1ra) levels as compared with phosphate-buffered saline (PBS) treated mice. n = 6 mice/group. * = $p<0.05$, ** = $p<0.01$.

The effect of DEX treatment on organ inflammation in a moderate CLP-induced sepsis model

Since lungs, liver, and kidneys are commonly affected during sepsis [29-31], we examined the effect of the different DEX dosages on cytokine and adhesion molecule mRNA expression levels in these organs at 6 hrs after CLP. In control mice, CLP strongly increased CCL2, IL-6, TNF-α, E-selectin, and ICAM-1 mRNA expression levels in lung, liver, and kidney tissue. DEX dose dependently reduced CCL2, IL-6, E-selectin, and ICAM-1 mRNA expression in all three organs, with the most powerful and significant reductions in M/DEX- and H/DEX-treated mice. None of the tested DEX dosages affected the CLP-induced increase of TNF-α mRNA in any of the organs tested (Fig. 3).

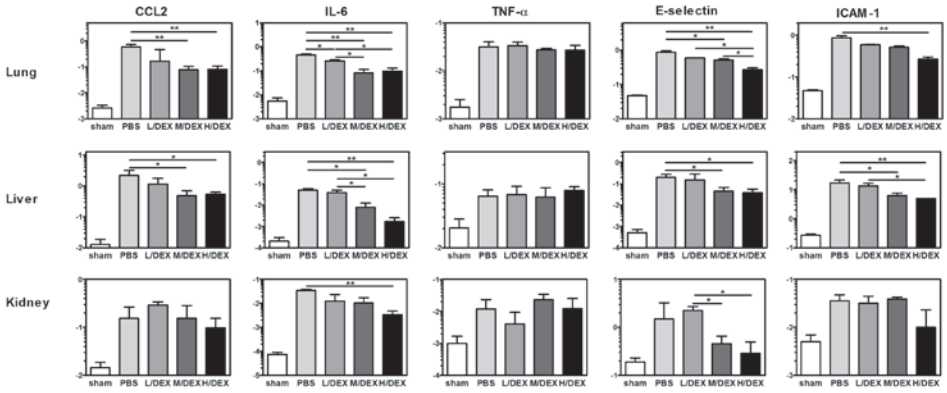

Figure 3. Effect of dexamethasone treatment on pulmonary, hepatic, and renal inflammation. Dexamethasone was administered intravenously 20 mins after moderate cecal ligation and puncture (CLP)-induced sepsis. Lung, liver, and kidney tissue was obtained at 6 hours following CLP. Relative interleukin (IL)-6, chemokine (C-C) motif ligand 2 (CCL2), tumor necrosis factor-α (TNF-α), E-selectin, and intercellular adhesion molecule (ICAM)-1 messenger RNA (mRNA) expression levels were determined in lung, liver, and kidney tissue. Low-dose DEX (L/DEX; 0.05 mg/kg body weight), medium-dose DEX (M/DEX; 0.25 mg/kg body weight), and high-dose DEX (H/DEX; 2.5 mg/kg body weight) treatment was associated with reduced IL-6, CCL2, E-selectin, and ICAM-1 mRNA expression levels compared with phosphate-buffered saline (PBS) treated mice. n = 3 mice/group. * = $p<0.05$, ** = $p<0.01$.

The effect of DEX treatment on bacterial load in a moderate CLP-induced sepsis model

To examine whether DEX treatment interfered with bacterial dissemination we determined bacterial loads in the peritoneal cavity and the blood at 24 hrs after CLP. None of the tested DEX dosages affected the amount of CFU present in the peritoneal fluid. L/DEX treatment significantly reduced the occurrence of bacteremia (50% vs. 90% in PBS-treated mice; $p<0.05$), whereas M/DEX and H/DEX treatment did not (Table 1).

Table 1. Effect of dexamethasone treatment of bacterial load at 24 hours after CLP

	Blood culture		Peritoneal lavage		
Treatment	% positive[1]	Mean CFU [Range] (Log_{10} CFU/ml)	% positive	Mean CFU [Range] (Log_{10} CFU/ml)	*n*
PBS	90 %	3.07 [0 – 7.21]	100 %	6.63 [0 – 7.21]	10
L/DEX	50 %*	2.33 [0 – 8.24]	100 %	5.42 [0 – 8.24]	12
M/DEX	91 %	2.69 [0 – 6.37]	100 %	6.00 [0 – 6.37]	11
H/DEX	91 %	2.69 [0 – 7.09]	100 %	6.05 [0 – 7.09]	11

[1] = % of mice with a positive culture. *= $p<0.05$
L/DEX = 0.05 mg/kg body weight, M/DEX = 0.25 mg/kg body weight and H/DEX = 2.5 mg/kg body weight

The effect of DEX treatment on oxidative burst capacity of monocytes

Because ROS generation is an important anti-bacterial defense mechanism we examined whether DEX treatment affected the oxidative burst capacity of blood monocytes at 24 hrs after CLP. Monocytes from PBS-treated and L/DEX-treated mice displayed a comparable capacity to produce ROS when stimulated with PMA. Monocytes from M/DEX- and especially H/DEX-treated mice displayed a diminished capacity to produce ROS after PMA stimulation, although this was not significant when compared with PBS- or L/DEX-treated CLP-mice (Fig. 4).

The effect of DEX treatment on plasma corticosterone levels

To examine whether DEX treatment affected adrenal function we analyzed plasma corticosterone levels in all experimental groups. Corticosterone plasma levels in all groups were significantly increased at 6 h after CLP, declined at 24 h after CLP, but remained significantly higher than levels in sham mice. The CLP-induced increase of plasma corticosterone levels at the time points tested was not affected by treatment with any of the DEX dosages (Fig. 5).

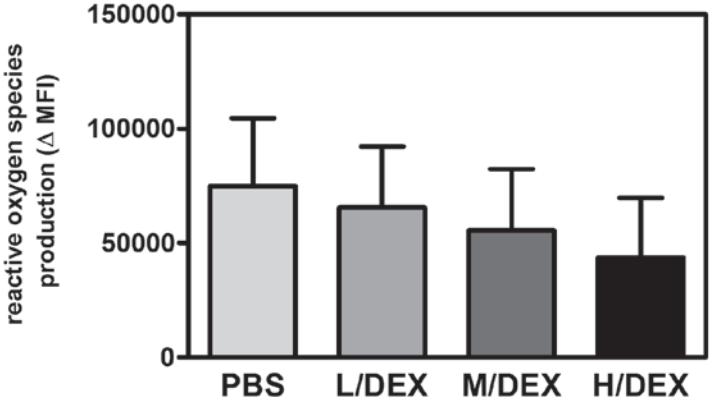

Figure 4. Effect of dexamethasone treatment on oxidative burst capacity of monocytes.
Dexamethasone was administered intravenously 20 mins after moderate cecal ligation and puncture (CLP)-induced sepsis and the oxidative burst capacity of blood monocytes was determined in whole blood obtained at 24 hrs after CLP. Monocytes from medium-dose DEX (M/DEX; 0.25 mg/kg body weight) and high-dose DEX (H/DEX; 2.5 mg/kg body weight) treated mice showed a diminished capacity to produce reactive oxygen species (ROS) after phorbol 12-myristate 13-acetate (PMA) stimulation compared with phosphate-buffered saline (PBS)-treated and low-dose DEX (L/DEX; 0.05 mg/kg body weight)-treated mice, however never significant. Data are presented as the difference in mean fluorescence intensity (MFI) between PMA stimulated and unstimulated cells. PBS n = 5, L/DEX n = 6, M/DEX n = 5, H/DEX n = 6.

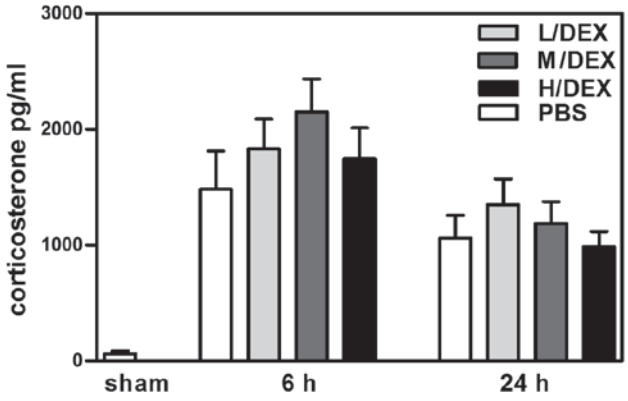

Figure 5. Effect of dexamethasone treatment on plasma levels of corticosterone.
Dexamethasone was administered intravenously 20 mins after moderate cecal ligation and puncture (CLP)-induced sepsis and corticosterone plasma levels were determined at 6 hrs and 24 hrs after CLP. In all treatment groups corticosterone plasma levels were significantly increased at 6 hrs when compared with sham operated mice, declined at 24 hrs after CLP, but remained significantly higher than levels in sham mice. Dexamethasone treatment did not influence the CLP-induced increase in corticosterone levels. Low-dose DEX (L/DEX; 0.05 mg/kg body weight), medium-dose DEX (M/DEX; 0.25 mg/kg body weight) and high-dose DEX (H/DEX; 2.5 mg/kg body weight). n = 6 mice/group.

The effect of DEX treatment in addition to fluid resuscitation and antibiotics in a severe CLP-induced sepsis model

We further evaluated the effect of different dosages of DEX treatment in combination with the standard of sepsis care, consisting of fluid resuscitation and antibiotics, in a severe CLP-induced sepsis model. In control mice that only received fluid resuscitation, the overall survival at day 21 was 17% (Fig. 6). In mice that received treatment with fluid resuscitation and antibiotics, survival significantly increased to 50% ($p<0.05$) at 21 days (Fig. 6). In this severe sepsis model H/DEX treatment in combination with fluid resuscitation and antibiotics did not result in a better survival than with fluid resuscitation and antibiotics alone (Fig. 6). L/DEX treatment in combination with fluid resuscitation and antibiotics increased survival to 92%, which was significantly ($p<0.05$) higher than with fluid resuscitation and antibiotics alone (Fig. 6).

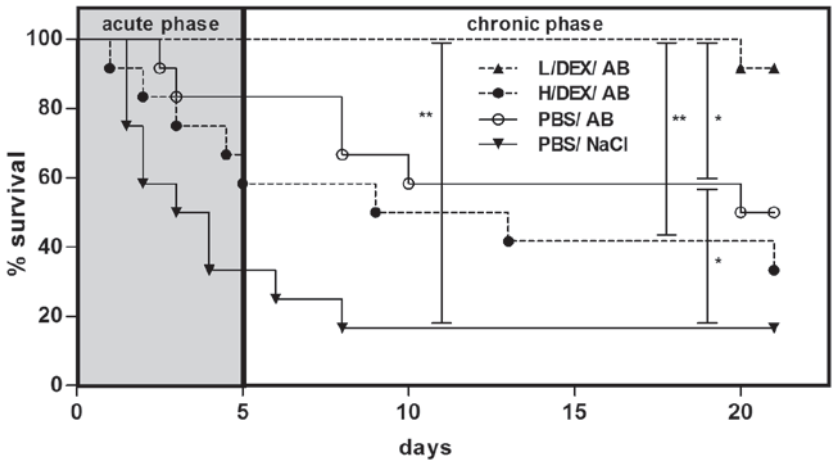

Figure 6. Effect of dexamethasone treatment in combination with standard sepsis care on survival.

Severe polymicrobial sepsis was induced by ligation of the cecum just below the ileocecal valve followed by a double puncture with an 18-G needle. DEX or phosphate-buffered saline (PBS) as control was administered intravenously 20 mins after cecal ligation and puncture, followed by 5 day treatment with antibiotics and fluid resuscitation or fluid resuscitation alone. Survival was monitored for 21 days after cecal ligation and puncture. PBS treatment followed by postoperative treatment with antibiotics and fluid resuscitation [PBS/ AB] was associated with improved survival compared with control mice treated with fluid resuscitation alone [PBS/ NaCl]. High-dose DEX (H/DEX; 2.5 mg/kg body weight) treatment in combination with antibiotics and fluid resuscitation [H/DEX/ AB] did not affect survival compared with control treatment. Low-dose DEX (L/DEX; 0.05 mg/kg body weight) treatment in combination with antibiotics and fluid resuscitation [L/DEX/ AB] did improve survival compared with treatment with antibiotics and fluid resuscitation alone. Presented results were obtained in two identical independent experiments. Log-rank survival analyses were performed as described in Materials and Methods. n = 12 mice/group. * = $p<0.05$, ** = $p<0.01$.

DISCUSSION

Sepsis and septic shock are major causes of morbidity and mortality within intensive care units. The incidence of these conditions is still increasing [1]. A generalized pro-inflammatory state is an early characteristic of sepsis and contributes to the severe pathology. This stresses the need for therapeutic approaches that modulate this proinflammatory response. In the present study, we show for the first time that treatment with L/DEX (0.05 mg/kg BW) significantly improves survival in CLP-induced polymicrobial sepsis in mice, while higher DEX dosages (M/DEX (0.25 mg/kg BW) and H/DEX (2.5 mg/kg BW)) showed no survival benefit. Remarkably, the prosurvival effect of L/DEX was associated with only a mild but consistent reduction of the inflammatory response, while both M/DEX and H/DEX were associated with significant down-regulation of the inflammatory response. In addition, L/DEX treatment was associated with a significantly

lower occurrence of bacteremia. Our data demonstrate that the modest reduction of the inflammatory response by L/DEX is associated with a significant survival benefit. Importantly, this effect of L/DEX was of additive value to the standard sepsis treatment with fluid resuscitation and antibiotics.

Glucocorticosteroids are potent anti-inflammatory agents that upon binding to an intracellular glucocorticoid receptor prevent activation of a variety of inflammatory genes [32]. Glucocorticosteroid treatment has been show to improve survival in LPS models of septic shock, which was associated with a marked reduction of the inflammatory response [33-36]. However, in the CLP model, which more accurately reflects the pathophysiology of human sepsis [37], conflicting results have been obtained with corticosteroid treatment. Some studies described that high-dose corticosteroid treatment (30 mg/kg methylprednisolone) resulted in survival improvement [38], while others found no effect of high-dose corticosteroid treatment (e.g. dexamethasone ≥2.5 mg/kg [35]). Here, we demonstrate that a single L/DEX administration 20 mins after the CLP procedure improved survival, while M/DEX and H/DEX did not. We observed no differences in the systemic corticosterone levels between the different treatment groups, suggesting that the effect of the different DEX dosages on mortality and inflammation was not related to induction of a relative adrenal insufficiency by the higher DEX dosages used.

Proinflammatory cytokines and chemokines are crucially important in the activation of host defense mechanisms against invading pathogens [39], since they facilitate immune cell recruitment and activation [40, 41]. Here, we found that L/DEX treatment was associated with a mild, nonsignificant suppression of the sepsis-related inflammatory response, as reflected by mild reduction of cytokines, chemokines, and adhesion molecule levels in plasma, lung, liver, and kidney tissue. In contrast, M/DEX and H/DEX treatment resulted in significant reduction of the inflammatory response. Our data therefore suggest that mild down-regulation of the proinflammatory response is associated with survival improvement, while strong immunosuppression is not. This is supported by the observation that marked suppression of the innate immune system before infection favors excessive bacterial replication [42, 43]. Deletion of signaling molecules, such as myeloid differentiation primary response gene (88), which orchestrate cytokine responses upon a septic stimulus, also enhances vulnerability to infection [44]. Also, despite the fact that IL-6 has been identified as a predictor of mortality in experimental sepsis [5] and in septic patients [45, 46], high dosage of IL-6-neutralizing antibodies did not improve survival following CLP while a lower dosage did [47]. In addition, IL-6 knockout mice showed an enhanced mortality upon CLP [48, 49], pointing at a protective function of IL-6 to a septic insult. Recently, we described that treatment with the human chorionic gonadotropin-related tetrapeptide LQGV (at a dose of 2 x 5 mg/kg BW) improved survival following CLP, while it only modestly reduced the inflammatory response [23]. Overall, this data

suggests that strong immunosuppressive therapy abrogates the protective functions of the inflammatory response, such as activation of host defense mechanisms, while mild anti-inflammatory therapy blocks the detrimental effects of SIRS but preserves the protective inflammatory response. This is supported by our observation that L/DEX significantly reduced the occurrence of bacteremia and better preserved the ROS producing capacity of monocytes. Despite the lower incidence of bacteremia, all L/DEX-treated CLP-mice had positive peritoneal lavage cultures with CFU counts comparable to that in all the other groups. This indicates that mild immunosuppressive therapy can prevent systemic bacterial dissemination from local sites but does not necessarily facilitate local clearance. Altogether, these data suggest the existence of a specific range in which the sepsis-related inflammatory response can be pharmacologically regulated to obtain survival improvement.

In the present study L/DEX improved survival in a moderate CLP-induced polymicrobial sepsis model. However, as fluid resuscitation and antibiotics are the cornerstones of severe sepsis and septic shock treatment, we also investigated the effect of different DEX dosages in combination with fluid resuscitation and antibiotics in a severe CLP-induced polymicrobial sepsis model. In line with previous observations [50], we found that fluid resuscitation and antibiotics improved long-term survival following CLP. When L/DEX was added to treatment with fluid resuscitation and antibiotics long term survival became significantly better than with fluid resuscitation and antibiotics alone. The highest DEX dosage tested in this study had no additive value to the treatment with fluid resuscitation and antibiotics alone. Others demonstrated that hydrocortisone treatment (in doses ranging from 2.5 – 15 mg/kg BW) added to the beneficial effects of antibiotics and fluids on short-term mortality in a pneumonia-associated sepsis model in mice, and that this was independent of the sepsis severity and risk of death [51]. However, high IL-6 plasma levels at 6 hrs after induction of CLP have been shown to predict subsequent mortality in mice. Administration of high dose DEX (2.5 mg/kg BW, two doses) in mice stratified according to the IL-6 plasma level (predict to die and predict to live) significantly improved survival whereas it did not when this stratification was not applied [25]. It may therefore well be that a high dose of DEX exerts a beneficial effect in our model following similar stratification. Also, in septic patients it appears that only low-dose corticosteroid treatment, defined as a total daily dosage of ≤ 300 mg hydrocortisone (or equivalent) and administered as replacement therapy for adrenal insufficiency, has a beneficial effect on short-term mortality [22]. Thus, mild reduction of the inflammatory response, as established by low-dose corticosteroids, further improves the therapeutic potential of the current standard sepsis care. The human equivalent dose of the DEX concentrations used in our study are 0.20 mg/kg for H/DEX, 0.020 mg/kg for M/DEX, and 0.0041 mg/kg for L/DEX [52], which suggests that DEX dosages currently administered

to septic patients [22, 53], can be reduced to gain further survival improvement.

We cannot exclude that other properties of DEX, such as its effect on the cardiovascular system, contributed to the survival benefit. Also, in our study the DEX treatment was given shortly (20 mins) after induction of the septic insult, which we consider preventive rather than therapeutic. Nevertheless, our data clearly demonstrate that mild reduction of the inflammatory response during the early phase of sepsis is associated with long-term survival improvement, which warrants further studies to determine the time-window as well as the dosage in which anti-inflammatory drugs can be of benefit in a septic setting.

CONCLUSIONS

Our results demonstrate that L/DEX treatment can reduce mortality following CLP-induced polymicrobial sepsis. This beneficial effect is associated with a mild (but non-significant) suppression of the CLP-induced inflammatory response and reduced systemic bacterial dissemination. We propose that the success of anti-inflammatory therapies in a septic setting fundamentally depends on finding the optimal level of immunosuppression that reduces SIRS-induced pathology but still allows an adequate host defense against invading pathogens. This implies the existence of a specific range in which reduction of the pro-inflammatory response will lead to survival benefit, while suppression beyond this range will not result in survival improvement. As the septic inflammatory response is complicated and differs among patients [29] insight into the immunological status of the individual patient will aid to optimize treatment success.

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V

Amelioration of renal ischemia-reperfusion injury by synthetic oligopeptides related to human chorionic gonadotropin

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ABSTRACT

Background: We have previously reported that small synthetic oligopeptides related to human β-chorionic gonadotropin (β-hCG) can reduce inflammation. Here we investigated whether such oligopeptides can reduce renal ischemia reperfusion injury in the mouse.

Methods: Ten different oligopeptides were administered 1 minute before induction of renal ischemia and 1 minute before reperfusion.

Results: Survival at 72 hours post reperfusion was significantly higher in mice treated with oligopeptides MTRV, LQG, VLPALPQ or AQGV as compared to placebo treated mice. Some oligopeptides were more effective than others. AQGV completely prevented mortality and best preserved kidney function. Next, AQGV was tested in a dose-escalating study in a range of 0.3 to 30 mg/kg. A survival gain was observed with all doses. Improvement of kidney function was observed from 1 mg/kg. Highest survival and best preserved kidney function was observed at 3 and 10 mg/kg.
Upon treatment with AQGV, a significantly lower influx of neutrophils was found, apoptosis was decreased, whereas tubular epithelial cell proliferation was significantly increased at 24 hours post-reperfusion. Serum levels of TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased at 24 hours post-reperfusion. E-selectin mRNA levels in kidneys were significantly decreased at 6 hours post-reperfusion. AQGV did not reduce mortality when treatment was started after reperfusion.

Conclusions: This study shows that small oligopeptides related to the primary structure of β-hCG, especially AQGV, are promising potential drugs for preventing the development of renal ischemia-reperfusion injury.

INTRODUCTION

Inflammation plays a major role in the pathophysiology of renal ischemic injury [1]. The initial ischemic injury results in up-regulation of adhesion molecules on activated endothelium and release of cytokines, reactive oxygen species (ROS) and eicosanoids. Leukocytes, recruited by chemokines and pro-inflammatory cytokines, potentiate injury by generating more ROS and eicosanoids, thereby further enhancing inflammation.

Human chorionic gonadotropin (hCG) is a hormone produced during pregnancy by placental trophoblasts [2], but is also produced by the pituitary gland and leukocytes in non-pregnant females and males [3,4]. It consists of an α- and β-chain. In human pregnancy urine and in commercial hCG preparations hCG occurs in a variety of forms, including breakdown oligopeptide products. During pregnancy, the urine contains increasing proportions of nicked hCG and hCG β-core fragments [5]. Nicked hCG has peptide bond cleavages in loop 2 of the β-chain between residues 44 and 52, whereas hCG β-core completely lacks the β-chain loop 2, which consists of amino acid residues 41-54.

We have previously shown that the 400–2000 Dalton peptide fraction of pregnancy urine, but not of normal female or male urine, is able to inhibit the development of diabetes in NOD mice, whereas fractions greater than 2000 Dalton, including hCG, did not have this activity [6]. We then reported that the synthetic hexapeptide VLPALP, which is part of the primary structure of the hCG β-chain loop 2, reduced mortality in a murine model of lipopolysaccharide (LPS) induced systemic inflammatory response syndrome [7,8].

Based on these findings, and known preferential cleavage sites of the hCG β-chain loop 2 [5,9-12], we selected six different synthetic oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ), which are part of the primary structure of loop 2 of the hCG β-chain, as well as four alanine variants of LQG and LQGV (AQG, LAG, AQGV and LAQV) (Fig. 1), and tested these in a murine model for their capacity to reduce renal I/R injury.

MATERIAL AND METHODS

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Ten different hCG-related oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG and LAGV) were evaluated for their capacity to reduce ischemia-reperfusion induced renal injury as compared to mice treated with phosphate-

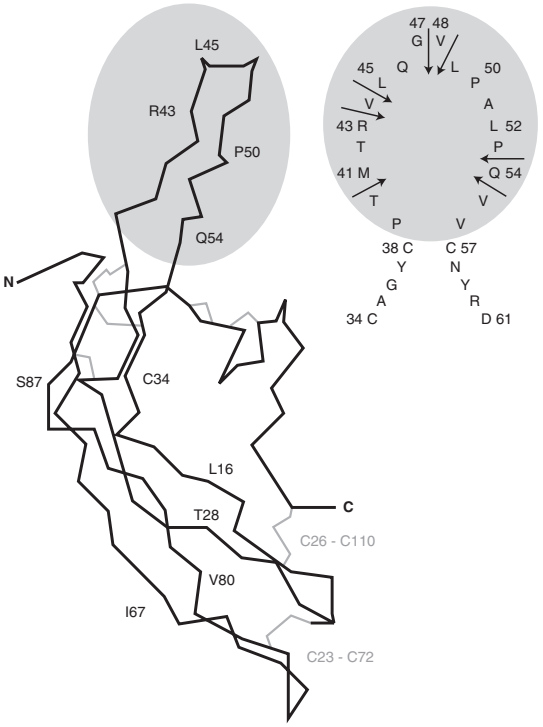

Figure 1.
Structure of β-hCG with loop 2 and the amino acid sequence of loop 2 indicated. Adapted from Lapthorn et al. [35]. Arrows indicate the preferential cleavage sites in loop 2.

buffered saline (PBS). Five mg/kg body weight of oligopeptide or PBS in a volume of 0.1 mL was administered intravenously (i.v.) 1 minute before clamping the kidney, and 1 minute before releasing the clamp. Subsequently a dose-escalating study was performed with AQGV. The AQGV was given in doses of 0.3, 1, 3, 10, and 30 mg/kg in a volume of 0.1 mL and was administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. Possible toxic side effects were studied by careful observation of control and peptide treated mice for signs of discomfort. Contra-lateral kidney samples were obtained for further analysis. At 24 and 72 hr post-reperfusion, mice were sacrificed and clamped kidneys were harvested and snap frozen for further analysis. Serum urea levels were measured to determine kidney function. Infiltrating cells were analysed using immunohistochemistry. In all groups survival was assessed and analyzed by Kaplan-Meier analysis.

In an additional experiment AQGV was given in a dose of 5 mg/kg BW in a volume of 0.1 mL and administered i.v. 1 minute before clamping, and 1 minute before

releasing the clamp. At 6 and 24 hr post-reperfusion, mice were sacrificed and blood was obtained for cytokine measurements in serum. From the 6hr post-reperfusion group the clamped kidney was harvested for determination of mRNA expression levels.

Furthermore, survival experiments were performed in which mice received PBS or AQGV (5mg/kg BW) at 12 and 24 hours, or at 6 and 12 hours post-reperfusion.

Mice

Male C57BL/6JOlaHsd mice of 12-16 weeks of age were obtained from Harlan (Horst, The Netherlands). Mice were kept under standard laboratory conditions (temperature 20-24ºC, relative humidity 50-60%, 12 h light/12 h dark) and were allowed free access to food (Hope Farms, Woerden, The Netherlands) and water.

Ischemia model

Mice were anaesthetized by isoflurane inhalation. Anesthesia was maintained using a mixture of N_2O/O_2/isoflurane. Blood was collected by retro-orbital puncture. Body temperature was maintained by placing the mice on heating pads. Following a midline abdominal incision, the left renal pedicle was localized and clamped for 25 mins using an atraumatic micro-vascular clamp. After inspection for signs of ischemia, the wound was covered with PBS soaked cotton and the animal was covered with a tin foil insulation sheet. After release of the clamp, restoration of blood-flow was inspected visually and a contra-lateral nefrectomy was performed. The abdominal wound was closed in two layers, and mice were given 0.5 ml PBS subcutaneously.

Oligopeptides

Selection was based on either the known preferential cleavage sites or known *in vivo* nick sites of the sequence MTRVLQGVLPALPQ (aa_{41-54}) of loop 2 of the β-subunit of hCG [14, 16-19]. Selected oligopeptides were MTR (aa_{41-43}), MTRV (aa_{41-44}), LQG (aa_{45-47}), AQG and LAG (alanine replaced oligopeptides of LQG), LQGV (aa_{45-48}), AQGV and LAGV (alanine replaced oligopeptide of LQGV), VLPALP (aa_{48-53}), VLPALPQ (aa_{48-54}). Oligopeptides were synthesized (Ansynth BV, Roosendaal, The Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support. Oligopeptides were dissolved in PBS at a concentration of 1 mg/ml and stored at -20°C in small aliquods.

Functional measurements

Serum urea and creatinine values were measured using a kinetic urease method where the decrease in NADH absorbance is measured photometrically, using an ELAN multi-analyzer (Eppendorf-Merck, Germany).

Immunohistochemistry

Primary antibodies used were rat-anti-mouse CD4, CD8, CD45, neutrophils, macrophages, CD54 (Serotec, Oxford, UK). Antibodies were diluted in PBS/5% BSA solution. Primary antibody was applied on for 30 min at RT and slides were subsequently incubated with a mixture of goat-anti-rat IgM+IgG (H+L) alkaline-phosphate conjugated antibody (Southern Biotech, Birmingham, USA) for 30 min at RT. Enzyme detection was performed using Naphthol AS-MX, New Fuchsine, sodium-nitrite and levamisol mixture in Tris-HCl pH8 as a substrate for 30 min at RT in the dark.

Formalin-fixed-paraffin sections (3 μm) were used for Ki-67 staining. Slides were deparaffinized and rehydrated and boiled for antigen retrieval in a 0.01M sodium citrate solution for 30 min in a microwave-oven. Endogenous peroxidase was blocked with a 0.03% H_2O_2 solution. The sections were incubated overnight at 4°C with rat-anti-mouse Ki-67 primary antibody (Dako Cytomation, Glostrup, Denmark) and subsequently incubated for 30 min at RT with rabbit-anti-rat IgG conjugated with HRP secondary antibody (Dako Cytomation, Glostrup, Denmark). Enzyme detection was performed using DAB as a substrate. Slides were rinsed in tap water, counterstained with hematoxylin and rinsed with tap water again. As a negative control the primary antibody was omitted. Positive cells were counted in 10 high power fields (400X) using a semi quantitative scoring system as follows: 0: no positive cells, 1: 1-10 cells, 2: 11-30 cells, 3: 30-60 cells, 4 > 60 cells.

Measurement of apoptotic cells

Formalin-fixed-paraffin sections were stained for apoptotic cells by TUNEL staining using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, USA) according to the manufacturer's instructions. Positive cells were counted in 10 fields at a magnification of 400x.

Cytokine measurements

TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MCP-1 were measured using a commercially available cytometric bead array (CBA) (BD Biosciences, San Jose, CA, USA) and a BD FACSArrayTM Bioanalyzer (BD Biosciences). Analysis of the data was performed using FCAP ArrayTM software (BD Biosciences). Assay sensitivity was 2.5 pg/ml.

Real-time quantitative (RQ)-PCR analysis

Sections of kidney were homogenized and RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). In total 1μg of RNA was reverse transcribed and RQ-PCR using an AppliedBiosystems 7700 PCR machine (Foster City, CA, USA) was performed as described previously [13]. In all 6h samples the mRNA transcript levels of TNF-α,

IFN-γ, IL-6, IL-10, IL-12, MCP-1, and the adhesion molecules E-selectin and ICAM-1 were determined. Transcript levels of these genes were quantified by normalization against ABL.

Statistical analysis

Survival data were compared by log-rank analysis. Other data were analyzed using ANOVA, followed by a Mann-Whitney *U*- test. Calculations were performed using SPSS v11.0 for Windows. A p value ≤0.05 was considered statistically significant. Data are presented as mean values ± standard error of the mean.

RESULTS

Effect of hCG-related oligopeptide treatment on survival

25 mins of warm renal ischemia and contra-lateral nefrectomy resulted in a survival of 50% in the control group at 3 days post-reperfusion (Table 1). The groups treated with oligopeptides MTR, LQGV, VLPALP, AQG, LAG and LAGV (5 mg/kg), had survival rates not significantly different from controls. Treatment with LQG led to a significant better survival (90%), while treatment with oligopeptides MTRV, VLPALPQ or AQGV totally prevented mortality.

Table 1. Effect of various hCG-related oligopeptides (5 mg/kg) on the survival of mice subjected to ischemia-reperfusion damage.

	Survival		
Treatment	**24h**	**72h**	**p**
PBS	90%	50%	
MTR	100%	60%	ns
MTRV	100%	100%	< 0.05
LQG	100%	90%	< 0.05
LQGV	100%	80%	ns
VLPALP	100%	70%	ns
VLPALPQ	100%	100%	< 0.01
AQG	100%	70%	ns
AQGV	100%	100%	< 0.01
LAG	100%	70%	ns
LAGV	90%	90%	ns

Survival at 24 and 72 hours post-reperfusion of mice subjected to 25 mins of left renal warm ischemia. Mice treated with PBS or hCG-related synthetic oligopeptide were compared by log-rank test (n = 10 animals / group).

Effect of hCG-related oligopeptide treatment on kidney function

Treatment of mice with oligopeptide MTRV, AQGV or LAGV provided significant ($p<0.05$) functional protection against renal I/R injury at both 24 and 72 hr, as measured by serum urea levels (Fig. 2). Although treatment with LQG resulted in significantly decreased serum urea at 24 hours post-reperfusion ($p<0.05$), at 72 hours no significant beneficial effect was found. While treatment with VLPALPQ did not cause a significant decrease in serum urea at 24 hours, at 72 hours it was significantly decreased as compared to the control group ($p<0.05$). Treatment with AQGV provided the most powerful protection against renal ischemia-reperfusion injury at both 24 hours ($p<0.01$) and 72 hours ($p<0.01$).

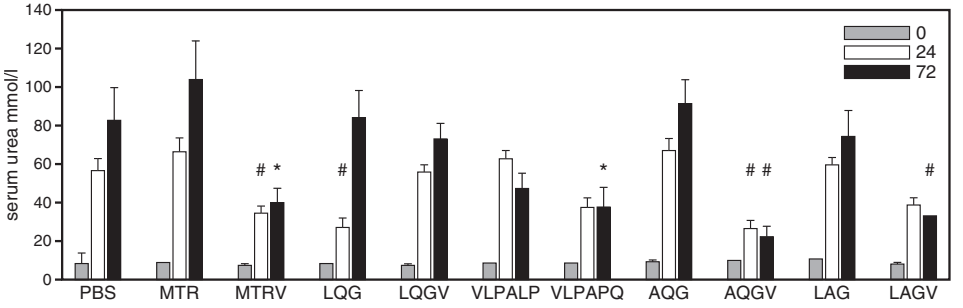

Figure 2.

Renal function as reflected by serum urea levels. Preoperative values and 24 and 72 hours post reperfusion values in the different oligopeptide treated groups were compared to PBS-treated controls. Treatment with MTRV and AQGV significantly reduced renal function loss both at 24 hours and 72 hours after ischemia reperfusion injury. Treatment with LQG reduced serum urea levels at 24 hours. Treatment with VLPALPQ and LAGV showed significantly reduced serum urea levels at 72 hours. *$p<0.05$ and #$p<0.01$ (n = 10 animals / group).

Effect of different doses of AQGV (0.3 – 30 mg/kg) on survival

Because AQGV showed the most powerful protection against warm renal I/R injury, we determined the optimal dose of this oligopeptide in a dose escalating study. Therefore, AQGV was administered in doses ranging from 0.3 to 30 mg/kg, and compared to mice treated with PBS. A survival rate of 60% was seen in the control group (Table 2). Although treatment with 0.3, 1 and 30 appeared to result in a survival benefit, no significant difference could be measured (80%, 90%, and 80%, respectively). The doses of 3 and 10 mg/kg totally prevented mortality ($p<0.05$).

Effect of different doses of AQGV (0.3 – 30 mg/kg) on kidney function

Treatment of mice subjected to renal I/R damage with 1, 3, 10 and 30 mg/kg AQGV resulted in significant reduction of serum urea levels at 72 hours ($p<0.05$). A dose of 3 mg/kg resulted in best preservation of kidney function, with return to normal function

already observed at 72 hrs ($p<0.01$). With 0.3 mg/kg no significant benefit was observed (Fig. 3). Creatinine values confirmed these data but in our model did not show the same level of responsiveness to the injury as urea.

Table 2. Effect of different doses of AQGV on the survival of mice subjected to ischemia-reperfusion damage.

	Survival		
Treatment (mg/kg)	**24h**	**72h**	**p**
PBS	100%	60%	
0.3	100%	80%	ns
1.0	100%	90%	ns
3.0	100%	100%	< 0.05
10	100%	100%	< 0.05
30	100%	80%	ns

Survival at 24 and 72 hours post-reperfusion of mice subjected to 25 mins of left renal warm ischemia and treated with increasing doses of the oligopeptide AQGV. AQGV- and PBS-treated mice were compared by log-rank test (n = 10 animals / group).

Effects of AQGV on cellular infiltration, apoptosis, and proliferation

To study the mechanism underlying the protective effect of AQGV, we investigated the cellular infiltrate and proliferation in the kidneys of mice treated with 5 mg/kg AQGV. At both 24 and 72 hr post reperfusion the neutrophil influx was significantly decreased in the AQGV-treated group ($p=0.03$ and $p=0.022$, respectively) (Fig. 4*A*). Additional staining for CD4+, CD8+ cells and macrophages revealed no differences between the two groups (data not shown). TUNEL staining identified apoptotic cells which were localized mainly in the tubular epithelium (Fig. 4*B*, middle and lower panels). The number of TUNEL-positive cells was significantly lower in AQGV treated animals 24 hours after reperfusion (Fig. 4*B*). Ki-67 staining showed a significantly higher proliferative activity of renal tubular epithelial cells in AQGV-treated mice at 24 hours (Fig. 4*C*). At 72 hours this difference had disappeared.

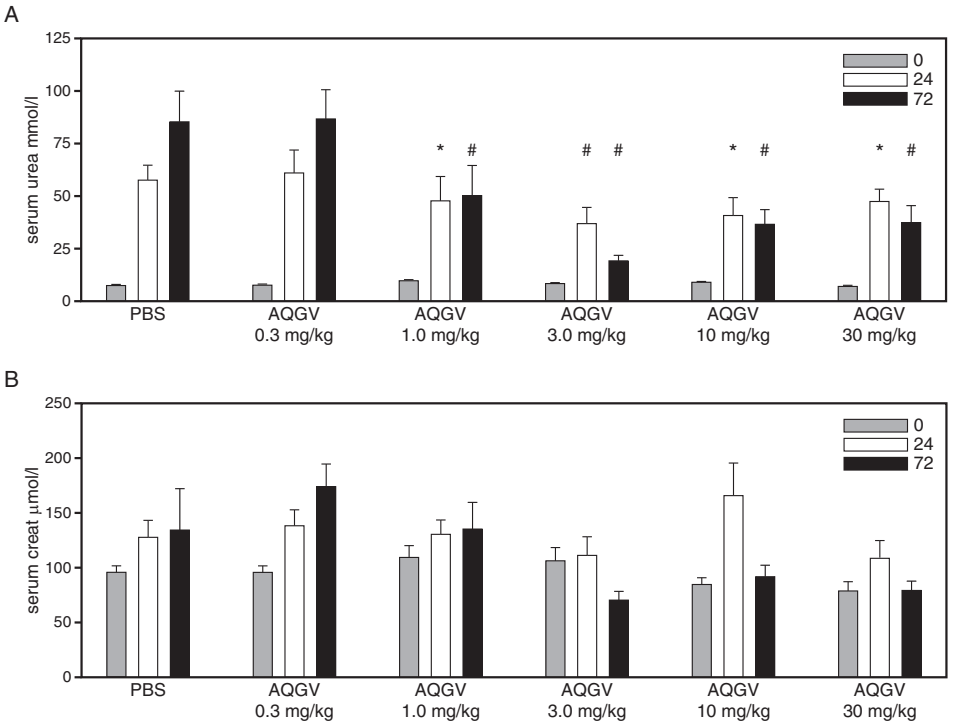

Figure 3.
Renal function as reflected by serum urea (A) and creatinine (B). Values are shown preoperative, and after 24 and 72 hours in groups treated with AQGV in a dose escalation study (0.3 – 30 mg/kg), and compared to a PBS-treated control group. Treatment with AQGV in a dose from 1 mg/kg up to 30 mg/kg significantly reduced renal function loss after renal ischemia-reperfusion injury. The dose of 3 mg/kg was the most potent. *$p<0.05$ and #$p<0.01$ (n = 10 animals / group).

Effects of AQGV on serum cytokine levels and renal mRNA transcript levels

Using the bead-array we determined serum cytokine levels at 6 and 24 hr post-reperfusion. MCP-1 was below the detection limit in all samples. No differences in serum TNF-α, IFN-γ, IL-6, IL-10, and IL-12 levels were observed at 6hrs post-reperfusion. At 24 hrs post-reperfusion the levels for all cytokines were decreased upon AQGV treatment, with IL-6, IL-10, IFN-γ ($p<0.05$), and TNF-α ($p<0.01$) being significantly lower (Fig. 5*A*).

AQGV treatment showed no effect on inflammatory cytokine mRNA levels 6 hrs post-reperfusion (data not shown). AQGV treatment did result in a significant ($p<0.05$) down regulation of renal E-selectin, but not ICAM-1 mRNA expression at 6 hr post-reperfusion as compared to PBS treated mice (Fig. 5*B*).

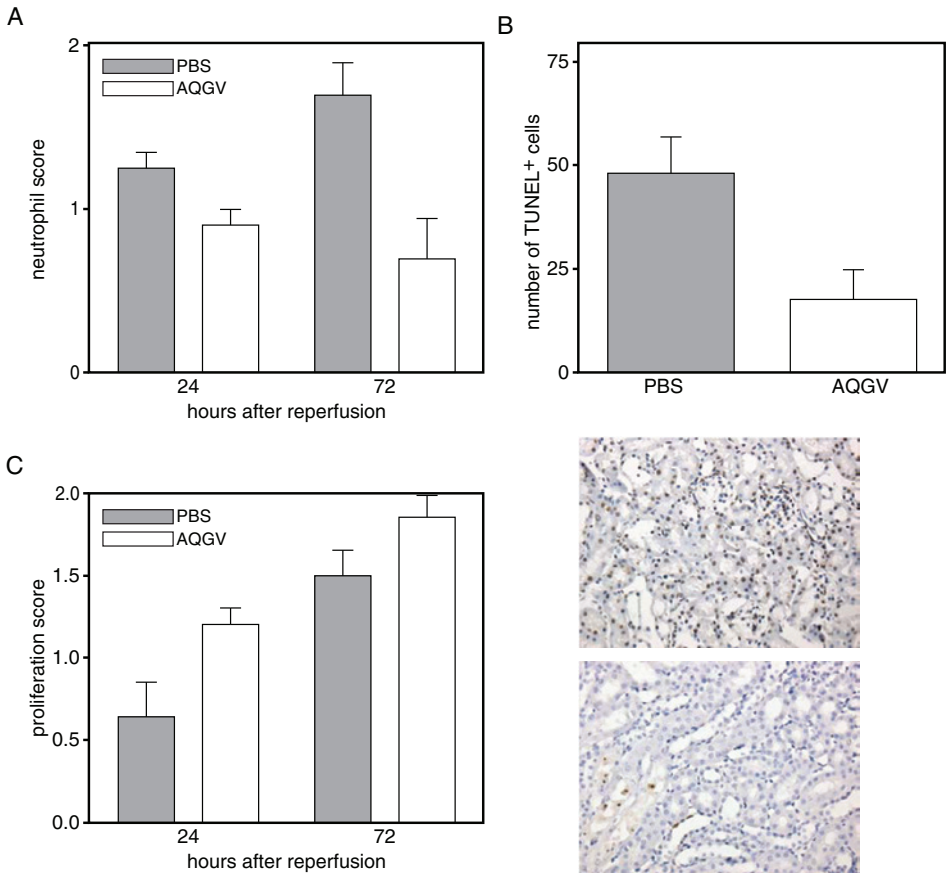

Figure 4.

(A) Renal neutrophil influx as assessed by immunohistochemical staining. AQGV treatment reduced neutrophil infiltration after 25 mins of renal warm ischemia as assessed at 24 and 72 hours post-reperfusion. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *p<0.05 (n = 10 animals / group). (B) AQGV treatment significantly reduced the number of apoptotic cells in the kidney 24 hours after renal ischemia reperfusion injury. *p=0.01 vs. PBS treated controls at 24 hours (n= 6 - 10 animals / group, upper panel). Middle and lower panels: representative photomicrographs of TUNEL stained control-, and AQGV treated kidneys respectively, 24 hours after reperfusion (200x). (C) Proliferation as assessed by Ki-67 immunohistochemistry. AQGV treatment significantly enhanced cellular proliferation at 24 hours after renal ischemia-reperfusion injury. Although a higher trend of proliferation was seen at 72 hours as well, no statistically significant difference was found. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *p<0.05 (n = 10 animals / group).

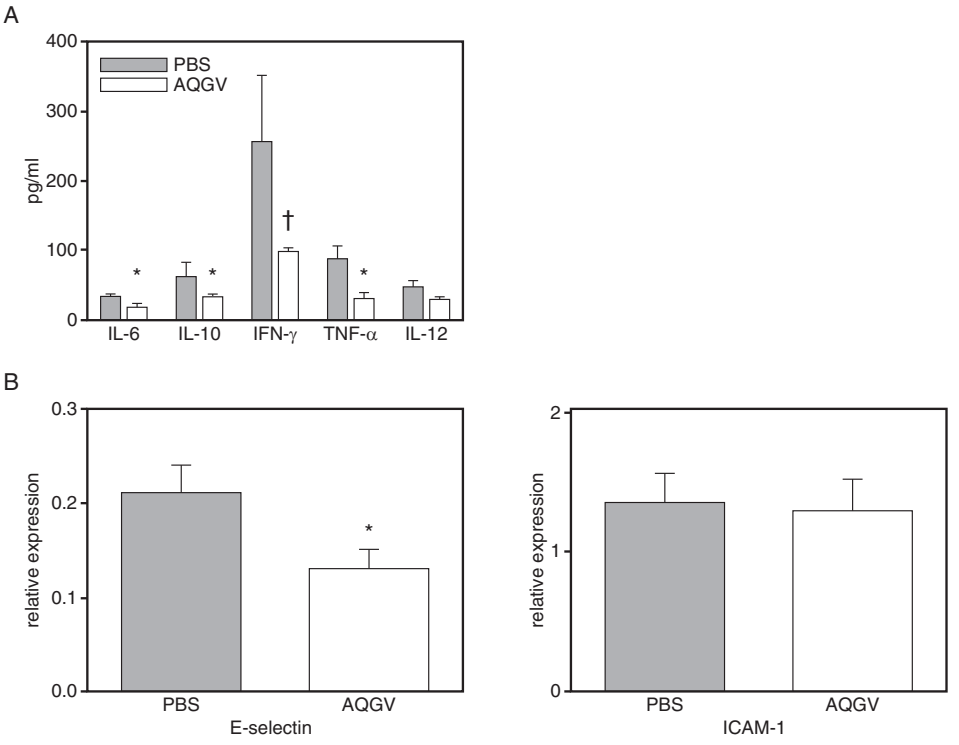

Figure 5.
(A) Treatment with AQGV reduces serum cytokine levels at 24 hours after renal ischemia reperfusion injury. *p<0.05, †p<0.01 (n = 5 per group). (B) Treatment with AQGV reduces renal E-selectin (left panel) but not ICAM-1 (right panel) mRNA levels at 6 hours after renal ischemia reperfusion injury. Data are presented as mean value ± sem. *p<0.05 (n = 6 animals / group).

Effect of post-reperfusion AQGV treatment on survival

AQGV treatment given either at 12 and 24 hr post-reperfusion or at 6 and 12 hr post-reperfusion did not improve survival (~50%) as compared to the control group (data not shown).

DISCUSSION

We investigated whether treatment with synthetic oligopeptides, consisting of 3 to 7 amino acids, based on the primary structure of hCG, was able to reduce warm ischemia-reperfusion injury of the kidney. We demonstrate for the first time that oligopeptides as small as three or four amino acids can significantly reduce mortality seen after severe renal I/R injury and improves kidney function as measured by serum urea levels. Especially AQGV showed superior results in enhancing survival and preservation of kidney function

after 25 mins of renal ischemia. A dose of 3-10 mg/kg proved to be the most potent with regard to reducing mortality as well as preserving kidney function. Furthermore, up to 30 mg/kg, no toxicity was observed. Also in rats, dogs and a human phase I study no harmful side effects of single and repeated AQGV administration were found. Data of these studies will be published elsewhere (manuscript in preparation).

Both natural hCG and commercial hCG preparations have been investigated for their role on the immune system, because of their putative immunomodulating role during pregnancy in protecting the fetus from rejection [14].

Our previous work [6] shows that short-term treatment of female NOD mice, with a hCG preparation purified from first trimester pregnancy urine, starting prior to the onset of hyperglycemic symptoms, inhibits the development of type I diabetes. Interestingly, however, the anti-diabetic activity of the used hCG preparation did not reside in the heterodimeric hCG molecule, or its subunits, but in a 400-2000 Dalton fraction.

Subsequently, we showed in a model of LPS-induced systemic inflammatory response syndrome in mice that treatment with this low weight molecular fraction was capable of inhibiting the septic shock morbidity as well as mortality [7]. The same beneficial effect was obtained with the synthetic oligopeptide VLPALP, which sequence is part of loop 2 of the β-chain of hCG [7]. Recently, we showed that hCG-related oligopeptides reduce inflammation and liver injury in a rat model of hemorrhagic shock and resuscitation [15].

During pregnancy, hCG occurs in a variety of forms and breakdown products in serum and urine, including intact hCG, α- and β-subunits, nicked hCG, hCG β-core fragment, and smaller peptide fragments. Both nicked hCG and the β-core subunit consist of a β-chain with a defective loop 2. This loop, consisting of the amino acid residues 41-54, is absent in β-core subunit, and is cleaved in nicked hCG [9-12]. Since the immunomodulatory activity of hCG resided in the low molecular weight fraction, we hypothesized that *in vivo* liberated breakdown products, such as those originating from the proteolytic cleavage of peptide bonds between amino acid residues 41-54, may have significant biological activity [8]. Based on known preferential cleavage sites [5,9,10,12], we tested synthetic oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and, based on alanine replacement mapping, the LQG and LQGV analogs AQG, LAG, AQGV and LAGV (Fig. 1). Of these oligopeptides, MTRV, LQG, VLPALPQ and AQGV appeared able to reduce mortality and decline in kidney function induced by warm renal ischemia-reperfusion injury, AQGV being the most effective (Table 1 and Fig. 1).

Cell migration plays an important role during the initial phase of renal I/R injury. Up-regulation of adhesion molecules on endothelial cells, induced by locally produced pro-inflammatory mediators, is amongst the first changes observed after renal I/R injury and is central to the pathogenesis of ischemic acute kidney injury [1,16]. Subsequently

leukocytes become activated by local pro-inflammatory factors, thereby facilitating adherence to endothelial cells and subsequent renal tissue infiltration [1]. Sequestered neutrophils induce parenchymal damage, followed by cytokine production by resident renal cells and infiltrating cells, which promotes further tissue damage [1,17]. It has been demonstrated that renal mRNA expression of the early adhesion molecule E-selectin peaks within 6 hours post-reperfusion, with neutrophils infiltrating in parallel. E-selectin blockage with the selectin specific ligand sPSGL has been shown to inhibit renal neutrophil infiltration after I/R and to preserve kidney function [18]. In mice treated with AQGV we observed decreased E-selectin mRNA levels 6 hrs post-reperfusion and decreased renal neutrophil infiltration at 24 hours post-reperfusion. Apoptotic cell death, an important determinant of cellular damage in ischemic kidneys [19] was also significantly reduced in AQGV treated mice. Additionally, serum levels of the inflammatory cytokines TNF-α, INF-γ, IL-6, and IL-10 were significantly decreased 24 hours post-reperfusion upon AQGV treatment. This data is indicative of decreased renal injury and fits with the preservation of kidney function we observed upon AQGV treatment. The lower levels of systemic cytokines observed upon AQGV treatment may be a reflection of reduced formation as well as better renal clearance of these cytokines [20]. The lower serum cytokine levels likely contribute to the decreased mortality by preventing systemic inflammation and subsequent complications in these animals [21].

Our data indicate that AQGV treatment protects against renal I/R injury by interfering with early E-selectin upregulation, thereby reducing neutrophil influx, parenchymal damage and possibly cytokine production. So far it is unclear what the molecular mechanism of action is by which AQGV exerts its effects. It is possible that AQGV mediates its effect by an as yet unidentified receptor. However, we cannot exclude the possibility that, due to the small size and molecular weight, AQGV penetrates the cell membrane [22] and exerts its action either by interfering with signaling cascades or the transcriptional machinery. E-selectin is expressed *de novo* on endothelial cells after transcriptional induction by pro-inflammatory agents [23]. Whether AQGV inhibits the local production of pro-inflammatory mediators that induce E-selectin or directly interferes with the intracellular signaling cascade involved in activating E-selectin transcription is not clear so far. In contrast to E-selectin, ICAM-1 expression was not altered by peptide treatment. The transcription factor HMGA1 is required for optimal activation of E-selectin gene transcription while it has no role in activating ICAM-1 transcription [24,25]. Therefore, it is possible that AQGV interferes specifically with pathways required for E-selectin transcriptional activation.

Although previous work revealed a pathophysiological role of the T-cell as mediator of ischemic acute renal failure [26,27], we did not find a significant difference between the AQGV and placebo treated mice in numbers of CD4+, CD8+ T-cells or

macrophages. Our data fit with the observation that RAG-1 deficient mice (lacking both T- and B-cells) are not protected from renal I/R injury [28].

Ki-67, a marker for cellular proliferation, is part of a nuclear protein complex expressed in the G1, S, G2 and M phases of the cell cycle in proliferating cells [29,30]. Mice treated with AQGV showed significantly increased numbers of Ki-67 positive renal tubular epithelial cells at 24 hr post-reperfusion, reflecting enhancement of the regenerative process [31]. The increase in proliferation is likely facilitated by a reduction in inflammation-induced tissue injury, since high levels of pro-inflammatory cytokines have been shown to suppress regeneration of ischemically damaged kidneys [32].

AQGV treatment, at a dose of 5 mg/kg BW, given at either 12 and 24 hr post-reperfusion or at 6 and 12 hr post-reperfusion was not associated with improved survival. Although we cannot formally exclude that these post-reperfusion treatment regimens improved kidney function, it appears that in the currently used model AQGV only prevents the onset of renal ischemia reperfusion injury. This may indicate that AQGV inhibits the activation of pathophysiological pathways involved in renal ischemia-reperfusion injury, but is unable to reverse these pathways once activated. However, since we do not exclude that higher doses of AQGV given post-reperfusion do reverse renal-ischemia reperfusion injury, detailed dose-response studies are warranted to gain full insight into the renoprotective effect of AQGV, and other hCG-related oligopeptides.

CONCLUSIONS

This study shows that treatment of mice with 5 mg/kg of either one of the hCG-related oligopeptides MTRV, LQG, VLPALPQ or AQGV shortly before and immediately after renal pedicle clamping can significantly reduce mortality and ameliorate kidney injury in a model of warm ischemia reperfusion injury. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased renal neutrophil infiltration, reduced numbers of apoptotic tubular epithelial cells, and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL-10. This data implies that AQGV interferes with the early renal inflammatory response induced by I/R and as such prevents parenchymal damage and organ dysfunction. These new renoprotective oligopeptides show great promise for preventing the development of renal ischemia-reperfusion injury and may well be used in clinical situations where renal I/R is foreseeable, such as semi-elective surgeries including kidney transplantation, cardiac surgery, and abdominal aorta surgery. So far, phase IA and phase 1B studies with AQGV (EA-230) have been successfully completed and phase II studies are underway [33,34].

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VI

Preoperative fasting induces protection against renal ischemia-reperfusion injury by a corticosterone-independent mechanism

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ABSTRACT

Background: Three days of fasting protects mice against lethal renal ischemia-reperfusion (I/R) injury. We hypothesize that the protection imposed by fasting is mediated by increased levels of corticosterone, induced by the stress of food deprivation.

Methods: C57BL/6 mice were fasted for 3 days after which serum corticosterone levels were determined. Mice underwent a bilateral adrenalectomy (ADX). Ten days later, they were either fasted or given a corticosterone receptor antagonist while fasting. Bilateral renal I/R injury was induced by clamping the artery and vein of the left and right kidney simultaneously for 37 mins. Survival and kidney function were determined.

Results: Fasting significantly increased corticosterone levels. Only 8% of the ADX mice which were fasted prior to I/R injury survived, whereas all sham-ADX operated mice survived I/R injury after fasting. After ADX and fasting, 70% of the mice subjected to sham I/R succumbed to the surgical procedure. After fasting with concomitant blockade of the glucocorticoid receptor all animals survived renal I/R.

Conclusions: Three days of fasting protects against I/R injury and increases serum corticosterone levels. ADX renders mice incapable of withstanding subsequent abdominal surgery. Glucocorticoid receptor blockade does not interfere with the protective effects of fasting. Thus, the protection against renal I/R injury induced by preoperative fasting is mediated by corticosterone-independent mechanisms.

INTRODUCTION

Renal transplantation is considered the treatment of choice for people with end-stage renal disease. One of the factors negatively influencing the outcome after kidney transplantation is ischemia-reperfusion (I/R) injury [1-2]. Delayed graft function is primarily a consequence of I/R injury and contributes to the loss of kidney grafts [3]. We have previously shown that dietary restriction protects against I/R injury [4]. Both 3 days of fasting and 2 weeks of reduced (30%) caloric intake prior to renal I/R resulted in protection against I/R injury in mice. Dietary restriction increased baseline levels of cytoprotective and antioxidant genes and resulted in a more expeditious and pronounced response of these genes to I/R injury [4-5]. The mechanism by which dietary restriction induces this protection remains elusive.

During short-term stress responses, activation of the hypothalamic-pituitary-adrenal axis stimulates the release of glucocorticoids from the adrenal gland. Glucocorticoids are one of the main mediators in these stress response pathways [6] and are essential in limiting and resolving inflammation [7]. I/R injury induces inflammation, which is responsible for its detrimental consequences [8]. Prolonged fasting acts as an acute stressor and increases levels of corticosterone in rodents [9]. We hypothesized that the protection against I/R injury imposed by fasting is mediated by increased systemic levels of corticosterone. We quantified serum corticosterone levels after three days of fasting and subjected mice to a bilateral adrenalectomy (ADX) and treatment with the glucocorticoid receptor antagonist Mifepristone during fasting. The effect of glucocorticoid receptor blockade on the increased expression of cytoprotective and antioxidant genes induced by fasting was determined to investigate the relationship between fasting, corticosterone, and the expression profile of these genes.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice with an average weight of 25 g were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 h light/dark cycle and were allowed food and water ad libitum. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Fasting protocol

Mice in the fed group were allowed unrestricted access to food. Mice in the fasting groups were transferred to a clean cage at 17:00 hrs and withheld food for 3 days. All animals were given continuous access to water or 0.9% NaCl (discussed next).

Bilateral adrenalectomy

Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2% with oxygen for maintenance). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. A small incision (0.5 cm) was made in the left and right flanks after which the adrenal glands were identified. Diathermy coagulation was performed to remove the adrenal glands from the surrounding tissue. Wounds were closed in two layers using 5/0 Safil (B.Braun Medical B.V., Oss, The Netherlands) sutures. Sham animals underwent the same procedure without removal of the adrenal glands. After surgery, 0.5 mL phosphate-buffered saline (PBS) was administered subcutaneously for maintenance of the fluid balance. Postoperatively, all animals were given access to 0.9% NaCl to ensure adequate salt balance. The experiments were resumed following a recovery period of 10 days. Corticosterone levels were determined as described below to confirm complete removal of the glands.

Bilateral renal I/R injury

All surgical procedures were conducted between 9.00 and 12.00 hrs. Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anaesthesia). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the renal artery and vein of both the left and right kidney were occluded simultaneously, by using atraumatic microvascular clamps, for 37 mins. In a previous study we showed that this ischemic time induces a mortality rate of 40% [4]. After macroscopic confirmation of ischemia (purple color), the incision was covered with PBS-soaked gauze and the animal was covered with an aluminum foil blanket to maintain body temperature. Following release of the vascular clamp, restoration of blood-flow was confirmed by the kidney returning to normal color. The abdominal wound was closed in two layers using 5/0 Safil sutures. Directly after closing the abdomen 0.5 mL of PBS at body temperature was injected subcutaneously for maintenance of fluid balance.

Glucocorticoid receptor blockage

Mifepristone is a potent glucocorticoid type II receptor antagonist that also blocks the progesterone receptor, albeit to a much lesser extent. Mifepristone (RU-38486, Sigma-Aldrich, St. Louis, MO,USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a final concentration of 500 mg/mL. This stock solution was diluted 850- or 85-fold with PBS before intraperitoneal injection yielding a final DMSO concentration of 0.12% or 1.16 %, respectively, to minimize the effect of DMSO on I/R injury [10]. As these treatments differ in final DMSO concentration, we used two vehicle solutions containing either 0.12% or 1.16% DMSO to correct for this difference.

Serum measurements

Blood samples were obtained under anaesthesia by retro-orbital venous plexus puncture (during the experiments) or heart puncture (at the end of the experiment). Serum urea levels were determined using a QuantiChrom assay kit, DIUR-500 (Gentaur, Brussels, Belgium). Serum corticosterone was determined using a corticosterone ELISA kit (Sigma Aldrich) according to the manufacturer's protocol. Corticosterone serum levels were determined from blood samples obtained between 9:00 and 10:00 hrs.

Influence of fasting and ADX on corticosterone levels

Animals were fed ad libitum or fasted for 1, 2, or 3 days (n = 6/group), after which they were scarified by exsanguination under anesthesia. Furthermore, blood samples were obtained from ADX and sham (ADX)-operated mice (n = 6/group) after a 3-day fast. Corticosterone levels were determined to confirm complete removal of the adrenal glands (Fig. 1*A*).

Survival following renal I/R injury after ADX and subsequent fasting

ADX mice (n = 13) or sham (ADX)-operated mice (n = 8) underwent three days of fasting and subsequent renal I/R. Animals were observed twice a day for one week to monitor survival. In addition, survival was assessed in fasted ADX mice that had been subjected to a sham I/R procedure (n = 8) (Fig. 1*B*).

Survival following renal I/R injury after mifepristone treatment

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after a 3-day fast several experiments were performed. First, either the vehicle (PBS containing 0.12% DMSO, n = 6) or mifepristone (10 mg/kg, n = 6) was injected intraperitoneally (i.p.) 30 mins prior to I/R after 3 days of fasting. Next, vehicle (n=6) or mifepristone (n=6) were administered once daily at 17.00 during the 3-day fast before renal I/R was applied. Finally, either vehicle (PBS containing 1.16% DMSO) (n = 8) or mifepristone in a ten times higher dose (100 mg/kg, n = 8) was injected daily i.p. during the 3-day fast before renal I/R was applied. To investigate the effects of mifepristone on renal I/R without preoperative fasting, vehicle (n = 6) or mifepristone (100 mg/kg, n = 6) was administered daily i.p. to ad libitum fed control mice (no ADX) during 3 days preceding I/R injury (Fig. 1*C*).

Mifepristone was administered in dosages that have been reported to effectively block all glucocorticoid receptors [11-12]. Serum corticosterone levels increase after administration of mifepristone [11] as a result of feedback inhibition of the pituitary gland. Therefore, increased corticosterone levels were used to indirectly assess blockade of the glucocorticoid receptors by mifepristone.

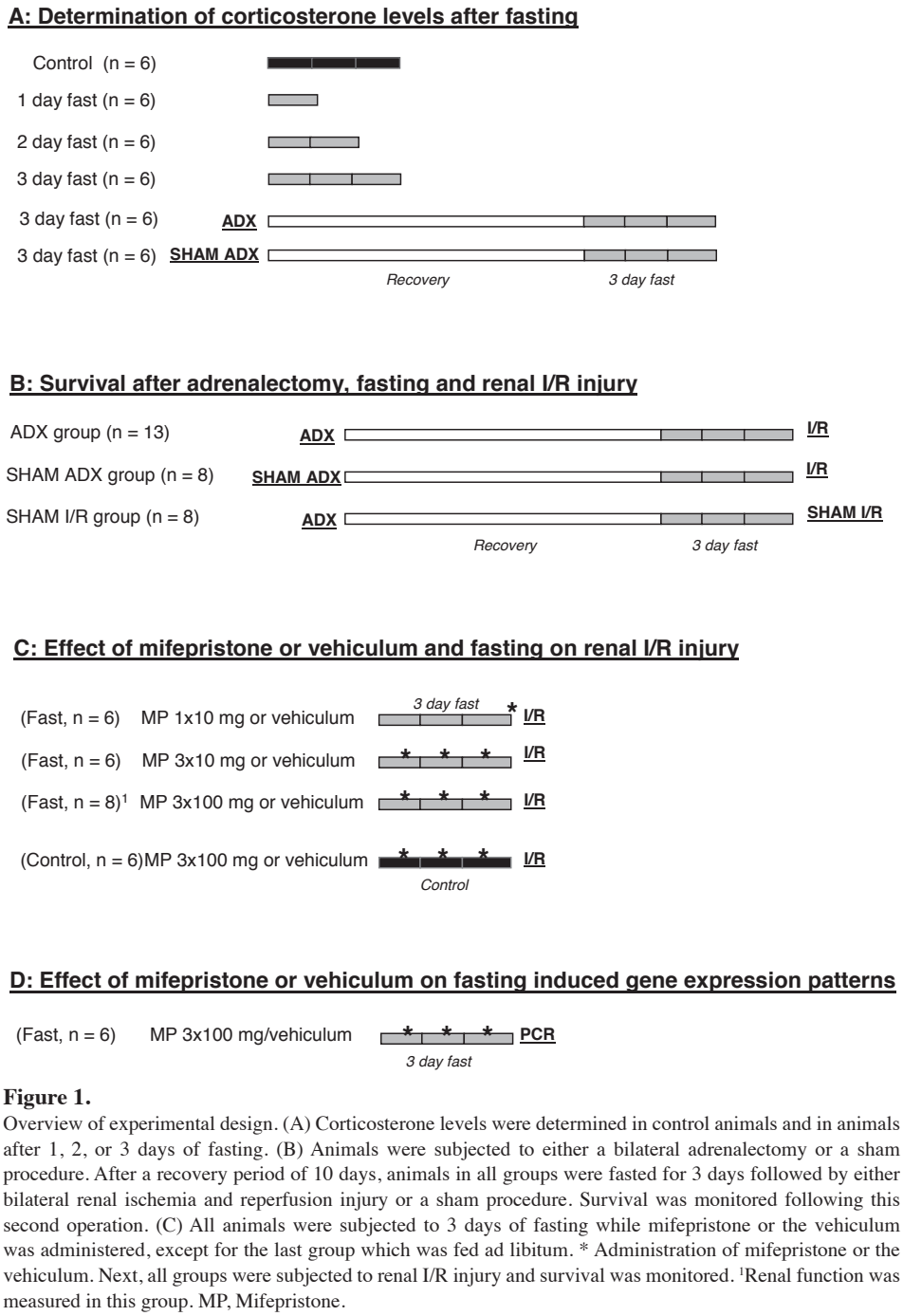

Figure 1.
Overview of experimental design. (A) Corticosterone levels were determined in control animals and in animals after 1, 2, or 3 days of fasting. (B) Animals were subjected to either a bilateral adrenalectomy or a sham procedure. After a recovery period of 10 days, animals in all groups were fasted for 3 days followed by either bilateral renal ischemia and reperfusion injury or a sham procedure. Survival was monitored following this second operation. (C) All animals were subjected to 3 days of fasting while mifepristone or the vehiculum was administered, except for the last group which was fed ad libitum. * Administration of mifepristone or the vehiculum. Next, all groups were subjected to renal I/R injury and survival was monitored. [1]Renal function was measured in this group. MP, Mifepristone.

Quantitative real-time PCR

During the 3-day fast, either vehicle (PBS containing 1.16% DMSO, n = 6) or mifepristone 100 mg/kg i.p. (n = 6) was injected daily i.p. (Fig. 1*D*). As the most robust upregulation of cytoprotective and antioxidant genes upon fasting was observed in the liver; we investigated the effect of mifepristone treatment on the expression of these genes in the liver. Livers were harvested and snap frozen in liquid nitrogen after the 3-day fast. For gene expression analysis, total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To prevent contamination by genomic DNA, the isolated RNA was purified by a DNase treatment (RQ1 Rnase-Free Dnase; Promega, Madison, WI, USA). Two μg of total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and Superscript II RT (Invitrogen) according to manufacturer's instructions. Quantitative real-time PCR was performed using a MyiQ Single-color Real-Time PCR Detection System (Bio-Rad Laboratories, Herculus, CA, USA) in combination with SYBR Green as DNA probe (Bio-Rad Laboratories). The following primers were used: B2m, forward 5'-TCACTGACCGGCCTGTATGC-3,' reverse 5'-GAGGCGGGTGGAA-CTGTGTT-3,' Hsp32/HO-1, forward 5'-GAAGGCTTTAAGCTGGTGATGG-3,' reverse 5'-CTTCGGTGCAGCTCCTCAGG-3,' SOD2, forward 5'-TCTG-GCGGGAGATGTTACAA-3,' reverse 5'-GGGCTCAGGTTTGTCCAGAAAAT-3,' GSR, forward 5'-CCGCCTGAACACCATCTAT-3,' reverse 5'-TTCCCATT-GACTTCCACCG-3,'. Relative mRNA expressions were calculated using the equation 2^ -(ΔCt, sample - ΔCt, control). Each sample was assayed in duplicate.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean ± SEM (normal distribution, assessed visually and by means of Shapiro-Wilks test) or median ± interquartile distance (no normal distribution). Means between two groups were compared using either the non-parametric Mann-Whitney *U*-test or the *t*-test for parametric data. Survival curves were compared using a log-rank (Mantel-Cox) test. A value of $p<0.05$ was considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL, USA).

RESULTS

Fasting induces increased levels of corticosterone

Baseline corticosterone levels were 298 ± 40 nmol/L. One, 2, and 3 days of fasting significantly increased corticosterone levels compared with baseline to 1135 ± 163 nmol/L ($p<0.01$), 1253 ± 234 nmol/L ($p<0.01$), and 1287±167 nmol/L ($p<0.01$), respectively (Fig.

2*A*). ADX in combination with 3 days of fasting led to significantly reduced corticosterone values of 2.6 ± 0.3 nmol/L, when compared to the sham (ADX)-operated group, who had corticosterone levels of 1186 ± 150 nmol/L ($p<0.01$) after a 3-day fasting period (Fig. 2*B*).

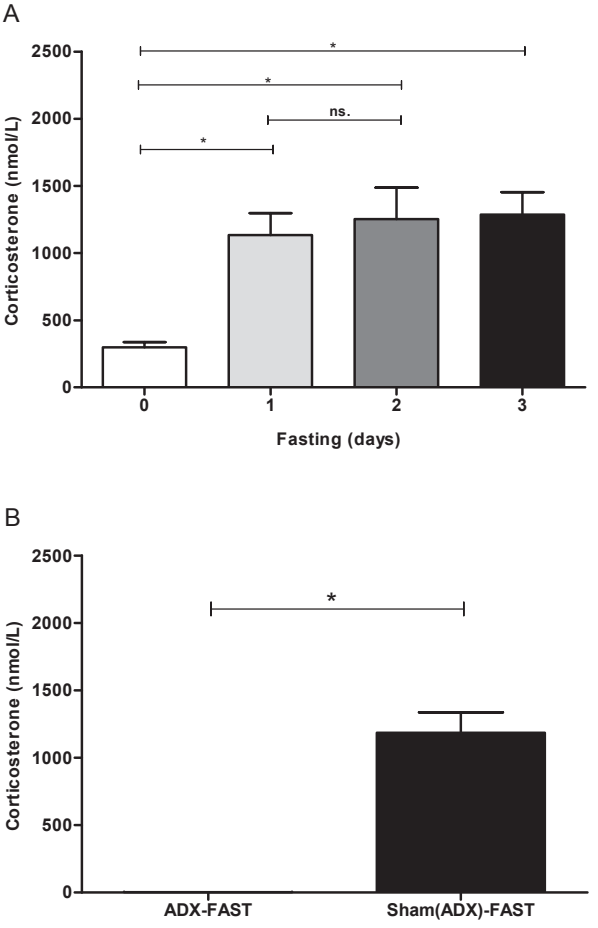

Figure 2.
(A) Corticosterone levels after fasting. Animals were fasted for 0,1,2, or 3 days after which the serum corticosterone levels were determined. Data are presented as mean ± SEM. An asterisk (*) designated a statistically significant difference between the indicated groups ($p<0.01$ for all comparisons). NS, not statistically different. (B) Corticosterone levels after adrenalectomy. ADX-FAST animals underwent an adrenalectomy 10 days prior to fasting and subsequent I/R. Sham (ADX)-FAST animals served as a control group. Animals in this group underwent a sham adrenalectomy 10 days prior to fasting. Data are presented as mean ± SEM. An asterisk (*) designates a statistically significant difference between the indicated groups ($p<0.01$).

ADX abolishes the protective effect of fasting on renal I/R injury

Mice recovered rapidly from the ADX as reflected by their return to preoperative weight on postoperative day 2. When ADX mice were subjected to a 3-day fast followed by renal I/R injury only 8% of the animals survived (Fig. 3*A*). In contrast, survival of sham (ADX)-operated mice after fasting and subsequent I/R was 100% ($p<0.01$). To determine whether the high mortality rate was resulting from I/R injury or the absence of adrenal glands, the survival of ADX mice subjected to a sham I/R procedure after 3 days of fasting was assessed (Fig. 3*B*). The 7-day survival in this group was 30%, similar to the ADX mice that had undergone renal I/R ($p=0.50$), indicating that mice are not able to withstand abdominal surgery after bilateral adrenalectomy.

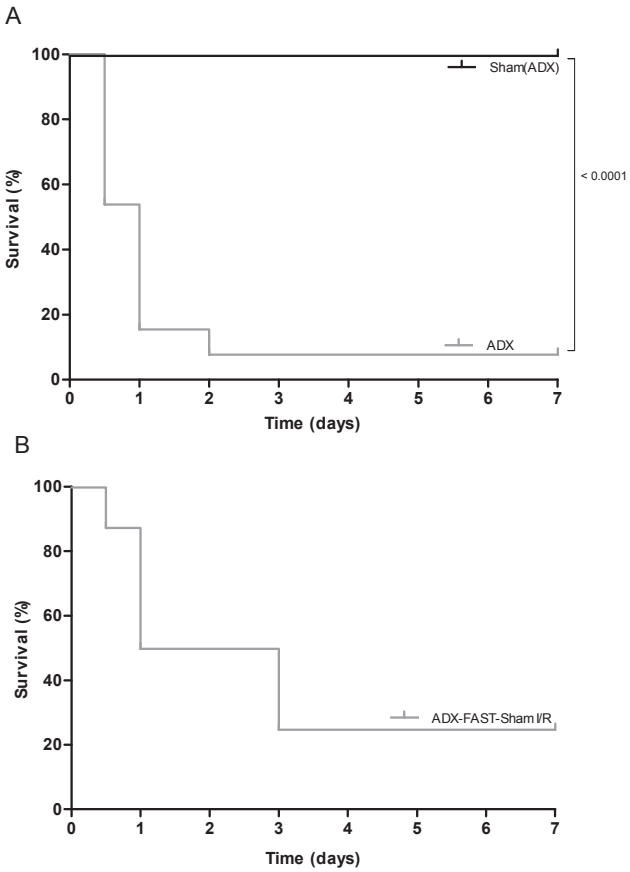

Figure 3.

(A) Survival of adrenalectomized (ADX) mice versus sham ADX mice after a 3-day fast and subsequent renal I/R injury. Survival in the sham-operated group is 100% vs. 8% in the adrenalectomized group ($p<0.01$). (B) Survival of adrenalectomized mice after a 3-day fast and subsequent sham I/R injury. Survival in the sham-operated group is 30%. This is not statistically different from the survival of the ADX group in Fig. 2A ($p=0.50$).

Glucocorticoid receptor blockade does not affect the benefits of fasting on renal I/R injury

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after and during a 3-day fast several experiments were performed. In the first experiment, mice received either 10 mg/kg mifepristone or vehicle after 3 days of fasting and 30 mins prior to renal I/R injury. In both groups survival was 100%. Subsequently, we increased the frequency of mifepristone administration to once daily during the 3-day fast preceding I/R injury. All animals survived the experiment. When a tenfold higher mifepristone dosage (100 mg/kg) was given, again all animals in the control and mifepristone groups survived I/R after the 3-day fast. Following the high dose of mifepristone, kidney function assessed by serum urea concentrations before and 24 and 48 hrs after I/R showed no differences between the two groups (Fig. 4). To rule out that mifepristone or the vehicle interfered with the renal I/R injury model, the 3-day treatment as described above was applied to ad libitum fed control mice (no ADX, only I/R injury). The survival of mifepristone and vehicle-treated mice (Fig. 5*A*) was similar to the survival of untreated (no mifepristone or vehicle), ad libitum fed mice [4]. To confirm effective glucocorticoid receptor blockade by mifepristone serum levels of corticosterone were measured. Corticosterone levels were significantly increased ($p<0.05$) in mifepristone-treated animals, confirming adequate blockade of the glucocorticoid receptors during fasting and subsequent I/R (Fig. 5*B*).

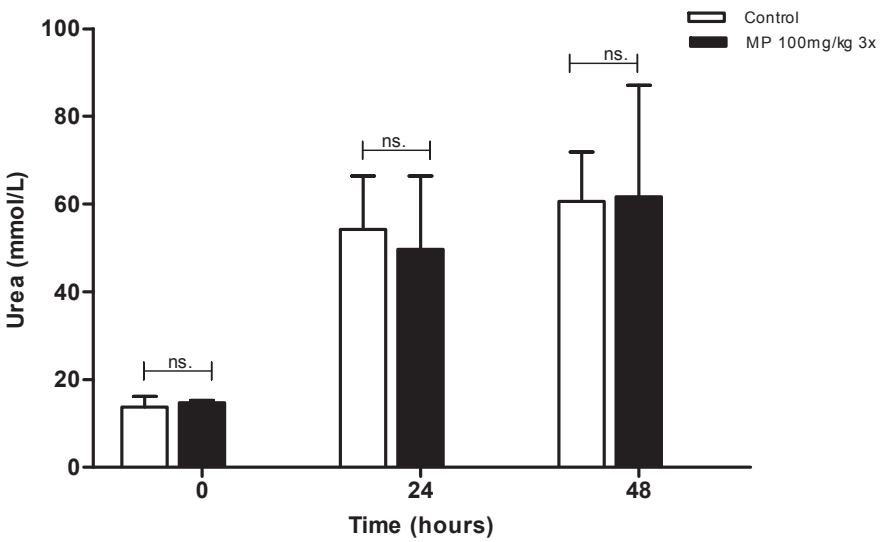

Figure 4.
Kidney function after renal I/R injury as indicated by serum urea values. Mifepristone treatment was given once daily (100 mg/kg) during the 3-day fast preceding I/R. The control group received a vehicle. There were no statistically significant differences between both groups.

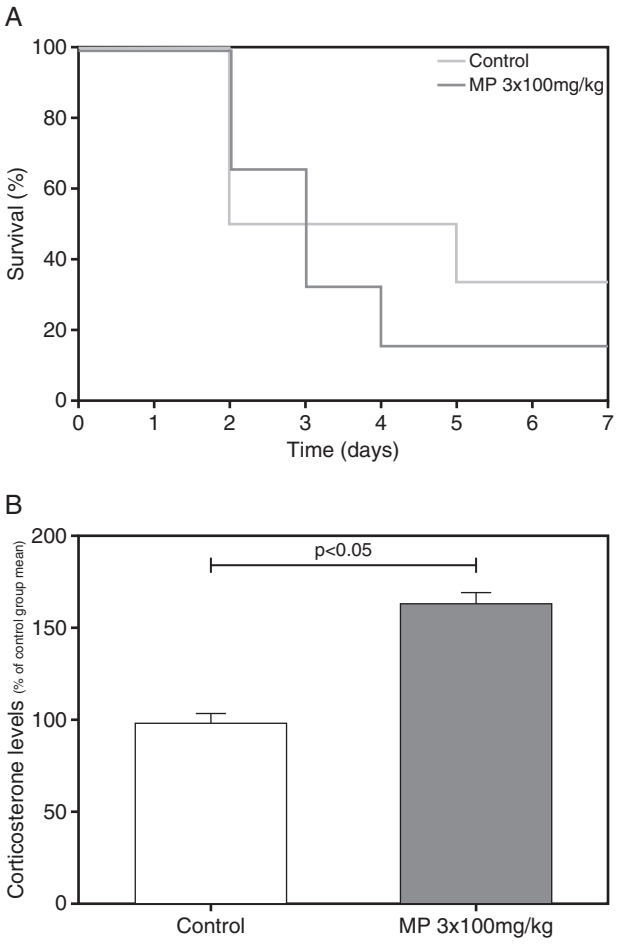

Figure 5.
(A) Survival of mifepristone-treated and vehicle-treated control animals after renal I/R injury. Mifepristone (100 mg/kg) was administered once daily, starting 3 days prior to I/R. Vehicle (PBS containing 1.16% DMSO) was administered to the control group. There was no significant difference in survival. Survival was similar to that observed in control mice without treatment[4] (data not shown). (B) Corticosterone levels of mifepristone-treated and control animals after a 3-day fast. Mifepristone (MP) (100 mg/kg) was administered once daily during the 3-day fast (n=4). After the 3-day fast, corticosterone levels were measured and expressed as a percentage of the control group. Corticosterone levels were significantly ($p<0.05$) higher in the mifepristone-treated group, when compared with the control group. This indicates total blockade of the glucocorticoid receptor.

The effect of mifepristone on fasting-induced upregulation of cytoprotective genes

We have previously shown that 3 days of fasting led to significantly higher baseline expression levels of antioxidant defense genes in the liver [4]. Here, we determined mRNA expression levels of hepatic tissue after 3 days of fasting with or without mifepristone treatment (3 days, 100mg/kg/day). No significant differences were observed in mRNA

expression levels of hemoxygenase-1, glutathione reductase, and superoxide dismutase, suggesting that corticosterone receptor inhibitor treatment did not interfere with the induction of cytoprotective and antioxidant genes by fasting (Fig. 6).

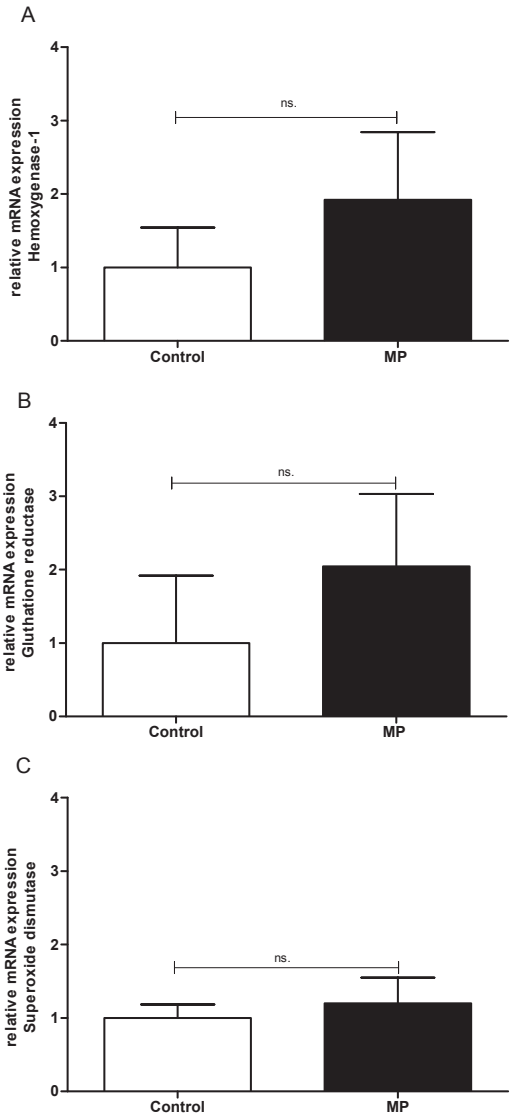

Figure 6.
Hepatic mRNA expression levels of hemoxygenase-1 (A), glutathione reductase (B), and superoxide dismutase-2 (C). Mifepristone treatment was given once daily (100mg/kg) during a 3-day fast after which the livers were harvested (n=6). The control group (n=6) received a vehicle. There were no statistically significant differences in mRNA expression between both groups.

DISCUSSION

Renal ischemia and reperfusion injury (I/R) negatively influences the outcome of kidney transplantation. Strategies to reduce I/R injury are important to improve patient survival as well as graft function and survival, as I/R is one of the main factors contributing to graft loss [3]. We have recently reported that fasting is able to protect both kidney and liver against I/R injury [4]. Current experiments were designed to investigate whether the protection afforded by fasting against I/R injury is mediated by increased levels of corticosterone. Fasting led to significantly higher levels of corticosterone when compared with ad libitum feeding [9]. Bilateral ADX was performed to investigate the effect of corticosterone on renal I/R injury. After ADX, mice exhibited higher mortality rates after I/R compared with control mice. However, survival after laparotomy in ADX mice without I/R injury resulted in similar mortality rates. These experiments did not address our hypothesis that the protection afforded by fasting is due to increased corticosterone levels.

Mifepristone, a glucocorticoid receptor antagonist, blocks the downstream signaling of the glucocorticoid receptor. The use of mifepristone therefore enables controlled studies on the effects of corticosterone on renal I/R injury without bilateral ADX. Glucocorticoid receptor blockade 30 mins prior to I/R injury did not abolish the protective effects of fasting on renal I/R injury. This suggests that either glucocorticoid receptor blockade does not interfere with the protective effects of fasting or that fasting induces its protection during the 3-day fast. The latter is supported by elevated levels of corticosterone already after 1 day of fasting. Therefore, mifepristone was administered daily during the 3-day fast. However, this regime did not affect the protective effect of fasting on renal I/R injury. Finally, a higher dosage of mifepristone was used based on previous studies [13]. Again, this regime did not abolish the protection afforded by fasting on renal I/R injury. Survival rates and kidney function were similar in both the treatment and the control group. We therefore conclude that fasting-induced protection against renal I/R injury is mediated by corticosterone/glucocorticoid receptor-independent pathways. This is partially in line with earlier reports indicating that mice subjected to social stress [13] or high physiological titers of endogenous glucocorticoids [14] exhibited exacerbated ischemic injury. In contrast, a study in rats concluded that bilateral ADX prevents renal I/R injury [15]. However, this protection is probably induced by the depletion of mineralocorticoid hormones only, as these rats were supplemented with dexamethasone, a potent exogenous glucocorticoid agonist. Administration of exogenous glucocorticoids is known to protect against cerebral [16], cardiac [17-18], and renal I/R injury [19]. In addition, clinical studies have shown that donor pre-treatment with steroids significantly decreased tissue (liver) and serum expression of proinflammatory cytokines [20] after I/R injury. A recent prospective randomized study investigated the effects of

donor pretreatment with methylprednisolone on organ function and outcome after liver transplantation. The use of steroids significantly reduced I/R injury and inflammation and improved graft function [21]. We did not administer exogenous glucocorticoids in our model because they are already known to improve I/R injury and because our hypothesis predicted the involvement of endogenous steroids.

If increased levels of endogenous corticosteroids do not mediate the protective effects of fasting, the question remains which mechanisms do contribute to the induced protection. In previous experiments we have shown that 3 days of fasting lead to significantly higher expression levels of cytoprotective and antioxidant defense genes in the kidney and liver [4, 22]. The strongest response to fasting was observed in the liver; therefore we investigated the effect of mifepristone treatment on the expression of these cytoprotective and anti-oxidant genes in the liver. This study demonstrated that mifepristone treatment did not interfere with the upregulation of antioxidant defense systems. It would be interesting to investigate whether exact mimicking of corticosterone induction by fasting, by corticosteroid administration, would be able to increase the expression of cytoprotective genes as well. However, as it is difficult, if not impossible, to duplicate the physiological response to fasting, we have not performed these additional experiments. Together, these data support a hypothesis that the up-regulated expression of these genes was instrumental in the protection afforded by fasting against I/R injury, but that these changes are independent of corticosterone. Future experiments are warranted to investigate the relation between these fasting-induced changes in gene expression patterns and I/R injury.

CONCLUSIONS

Our data demonstrate that fasting increases serum corticosterone levels. However, the protective effect of fasting on I/R injury is induced independently of corticosterone levels and glucocorticoid receptor availability. The upregulation of antioxidant genes is independent of the availability of the glucocorticoid receptor. The latter may represent an important clue to elucidate the mechanisms by which fasting affords protection against I/R injury.

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VII

Preoperative dietary restriction reduces hepatic tumor load by reduced E-selectin-mediated adhesion in mice

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ABSTRACT

Background: Inflammatory responses facilitate metastases by increasing expression of adhesion molecules. Dietary restriction (30% reduction in daily calorie intake) reduces the expression of adhesion molecules and protects against surgically induced inflammation. DR might therefore beneficially interfere with surgery-induced inflammation and subsequent adhesion of circulating tumor cells.

Methods: BALB/c mice were subjected to 2 weeks dietary restriction prior to inoculation with tumor cells. Intrasplenic injection of 5.0 x 10^4 C26-colon carcinoma cells was followed by splenectomy. Hepatic tumor load was scored after 10 days as a percentage (tumor surface/total liver surface) on haematoxylin and eosin stained sections. Liver mRNA expression of adhesion molecules was determined and the effect of serum from dietary restriction mice on *in vitro* tumor growth and adhesion capacity was assessed.

Results: Preoperative dietary restriction significantly reduced mRNA expression levels of E-selectin (p=0.0087) and hepatic tumor load (p=0.036). Dietary restriction serum did not affect *in vitro* cell growth but reduced *in vitro* adhesion of C26 cells to endothelial cells (p=0.0043).

Conclusions: Preoperative dietary restriction reduces hepatic tumor load after injection with tumor cells. Reduced adhesion to endothelial cells and reduced mRNA expression of E-selectin suggest that dietary restriction reduces tumor load by lowering the adhesion of circulating tumor cells to hepatic vascular endothelium.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide, with a cumulative lifetime risk of approximately 5% in the United States. Each year approximately 150,000 patients present with colorectal cancer and over 55,000 deaths are attributed to this disease [1]. In addition, in Europe almost 200,000 new cases of colorectal cancer occur every year [2] and this incidence is estimated to increase with 45% in the next two decades [3]. Surgical resection of the primary tumor remains the treatment of choice. Unfortunately, 30-50% of all patients undergoing curative resections subsequently develop either a local recurrence or distant metastases, predominantly in the liver, resulting in increased mortality [4]. Most recurrences are observed within 2 years after an operation. It is hypothesized that viable circulating tumor cells (CTC) play an important role in the pathogenesis of distant metastases. CTC were first detected in colorectal cancer patients more than 50 years ago [5] and are mainly detected in portal blood [6]. Recently, a meta-analysis showed a significantly increased hepatic metastases rate of 21% in CTC-positive patients compared with 8% in negative patients which emphasizes the influence of CTC on hepatic metastases formation [7]. Surgery increases the number of CTC due to tumor handling. In addition, it enhances the metastatic potential of preexisting or intra-operatively spilled CTC due to several factors. First, surgery inevitably leads to tissue trauma which evokes an inflammatory reaction with elevated levels of local and systemic proinflammatory cytokines. These cytokines subsequently result in the up-regulation of adhesion molecules, such as E-selectin, on liver endothelial cells, which may promote metastases outgrowth by facilitating tumor cell adhesion. Secondly, the induction of a pronounced immunosuppressive period after major surgery may impair the innate effector cell function of Kupffer cells and natural killer cells. These cells have an important role in eradication of tumor cells retained in the liver vasculature. Impairment of their activity may result in an increased risk of hepatic metastases development [8-10].

Several interventions have demonstrated their beneficial effects on perioperative tumor metastases, that is, the "no touch technique" [11, 12], the use of immunosuppressive drugs to blunt the proinflammatory cytokine response [13], and blockade of alpha2 integrins on tumor cells to reduce adhesion on endothelial cells [8]. The perioperative period may provide a window of opportunity in which the adhesion and outgrowth of circulating tumor cells in the liver can be reduced, leading to less metastatic lesions and possibly lower patient morbidity and mortality rates.

We investigated the effect of preoperative dietary restriction (DR) on perioperative adhesion and outgrowth of CTC. DR, reduced food intake without causing malnutrition, is associated with extended longevity [14] and reduced cancer incidence [15-19]. Short-term preoperative DR for 1 week reduces angiogenesis and growth in a mouse brain tumor model [20]. Recently, we reported that short-term DR prior to both renal

and hepatic ischemia and reperfusion injury reduces the expression of pro-inflammatory cytokines and adhesion molecules [21]. Here, we used a murine model to determine the effect of short-term preoperative DR on tumor cell adhesion and hepatic tumor load after inoculation with tumor cells. We demonstrate that preoperative DR reduces hepatic tumor load. Furthermore, we demonstrate that a 2-week DR regimen reduces the hepatic expression of the endothelial cell specific adhesion molecule, E-selectin. *In vitro*, serum from DR mice was able to reduce the adhesion of tumor cells to endothelial cells. Our results indicate that DR might be a valuable addition to the multimodality treatment of patients with colorectal malignancies.

MATERIALS AND METHODS

Animals

Male BALB/c mice (± 25 gram) were purchased from Charles River (Maastricht, The Netherlands). Mice were housed separately under standard laboratory conditions and allowed to acclimatize for 1 week. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

Dietary restriction

After 1 week of acclimatization, food intake was measured daily during 1 week. Thereafter, mice were randomized to either the control or the experimental group. Control mice were fed standard rodent chow (SDS, Hope Farms, Woerden, The Netherlands) *ad libitum* (= AL group). Experimental mice received only 70% of the daily caloric intake by means of standard rodent chow leading dietary restriction (= DR group). Mice were subjected to 2 weeks of DR prior to the surgical intervention. Postoperatively, all mice were allowed *ad libitum* access to food.

Cell culture

The murine colon carcinoma cell line C26 (kindly provided by Dr. R. Schiffelers, Utrecht University, The Netherlands) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 U/mLl) in a 5% carbon dioxide environment. Near confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% ethylenediamine tetra-acetic acid (EDTA)). For the surgical procedure, cells were harvested and after centrifugation, single-cell suspensions were prepared in phosphate buffered saline (PBS) to a final concentration of 5.0×10^4 cells/100 µL or 10.0 x 10^4 cells/100 µL. Cell viability was determined by trypan blue staining, and was always at least 98%.

Induction of circulating tumor cells

For induction of hepatic tumor growth mice were anaesthetized with isoflurane inhalation. Surgical procedures were performed under aseptic conditions. Body temperature was maintained by placing the mice on heating pads. Following a left lateral flank incision, the spleen was localized and C26 colorectal carcinoma cells were injected into the splenic parenchyma (total volume 100μL, n = 6 per group). This experiment was divided into two sub-experiments. In sub-experiment 1: 5.0×10^4 cells were injected, in sub-experiment 2: 10.0 x 10^4 cells were injected intra-splenically. After 10 mins, the spleen was removed to prevent intra-splenic tumor growth. Single tumor cells reach the liver through the portal vein, where a subset grows out to form intrahepatic micrometastases. Metastases were allowed to develop for 10 days. Morphological assessment of tumor growth was performed on right lower liver lobes harvested 10 days after tumor induction.

Determination of hepatic tumor load

Intra-hepatic tumor load was scored as the percentage of hepatic tissue that has been replaced by tumor cells (hepatic tumor percentage, HTP). Digital images were captured from two non-sequential hematoxylin-eosin–stained sections of the right lower liver lobe using virtual microscopy (NanoZoomer, Hamamatsu photonics, Hamamatsu Cit, Japan). Using specific software (NanoZoomer Digital Pathology, NDP) the HTP ratio was determined by two independent observers blinded to treatment (Fig. 1). We obtained <5% intra- and inter-observer variability. The mean HTP per slide was used to compare the AL-group and the CR-group.

Serum collection for *in vitro* assays

Blood was obtained from mice after 2 weeks DR or AL access to food by means of cardiac puncture under general anesthesia. Serum was stored at -80ºC until further analysis, without pooling of the serum.

***In vitro* growth curves**

C26 colon carcinoma cells were cultured in DMEM containing 10% fetal calf serum and 200 U/mL penicillin/streptomycin. Cells were suspended in serum-free DMEM containing a concentration of 5.25 x 10^4 cells/mL. Subsequently, 10% mouse serum obtained from individual AL or DR mice was added to reach a final concentration of 5.0 x 10^4 cells. These cells were plated in triplicate on 5 different 96-wells plates. Plates were analyzed 6, 24, 48, 72, and 96 hrs after seeding. Cell proliferation was determined by a colorimetric assay using tetrazolium salt (XTT**,** Sigma- Aldrich). XTT was dissolved in DMEM until a final concentration of 1.0 mg/ml was obtained. N-methyl dibenzopyrazine methyl sulfate (PMS, Sigma-Aldrich) was added to the XTT solution to achieve a final

concentration of 7.6 μg/ml. Subsequently, 50 μL of the XTT-solution was added to the experimental wells followed by 1 hr incubation at 37ºC. Absorbance of the samples was measured spectrophotometrically (ELISA plate reader, Victor, Perkin Elmer, Groningen, The Netherlands) and cell content was expressed as the optical density at wavelength of 490 nm.

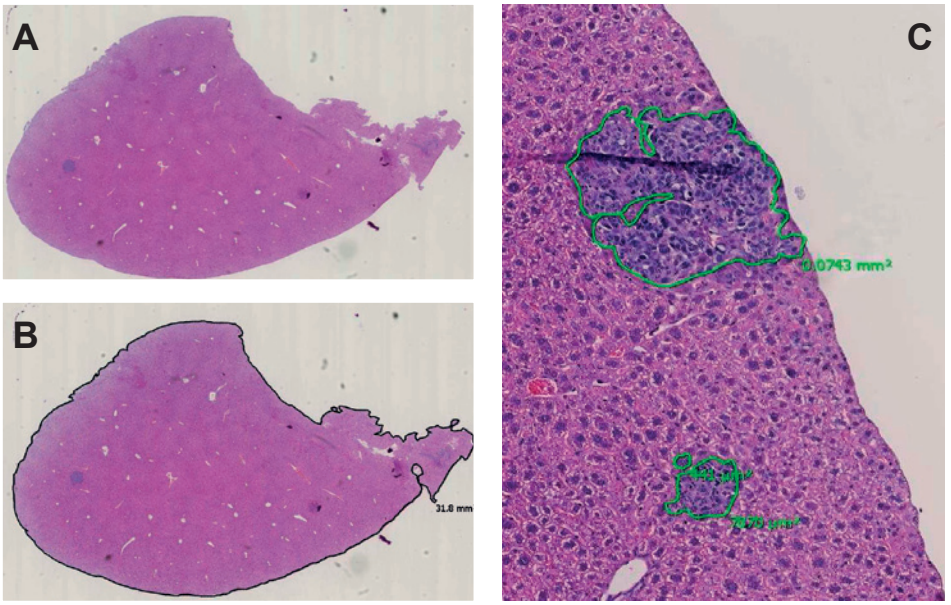

Figure 1. Histological analysis of tumor load in liver tissue.
Hepatic tumor growth was induced in BALB/c mice by intrasplenic injection of C26 tumor cells. Histopathologic analyses were performed on hematoxylin and eosin-stained liver sections. Digital images of stained sections were captured using a virtual microscopy system. Intra-hepatic tumor load was determined as the percentage of hepatic tissue that has been replaced by tumor cells. (A) Microscopic appearance of a liver section (magnification 2x). (B) Manual indication of the total liver section surface using the computerized system. Surface indicated in black. (C) Microscopic appearance of C26 tumor areas present in liver tissue using the computerized system, tumor indicated as green (magnification 20x).

In vitro adhesion assay

Human umbilical vascular endothelial cells (HUVEC) (kindly provided by dr. A. Seynhaeve, Erasmus MC, University Medical Center, Rotterdam, The Netherlands) at passage 2 were maintained in EGM-2-MV Bullet kit medium (Sigma-Aldrich). Confluent monolayers were passaged by 0.025% trypsin/0.01% EDTA and cells were used up to passage six. C26 colon carcinoma cells were cultured as described earlier. To quantify C26 tumor cell adhesion to HUVEC, a standardized cell adhesion assay was used as described previously [22]. Briefly, endothelial monolayers were established in 96-well microtiter plates (Perkin Elmer). Confluent HUVEC were trypsinized and 2×10^4 endothelial cells

were plated in each well followed by incubation at 37°C, 95% relative humidity, 5% CO_2. Medium was daily replaced by fresh medium until HUVEC reached confluence in 3-4 days, confirmed by light microscopy.

To quantify tumor cell adhesion, trypsinized tumor cells (1×10^6 cells/ml) were labeled with calcein-AM (Molecular Probes, The Netherlands, Leiden) and 3×10^4 C26 cells were added to the HUVEC monolayer in the presence of 10% mouse serum, obtained from individual AL or CR mice. Assays were performed in triplo. Thereafter plates were centrifuged for 1 min at 80*g* in a Heraeus centrifuge and incubated at 37°C for 1 hr. After this, wells were washed twice with medium to remove non-adherent tumor cells. The remaining fluorescence per well was measured on a Perkin Elmer plate reader using 485 nm excitation and 530 nm emission filters.

Real time quantitative PCR

RNA was isolated from liver tissue obtained after 2 weeks of 30% DR or *ad libitum* access to food. For gene expression analysis, total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To prevent contamination by genomic DNA the isolated RNA was purified by a DNase treatment (RQ1 Rnase-Free Dnase; Promega, Madison, WI, USA). Two micrograms of total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and Superscript II RT (Invitrogen) according to manufacturers instructions.

E-selectin mRNA expression level was determined by real-time quantitative PCR (RT-PCR) using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA) and quantified by normalization against ABL as previously [23]. Each sample was tested in duplicate. All values were normalized to the mean relative expression calculated for the AL group, which was assigned a value of 1.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean ± SEM (normal distribution, assessed visually and by means of Shapiro-Wilks test) or median ± interquartile distance (no normal distribution). Means between two groups were compared using either the non-parametric Mann-Whitney *U*- test or the *t*-test for parametric data. Mixed models are used to analyze repetitive measurements. P-values of <0.05 were considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL, USA).

RESULTS

Dietary restriction

In both groups mean daily food intake was 4.0 gram (95% CI 3.9-4.1). Dietary restriction was performed by reducing the intake to 70% of the *ad libitum* intake, which is 2.8 gram per day/mouse. During dietary restriction, the intake of the control group remained constant (Fig. 2*A)*. Weight loss, an objective measurement of decreased caloric intake, was 15.6 ± 3.3% in the intervention group during DR, while the control group gained 5.0 ± 1.3% bodyweight during the same period (Fig. 2*B)*. After intra-splenic tumor injection, all mice were allowed to eat *ad libitum*, resulting in "catch up" intake of the DR group (Fig. 2*A*).

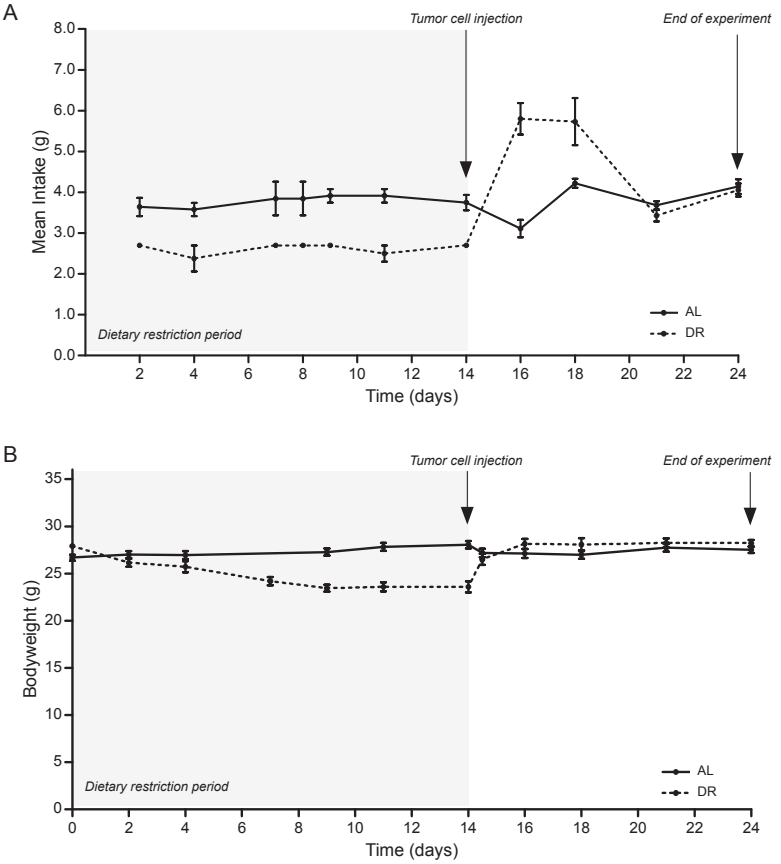

Figure 2. Food intake and bodyweight during the experimental period.
Mice were fed ad libitum or dietary restricted to 70% of the daily caloric intake during 14 days prior to surgery. After intrasplenic injection all mice were allowed to eat ad libitum. (A) Daily food intake was monitored from the onset of diet untill the mice were sacrificed. In mice randomized to dietary restriction a 70% caloric intake was achieved during the dietary period. After surgery caloric intake showed a rapid increase in mice subjected to dietary restriction. (B) Mean body weight was monitored during the experiment. All mice regained their predietary weight within 2 days after surgery.

Hepatic tumor load

We examined whether preoperative dietary restriction affected hepatic tumor load after inoculation with CTC. Therefore, 5.0 x 10^4 tumor cells were injected intrasplenically. In mice who underwent DR prior to tumor inoculation hepatic tumor load was significantly reduced to 0.11% as compared to 0.62% in the control group ($p<0.05$) (Fig. 3*A*). Next we performed the experiments by injection of a larger tumor volume (1.0 x 10^5) to increase the number of CTC. In both control and DR mice higher tumor loads were found (Fig. 3*B*). Although hepatic tumor load in DR mice was consistently lower this did not reach statistically significance ($p=0.41$). Collectively these data suggest that preoperative DR is able to reduce hepatic tumor load after inoculation with CTC.

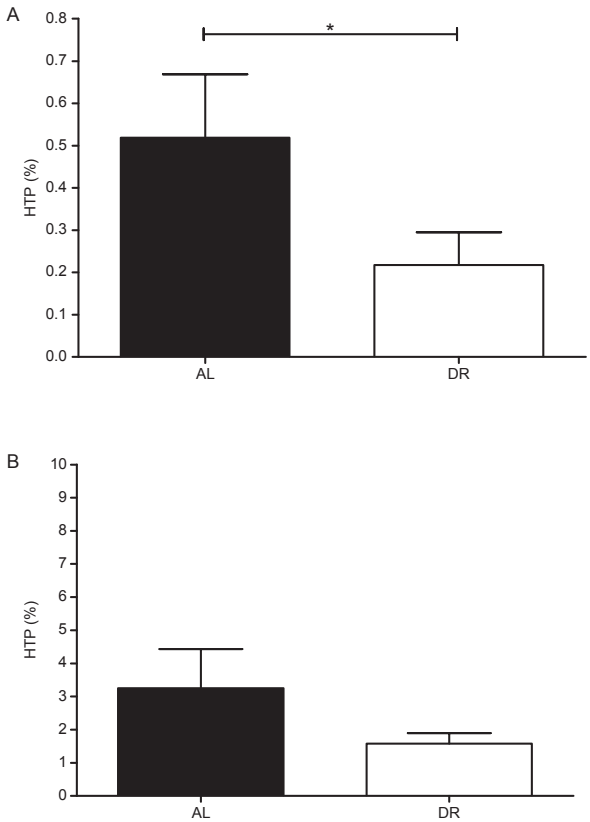

Figure 3. Effect of caloric restriction on hepatic tumor growth.

Hepatic tumor growth was induced in BALB/c mice by intra-splenic injection of tumor cells followed by splenectomy. Mice were fed ad libitum or dietary restricted to 70% of the normal daily caloric intake during 14 days prior to surgery. Intra-hepatic tumor load was determined in liver sections obtained 10 days after surgery. (A) Caloric restriction was associated with reduced intra-hepatic tumor load after intra-splenic injection of 5.0 x 10^4 tumor cells. (B) Caloric restriction did not reduce intra-hepatic tumor load after intra-splenic injection of 1.0 x 10^5 tumor cells. (n = 2 sections per mice, 5 mice/group, *$p<0.05$). Data are presented as median ± interquartile distance.

In vitro experiments

To determine the effect of dietary restriction on the *in vitro* growth rate of C26 colon carcinoma cells, we evaluated the effect of serum obtained from *ad libitum* or DR mice on *in vitro* growth curves of C26 colon carcinoma cells (Fig. 4*A*). Serum obtained from both groups showed no significant differences on the *in vitro* growth rates of C26 cells. Next, we evaluated the effect of DR on adhesion of C26 colon carcinoma cells to endothelial cells *in vitro*. Therefore, we determined the effect of serum from either DR or AL mice on the capacity of tumor cells to adhere to a HUVEC monolayer. C26 cells in the presence of DR serum displayed a significantly ($p<0.01$) reduced capacity to adhere to HUVEC as compared to C26 cells in the presence of AL serum (Fig. 4*B*).

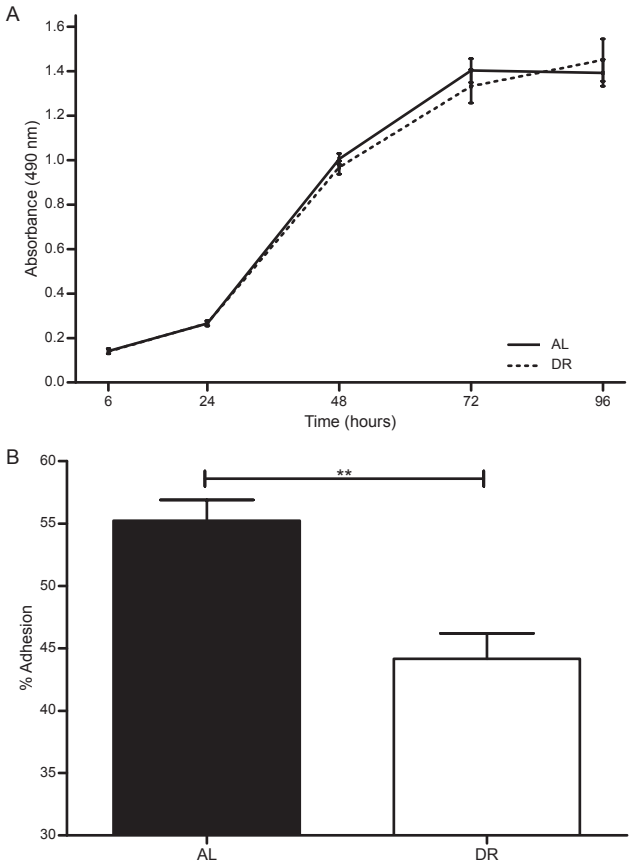

Figure 4. Effect of caloric restriction on *in vitro* tumor cell growth and adhesion.
(A) C26 colon carcinoma cells were cultured in medium combined with serum obtained from ad libitum or dietary restriction mice. Dietary restriction did not affect *in vitro* growth of C26 colon carcinoma cells. (B) We used an *in vitro* adhesion assay to determine the effect of serum obtained from dietary restriction mice on adhesion of C26 tumor cells to a HUVEC monolayer. Dietary restriction was associated with reduced adhesion of C26 tumor cells to HUVECs (**$p<0.01$).

Real-time Quantitative PCR

The endothelial cell specific adhesion molecule E-selectin has an important role in the process of tumor cell adhesion to endothelial cells. Therefore, we examined the effect of DR on hepatic E-selectin mRNA expression levels. DR resulted in a significant ($p<0.01$) reduction of E-selectin mRNA expression (Fig. 5).

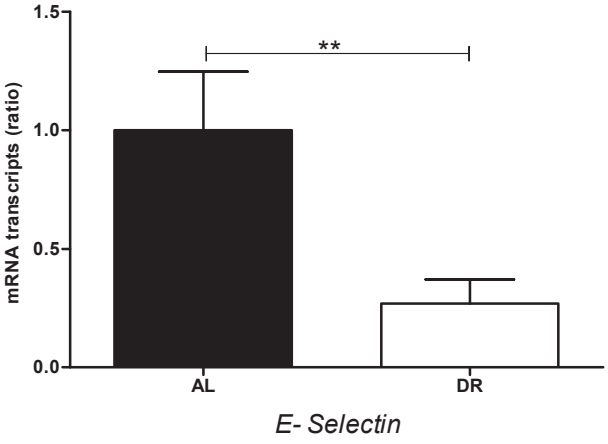

Figure 5. Effect of caloric restriction on hepatic E-selectin mRNA expression.
Mice were fed ad libitum or dietary restricted to 70% of the normal daily caloric intake during 14 days prior to surgery. Hepatic tissue was obtained after 14 days of dietary restriction or ad libitum diet. Relative hepatic E-selectin expression level was determined. Dietary restriction was associated with reduced E-selectin expression level. (**$p<0.01$).

DISCUSSION

For most patients with colorectal malignancies, surgical resection is the cornerstone of any potentially curative treatment. Surgical trauma results in systemic inflammation as reflected by cytokine release [24, 25] and in postoperative cellular immunosuppression [26]. There is emerging evidence suggesting that these surgery-induced processes facilitate tumor metastases [27]. Furthermore, surgical procedures induce hematogenic tumor cell dissemination as reflected by increased circulating tumor cells (CTC) present during surgical procedures [9]. The importance of CTC is underlined by an increased hepatic metastases rate in CTC-positive patients, when compared to CTC-negative patients [7]. There are two major schools of thought regarding tumor cell metastases and extravasation. On the one hand, it is believed that CTC arrest in narrow capillaries is due to size restriction, on the other hand adhesion of CTC to the microvascular endothelium is considered one of the most important steps [28]. Preventing adhesion of CTC to the endothelium of distant organs during the perioperative period may be a potential effective

treatment to reduce metastases rates after curative surgery. In the current study, we show that preoperative dietary restriction lowers the expression of E-selectin in the liver reduces hepatic tumor load after exposure to CTC.

Selectins mediate tethering, rolling, and adhesion of several types of cells. E-selectin, an endothelial cell specific adhesion molecule, is expressed de novo on endothelial cells, such as liver endothelial cells, after transcriptional induction by proinflammatory cytokines [29]. These activated endothelial cells express E-selectin, which mediates tumor cell adhesion and subsequent liver metastases [29]. Here, we demonstrate that dietary restriction (DR) reduces mRNA expression of E-selectin and hepatic tumor load. It is known that tumor cells trigger the induction of E-selectin expression on endothelial cells [30]. We show that serum obtained from DR mice reduces *in vitro* adhesion of C26 colon carcinoma cells to HUVEC. Although we do not show a direct correlation, it has already been demonstrated that E-selectin expression plays a crucial role in the process of liver metastases formation in the murine BALB/c-C26 colon carcinoma cell model as direct blockage of E-selectin was associated with lower numbers of liver metastases [31].

The protective effect of DR on HTP was statistically significant after inoculation with 5.0×10^4 cells; while only a trend was observed after injection of 1.0×10^5 cells. These data suggest that if an overwhelming amount of tumor cells has been injected the positive effect of DR on CTC adhesion is blunted. However, this situation is unlikely to be encountered in the clinical situation as the amount of CTC is much lower (1-10 CTC per 7.5 mL blood) [32] than the supra-physiological amounts of CTC used in our model. The model does not fully represent the clinical situation, as a primary tumor is absent and the level of (pre-) and post-operative inflammation may be different. But in this model, where higher concentrations of CTC than ever encountered in the clinical situation are induced, a beneficial effect of DR is observed.

Although a reduced HTP was observed after DR, we cannot rule out that the difference in postoperative calorie intake contributes to this reduction. However, we assume this unlikely as the adhesion assay and hepatic E-selectin mRNA expression level were performed with serum samples obtained directly after DR, thus unaffected by postoperative calorie intake. Translation of preoperative DR to the clinical setting also poses a challenge. Although in literature periods of much longer than 2 weeks 30% DR have been reported [33]. We must take into account that several patients suffering from colorectal disease may be malnourished. Interestingly, a diet consisting of protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice as well [34]. Although the magnitude of these increases is around 30–40% of that of DR, neither carbohydrate [35] nor lipid restriction [36, 37] exerted these effects. Restriction of proteins could therefore be another way to induce the beneficial effects

seen after DR and overcome the problem of reducing calorie intake. In addition, the use of DR mimetics may be a way to overcome the problems associated with DR in surgical patients. A DR mimetic can be loosely defined as any pharmacological intervention that produces beneficial effects of DR without causing or requiring a significant reduction in calorie intake. In clinical practice, a DR mimetic might be a powerful addition to standard cancer treatment. One compound that has received considerable attention as DR mimetic is resveratrol, a naturally-occurring polyphenol found in red wine. Resveratrol induces, at doses that can be readily achieved in humans, genomic changes which resemble many of the genetic alterations induced by DR [38]. Furthermore, evidence supporting the use of resveratrol in the treatment of malignancies is emerging [39-41].

The question remains why DR lowers E-selectin expression. We recently reported that DR robustly down regulates the production of proinflammatory cytokines and adhesion molecules in models of renal and hepatic ischemia-reperfusion injury. In addition, DR induced the expression of cytoprotective and anti-oxidant genes, leading to a reduced formation of reactive oxygen species [42]. As surgical trauma causes oxidative stress [43, 44], the increased protection against oxidative stress and the subsequently reduced inflammatory response, may explain why lower levels of E-selectin are encountered. Microarray analyses are currently being performed, aiming to elucidate how DR induces this protective response. In addition, future experiments need to identify the optimal regimen of DR, in terms of percentage of DR and duration. These should focus on combining the beneficial effects of preoperative fasting, which protects against the side effects of chemotherapy [45, 46], with those of DR found in the present study, to a regimen that induces the protection against both.

CONCLUSIONS

In conclusion, our data demonstrate that preoperative DR is able to reduce hepatic tumor load 10 days after inoculation with CTC. This beneficial effect appears to be mediated by reduced vascular E-selectin expression and a subsequent decreased tumor cell-endothelial cell adhesion, as lower E-selectin levels were related to less hepatic metastases. This may be a mechanism by which DR inhibits hepatic metastases. Therefore, DR may provide a new strategy in the multimodality treatment of patients with colorectal cancer.

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VIII

General discussion

Inflammation plays a major role in the pathophysiology of several severe conditions such as sepsis, renal ischemia-reperfusion (I/R) injury, and cancer. Mortality and morbidity related to these illnesses remain a large problem, which warrants studies on new therapeutic approaches targeting the inflammatory response.

Up to now, numerous immunomodulatory approaches have been investigated in experimental animal models of lipopolysaccharide (LPS)-induced septic shock, cecal ligation and puncture (CLP)-induced polymicrobial sepsis, colon ascending stent peritonitis (CASP), renal ischemia-reperfusion (I/R) injury, and hepatic tumor metastases growth. The research described in this thesis was initiated to increase the knowledge on both the effects and the mechanistic aspects of two novel immunomodulatory therapeutic approaches, namely oligopeptides related to the primary structure of the β-chain of human Chorionic Gonadotropin (β-hCG) and dietary restriction (DR), in animal models of sepsis, renal I/R injury, and hepatic tumor metastases. In addition, the frequently used anti-inflammatory agent dexamethasone was used to gain insight into the optimum treatment dose for modulation of the sepsis-related inflammatory response.

8.1 SEPSIS AND TREATMENT: WHAT DO WE LEARN FROM ANIMAL MODELS?*

Septic shock is a complex syndrome that results from an insufficient and dysregulated immune response to invading pathogens [1]. Different insults, such as primary infections, burns, trauma, and hemorrhagic shock, can progress toward septic shock, acute lung injury, renal failure, and multiple organ dysfunction syndrome (MODS) [2]. During the last decades the incidence of sepsis, sepsis-related hospitalization, and sepsis-related mortality substantially increased [3, 4], emphasizing the need for new therapeutics. Despite advances in our understanding of the immunobiological processes involved, many immunoregulatory therapies that were successful in experimental sepsis failed in clinical sepsis. Here, we discuss the immunobiology of the septic response, with special emphasis on immune regulation and potential novel therapeutic targets. We also discuss critical factors that need to be considered to facilitate successful translation of immunomodulatory therapies from animal studies to the human setting.

PATHOPHYSIOLOGY

Sepsis provokes an inflammatory response that is initially characterized by excessive release of pro-inflammatory mediators relative to anti-inflammatory mediators, which

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is defined as the systemic inflammatory response syndrome (SIRS) (Fig. 1) [2, 5]. As a result of this hyperinflammatory state, hypoperfusion, tissue hypoxemia, MODS, and death can occur. However, when sepsis persists, a progressive immunosuppressive response, characterized by increased production of anti-inflammatory mediators relative to pro-inflammatory mediators as well as apoptosis can occur [5-8]. Eventually, this may develop into the compensatory anti-inflammatory response syndrome (CARS) (Fig. 1), characterized by immunoparalysis and subsequent failure to clear the primary infection, and increased susceptibility to new infections [6, 8]. Although septic patients may die during the uncontrolled hyperinflammation, most deaths occur during the immunosuppressive phase [6, 7, 9]. These findings have contributed to the consensus that a well controlled balance between the pro-inflammatory and anti-inflammatory responses is vital for surviving sepsis.

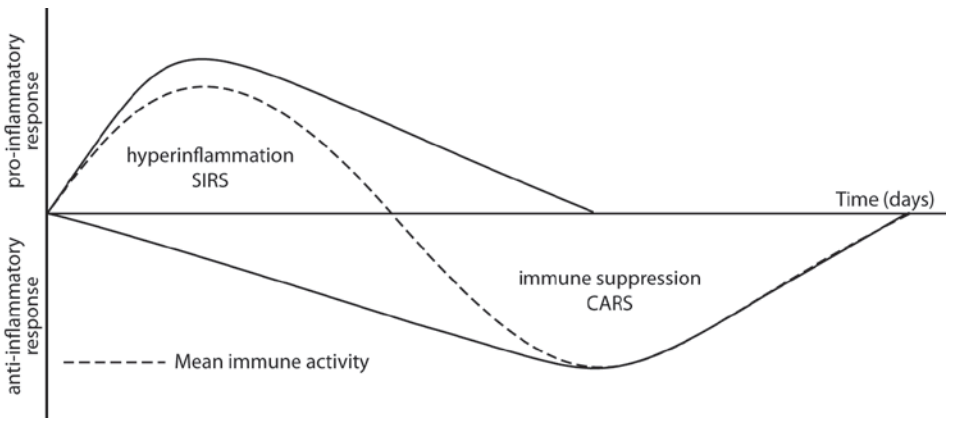

Figure 1. The inflammatory response in sepsis over time.
Concomitant with the development of a pro-inflammatory response which results in the systemic inflammatory response (SIRS), an anti-inflammatory response, which eventually results in the compensatory anti-inflammatory response syndrome (CARS), begins. First, the SIRS phase predominates but as sepsis progresses CARS becomes predominant. The balance between pro-inflammatory mediators and anti-inflammatory mediators determines the inflammatory status of each individual.

Much of the insights into the pathophysiology of sepsis and septic shock have been obtained by the use of animal models. Many models for sepsis and septic shock exist, including injection of live or dead bacteria, intravenous infusion of endotoxin (e.g. LPS), the administration of fecal material or live micro-organisms into the peritoneal cavity, the placement of foreign material into the soft tissue of extremities, and damaging the gastrointestinal tract [10-12]. Although models involving single organisms or for instance LPS are useful to characterize response patterns and to evaluate new treatment modalities, they are of dubious relevance to clinical sepsis, which is commonly polymicrobial,

encompassing gram negative and gram positive bacteria, as well as aerobic and anaerobic species [10]. Moreover, infection leading to sepsis typically begins as a focus of infection that subsequently becomes systemic rather than originating in the periphery *de novo*. Also, in the context of trauma, the bacteria causing the sepsis often originate from the individual's own flora finding their way into puncture wounds, gut perforation, or microbial translocation from the gut. The CLP model has the advantage that it has a focal infection origin, the microbes involved are mixed and of host origin, septicemia develops over a period of time that allows the animal to respond to the insult, the involvement of necrotic (gut) tissue, and can show a full spectrum of sepsis severity, ranging from acute to chronic [10]. Because the LPS model is often used to examine new therapeutics and the CLP-model is regarded as the gold-standard sepsis animal model as its mimics human sepsis [12], we refer in this review mostly to studies that used these models.

Innate immunity and inflammation during early sepsis

Pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and nucleotide binding oligomerization domain (NOD)-like receptors (NLR), recognize conserved microorganism-expressed molecular patterns (pathogen-associated molecular patterns (PAMP)) and danger-associated molecular patterns (DAMP) released from damaged cells [13-17]. PAMP- and/or DAMP-induced PRR activation of leukocytes, such as neutrophils and monocytes, initiate host defense responses by activating a signaling cascade with subsequent activation of transcription factors such as nuclear factor-κB (NF-κB) [18], a key mediator of sepsis-related multiple organ inflammation [19]. Activated NF-κB regulates transcriptional activity of genes encoding pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-12, IL-17, HMGB-1, MIF, TNF-α), chemokines (e.g., CCL2, CXCL1, CXCL2), and adhesion molecules (e.g., E-selectin, ICAM-1, VCAM-1). Pro-inflammatory cytokines activate endothelial cells and leukocytes thereby facilitating leukocyte migration into infected or damaged sites [20, 21]. Here, the leukocytes release mediators (e.g. defensins, cathelicidins, myeloperoxidase, bacterial permeability increasing protein, elastase, cathepsin G, proteinase 3, and azurocidin) that combat infections [22, 23]. Excessive release of such mediators cause tissue damage. Activated T cells and NK cells produce IFN-γ which, together with IL-12, skew differentiation of $CD4^+$ T cells into a Th1 phenotype, which are required to efficiently combat infections [21, 24]. Recently, IL-17A producing γδ T cells were demonstrated to play a pivotal role in defense during the early phase of bacterial infection [25].

Shift to immunosuppression in late sepsis

The hyperinflammatory septic response is followed by an immunosuppressive reaction during which the initial Th1 response is skewed toward a Th2 response with production

of anti-inflammatory cytokines (e.g., IL-4, IL-10, and TGF-β) [7, 26]. The role of anti-inflammatory cytokines and the subsequent inability of the immune system to raise an adequate anti-microbial response is nicely illustrated by a study in which IL-10 blockade improved survival in mice that underwent CLP followed by a secondary infection with *Pseudomonas aeroginosa* [27].

Apoptotic cells promote anti-inflammatory cytokine release and suppress pro-inflammatory cytokine release by activated monocytes [28]. Apoptosis of immune cells thereby contributes to Th2 skewing and impaired innate and adaptive immune responses in sepsis [26, 29, 30]. The role of apoptosis in the septic reaction is also evident from studies showing that overexpression of the anti-apoptotic protein Bcl-2 decreased lymphocyte apoptosis and improved survival following CLP [31].

CURRENT STANDARD CARE OF SEPSIS AND SEPTIC SHOCK

Septic patients are mainly treated with broad-spectrum antibiotics, resuscitation, and lung-protective ventilation [32, 33]. Maintaining glucose levels <150 mg per deciliter is recommended, but the relevance of this is unclear [33-35].

Glucocorticosteroids were one of the first anti-inflammatory drugs tested in septic patients. Initially a reduced mortality was found when patients with septic shock received a bolus injection of dexamethasone (3 mg/kg body weight (BW)) or methylprednisolone (30 mg/kg BW) [36]. Subsequent clinical trials and experimental animal studies, however, yielded conflicting results. Glucocorticosteroids appeared beneficial in LPS-induced shock in primates [37] and CLP-induced sepsis in rats [38], while others found no such effect [39]. Other studies reported that high dose glucocorticosteroid (e.g. 30 mg/kg BW methylprednisolone) treatment did not improve survival of septic patients, and sometimes even worsened outcome due to increased secondary infection [40]. Consequently, the consensus emerged that high-dose glucocorticosteroid treatment should be avoided in septic patients and the attention shifted to the use of lower doses of glucocorticosteroids. A systematic review reported that prolonged and severely ill septic patients may benefit from low doses of glucocorticosteroids [41]. More recently, an extensive meta-analysis suggested that prolonged low-dose glucocorticosteroid treatment, given as replacement therapy for adrenal insufficiency, improved short-term mortality in patients with vasopressor-dependent shock [42]. Currently, “stress-dose” glucocorticosteroid therapy is recommended for patients with septic shock and poor responsiveness to fluid and vasopressor therapy [33].

NOVEL IMMUNOMODULATORY APPROACHES AND IMMUNE TARGETS

The development of novel immunomodulatory approaches and identification of novel therapeutic targets are major goals in septic shock research. During the last decades several novel anti-inflammatory drugs have been found to improve the outcome in animal sepsis

and septic shock models. Of these, recombinant human activated protein C (rhAPC), which exerts anti-inflammatory, anticoagulant, and fibrinolytic effects, is the only agent that has so far been incorporated into treatment protocols of septic patients [33, 43]. Because hemorrhage is a side-effect, rhAPC treatment is only approved for septic patients with a high death expectancy. Several other new therapeutic targets and approaches to modulate the septic inflammatory response are summarized in Table 1. These will be shortly discussed hereunder. In the subsequent section we will discuss the possibilities and limitations of these modalities for effective treatment of patients with septic shock.

Complement-directed therapy

Robust complement activation is part of the septic response. Increased C3a, C4a, and C5a concentrations have been linked to poor outcome and survival [7, 93]. During the early hyperinflammatory phase of sepsis C5a modulates coagulation cascade activity, TLR-4 mediated responses, cytokine release, neutrophil responses, and apoptosis [7, 94]. C5a also activates the intracellular signaling enzyme sphingosine kinase 1 (SphK1), which is increased in peritoneal phagocytes of septic patients [50]. C5a neutralization by specific antibodies and C5a-receptor blockade reduced CLP-induced systemic cytokine levels and mortality in rats and mice, and protected against *Escherichia coli* induced mortality and development of acute respiratory distress syndrome in non-human-primates [46-49, 95].

Cytokine blockade

Interleukin-6

High IL-6 levels predict mortality in sepsis [96-98]. Therefore IL-6 seems an attractive therapeutic target. Pretreatment of mice with an anti-IL-6 antibody prevented *Escherichia coli* induced mortality [58]. Anti-IL-6 antibody treatment also improved survival when sepsis was induced by oral administration of *Escherichia coli* combined with thermal injury [99]. This effect of IL-6 blockade may be dose dependent, as high or low anti-IL-6 antibody dosages resulted in modest survival improvement, while an intermediate dose optimally improved survival following CLP [59]. In addition, IL-6 knockout mice showed increased mortality following CLP [100, 101].

Interleukin-17A

Gamma-delta T cells are an important source of IL-17 in CLP-induced sepsis [61]. IL-17 can amplify inflammation through induction of cytokine and chemokine production and neutrophil activation [102, 103]. IL-17 receptor deficient mice were found to have impaired neutrophil migration toward the infection focus, enhanced bacteremia, and showed increased mortality following CLP [104]. In contrast, anti-IL-17A antibody therapy improved survival following CLP, which was associated with reduced cytokine plasma levels and less bacteremia [61].

Table 1. Immunoregulatory therapies for sepsis in experimental models and humans

Pathway	Mediator	Treatment	Endotoxemia	CLP	Results in humans
Endocrine	Adrenal insufficiency	Corticosteroids			Mixed [36, 42, 44]
	Hyperglycemia	Intensive insulin therapy			Mixed [34, 35]
Coagulation	Protein C	rhAPC	Positive [45]		Positive [43]
Complement	C5a	Anti-C5a	Positive [46]	Positive [47-49]	Not evaluated
	C5a	SphK1-inhibitor 5c		Positive [50]	Not evaluated
Apoptosis	Lymphocyte apoptosis	Caspase inhibitor		Positive [51-53]	Not evaluated
		Protease inhibitor		Positive [54]	Not evaluated
		IL-10 overexpression		Positive [55]	Not evaluated
		Anti-PD-1		Positive [56]	Not evaluated
		Recombinant IL-7		Positive [57]	Not evaluated
Pro-inflammatory	IL-6	Anti-IL-6	Positive [58]	Positive [59, 60]	Not evaluated
	IL-17A	Anti-IL-17A		Positive [61]	Not evaluated
	IL-27	IL-27 receptor antagonist[a]		Positive [62]	Not evaluated
	TNF-α	Anti-TNF-α	Positive [63-65]	Negative [65, 66]	Negative [67, 68]
	TLR-4-signaling	TAK-242	Positive [69]		Negative [70]
	IL-1	rhIL-1 receptor antagonist	Positive [71, 72]		Negative [73]
	IL-33	rhIL-33		Positive[74]	Not evaluated
	HMGB-1	Anti-HMGB-1	Positive [75]	Positive [76, 77]	Not evaluated
	MIF	Anti-MIF	Positive [78, 79]	Positive [79]	Not evaluated
	TLR signaling	Glucan		Positive [80, 81]	Not evaluated
	TLR-9	Anti-TLR-9		CLP [82]	Not evaluated
		High dose corticosteroids	Positive [37]	Mixed [38, 39]	Mixed [36, 42, 83, 84]
anti-inflammatory	IL-10	IL-10	Positive [85, 86]	Negative [87]	Not evaluated
Immune-modulatory		MSC		Positive [88, 89]	Not evaluated
		hCG related oligopeptides	Positive [90, 91]	Positive [92]	Not evaluated

CLP =cecal ligation and puncture. rhAPC=recombinant human activated protein C. IL=Interleukin. Anti-PD-1=Anti-programmed death-1. TNF-α=Tumor necrosis factor alpha. TLR-4= Toll like receptor-4. HMGB-1= High-mobility group protein B1. MIF=macrophage migration inhibitory factor. MSC=mesenchymal stem cells. hCG=human chorionic gonadotropin

Interleukin-27

IL-27 controls T cell-dependent immune responses [105]. Its expression was elevated in macrophages and neutrophils following CLP [62]. Mice deficient for the EBI3 subunit of IL-27 showed better survival and enhanced neutrophil migration and bacterial clearance following CLP [62]. The contrary was found after recombinant IL-27 administration [62]. Also IL-27 neutralization with a soluble IL-27 receptor fusion protein improved survival following CLP [62].

High-Mobility Group Box-1 Protein

High-mobility group box-1 protein (HMGB-1) is a pro-inflammatory cytokine that is secreted by activated macrophages, monocytes, neutrophils, necrotic, and apoptotic cells, and occurs in elevated concentrations during later stages of disease in septic patients [75, 76, 106]. HMGB-1 activates the receptor of advanced glycation end products (RAGE), mitogen activated protein kinase (MAPK) signaling, and TLR-2 and TLR-4, thereby boosting the inflammatory response [107-109]. Anti-HMGB-1 antibody treatment reduced both LPS-induced and CLP-induced mortality [75-77].

Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF), expressed by macrophages, monocytes, B cells, and T cells, activates macrophages and T cells. High MIF plasma levels predict mortality in septic patients [79, 110, 111]. MIF deficient mice are less susceptible to LPS and *Staphylococcus aureus* induced septic shock [112]. Anti-MIF antibodies protect against LPS-induced mortality, whereas mortality is enhanced by MIF administration [78]. MIF blockade also reduces sepsis severity and mortality in mice following *Escherichia coli* infusion or CLP [79].

Cytokine supplementation

Interleukin-7

IL-7 is an anti-apoptotic cytokine that enhances the proliferation of $CD4^+$ T cells and $CD8^+$ T cells as well as the Bcl-2 expression [78, 79]. IL-7 treatment improves survival following CLP-induced sepsis [57].

Interleukin-33

IL-33 binds to the ST2 receptor, which is mainly expressed by Th2 cells, controls Th2 effector functions, and negatively regulates TLR activity [113-115]. Recombinant IL-33 enhances peritoneal neutrophil recruitment and bacterial clearance, and reduces mortality after CLP [74]. IL-33 treatment also reduced the systemic proinflammatory response, maintained CXCR2 (a receptor crucial for neutrophil recruitment) expression, and blocked TLR-4 mediated downregulation of chemotaxis [74].

Toll-Like receptor blockade

TLR are attractive targets for sepsis treatment as their activation orchestrates many inflammatory responses. MyD88 is a protein involved in TLR-signaling, and MyD88 deficient mice are insensitive to LPS [116]. Mice that lack IRAK, another protein involved in the TLR-MyD88 signaling pathway, are less susceptible to LPS-induced septic shock [117]. MyD88 deficiency was also associated with better survival following colon ascendens stent peritonitis (CASP)-induced sepsis [118]. MyD88 deficiency did, however, diminish survival following CLP-induced sepsis, which was associated with decreased apoptosis and decreased serum cytokine levels [119]. On the other hand, TLR-9 deficiency or blockade improved survival, reduced serum cytokine levels, reduced tissue TLR-2 and TLR-4 expression levels, and enhanced bacterial clearance after CLP-induced sepsis [80-82].

Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multipotent stromal cells that exert immunosuppressive activity through anti-inflammatory cytokine secretion and direct cellular interactions [120, 121]. MSC administration has been found to reduce serum cytokine levels, and to improve organ function and survival following CLP [88, 89].

Human chorionic gonadotropin related oligopeptides

During pregnancy the β-chain of human chorionic gonadotropin (β-hCG) is partly degraded, leading to an array of products, a.o. oligopeptides from loop-2 [122]. Several oligopeptides from loop-2 exert immunomodulatory activity. For instance, synthetic LQGV and VLPALP protected mice from LPS-induced shock [90, 91], while a combination of LQGV, VLPALP, and AQGV (the alanine replacement variant of LQGV) diminished the clinical signs of septic shock and reduced organ pathology in rhesus monkeys after *Escherichia coli* infusion [91]. Furthermore, *in vivo* LQGV administration reduced the capacity of splenocytes to produce IL-6 and TNF-α upon *in vitro* stimulation with either LPS or heat killed *Listeria monocytogenes*, and reduced the proliferative capacity of splenocytes to *in vitro* LPS stimulation [91, 123], indicating that LQGV reduces TLR-driven immune activation. Recently we showed that perioperative administration of a moderate dose (2 x 5 mg/kg BW) of LQGV alone, or in combination with fluid resuscitation and antibiotics, improved survival following CLP-induced sepsis in mice. This survival improvement was associated with a moderate reduction of the inflammatory response [92]. Although further research is necessary to investigate optimal time points, dosage, and duration of LQGV treatment, this data shows that particular hCG-related oligopeptides should be considered as potential treatment modalities for septic patients.

Anti-apoptotic therapy

Apoptosis contributes to sepsis-induced morbidity and mortality [26, 28, 30, 31] and should therefore be considered as a major treatment target. Apoptosis inhibition by inhibitors of caspase or other proteases improved survival following CLP-induced sepsis [51-55]. Programmed cell death 1 (PD-1) is a cell surface molecule that regulates the adaptive immune response and is overexpressed in apoptotic cells [124]. Recently it was found that anti-PD-1 antibodies administered 24 hours after CLP prevented sepsis-induced depletion of lymphocytes and dendritic cells (DC), increased Bcl-2 expression, blocked apoptosis, and improved survival [56].

CRITICAL EVALUATION OF EXPERIMENTAL SEPSIS MODELS IS NECESSARY BEFORE TRYING TO TRANSLATE NOVEL TREATMENTS INTO THE CLINIC

Despite the recognition of new therapeutic targets and the development of new therapeutic approaches to modulate the inflammatory response in animal models, those tested in a clinical setting were mostly unsuccessful. Several factors may account for the failure of these therapies in human sepsis, as will be discussed hereunder.

Limitations of experimental animal models

Successful translation of novel therapies to the clinic may depend on the animal models in which they were tested. This is nicely illustrated by the observation that recombinant human IL-1 receptor antagonist and anti-TNF-α monoclonal antibodies improved survival in LPS-induced septic shock models [63-65, 71, 72], but not in CLP-induced sepsis [65, 66] and septic patients [67, 68, 73]. Also, the TLR-4 inhibitor TAK-242 improved survival following LPS administration in mice but failed in septic patients [69, 70]. Thus, specific treatment strategies that are effective in LPS models may be ineffective in true infection models and septic patients, indicating that the choice of the animal model is of great importance for evaluation of potentially effective new treatments.

The discrepancy between the results obtained in the LPS model on the one hand and CLP and human sepsis on the other hand may be related to differences in cytokine kinetics. LPS induces a rapid but transient increase of systemic cytokines, while CLP and clinical sepsis originate from bacterial infection and have a prolonged, but generally less pronounced increase of systemic cytokines [12]. Although endotoxin models are suitable for a first screening of anti-inflammatory therapies, they represent a sterile inflammation that is not comparable to septic shock.

A drawback of mice models is that the animals are mostly between 6 – 16 weeks of age. This corresponds to a human age of 10 – 17 years [125], while septic shock in humans mainly occurs in the elderly (mean age of 65 years) [4]. In this context it is

relevant to note that aged mice (20-24 months) are more susceptible to CLP- and LPS-induced septic shock than young mice [126, 127]. Also, mostly inbred mice strains are used, which do not represent the genetic heterogeneity of the human population. Use of species genetically more closely linked to humans may increase the validity of the experimental data for the clinic. However, emotional, ethical, and financial limitations restrict the use of nonhuman primates in biomedical research, especially in septic shock studies. Therefore, the use of older animals and multiple inbred strains or outbred mice may reduce some of the limitations of the current models.

Septic patients often suffer from co-morbidities [128]. Therefore, introduction of co-morbidities into animal models would add to the validity of these models [129]. Also, implementation of critical care management (e.g. antibiotics, fluid resuscitation and mechanical ventilation) into the employed animal models is useful to mimic the clinical situation while evaluating the efficacy of novel therapeutics.

Although the CLP model most accurately mimics the human septic inflammatory response, and therefore is considered the model of choice to investigate therapeutic approaches for sepsis [12], testing of novel therapeutic approaches in different experimental sepsis models, in animals of different ages, with and without co-morbidities, and supported with standard clinical sepsis care, is recommended. This will generate more insight into the diverse aspects of modulation of SIRS and septic shock by the new drugs, and facilitate the translation of the animal data to the human setting.

Cytokine kinetics and level of immunosuppression

Multiple immune-directed therapeutic approaches have been evaluated. Although several of them appeared beneficial in different septic shock models, including CLP, the data still need to be critically evaluated before application in human sepsis patients can be considered.

The cytokine response to a septic insult is highly dynamic as the 'cytokine profile' changes overtime [5, 92, 123, 130]. This implies the existence of a therapeutic time window for a specific cytokine to be considered an attractive target for therapy. For example, anti-HMGB-1 antibody treatment improved survival when treatment was initiated between 12 to 24 hours after CLP, but not at a later time point [76, 77]. Other therapies, such as Sphk1 inhibitor 5c and anti-IL-17A antibody treatment, improved survival when administered directly after CLP up to 12 hours after the CLP procedure [50, 61]. Anti-IL-27 treatment improved survival when administered up to at least 2 hours after CLP [62]. Anti-IL-6 antibody treatment improved survival when given at the time of CLP, but not when given 6 hours later [59, 60]. This data demonstrates that specific cytokine targeting may only be beneficial within a certain time window of disease, the window of opportunity for a specific therapy. This window of opportunity not only seems

to exist for cytokine blocking therapies, but also for other therapies. For example IL-33 supplementation improved survival when given up to 3 hours after CLP, but was no longer of benefit when given ≥ 6 hours after CLP [74]. In addition, IL-7 supplementation improved survival when initiated within 2 hours after CLP [57], while MSC were effective when administered 6 hours after CLP [89]. So far it is unclear whether IL-7 or MSC treatment is also effective when given at other time points. Altogether this data indicates that detailed studies on cytokine kinetics in animal models and human sepsis are needed to determine the time window in which specific targeted therapies may be effective. Possibly combinations of several biomarkers may further facilitate treatment guidance [131].

Not only knowledge on the kinetics of the inflammatory profile (e.g. cytokines) is important for successful intervention in the excessive inflammatory response in sepsis, also the acquired level of suppression may be an important determinant. The fact that IL-6 or IL-17 receptor knock-out mice were not protected from CLP but even displayed increased mortality [100, 101, 104] points at a protective function of the sepsis-related inflammatory response, and a role for IL-6 and IL-17 signaling herein. Indeed, administration of high or low doses of IL-6 neutralizing antibodies only resulted in modest survival improvement following CLP, while an intermediate dose of the IL-6 neutralizing antibody significantly improved survival [59]. In our own studies we found that treatment with a high dose (50 mg/kg BW) of the anti-inflammatory β-hCG-related peptide LQGV strongly suppressed immune activation and reduced LPS-induced mortality in C57BL/6 mice but enhanced their susceptibility to infection with *Listeria monocytogenes* [123, 132]. Moreover, we found that treatment with a low dose of LQGV (2 x 5 mg/kg BW) only moderately suppressed the inflammatory response but did result in significant survival improvement following CLP, while strong inhibition of the inflammatory response by treatment with a dexamethasone dosage of 2.5 mg/kg BW did not result in survival improvement [92]. Altogether this data suggests that, in case of microbial infection, moderate downregulation of the sepsis-related inflammatory response results in survival gain while vigorous immunosuppression does not. This notion is supported by a recent study we conducted in which we found that low dose dexamethasone treatment (0.05 mg/kg BW) only moderately suppressed CLP-induced cytokine production but did result in survival improvement following CLP, while higher dexamethasone dosages (0.25 mg/kg BW and 2.5 mg/kg BW), although these strongly suppressed the CLP-induced inflammatory response, did not result in survival improvement [133]. Moreover, low dose dexamethasone treatment significantly reduced the occurrence of bacteremia, while the capacity of monocytes to produce reactive oxygen species upon stimulation with phorbol 12-myristate 13-acetate was better preserved [133].

From this data we conclude that the protective function of the sepsis-induced

inflammatory response should not be neglected when anti-inflammatory therapies are applied. The insight is emerging that only moderate downregulation of the sepsis-related inflammatory response results in survival gain. From the studies reported in this theses we conclude that the success of anti-inflammatory therapies in a septic setting fundamentally depends on finding an optimal level of immunosuppression that reduces SIRS-induced pathology but still allows an adequate host defense against the invading pathogens. This implies the existence of a specific range in which reduction of the pro-inflammatory response will lead to survival benefit, while suppression beyond this stage will not (Fig. 2). Thus, dosing of a specific drug with regard to the level of immunosuppression to be achieved is of great importance. Establishing the optimum dose and time window for new treatment modalities (e.g. therapeutic antibodies and hCG-related oligopeptides) or older treatment modalities (e.g. glucocorticosteroids) is an urgent challenge.

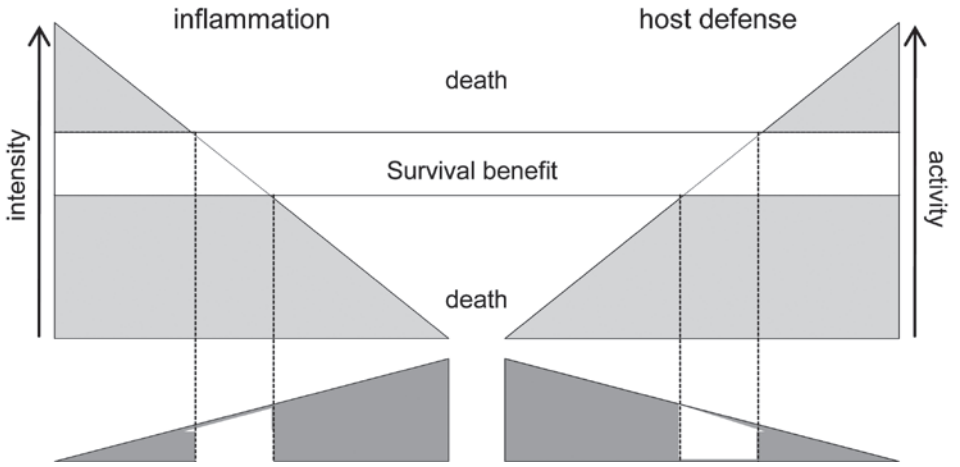

Figure 2. Model explaining how anti-inflammatory therapy can be successfully applied in a septic setting.
The figure represents two pillars of the sepsis-related inflammatory response, i.e. intensity of the inflammation (left upper triangle) and host defense (right upper triangle). The success of anti-inflammatory therapies in a septic setting fundamentally depends on establishing a critical level of immunosuppression (determined by the dosage of the used anti-inflammatory drug (indicated by the lower triangles)) that prevents inflammation-involved pathology but still allows an adequate host defense against invading pathogens. No inhibition of the inflammatory response will increase the risk of death. Robust anti-inflammatory therapy strongly inhibits the inflammatory response (left upper triangle) but also diminishes activation of crucial host defense mechanisms (reflected by the right upper triangle) and will therefore also increase the risk of death. In contrast, mild anti-inflammatory therapy does ameliorate the detrimental effect of the sepsis-related inflammatory response while it maintains the ability of the immune system to raise an adequate host defense. Therefore, mild anti-inflammatory therapy will improve survival outcome in sepsis. This model suggests the existence of a specific range in which the sepsis-related inflammatory response can be modulated to obtain survival improvement.

CONCLUSIONS

Despite advances in our pathobiological knowledge of sepsis, promising experimental therapeutic approaches that interfere with the sepsis-related inflammatory response in animal models were mostly unsuccessful in septic patients so far. Successful implementation of such promising therapeutics in the clinic requires careful consideration of the appropriateness of the animal models used, the optimum level of immunosuppression, and the window of opportunity for a specific therapy. The latter requires careful staging of the inflammatory response of the individual sepsis patient, being either hyperinflammatory or immunosuppressive.

8.2. RENAL ISCHEMIA-REPERFUSION INJURY

I/R injury is initiated by a lack of blood flow (ischemia) which results in a state of tissue oxygen deprivation, followed by restoration of blood flow (reperfusion). The latter causes further damage, at first by inappropriate activation of cellular oxidases and subsequently by the release of inflammatory mediators in response to tissue damage [134]. The inflammatory response induced by renal I/R injury is associated with the production of pro-inflammatory mediators, the upregulation of adhesion molecules on endothelial cells, and tissue infiltration by immune cells.

It is known that reduction of the I/R related inflammation by anti-inflammatory therapies, such as treatment with dexamethasone or neutralizing antibodies directed against cytokines or adhesion molecules, protects against renal I/R injury in animal models. Earlier, it was demonstrated that both preoperative fasting and dietary restriction (DR) improve survival and kidney function following renal I/R injury. Although the mechanism by which fasting and DR protect the kidney against I/R injury is still unknown, it was associated with reduced oxidative injury, reduced cell death, and less inflammation [135].

A stress response is characterized by activation of the HPA-axis and subsequent release of cortisol by the adrenal glands in humans, and corticosterone in rodents. Glucocorticoids bind to an intracellular glucocorticoid receptor, thereby preventing activation of a variety of inflammatory genes, which is essential in limiting and resolving inflammation [136, 137]. This thesis describes that fasting results in an increase in plasma corticosterone levels in mice. However, blockade of the glucocorticoid receptor, during a fasting period prior to renal I/R did not abolish the protection afforded by the fasting. This data strongly suggests that DR-induced protection against renal I/R injury is mediated by a corticosterone or glucocorticoid receptor independent pathway. Earlier, it was demonstrated that preoperative fasting increased expression levels of cytoprotective and

antioxidant defense genes in the kidney [135]. It would be interesting to investigate if the upregulation of these cytoprotective genes is corticosterone mediated.

Comparable to DR, LQGV administration to naive mice significantly increased plasma corticosterone levels, and blockade of the glucocorticosteroid receptor signaling abolished the protective effect of LQGV on LPS-induced mortality [132]. Remarkably, perioperative treatment with LQGV (2 x 5 mg/kg BW) did not improve survival and kidney function following renal I/R in mice, whereas perioperative treatment with the same dose of other β-hCG-related oligopeptides, amongst which AQGV, MTRV, LQG, or VLPALPQ, did result in survival improvement. This suggests that LQGV in this model does not have the same mechanism of action as the other hCG-related oligopeptides tested. This is supported by the observation that LQGV (5 mg/kg BW) given 24 hours after LPS-injection did not protect against LPS-induced mortality, while the same dose of AQGV did reduce mortality [91]. AQGV, however, did not protect against renal I/R injury when treatment was initiated at 6 or 12 hours after the insult. This suggests the existence of different regulatory mechanisms of the inflammatory processes induced by LPS and renal I/R injury due to which AQGV is ineffective in renal I/R injury when treatment is initiated at later time points. This data also illustrates that, besides different mechanisms of action of these hCG-related oligopeptides, these hCG-related oligopeptides also have a different time window of opportunity for treatment of renal I/R injury and LPS-induced inflammation. This time window of opportunity may be influenced by the nature of as well as the peptide dose used [90]. Nonetheless, the data so far suggests that AQGV, but may be also some other β-hCG-related oligopeptides, might be effective in a clinical setting which requires prevention of I/R-induced kidney injury.

8.3. INFLAMMATION AS TREATMENT TARGET IN CANCER

Immune mediators as well as activated immune cells within the tumor microenvironment direct the balance between tumor-promoting inflammation and antitumor immunity. It is generally agreed that inflammation provides an ideal setting for carcinogenesis because inflammatory cells, growth factors, and genotoxic agents work in concert to potentiate and promote neoplastic progression [138]. Therefore, malignancies may arise from areas of infection and inflammation [138]. It is estimated that underlying infection and inflammatory responses are linked to 15-20% of all deaths from cancer worldwide [139]. The strongest association between chronic inflammation and malignant diseases is the occurrence of colon carcinogenesis in individuals with inflammatory bowel disease such as ulcerative colitis and Crohn's disease. In addition, hepatitis C infection predisposes to liver carcinoma, while chronic *Helicobacter pylori* infection is the world's leading cause of stomach cancer [138]. Also morbid obesity, which is characterized by low

grade chronic inflammation [140], promotes the development of malignancy such as hepatocellular carcinoma [141].

Inflammation can promote cancer development through multiple mechanisms, for instance through the anti-apoptotic effect of NF-κB, the induction of mutations through oxidative damage to DNA, and the induction of a tissue repair response [142]. Following tissue injury cellular proliferation is enhanced to facilitate tissue regeneration. If these proliferating cells sustain DNA damage or continue to proliferate in a microenvironment rich of inflammatory cells and growth factors, growth of tumor cells will be supported [138]. Key inflammatory mediators involved in carcinogenesis comprise cytokines such as IL-1β, IL-6, IL-23, and TNF-α, as well as specific transcription factors (e.g. NF-κB and STAT3) [143]. Cytokines produced by tumor-infiltrating immune cells may activate pro-tumorigenic processes such as growth, invasion, proliferation, and survival. Angiogenesis, a process crucial for the survival of solid tumors, is stimulated by factors such as VEGF that is produced by tumor cells and inflammatory cells. Furthermore, tumor cells produce chemokines that attract additional inflammatory cells to maintain tumor inflammation [144].

As inflammation seems to play an important role in carcinogenesis and cancer progression, modulation of inflammation may be a therapeutic option to prevent or treat cancer. TNF-α, for instance, has tumor-promoting activity as it induces expression of the promalignant gene secretory leukocyte protease inhibitor in a Lewis Lung Carcinoma (LCC) model in mice and stimulates LCC growth. In addition, TNF-α deficiency reduced skin tumor development in mice, indicating that TNF-α can play a pivotal role in tumor promotion [145]. Treatment with the TNF-α blockers Etanercept or Infliximab resulted in disease stabilization and sometimes partial responses in patients with advanced cancer [146-148], particularly in patients with renal-cell carcinoma [146]. Lenalidomide, a thalidomide derivative which inhibits the activity of several inflammatory cytokines, appeared active against advanced myeloma when combined with dexamethasone [149]. In addition, COX2 inhibitors, which inhibit TNF-α induced NF-κB activation [150], prevented the recurrence of colon adenomas in patients with a genetic predisposition [151]. It is described that the hCG-related oligopeptide LQGV reduces the capacity of cells to produce TNF-α and IL-6 [123]. Previously, LQGV was found to inhibit LCC outgrowth in mice [152]. Possibly inhibition of TNF-α and other cytokine production by LQGV treatment contributed to its inhibitory effect on LCC outgrowth. This, however, was not investigated yet.

Surgical resection still is the cornerstone of curative treatment for colorectal malignancies. However, surgical trauma induces an inflammatory response characterized by increased levels of pro-inflammatory mediators, adhesion molecules, activated leucocytes, and finally postoperative cellular immunosuppression [153, 154]. The

surgery-induced inflammatory response may facilitate tumor metastases by enhancing the adhesion of circulating tumor cells to adhesion molecules on endothelial cells. Earlier, it was demonstrated in animal models that DR reduces the expression of pro-inflammatory cytokines and adhesion molecules following I/R injury [135]. In this thesis the therapeutic use of short-term DR was extended to oncologic surgery and it was demonstrated that 2 weeks of 30% DR prior to inoculation of C26 colon carcinoma cells in the liver reduced hepatic tumor metastases outgrowth in mice. The adhesion molecule E-selectin plays a crucial role in the process of C26 colon carcinoma liver metastases formation as E-selectin blockade in mice reduced the frequency of liver metastases [155]. The reduced capacity of the inoculated C26 colon carcinoma cells to metastasize to the liver after DR was associated with a reduction in hepatic E-selectin mRNA expression. In addition, it was observed that plasma obtained from DR mice reduced adhesion of C26 colon carcinoma cells to human vascular endothelial cells *in vitro*. This data suggests that the effect of DR is, at least partly, mediated by one or more secondary mediators released into the blood. DR was found to stimulate corticosterone release. Whether this contributed to the inhibitory effect of plasma from DR mice to prevent the adhesion of C26 colon carcinoma cells to human vascular cells *in vitro* has not been explored so far. Also it remains unexplored whether DR-induced release of corticosterone contributed to the inhibitory effect of DR on the occurrence of hepatic tumor metastases in the mice model used. As discussed earlier, increased expression levels of cytoprotective and antioxidant defense genes were found in the liver of mice after DR [135]. This possibly contributes to the beneficial effect of DR in preventing tumor metastases as well. The mechanism how DR exerts its function has still to be determined.

LQGV treatment reduced the CLP-induced upregulation of E-selectin on pulmonary endothelial cells in mice as well as on hepatic epithelial cells following hemorrhagic shock and resuscitation in rats [156]. Also, treatment with AQGV was found to reduce renal and hepatic expression of E-selectin following renal I/R and hemorrhagic shock and resuscitation, respectively. The expression of ICAM-1, another molecule that facilitates the adhesion of tumor cells to endothelial cells, might be inhibited by LQGV and AQGV. Therefore, it is of interest to investigate whether hCG-related oligopeptides such as LQGV and AQGV can prevent surgery induced tumor adhesion and outgrowth.

The application of DR in humans was demonstrated by van Ginhoven *et al*. They investigated both the feasibility and the effect of preoperative DR in live-kidney donors [157, 158]. These studies revealed that DR is applicable in healthy surgical patients regarding logistics, well-being, appetite, and ability to perform daily tasks. However, only a marginal inhibitory effect on the postoperative acute phase response was observed, which was most likely related to the relative mildness of the surgical intervention in this study [158]. DR by itself is unlikely to be applied in weakened patients, for instance

those with cancer. Elucidation of the mechanism by which DR exerts its beneficial effects may aid to the design of pharmacological mediators that mimic the effect of DR and may finally prove to be applicable in weakened (cancer) patients as well.

8.5 FUTURE DIRECTIONS

This thesis describes the effect of three potential therapeutic approaches on inflammation in experimental animal models of sepsis, renal ischemia-reperfusion (I/R) injury, and cancer, namely hCG-related oligopeptides, low dose dexamethasone, and dietary restriction (DR). Although the data shows that particular hCG-related oligopeptides as well as a low dose of corticosteroids should be considered as potential treatment modalities for septic patients, further studies are required to facilitate optimal implementation in the clinic. In addition, the data shows that DR should be considered as a potential treatment modality for renal I/R and surgery-induced metastatic outgrowth.

The data suggests that potential anti-inflammatory therapeutics may only be beneficial within a defined time window of disease. Administration of anti-inflammatory agents such as hCG-related oligopeptides and dexamethasone improved survival following cecal ligation and puncture-induced sepsis when administered in a preventive setting, that is when given around the time of sepsis induction. Although these are promising results, administration at a time point of already existing sepsis is far more relevant to daily clinical practice. Most patients with a severe inflammation or sepsis already suffer from the disease for some time before admission to the hospital. Thus, it would be of clinical interest to evaluate the effect of the here described anti-inflammatory therapies when administered at later time points, after induction of sepsis and in a prolonged scheme. For example, a one week regimen of hCG related oligopeptides or dexamethasone starting at 24 hours after induction of sepsis. Studies like these will help to increase insight into the therapeutic window of opportunity for a specific treatment.

The data presented in this thesis demonstrate that the level of immunosuppression achieved during sepsis is critical to survival improvement. The optimum dose and time window can be established by dose response studies in which different dosages of a potential anti-inflammatory agent are administered for different periods.

In general, animal models for sepsis research suffer from serious drawbacks that may contribute to the failure to successfully implement potential novel treatments into clinical practice. For instance, in humans septic shock mainly occurs in elderly. Therefore, the use of older animals may improve the value of animal studies. Also, the septic inflammatory response may greatly differ among patients, which is most likely related to the genetic heterogeneity of the human population. Therefore, the effect of LQGV and low dose dexamethasone should also be investigated in other mice strains than

C57BL/6 as well as in outbred mice. In addition, to more accurately mimic septic patients, introduction of co-morbidities into animal models is of clinical relevance, for instance, sepsis induced in NOD mice (mice that spontaneously develop autoimmune diseases such as diabetes) or mice suffering van renal insufficiency. It would also be of interest to evaluate the effect of LQGV and low dose dexamethasone in a double hit model, as many patients admitted to an intensive care unit suffer multiple illnesses. For instance, this can be achieved by a model of burns or hemorrhagic shock/fluid resuscitation followed by a CLP-induced septic shock. In this context the use of optimal critical care management by fluid resuscitation and antibiotics is vital.

Anti-inflammatory therapies may be more effectively applied when detailed insight in the ongoing immune activity of the patient is available. Therefore, further research is needed to define biomarkers that allow clinical staging of the patient and treatment guidance.

The data described in this thesis demonstrate that hCG-related oligopeptides exert anti-inflammatory activity in different experimental animal models. However, their mechanisms of action remain largely unclear. Data suggests that LQGV acts through stimulation of the adrenal axis as LQGV stimulated adrenal glucocorticosteroid production and subsequent glucocorticoid receptor activation. However, it cannot be excluded that LQGV exerts other functions as well. Therefore, further studies should be conducted to provide a detailed insight into its mechanism of action as well as that of other hCG related oligopeptides.

DR influences inflammatory responses which results in survival improvement following renal I/R injury in mice. The mechanism by which fasting and DR protect the kidney against I/R injury is still unknown. DR was associated with reduced oxidative injury, reduced cell death, and less inflammation. The protective effect of DR proved to be not mediated by corticosterone. Further research may focus on genes involved in the effects of DR as well as on the role of specific dietary components. A diet without particular components, for example lacking proteins, might enhance the beneficial effects of DR.

This thesis demonstrates that DR inhibits hepatic tumor outgrowth which may be related to the inhibition of E-selection expression. The data suggests that the effect of DR is mediated by secondary mediators. DR was found to stimulate corticosterone release, but whether this contributed to the inhibitory effect of DR on hepatic tumor metastases has not been explored so far. Increased expression levels of cytoprotective and antioxidant defense genes were found in the liver of mice after DR, which possibly contributed to the beneficial effect of DR in preventing tumor metastases. This, however, needs further studies.

Besides research targeting the mechanism of action of DR and specific dietary components the development of a pharmacological compound that mimics the effect of DR also is an interesting target for further research. This being so because surgical patients, especially cancer patients, may be malnourished and therefore may not tolerate or respond to DR as healthy individuals and experimental animals do. The use of a DR mimetic may increase the applicability of DR related therapy as weakened patients may benefit of this therapy as well. Recently it was demonstrated that ghrelin, a ligand from the growth hormone secretagogue receptor, which release increases after fasting, does not mediate protection against renal I/R injury.

This thesis describes that a reduction of adhesion molecule expression is associated with reduced occurrence of hepatic tumor metastases. As the upregulation of adhesion molecules (E-selectin and ICAM-1) was inhibited by the hCG-related oligopeptides LQGV and AQGV in murine renal I/R injury and a rat model of hemorrhagic shock and resuscitation, it is worth to examine whether these oligopeptides are effective in preventing surgery-induced adhesion and outgrowth of circulating tumor cells. In addition, as LQGV was found to inhibit LCC outgrowth it would be of interest to more extensively evaluate the effect of hCG-related oligopeptides on tumor outgrowth.

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IX

Summary
Nederlandse samenvatting

SUMMARY

Inflammation can result from several different insults, amongst which microbial infection and tissue injury. The inflammatory response serves a protective function of the body to the insult, and when it is well controlled it ensures removal of detrimental stimuli and stimulates the repair of damaged tissues.

Inflammation can also develop out of control. This can occur in several illnesses such as sepsis, ischemia-reperfusion (I/R) injury, and cancer, and then is a major pathophysiological component. It is generally thought that a controlled inflammatory response is beneficial but that it becomes detrimental if dysregulated. Therefore, restoring control of inflammatory reactions generally is of benefit in pathologic inflammatory conditions. The research described in this thesis was initiated to investigate the immunomodulatory effects of human chorionic gonadotropin (hCG)-related oligopeptides, dexamethasone, and dietary restriction in animal models of sepsis, renal I/R injury, and cancer metastases, in which dysregulated inflammation plays a central pathophysiological role.

Several studies in this thesis describe the immunomodulatory effects of the hCG-related oligopeptide LQGV. In **Chapter 2** we used the cecal ligation and puncture (CLP) model to explore the effect of LQGV on sepsis-induced mortality and inflammation. LQGV (2 x 5 mg/kg body weight) was administered around the time of sepsis induction, which resulted in a significant survival benefit compared to PBS-treated control mice. This survival benefit appeared to be associated with a modest reduction of the acute inflammatory response, as reflected by slightly decreased plasma cytokine levels, reduced cytokine and adhesion molecule mRNA levels in lung, kidney, and liver tissue, and better preservation of lung tissue integrity. Likely, this effect of LQGV is mediated through inhibition of nuclear factor-κB-dependent gene activation. This study also demonstrated that LQGV administration, around the time of sepsis induction, in combination with a 5 day regimen of fluid resuscitation and antibiotics, resulted in a significant better survival than treatment with fluid resuscitation and antibiotics alone. Apparently the employed treatment with a modest dose of LQGV acts as an appropriate anti-inflammatory approach in CLP-induced sepsis in mice. LQGV therefore might be a valuable add-on therapy next to the standard sepsis care with fluid resuscitation and antibiotics.

Subsequently, potential mechanisms of action by which LQGV exerts its function were investigated. As cortisol (of which the rodent analogue is corticosterone) serum levels increase during the late second trimester and the third trimester of pregnancy, the phase wherein the highest degree of cleavage of β-hCG into small oligopeptides occurs, it was examined whether LQGV exerts anti-inflammatory effects through the stimulation of glucocorticosteroid production and subsequent glucocorticosteroid receptor signaling. In **Chapter 3** it is described that LQGV dose-dependently stimulates the ACTH receptor and subsequent adrenal glucocorticosteroid production. This results in a prolonged increase

of corticosterone plasma levels with consequent glucocorticoid receptor activation and immunosuppression. Blocking *in vivo* glucocorticosteroid receptor signaling with mifepristone, a glucocorticosteroid receptor antagonist, reduced the pro-survival effect of high dose LQGV (50 mg/kg body weight) upon LPS administration. These observations suggest that glucocorticoid receptor activation contributes to the protective effect of high-dose LQGV treatment against LPS-induced mortality in mice. Whether such a mechanism also accounts for lower doses of LQGV (e.g. 5 mg/kg body weight) and in other models for septic shock (e.g. CLP) requires further investigation. The possibility, however, that LQGV can also exert its effects through mechanisms other than glucocorticoid receptor signaling can not be excluded.

The data described in **Chapter 2** suggests that mild downregulation of the inflammatory response during early sepsis may be beneficial while extensive reduction of the inflammatory response is not, which is in-line with recent literature. Therefore, the effect of different dosages of the commonly used anti-inflammatory drug dexamethasone (dosages ranging from 0.05 mg/kg body weight to 2.5 mg/kg body weight) on CLP-induced inflammation and mortality was also investigated **(Chapter 4)**. This study clearly revealed that modest downregulation of the sepsis associated inflammatory response by perioperative treatment with low dose dexamethasone (0.05 mg/kg) improves survival while extensive downregulation with high dose dexamethasone (0.25 mg/kg and 2.5 mg/kg) does not. Moreover, this study demonstrates that treatment with low dose dexamethasone reduces the occurrence of bacteremia and preserves the ROS producing capacity of monocytes compared with treatment with high dose dexamethasone. In addition, this study demonstrates that perioperative treatment with low dose dexamethasone in combination with a 5 day regimen of fluid resuscitation and antibiotics resulted in a significant better survival than treatment with high dose dexamethasone in combination with fluid resuscitation and antibiotics or fluid resuscitation and antibiotics alone. Low dose dexamethasone treatment therefore might be a valuable addition next to the standard sepsis care with fluid resuscitation and antibiotics.

From the observations in **chapter 2**, **chapter 4,** and available literature (as discussed in **Chapter 8**) it can be concluded that the important protective function of the sepsis-related inflammatory response should not be neglected when anti-inflammatory therapies are applied. Hence, the concept emerges that the success of anti-inflammatory therapies in a septic setting fundamentally depends on finding a treatment balance that reduces the hyperinflammation-induced pathology but still allows adequate defense against the involved pathogens. This implies the existence of a specific range in which reduction of the pro-inflammatory response will lead to survival benefit, while suppression beyond this range will not.

HCG-related oligopeptides other than LQGV, for example VLPALP, MTRV, and AQGV (the alanine replacement variant of LQGV), have previously been found to exert immunomodulatory properties as well. In **Chapter 5** the effect of 10 different β-hCG-related oligopeptides was explored in a renal ischemia-reperfusion (I/R) model in mice. This study shows that perioperative treatment with AQGV, LQG, MTRV, and VLPALPQ (2 x 5 mg/kg body weight) improved survival after renal I/R while LQGV did not. Of the tested oligopeptides AQGV was the most effective. Treatment with this peptide was associated with reduced systemic inflammation, decreased apoptosis, and reduced renal E-selectin mRNA levels. However, AQGV did not protect against renal I/R injury when treatment was initiated at 6 or 12 hours after the insult, which indicates that AQGV is only applicable in a preventive setting, when administered at the time of I/R injury induction. Though, the possibility is not excluded that higher doses of AQGV are effective when given in this model at later time points. Overall, this study shows that hCG-related oligopeptides can have a beneficial anti-inflammatory effect in a renal I/R model. Therefore, these small oligopeptides are promising potential drugs for preventing the development of renal ischemia-reperfusion injury.

Caloric restriction (CR), another promising immunomodulatory strategy, which may be achieved by various regimens such as dietary restriction (DR) and fasting, can also reduce inflammatory responses and improve survival in the employed murine model of renal I/R injury. To explore the mechanism by which fasting exerts its function we tested whether the protection imposed by fasting is mediated by corticosterone, a mediator produced by the adrenal glands. High serum levels of corticosterone can be induced by the stress of food deprivation **(Chapter 6)** as mice fasted for 1 to 3 days showed increased serum levels of corticosterone. Bilateral adrenalectomy (ADX) was performed to investigate the effect of corticosterone on renal I/R injury. ADX resulted in higher mortality rates after I/R compared with control mice. Laparotomy in ADX mice without I/R injury resulted in a similar mortality rate. Overall, this indicates that bilateral adrenalectomy in mice is an unsuitable model to investigate whether the protection against I/R injury may be due to increased corticosterone levels. Therefore, we used mifepristone which was administered daily during the 3-day fast. Treatment with mifepristone did not eradicate the beneficial effect of fasting on renal I/R injury. This demonstrates that fasting protects against I/R injury independent of the stimulation of glucocorticoid receptor signaling. Remarkably, LQGV, which stimulates corticosterone release and glucocorticoid receptor signaling **(Chapter 3)** also did not protect against renal I/R injury **(Chapter 5)**.

In **Chapter 7** we studied the effect of DR in a murine hepatic metastases model. The cornerstone of treatment of colorectal malignancies remains surgical resection, which is accompanied by a temporary systemic inflammation and cellular immunosuppression.

The postoperative inflammatory response is characterized by increased expression of adhesion molecules and thereby may facilitate metastases by circulating tumor cells. We explored whether DR interferes with surgery-induced inflammation and adhesion of circulating tumor cells. Using the BALB/c mouse C26 colon carcinoma cell line model, we found that a regimen of 2 weeks of preoperative DR reduced hepatic tumor outgrowth after injection of tumor cells. DR also reduced the expression of E-selectin in the liver. In addition, serum from DR mice reduced adhesion of tumor cells to human vascular endothelial cells *in vitro*. This suggests that DR induces a plasma factor which can reduce the tumor load by diminishing the adhesion of circulating tumor cells to hepatic vascular endothelium.

In **Chapter 8** the studies performed in **Chapters 2 - 7** are discussed in the context of the available literature. Also, directions for further studies are shortly pointed out. In this chapter we conclude that successful implementation in the clinic of novel therapeutic approaches for septic shock requires careful consideration of the animal models used, the level of immunosuppression achieved, and the window of opportunity for that specific therapy. Careful staging of the inflammatory response of the individual sepsis patient will aid significantly to treatment success, but requires the identification of suitable biomarkers for this purpose.

In conclusion, the studies described in this thesis show that (**1**) hCG-related oligopeptides exert anti-inflammatory properties in several murine inflammatory models; (**2**) LQGV can improve survival following CLP; (**3**) low dose dexamethasone treatment can improve survival following CLP while high dose dexamethasone treatment does not, which is related to the level to which the CLP-induced inflammatory response is reduced; (**4**) LQGV can exert anti-inflammatory action and provide protection against LPS-induced mortality by stimulating adrenal glucocorticosteroid production and subsequent glucocorticoid receptor activation; (**5**) DR can exert anti-inflammatory effects independent of corticosterone in a renal I/R model; and (**6**) DR can reduce hepatic tumor outgrowth after injection of tumor cells, likely by diminishing E-selectin mediated adhesion of tumor cells to hepatic vascular endothelium.

NEDERLANDSE SAMENVATTING

Een ontstekingsreactie is een reactie van het lichaam op bijvoorbeeld een microbiële infectie of weefselschade. Een ontstekingsreactie heeft eigenlijk een beschermende functie en resulteert, indien goed gecontroleerd, in het opruimen van het schadelijke agens en in weefselherstel. Als een ontstekingsreactie ongecontroleerd plaatsvindt is dit schadelijk voor het lichaam. Ongecontroleerde ontstekingsreacties spelen een belangrijke pathofysiologische rol bij verschillende aandoeningen zoals sepsis, ischemie en reperfusie (I/R) schade, en kanker. Het corrigeren van zulke ontregelde ontstekingsreacties heeft een gunstig effect. Het onderzoek dat in dit proefschrift wordt beschreven, werd uitgevoerd om het immunomodulatoire effect te onderzoeken van oligopeptiden waarvan de structuur gebaseerd is op de aminozuur volgorde van humaan choriogonadotrofine (hCG), van dexamethason, en van calorische restrictie in diermodellen voor sepsis, renale I/R schade, en kanker.

Verschillende studies in dit proefschrift onderzoeken het immunomodulatoire effect van het hCG-gerelateerde peptide LQGV. In **hoofdstuk 2** is het cecum ligatie en punctie (CLP) model gebruikt om sepsis te induceren in muizen en het effect van LQGV op de sepsis-geïnduceerde ontsteking en mortaliteit te onderzoeken. Perioperatieve toediening van LQGV (2 x 5 mg/kg lichaamsgewicht), vlak voor en vlak na de inductie van sepsis, resulteerde in een significante verbetering van de overleving ten opzichte van controlemuizen die op dezelfde tijdspunten werden behandeld met een controlevloeistof (PBS). De verbeterde overleving door LQGV behandeling was geassocieerd met een milde reductie van de acute ontstekingsreactie, gekenmerkt door een milde afname van de cytokinen concentraties in het plasma. Daarnaast was er in de longen, de nieren en de lever sprake van een afname van de hoeveelheden messenger RNA's coderend voor cytokinen en adhesiemoleculen. Ook bleef de integriteit van het longweefsel beter behouden. In dit CLP-model wordt het effect van LQGV waarschijnlijk gemedieerd door remming van de activiteit van de transcriptiefactor nuclear factor kappa B (NF-κB), een transcriptiefactor die betrokken is bij de activatie van een heel scala aan genen die bij immuun- en ontstekingsreacties zijn betrokken. Bovendien laat deze studie zien dat perioperatieve LQGV toediening gevolgd door een 5-daagse behandeling met vocht en antibiotica de overleving van muizen na CLP significant verbetert ten opzichte van een behandeling met alleen vocht en antibiotica. Deze resultaten impliceren dat LQGV een waardevolle aanvulling zou kunnen zijn op de al bestaande sepsisbehandeling met antibiotica en vloeistoftoediening.

Het bijnierhormoon cortisol, waarvan corticosteron het analoog is in knaagdieren, heeft een ontstekingsremmend effect. De serumconcentratie van cortisol stijgt sterk gedurende de laatste fase van de humane zwangerschap, het moment waarop tevens de meeste afbraakproducten van β-hCG, waaronder bijvoorbeeld

LQGV, gevormd worden. Daarom werd in **hoofdstuk 3** onderzocht of LQGV zijn ontstekingsremmende effect in muizen uitoefent via corticosteronproductie, en activatie van de glucocorticosteroïdreceptor. Uit deze studie bleek dat LQGV op dosis-afhankelijke wijze de *in vitro* corticosteronproductie door de bijnieren stimuleerde, en dat activatie van de ACTH-receptor hierbij betrokken was. *In vivo* LQGV toediening (50 mg/kg lichaamsgewicht, een relatief hoge dosering LQGV) resulteerde in een langdurige toename van de corticosteronconcentratie in het plasma en een verminderde capaciteit van miltcellen om cytokinen te produceren na *in vitro* stimulatie met microbiële componenten. Dit laatste was het gevolg van glucocorticosteroïdreceptor activatie. *In vivo* blokkade van de glucocorticosteroïdreceptor verminderde het pro-overlevings effect van LQGV (50 mg/kg lichaamsgewicht) na LPS toediening. Deze waarnemingen suggereren dat glucocorticosteroïdreceptor activatie bijdraagt aan het beschermende effect van hoge dosis LQGV behandeling op LPS-geïnduceerde mortaliteit in muizen. Of dit mechanisme ook verantwoordelijk is voor de gunstige effecten van lagere doseringen LQGV (bijvoorbeeld 5 mg/kg lichaamsgewicht) in andere ontstekingsmodellen (zoals het CLP model), vereist aanvullend onderzoek. Het is niet uitgesloten dat LQGV ook nog andere werkingsmechanismen heeft.

De resultaten die beschreven worden in **hoofdstuk 2** suggereren dat milde remming van de vroege sepsis-gerelateerde ontstekingsreactie in muizen een gunstig effect heeft op de overleving, terwijl sterke remming van deze ontstekingsreactie dit niet heeft. Daarom werd het effect van verschillende doseringen van het ontstekingsremmende medicijn dexamethason op CLP-geïnduceerde ontsteking en mortaliteit onderzocht **(hoofdstuk 4)**. Milde remming van de sepsis-gerelateerde ontstekingsreactie middels perioperatieve behandeling met een lage dosis dexamethason (0,05 mg/kg lichaamsgewicht) leidde tot een verbeterde overleving, terwijl sterke suppressie, met een hogere dosis dexamethason (0,25 of 2,5 mg/kg lichaamsgewicht), geen gunstig effect op de overleving had. Bovendien werd gevonden dat de behandeling met 0,05 mg/kg lichaamsgewicht dexamethason het ontstaan van bacteriëmie na CLP verminderde, en dat de capaciteit van monocyten om zuurstofradicalen te produceren, bij deze behandeling in stand bleef. Combinatiebehandeling bestaande uit perioperatieve toediening van een lage dosis dexamethason (0,05 mg/kg lichaamsgewicht) gevolgd door een 5-daagse behandeling van vocht en antibiotica bleek de overleving zeer sterk te verbeteren ten opzichte van behandeling met een hoge dosis dexamethason in combinatie met vocht en antibiotica, of behandeling met alleen vocht en antibiotica.

Uit de bevindingen van **hoofdstuk 2** en **hoofdstuk 4**, en reeds beschikbare literatuur (zoals bediscussieerd in **hoofdstuk 8**) kan geconcludeerd worden dat bij ontstekingsremmende behandelingen de belangrijke beschermende functie van de ontstekingsreactie in stand moet blijven. Uit onze resultaten komt het inzicht naar voren

dat het succes van ontstekingsremmende behandelingen in sepsis afhankelijk is van het bereiken van een balans waarbij ontsteking-gedreven pathologie gereduceerd wordt, terwijl een adequate verdediging tegen pathogenen gewaarborgd blijft. Dit impliceert het bestaan van een bandbreedte waarbinnen reductie van de ontstekingsreactie tot een verbeterde overleving leidt terwijl een sterke remming van de ontstekingsreactie schadelijk is. Hieruit blijkt dat zorgvuldige evaluatie van de huidige in de kliniek toegepaste doses ontstekingsremmende medicatie en het daarmee behaalde niveau van ontstekingsremming, noodzakelijk is om de behandeling van septische patiënten te optimaliseren.

Eerder onderzoek heeft aangetoond dat naast LQGV ook andere β-hCG gerelateerde oligopeptiden, zoals VLPALP, MTRV en AQGV (een alanine variant van LQGV), immunomodulatoire eigenschappen hebben. In **hoofdstuk 5** is het effect van 10 verschillende β-hCG gerelateerde oligopeptiden in een renaal I/R model in muizen onderzocht. Deze studie laat zien dat perioperatieve behandeling met AQGV, LQG, MTRV en VLPALPQ (2 x 5 mg/kg lichaamsgewicht) de overleving na renale I/R verbetert terwijl dit niet het geval is indien LQGV wordt toegediend. Van de geteste oligopeptiden was AQGV het meest effectief. De verbeterde overleving was geassocieerd met een reductie van de systemische ontstekingsreactie, een reductie van renale E-selectine mRNA niveaus, een verminderde neutrofiel influx in de nier, een verminderde apoptose en een verhoogde proliferatie van renale tubulaire epitheelcellen. Wanneer de behandeling met AQGV gestart werd op 6 of 12 uur na de I/R trad geen bescherming op. Dit geeft aan dat AQGV in de gebruikte dosis bij deze vorm van nierschade mogelijk alleen toepasbaar is bij een preventieve behandeling, dus ten tijde van inductie van I/R schade. Het is niet uitgesloten dat het toedienen van hogere doseringen AQGV op de latere tijdstippen wel effectief is. Deze studie laat zien dat β-hCG-gerelateerde oligopeptiden mogelijk ook bij de mens toepasbaar zijn, ter preventie van renale I/R schade, zoals deze kan optreden bij abdominale aorta chirurgie, cardiothoracale chirurgie en niertransplantatie.

Verschillende studies in dit proefschrift onderzoeken het immunomodulatoire effect van calorische restrictie (CR). CR, bijvoorbeeld doormiddel van dieet restrictie (DR) en vasten, onderdrukt de ontstekingsreactie die optreedt ten gevolge van renale I/R in muizen. DR kan leiden tot een sterk verbeterde overleving van muizen na renale I/R. Om te bepalen via welk mechanisme vasten de renale I/R geïnduceerde ontsteking en mortaliteit remt in muizen werd onderzocht of corticosteron en glucocorticosteroïdreceptor activatie hierin een rol spelen **(hoofdstuk 8)**. Eén tot 3 dagen preoperatief vasten resulteerde in een verhoogde plasma concentratie van corticosteron in muizen. *In vivo* blokkade van de glucocorticosteroïdreceptor ten tijde van het vasten leidde niet tot het verdwijnen van het beschermende effect van vasten op de renale I/R schade, wat aantoont dat glucocorticosteroïdreceptor signalering geen rol speelt in de door vasten

geïnduceerde bescherming. Opvallend is dat LQGV, dat zowel de corticosteronproductie als glucocorticosteroïdreceptor signalering stimuleert **(hoofdstuk 3)**, geen bescherming bood tegen renale I/R schade in dit muizenmodel **(hoofdstuk 5)**.

Chirurgische resectie is de hoeksteen van de behandeling van colorectale maligniteiten. Een chirurgische resectie gaat gepaard met een tijdelijke systemische ontstekingsreactie en cellulaire immuunsuppressie. De postoperatieve ontstekingsreactie wordt gekarakteriseerd door een verhoogde expressie van adhesiemoleculen en faciliteert daardoor mogelijk het ontstaan van metastasen vanuit circulerende tumorcellen na oncologische chirurgie. Aangezien DR in staat is om een ontstekingsreactie te remmen, werd onderzocht of DR interfereert met de door chirurgie geïnduceerde ontstekingsreactie en de daarmee gepaard gaande verhoogde expressie van adhesiemoleculen, en de adhesie van tumorcellen aan endotheel **(hoofdstuk 7)**. Een twee weken durend preoperatief dieet gevolgd injectie van de tumorcellen (C26 colon carcinoom cellen) in de milt van muizen leidde tot een verminderde uitgroei van tumorcellen in de lever 10 dagen na injectie. Daarnaast werd na een twee weken durend dieet een verlaging van de expressie van het adhesiemolecuul E-selectine in de lever waargenomen. Deze studie toonde tevens aan dat serum van DR muizen leidt to minder adhesie van tumorcellen aan humane vasculaire endotheelcellen *in vitro*. De verminderde adhesie van tumorcellen onder invloed van serum van DR muizen suggereert dat DR een factor in het bloed induceert die interfereert met cellulaire adhesie, mogelijk via het remmen van de adhesiemolecuul-expressie. Remming van de adhesie van circulerende tumorcellen aan hepatisch vasculair endotheel is dan ook een mechanisme via welke DR het ontstaan van metastasen na chirurgische resectie van tumoren zou kunnen remmen.

Hoofdstuk 8 beschrijft de bevindingen uit de **hoofdstukken 2 – 7** in de context van de beschikbare literatuur over ontsteking, sepsis, glucocorticosteroiden, renale I/R en uitgroei van tumor metastasen in de lever. Tevens worden enkele mogelijkheden voor verder onderzoek aangegeven.

Kort samengevat laten de studies in dit proefschrift zien dat **1)** hCG-gerelateerde oligopeptiden ontstekingsremmende eigenschappen hebben in verschillende ontstekingsmodellen in de muis; **2)** lage-dosis LQGV behandeling leidt tot een significante toename van de overleving na CLP; **3)** behandeling met een lage dosis dexamethason leidt tot een significante toename van de overleving na CLP terwijl behandeling met een hoge dosis dexamethason dit niet doet; **4)** LQGV een ontstekingsremmende werking heeft en kan beschermen tegen LPS-geïnduceerde mortaliteit via glucocorticosteroïd-receptor activatie; **5)** DR ontstekingsremmende effecten heeft die in een renaal I/R model in muizen op een corticosteron-glucocorticosteroïdreceptor onafhankelijke wijze tot stand komen; **6)** DR de capaciteit van tumorcellen reduceert om aan bloedvaten te hechten en DR op die wijze in staat is om tumor-uitgroei in de lever, na injectie van tumorcellen in de milt, te remmen.

X

List of abbreviations
Dankwoord
Curriculum Vitae
List of publications
PhD portfolio

ABBREVIATIONS

AB	antibiotics
ACTH	adrenocorticotropic hormone
ADX	adrenalectomy
AP-1	activator protein 1
ARF	acute renal failure
ATP	adenosine triphosphate
BW	body weight
cAMP	cyclic adenosine monophosphate
CARS	compensatory anti-inflammatory response syndrome
CASP	colon ascending stent peritonitis
CBG	corticosteroid-binding globulin
CCL2	chemokine (C-C motif) ligand 2
CFU	colony-forming units
CIP	corticotropin-inhibiting peptide
CLP	cecal ligation and puncture
CR	caloric restriction
CTC	circulating tumor cells
CXCL1	chemokine (C-X-C motif) ligand 1
CX3CL1	chemokine (C-X3-C motif) ligand 1
DAB	diaminobenzidine
DAMP	danger-associated molecular patterns
DC	dendritic cells
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DR	dietary restriction
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GR	glucocorticosteroid receptor
hCG	human chorionic gonadotropin
H/DEX	high-dose dexamethasone (2.5 mg/kg BW)
HE	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKLM	heat killed *listeria monocytogenes*
HMGB-1	high-mobility group box-1 protein
HPA	hypothalamic pituitary adrenal

HRP	horse radish peroxidase
HTP	hepatic tumor percentage
HUVEC	human umbilical vascular endothelial cells
ICAM-1	intracellular adhesion molecule 1
IFN-γ	interferon gamma
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IP	intraperitoneally
IRAK	interleukin-1 receptor-associated kinase
I/R	ischemia-reperfusion
IV	intravenous
LCC	Lewis Lung carcinoma
L/DEX	low-dose dexamethasone (0.05 mg/kg BW)
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP	monocyte chemotactic protein
M/DEX	medium-dose dexamethasone (0.25 mg/kg BW)
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MODS	multiple organ dysfunction syndrome
MSC	mesenchymal stem cells
MyD88	myeloid differentiation primary response gene 88
NK	natural killer
NLR	nucleotide binding oligomerization domain-like receptors
NOD	nonobese diabetic
NF-ϰB	nuclear factor-ϰB
OCT-1	organic cation transporter-1
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PD-1	programmed cell death 1
PMA	phorbol 12-myristate 13-acetate
PMS	n-methyl dibenzopyrazine methyl sulfate
PRR	pattern recognition receptors
RAGE	receptor of advanced glycation end products
rhAPC	recombinant human activated protein C
RQ-PCR	real time quantitative polymerase chain reaction
ROS	reactive oxygen species
RT	room temperature

SC	subcutaneous
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SphK1	sphingosine kinase 1
STAT3	signal transducer and activator of transcription 3
sTNF-r1	soluble tumor necrosis factor-receptor 1
TGF	transforming growth factor
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
XTT	tetrazolium

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CURRICULUM VITAE

Jan Willem van den Berg was born on November the 5th of 1981 in Geleen, The Netherlands. After graduating from high school at Graaf Huyn College in Geleen in 2000, he attended Pharmacy School at the University of Utrecht. In 2001 he started Medical School at the Erasmus University Rotterdam. In 2005 he performed research at the Department of Surgery, Laboratory of Experimental Oncology, University Medical Center Utrecht (Prof. Dr. I.H.M. Borel Rinkes). In 2007, he graduated from Medical School. Next, he worked as a surgical resident at the Department of Surgery of the Erasmus MC, University Medical Center Rotterdam (Prof. Dr. J.J.B. van Lanschot). In January 2008 he started as a PhD student at the Departments of Immunology and Surgery of the Erasmus MC, University Medical Center Rotterdam, under supervision of Prof. Dr. R. Benner, Prof. Dr. J.N.M. IJzermans, Dr. W.A. Dik, and Dr. R.W.F. de Bruin. His studies focused on novel therapeutic approaches to modulate inflammation in animal models of severe illnesses. In October 2010 he started as surgical resident at the Maasstad Hospital in Rotterdam (Dr. E. van der Harst). In July 2011, he will start his surgical training at the Maasstad Hospital (Dr. E. van der Harst) and the Erasmus MC (Prof. Dr. J.N.M. IJzermans).

PUBLICATIONS

J.W. van den Berg, R. Benner, J.N.M. IJzermans, R.W.F. de Bruin, W.A. Dik.
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Submitted.

J.W. van den Berg*, M. van der Zee*, C. van Holten-Neelen, J. Bastiaans, N.M.A. Nagtzaam, R. Benner, J.N.M. IJzermans, R.W.F. de Bruin, W.A. Dik.
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Nephrol Dial Transplant 2009, 24: 2701–2708.

PHD PORTFOLIO

Name PhD student:	J.W. van den Berg	PhD period:	Jan 2008 – Sept 2010
Erasmus MC Departments:	Immunology	Promotores:	Prof. Dr. R. Benner
	Surgery		Prof. Dr. J.N.M. IJzermans
		Copromotores:	Dr. R.W.F. de Bruin
			Dr. W.A. Dik
Postgraduate School:	Molecular Medicine		

PhD training	Year	Workload (ECTS)
Courses		
Laboratory animal science	2008	5.7
Biomedical research techniques	2008	1.6
Academic writing in English for PhD students	2008	1.6
Classical methods for data-analysis	2009	5.7
Molecular immunology	2009	3.0
Seminar and workshops		
Journal club	2008-2010	2.0
Presentations		
National conferences	2009	3.0
	2010	1.0
International conferences	2009	2.0
	2010	2.0

Teaching	Year	Workload (ECTS)
Supervising practicals		
Histology for medical students	2008	3.0
Immunology cases for medical students	2009	3.0
Bachelor thesis (Higher laboratory education)	2008	2.5