Regulation of Inflammatory Responses in Shock-Related Syndromes by Synthetic Oligopeptides and Steroids

Regulatie van ontstekingsreacties in shock-gerelateerde syndromen door synthetische oligopeptiden en steroïden

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I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. Inflammation

Inflammation is the body's way of responding to disturbances in homeostasis. Depending on the triggering event and the site of inflammation, the inflammatory response has different physiological purposes and pathological consequences (Figure 1) [1]. Inducers of inflammation are either foreign molecules or molecules derived from the body itself (self-molecules). These molecules, of which a selection is listed in Table 1, often contain highly conserved molecular patterns that are recognized by specific receptors that are expressed by cells of the immune system, but also by other cell types. Receptor binding of these molecularly conserved patterns leads to the production and secretion of inflammatory mediators that alter tissue functionality such that the tissue adapts to the harmful insult and homeostasis can be restored [1].

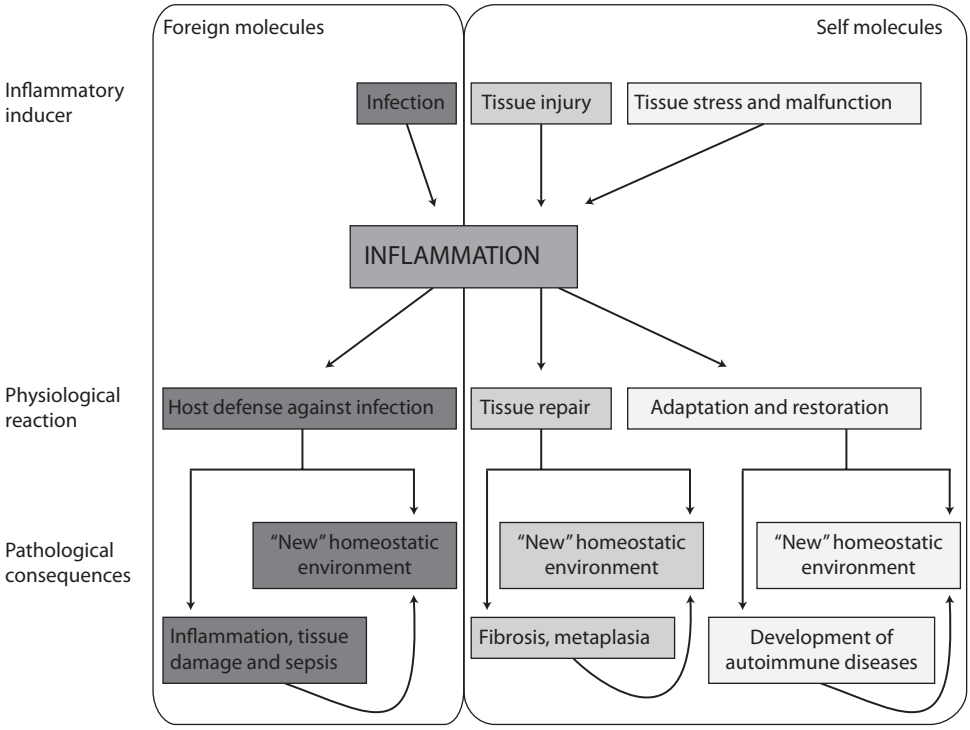

Figure 1. Inducers of inflammation.
Inducers of inflammation are either foreign molecules or molecules derived from the body itself (self-molecules) and can have different physiological purposes and pathological consequences. Modified from Medzhitov [1].

Table 1. A selection of inducers of inflammation.

Foreign molecules (pathogen-associated molecular patterns)	Reference	Self-molecules (danger-associated molecular patterns)	Reference
Cell membrane-derived		*Cell and tissue-derived*	
peptidoglycan	95,96	lipoprotein	97
zymosan	98	heat shock protein	99
lipopolysaccharide	96	high-mobility group box 1	100
mannan	101		
		Plasma-derived	
Intracellular-derived		complement	102
bacterial and viral nucleic acid	104,105	Hageman factor	103
Secreted factors		*Extra cellular matrix-derived*	
super antigen	106	collagen	107
pore forming exotoxin	108	hyaluronan	109
proteases	110	fibronectin	111,112
fMLP	113		

fMLP, formyl-methionyl-leucyl-phenylalanine.

2. The systemic inflammatory response syndrome and the compensatory anti-inflammatory response syndrome

The systemic inflammatory response syndrome (SIRS) is a complex host response inflicted by a variety of clinical insults, such as severe hemorrhage, ischemia-reperfusion injury, pancreatitis, and sepsis [2]. SIRS is characterized by (a) hyperthermia or hypothermia; (b) tachycardia; (c) tachypnea or hyperventilation; (d) leukocytosis; and (e) excessive production of pro-inflammatory mediators, of which a selection is listed in Table 2 [3]. The incidence of SIRS in the Western world is high and is even expected to increase in the near future [4,5]. SIRS occurs in more than one third of all in-hospital patients, in 50% of all intensive care unit (ICU) patients, and in 80% of all surgical ICU patients [4,5]. In developing countries the incidence of SIRS is even higher than in the Western world [6].

The SIRS-associated uncontrolled and excessive production of pro-inflammatory mediators can lead to multiple organ dysfunction syndrome (MODS) and death. Anti-inflammatory mediators, of which a selection is also listed in Table 2, are produced nearly concomitantly with the initial production of pro-inflammatory mediators [7,8]. The production of anti-inflammatory mediators is known as the compensatory anti-inflammatory response syndrome (CARS) and is essential to control SIRS (Figure 2) [8,9]. Initially, the pro- and anti-inflammatory responses take place locally in the affected

Table 2. A selection of pro- and anti-inflammatory cytokines that are involved in SIRS and CARS.

Inflammatory cytokines		Origin	Target	Reference
Pro-	Anti-			
IL-1β		monocytes,macrophages, keratinocytes	macrophages, lymphocytes, fibroblasts, endothelial cells	114-116
	IL-1RA	endothelial cells, myeloid cells, hepatocytes	myeloid cells, hepatocytes	117-121
	IL-2	T-cells	T-cells	122,123
	IL-4	T-cells, mast cells	B-, T-cells	124-127
IL-6	IL-6	T-cells, macrophages, endothelial cells	B-, T-cells, hepatocytes	128,129
	IL-6SR	monocytes, macrophages, T-cells	T-cells, endothelial cells	128
	IL-10	monocytes, macrophages, T-cells	monocytes, macrophages, T-cells	130,131
IL-12		NK-, T-cells	NK-, T-cells	132
	IL-13	T-cells	phagocytes	133,134
IL-18		macrophages	NK-, T-cells	135
	IL-22	NK-, T-cells	fibroblasts	136,137
IL-23		T-cells	T-cells	138
LT-α		T-cells	endothelial cells, T-cells	139
TNF-α		monocytes, macrophages	monocytes, macrophages, hepatocytes	140
	TNF-SRI	stromal cells, myeloid cells, B- and T- cells	stromal cells, myeloid cells, B- and T- cells	7,141-144
	TNF-SRII	stromal cells, myeloid cells, B- and T- cells	stromal cells, myeloid cells, B- and T- cells	7,141-144
	TGF-β	monocytes, dendritic cells, T-cells, fibroblasts, epithelial cells	fibroblasts, endothelial cells, epithelial cells	145-148
IFN-γ		lymphocytes	monocytes, macrophages	149

Abbreviations: IL, interleukin; TNF, tumor necrosis factor; SR, soluble receptor; IFN, interferon; RA, receptor antagonist; LT, lymphotoxin.

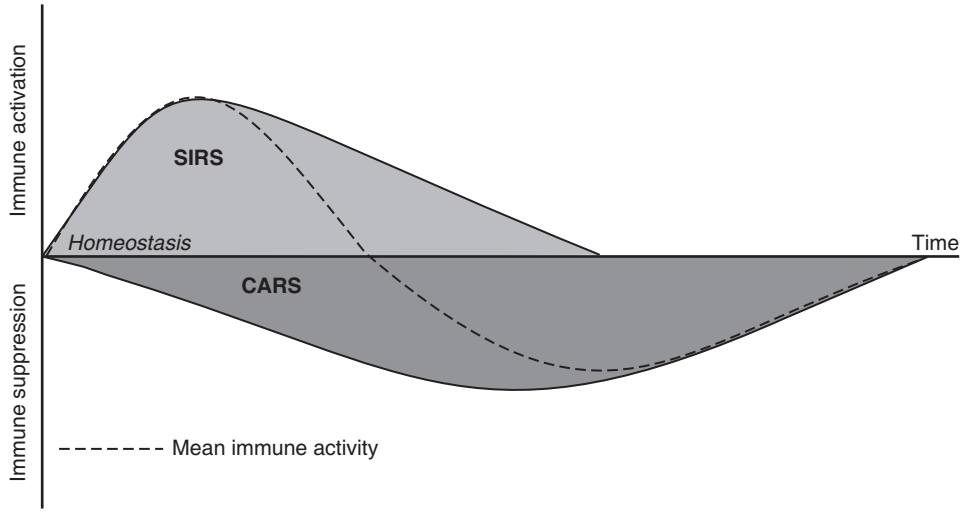

Figure 2. Schematic representation of SIRS and CARS following a severe clinical insult.
Nearly concomitant with the initial production of pro-inflammatory mediators starts the production of anti-inflammatory mediators. The balance between pro- and anti-inflammatory mediators determines the inflammatory status of the individual.

tissue and can be viewed as a battle between opposite forces. When the level of mediators is correctly balanced, homeostasis is restored. However, in case of excessive production of pro-inflammatory mediators in the affected tissue, systemic spillover of pro-inflammatory mediators can occur that affects other organs and induces pathology. When CARS is too strong and anti-inflammatory mediators are detected systemically, a profound state of immune paralysis can develop that makes the individual highly susceptible to infections [8,9].

2.1 Immune cells and acute systemic inflammation

Systemic spillover of pro-inflammatory mediators activates endothelial cells as well as other cells, including neutrophils and monocytes [10]. Subsequently, these cells start producing inflammatory mediators that activate other leukocytes, such as NK-, T-, and B-lymphocytes, leading to an uncontrolled and massive production of inflammatory mediators (Figure 3) [11,12]. Thereafter, leukocytes migrate from the circulation into vital tissues.

The extravasation process of leukocytes requires a multistep cascade of adhesive and migratory events that are mediated by three classes of adhesion molecules, the selectins, integrins and adhesion receptors of the immunoglobulin super family. The extravasation process of leukocytes can be divided into five sequential stages: (a)

selectin-mediated leukocyte attraction; (b) integrin-mediated intravascular crawling; (c) firm arrest of leukocytes to the endothelium; (d) chemokine-induced leukocyte activation; and finally (e) transendothelial migration [13,14]. Subsequently, through a chemokine gradient, leukocytes migrate to the inflamed loci, where they release their contents that affect local cell and tissue function [10,13,15]. Depending on the intensity of this response, the tissue is either completely shut down or an attempt is made to restore homeostasis, with some pathological changes [16].

Only a small percentage of SIRS patients die during the initial stage of inflammation, as a result of severe tissue dysfunction and destruction [10,17]. The primary tissues affected during the initial stage of SIRS are sequentially lungs, gut, liver, kidneys, and eventually the central nervous system [18]. Most patients that survive the hyper-inflammatory response enter a protracted stage of immunosuppression that has been termed immune paralysis [19].

2.2 Immune cells and immune paralysis

Immune paralysis is the inability to generate an immune response against foreign molecules and molecules derived from the body itself [20,21]. Several cellular mechanisms, such as the generation of regulatory T-lymphocytes and myeloid suppressor cells contribute to this process [22,23]. However, the most important contributing mechanism is apoptosis-induced depletion of immune cells, including antigen presenting cells (APC), B-, and T-lymphocytes (Figure 4) [24,25]. It is not only the apoptosis-induced loss of immune cells that contributes to immune paralysis. Apoptosis also induces anergy in surviving cells, as ingestion of apoptotic bodies by phagocytes reduces the expression of co-stimulatory molecules, such as CD80, CD86 and CD40L, on these cells [26]. Furthermore, phagocytosis of apoptotic bodies stimulates the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, by these cells [20]. Anti-inflammatory cytokine production can also be induced through direct cell contact with apoptotic cells [27]. In addition, apoptosis-induced loss of gastro-intestinal epithelial cells increases gut permeability with consequent translocation of microbial flora from the intestine into the circulation [28]. This, in combination with the inability of the immune system to respond appropriately against invading pathogens, increases the susceptibility to sepsis and septic shock [12,17,20]. Recent studies show that the prevention of immune cell apoptosis improves survival in a murine poly-microbial induced sepsis model [20].

In summary, apoptotic depletion of immune cells is a key pathological process that dampens the pro-inflammatory response which, in combination with the immunosuppressive effect of apoptotic cells, can induce a profound state of immune paralysis that is a major cause of morbidity and mortality.

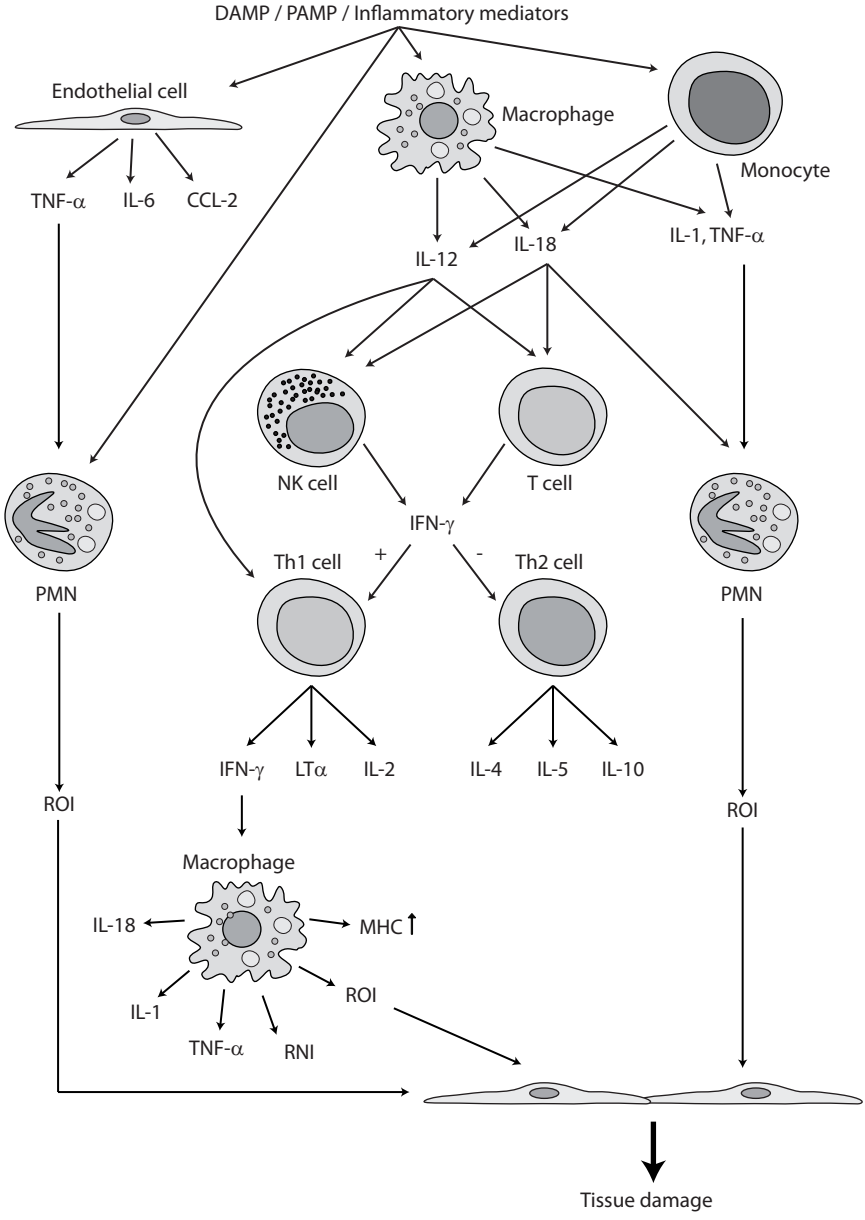
DAMP / PAMP / Inflammatory mediators
Endothelial cell
Macrophage
Monocyte
TNF-α
IL-6
CCL-2
IL-12
IL-18
IL-1, TNF-α
NK cell
T cell
IFN-γ
+
-
Th1 cell
Th2 cell
PMN
PMN
IFN-γ
LTα
IL-2
IL-4
IL-5
IL-10
ROI
ROI
Macrophage
IL-18
MHC ↑
IL-1
TNF-α
RNI
ROI
Tissue damage

Figure 3. Activation of host defense by pro-inflammatory cytokines.
Upon DAMP or PAMP recognition by endothelial cells and immune cells these cells become activated and secrete cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-12, and IL-18. Subsequently these cytokines activate T-, and natural killer (NK)-cells, and neutrophils. The activated T- and NK-cells produce interferon (IFN)-γ and together with IL-12, they induce the activation of $CD4^+$ Th1 cells, characterized by the production of IFN-γ, IL-2 and lymphotoxin (LT)-α. In addition, these mediators enhance the bactericidal activity in immune cells through the production of TNF-α, radical oxygen intermediates (ROI), and radical nitrogen intermediates (RNI), and increased MHC expression. IFN-γ inhibits the activation of $CD4^+$ Th2 cells, which can produce and secrete amongst others IL-4, IL-5, and IL-10. The latter is a potent inhibitor of Th1-derived cytokines. Modified from Netea *et al*. [11].

2.3 Danger recognition and molecular pathways involved in inflammation

Specific cell membrane-bound and secreted receptors initiate inflammatory responses as these receptors survey the host for potential danger [29]. These receptors are highly conserved throughout evolution and are referred to as pattern recognition receptors (PRR), of which a selection is listed in Table 3 [29]. PRR recognize a series of molecularly preserved patterns that are expressed on micro-organisms (pathogen-associated molecular patterns (PAMP)) and on intracellular proteins, DNA and RNA derived from dying host cells (danger-associated molecular patterns (DAMP)) [30]. In addition, phagocytosis of apoptotic cells can lead to the release of large amounts of DNA and RNA, which can interact with intracellular PRR [31].

Most PRR are expressed on extracellular and intracellular membranes of many cell types [29]. Cell membrane-bound PRR, such as most Toll-like receptors (TLR) and C-type lectins, are involved in the recognition of extracellular pathogens, while intracellular PRR are involved in the recognition of intracellular pathogens [29,32]. Intracellular PRR such as TLR-3, TLR-7, TLR-8, TLR-9, RNA helicase family (RLR), retinoic acid inducible gene protein 1 (RIG-1), and melanoma differentiation-associated gene 5 (MDA5) are involved in the recognition of different groups of RNA viruses and intracellular bacteria [33,34]. The nucleotide oligomerization-binding (NOD)-domain-like receptor (NLR) family consists of a group of intracellular PRR that include NLR-domain leucine rich repeat and pyrin domain-like receptors (NALP), IL-1β-converting enzyme (ICE)-protease activating factor (IPAF), and neuronal apoptosis inhibitor factors (NAIP) [30]. NLR survey the cytoplasm for the presence of intracellular pathogens [33]. Recently it was shown that NOD2 recognizes cytoplasmic viral RNA by triggering the activation of interferon-regulatory factor 3 (IRF-3) and the subsequent production of interferon (IFN)-β [35]. Secreted PRR are involved in the opsonization of invading micro-organisms and comprise amongst others the complement factors C3a and C1q, as well as the lipopolysaccharide (LPS)-binding protein (LBP) [36,37].

PAMP or DAMP recognition by PRR, such as by TLR, activates several intracellular signaling cascades, in which adaptor molecules are key players [38]. The most

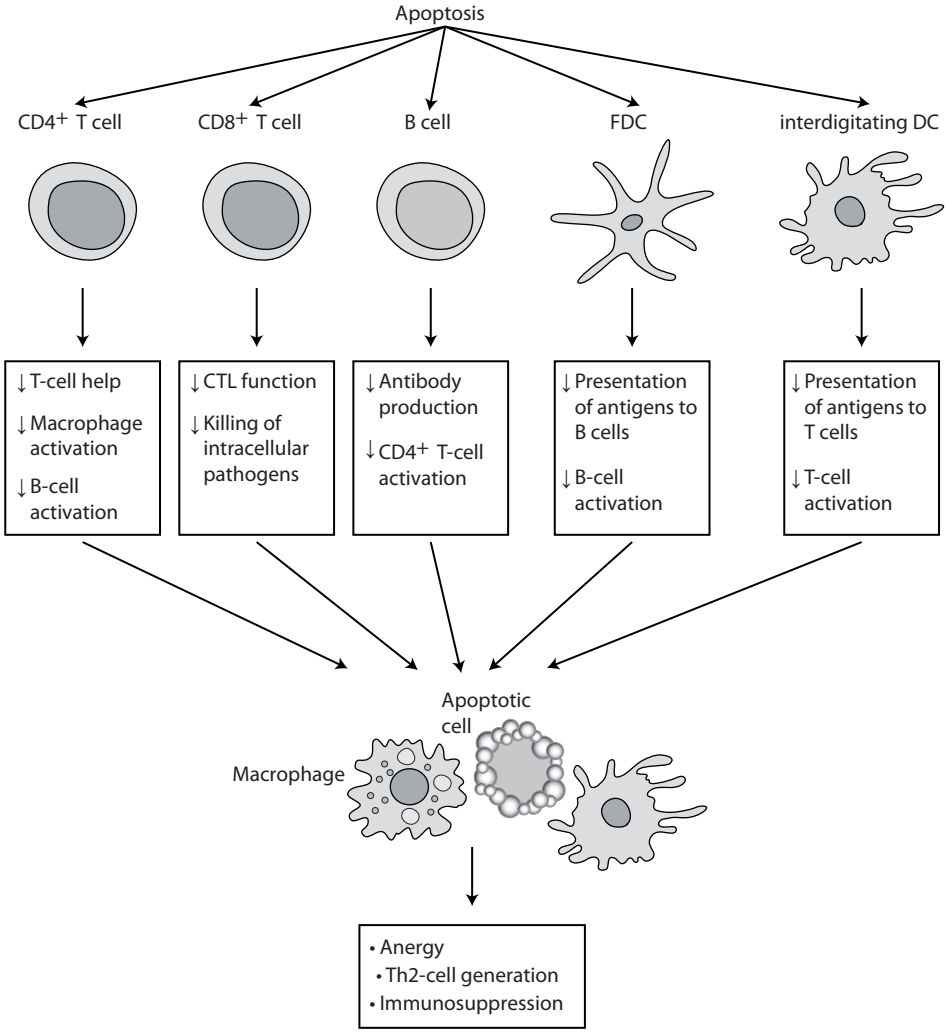

Figure 4. Impact of apoptosis on immune function.
Apoptosis-induced depletion of immune cells impairs the host anti-microbial defenses. In addition to the immune cell depletion, apoptosis also induces anergy in surviving cells as ingestion of apoptotic bodies by phagocytes shifts their pro-inflammatory state to an anti-inflammatory phenotype or these cells become anergic. CTL, cytotoxic T lymphocytes; FDC, follicular DC. Modified from Hotchkiss *et al.* [20].

Table 3. A selection of pattern recognition receptors and their ligands.

Pattern receptor	recognition	Ligand	Reference
TLR	TLR-1	triacyl lipoproteins	150
	TLR-2	lipoproteins, PG, zymosan, hsp, hyaluronan, HCMV, HSV-1	97 95,96 98 99 109 151
	TLR-3	dsRNA, poly I:C	104
	TLR-4	LPS hsp fibrinogen fibronectin	96 99,152 112 111,112
	TLR-5	flagellin	153
	TLR-6	lipoteichoic acid zymosan	154 98]
	TLR-7	ssRNA	105
	TLR-8	ssRNA	105
	TLR-9	demethylated CpG	155
	TLR-10	unknown	156
	TLR-11	profilin	157
	TLR-12	unknown	158
	TLR-13	unknown	158
NLR	NAIP1	anthrax lethal toxin	159,160
	NAIP2	muramyldipeptide	161
	NAIP3	bacterial RNA	160
	NAIP4	unknown	162
	NOD1	murodipeptide	163
	NOD2	mitochondria viral ssRNA	35 35
RLR	RIG-1	5'-triphosphate ssRNA poly I:C	164 164
	MDA5	MHV poly I:C	165 164
Complement	C3	OH-groups on carbohydrates and proteins	36
	C1q	immune complexes	36
Others	LBP	LPS	37

Abbreviations: TLR, Toll-like receptor; NOD, nucleotide oligomerization-binding; NLR, NOD-domain-like receptor; RIG-1 retinoic acid inducible gene-1; RLR, RIG-1-like receptor; NAIP, NLR-domain leucine rich repeat and pyrin domain-like receptors; MDA5, melanoma differentiation-associated gene 5; MHV, murine hepatitis virus; PG, peptidoglycan; LPS, lipopolysaccharide; hsp, heat shock protein; ds, double stranded; ss, single stranded; RNA, ribonucleic acid; HCMV, human cytomegalovirus; HSV-1, herpes simplex virus type-1; LBP, LPS-binding protein; CpG, cystine purine repeat in DNA.

important adaptor molecules in TLR signaling are myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), MyD88 adaptor-like (MAL), and TIR domain-containing adaptor-inducing IFN-β (TRIF) [38]. TLR mostly signal in a MyD88-dependent manner, with the exception of TLR-3 which signals through TRIF [39]. Recruitment of adaptor molecules to PRR leads to the initiation of intracellular signaling cascades that activate multiple transcription factors (discussed below), followed by transcriptional activity of a variety of inflammatory genes that encode cytokines, chemokines and adhesion molecules [40]. Together, these inflammatory molecules orchestrate the inflammatory response [40].

Based on the mode of action and function, the transcription factors downstream of PRR signaling can be divided into three categories [41]. The first category of transcription factors, which comprises nuclear factor (NF)-κB and IRF, are constitutively expressed by all cell types and are retained in an inactive form in the cytoplasm [42,43]. Activation of these transcription factors occurs by post-translational modifications, such as phosphorylation and inactivation of inhibitor molecules, after which they translocate into the nucleus to control gene transcription [41,44]. The second category of transcription factors, to which CCAAT/enhancer-binding protein (C/EBP)-δ belongs, regulate the waves of gene activation that occur after the primary response and they do so over a prolonged period of time [45,46]. The third category comprises transcription factors, such as PU.1 and runt-related transcription factor (RUNX)-1, that are well known for their role in hematopoiesis, but upon PRR activation these transcription factors can also enhance the transcriptional activity of transcription factors belonging to the first category [47-49].

Collectively, PRR activation initiates several intracellular signaling cascades that activate multiple transcription factors, which can initiate the transcription of genes that shape the inflammatory response.

3. Sepsis and septic shock

The term sepsis originates from the Greek word *sepo*, meaning decomposition of animal or vegetable organic matter in the presence of bacteria or putrefaction [50]. Nowadays, sepsis is defined as SIRS with a suspected or proven infection [51]. In clinical routine diagnostics it may, however, be difficult to detect the pathogen in blood or other body materials [52]. This makes the differential diagnosis between infectious and noninfectious SIRS, as well as the decision for a successful treatment strategy, difficult [52,53]. Therefore, a lot of research is performed to identify biomarkers that can discriminate between infectious and non-infectious SIRS. Several biomarkers with varying degrees of specificity and sensitivity to discriminate between infectious and noninfectious SIRS have been described. These include procalcitonin (PCT), C-reactive protein (CRP), soluble CD14, LBP, differential blood cell count and extracellular

phospholipase A2 [7,54-58]. However, the multi-factorial character of SIRS, as it depends amongst others on the nature of the inciting agent and the inflammatory status of the patient, makes the identification of one biomarker to discriminate between infectious and non-infectious SIRS difficult [7-9,19,59,60]. This means that a panel of markers should be used to discriminate between infectious and noninfectious SIRS.

Despite great improvements in our knowledge on the pathobiological processes involved in SIRS and sepsis, as well as the identification of several biomarkers, the incidence of sepsis still increases [17]. A shift in bacterial species that cause sepsis is thought to contribute to this. During the last decade the incidence of Gram-positive sepsis steadily increased, while before the 1980s Gram-negative bacteria were responsible for most cases of sepsis [17,61]. Nowadays, the most commonly isolated Gram-positive pathogens from septic patients are *Staphylococcus aureus* and *Streptococcus pneumoniae*, while the most common Gram-negative pathogens are *Escherichia coli, Klebsiella* species and *Pseudomonas aeruginosa* [61].

Annually, in the United states more than 200.000 people die from sepsis [17]. Only a small percentage of septic patients will die from SIRS [17]. The majority of septic patients that survive the initial insult, subsequently develop a profound state of immune paralysis that is manifested by the inability to eradicate the primary infection and/or the development of secondary infections [19]. This is also the phase in which most septic patients die [20]. The fact that many of the pathogens responsible for the fatal secondary infections, such as *Stenotrophomonas* species*, Acinetobacter* species*,* and *Candida albicans,* are not particularly virulent in individuals with a normal, competent immune system highlights the severe degree of immunosuppression that can occur in patients with SIRS and sepsis.

4. Treatment of infectious and non-infectious SIRS

The standard care for sepsis is the intravenous administration of a broad spectrum of antibiotics that eradicate the inciting organism. This treatment should be reevaluated daily to optimize efficacy, prevent antibiotic resistance, and avoid toxicity [62]. Over the last decades several anti-inflammatory therapeutics, such as corticosteroids, have been investigated in infectious and noninfectious SIRS. The results from these studies show that corticosteroids can inhibit the inflammatory response and pathology during a noninfectious SIRS, while during an infectious SIRS corticosteroid treatment did not improve survival. Despite this fact, it is not uncommon that clinicians treat sepsis patients with corticosteroids, such as hydrocortisone at a dose of 200-300 mg/day, for seven days [18]. Also activated protein C, which exerts anti-coagulant, anti-inflammatory, and fibrinolytic effects, is used to treat septic patients [18]. Furthermore, hyperglycemia is common in septic patients and there is evidence that maintaining blood glucose levels within 4.4-6.1 mmol/L can reduce morbidity and mortality [18]. However, maintaining

the blood glucose concentration at such a level is difficult and therefore hypoglycemic events can occur.

Other therapeutics have also been investigated and were focused on reducing systemic and local inflammatory responses with monoclonal antibodies that neutralize cytokine activity or inhibit inflammatory mediator production. Although such studies showed a remarkable survival improvement in LPS-models of septic shock in animals [63,64], no such beneficial effect on survival was observed in the murine poly-microbial induced sepsis model [65-67] as well as in clinical trials involving septic patients [68-70]. Therefore, more studies are needed to investigate and understand the effectiveness of anti-inflammatory therapies on morbidity and mortality during sepsis and septic shock.

5. Pregnancy and immunoregulation

Pregnancy remains to some extent an immunological enigma; the fetus inherits histocompatibility antigens from the father and yet coexists within the mother's uterus in harmony throughout pregnancy. However, when fetal tissues are grafted onto a third party they do induce an immunological response [71-73]. This suggests that the maternal immune system is regulated in such a way that immune responses against the fetal tissues are prevented to facilitate pregnancy success [74,75].

During pregnancy, the maternal immune response shifts from a type-1 (cell-mediated) response towards a type-2 (humoral) response [76]. This shift is most pronounced at the maternal-fetal interface, but it may also modulate systemic immunity to some extent. Consequently, during pregnancy symptoms of type-1 associated autoimmune diseases (e.g. rheumatoid arthritis and multiple sclerosis) decline, but they return to pre-pregnancy levels post-partum. In contrast, symptoms of systemic lupus erythematosus, which is considered as a type-2 associated autoimmune disease in which the principal pathology is autoantibody driven, may flare up during pregnancy [76]. Also, as a result of the pregnancy-associated immunoregulation, the susceptibility to certain pathogens, including *Listeria monocytogenes*, *Toxoplasma gondii, Leishmania major*, and *Plasmodium species* increases [77-80]. Likely, a particular pregnancy-associated hormonal environment contributes to the shift in immunity [76,81].

Characteristic for human pregnancy is the production of the hormone human chorionic gonadotropin (hCG) by syncytiotrophoblasts in the placenta [82]. hCG is a member of the glycoprotein hormone family to which also belong luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) [83]. Hormones from this family consist of similar heterodimeric structures of an identical α-subunit, but a unique β-subunit that confers the hormonal biological specificity [84]. It has been recognized that hCG, besides its endocrine function, has profound immunosuppressive effects [78-80,85-87]. There is also evidence that hCG-derived peptides have immunoregulating properties [see below].

5.1 hCG-derived oligopeptides

During pregnancy, hCG exists in a variety of forms in serum and urine. These forms include heterodimeric hCG and its separate α and β subunits, but also nicked hCG and hCG β core, which both consist of a β-chain with a defective loop 2 [88-90]. It has been demonstrated that a 400-2000 Dalton fraction from pregnancy urine prevented the onset of autoimmune diabetes in nonobese diabetic (NOD) mice [77,91]. In addition this fraction was able to reverse established pancreatic inflammation in these mice [91]. Considering these data it has been hypothesized that oligopeptides originating from loop 2 cleavage of the β-chain of hCG have immunoregulatory activity [92]. Therefore, based on known preferential cleavage sites within the loop 2 sequence MTRVLQGVLPALPQVVC (Figure 5) seven different oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, and VVC) were synthesized, as well as two alanine replacement variants of LQGV, namely AQGV and LAGV [92,93]. Several of these synthetic oligopeptides were tested in different animal models that are characterized by excessive immune activation [93,94]. In this thesis the immunoregulatory effects of the oligopeptides LQGV, VLPALP, AQGV, and LAGV were further explored in *in vivo* models associated with infectious and noninfectious inflammation. Also mechanistic studies of LQGV were explored in *in vivo, ex vivo,* and *in vitro* experimental settings.

6. Aim of the thesis

The overall aim of this thesis was to examine the anti-inflammatory effects of LQGV, VLPALP, AQGV, and LAGV in animal models associated with SIRS. Infectious as well as non-infectious animal SIRS models were used. The underlying mechanisms, by which LQGV can exert anti-inflammatory activity were also investigated. **Chapter II** demonstrates the effects of high dose treatment with LQGV, VLPALP, and AQGV (50 mg/kg BW) on early innate immune activation in a *L. monocytogenes* infection model in mice. In **chapter III** the *in vivo* effects of the same high dose of LQGV (50 mg/kg BW) on leukocyte subpopulations was investigated. In **chapter IV** mechanistic studies on the mode of action of LQGV (5 and 50 mg/kg BW) were explored by using an *in vivo* LPS-induced shock model in mice and *ex vivo* murine adrenal gland cultures. In **chapter V** the effects of LQGV, AQGV, and LAGV (5 mg/kg BW) was explored on hemorrhagic shock and resuscitation induced inflammation and subsequent liver damage in rats. **Chapter VI** demonstrates the effect of LQGV (5 mg/kg BW) in a polymicrobial sepsis model in mice. This study explores whether LQGV treatment is of additive value to the standard treatment in sepsis (antibiotics and fluid administration). **Chapter VII** demonstrates the effect of high, median, and low dose dexamethasone treatment on mortality and inflammation in a polymicrobial sepsis model in mice. Also the effect of low dose dexamethasone treatment in combination with antibiotics and fluid administration was explored. **Chapter VIII**, the general discussion of the experimental work of this

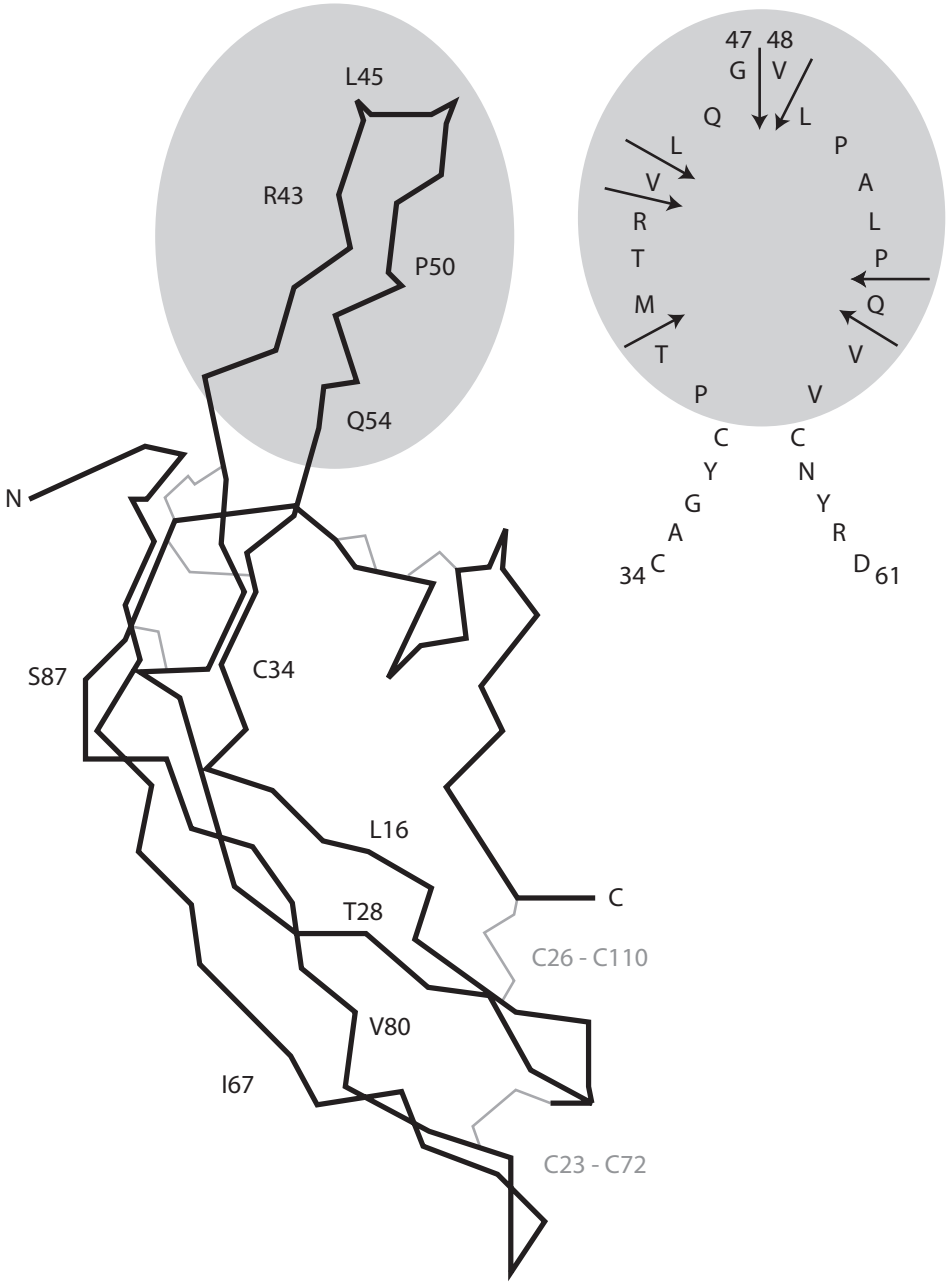

Figure 5. Structure of β-hCG with loop 2 (left) and the amino acid sequence of loop 2 (right). Adapted from Lapthorn *et al.* [90]. Arrows indicate the preferential cleavage sites in loop 2.

thesis, deals with the implications of the studies in the context of available literature. Also suggestions are made for future research in this area.

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II

SYNTHETIC HUMAN CHORIONIC GONADOTROPIN-RELATED OLIGOPEPTIDES IMPAIR EARLY INNATE IMMUNE RESPONSES TO *LISTERIA MONOCYTOGENES* IN MICE

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ABSTRACT

Background: Synthetic human chorionic gonadotropin (hCG)-related oligopeptides are potent inhibitors of pathogenic inflammatory responses induced by *in vivo* lipopolysaccharide exposure or hemorrhagic shock-induced injury. In this study, we tested whether hCG-related oligopeptide treatment similarly altered inflammatory responses and innate host defenses in mice during experimental *Listeria monocytogenes* infection.

Methods: Mice were infected with *L. monocytogenes* and treated with hCG-related oligopeptides (LQGV, VLPALP, AQGV) or phosphate-buffered saline. Subsequently, mice were analyzed for bacterial loads, cytokine and chemokine responses, and inflammatory cell infiltrates in target organs.

Results: Oligopeptide administration increased bacterial numbers in spleen and liver at 6 h after infection. Simultaneously, CXCL1/KC and CCL2/MCP-1 plasma levels as well as neutrophil numbers in spleen, blood, and peritoneal cavity decreased. In contrast, at 18 h after infection, systemic tumor necrosis factor-α, interleukin-12p70, interleukin-6, and interferon-γ levels increased statistically significantly in oligopeptide-treated mice compared to controls, which correlated with increased bacterial numbers.

Conclusion: These data show that treatment with hCG-related oligopeptides (LQGV, VLPALP, AQGV) inhibits early innate immune activation by reducing initial chemokine secretion following infection. This leads to bacterial overgrowth with subsequent enhanced systemic inflammation. Our data underscore the importance of early innate immune activation and suggests a role for hCG-derived oligopeptides at the placenta that increases the risks to *L. monocytogenes* infections.

INTRODUCTION

Listeria monocytogenes is an intracellular bacterium with a predilection for causing invasive infection in individuals with compromised host defenses [1]. It is also a model pathogen used for understanding innate and adaptive immune responses to bacterial infection [1]. Innate immune receptors, such as Toll-like receptors and nucleotide oligomerization-binding domain-like receptors, mediate the earliest recognition of *L. monocytogenes* components and trigger signaling pathways that induce specific chemokine and cytokine responses [2-4]. Chemokines are critical for directing cellular recruitment to infected tissues, and cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-12 are crucial for eradicating *L. monocytogenes* through activation of NK cells and organ-specific phagocytes [5-6]. Accordingly, mice lacking key chemokines, cytokines, or their cognate receptors and

downstream signaling proteins are highly susceptible to *L. monocytogenes* infection [7-11].

Pregnancy is one condition that increases the risk of invasive listeriosis [1]. Pregnancy transiently biases immune responses of the maternal host toward a type 2 (humoral) and away from a type 1 (cell-mediated) phenotype [12]. This shift is most pronounced at the maternal-fetal interface, but it may also modulate systemic immunity to some extent. Consequently, pregnancy has deleterious effects on the outcome of infections such as leishmaniasis, malaria, toxoplasmosis, and listeriosis [13-16]. In addition, autoimmune diseases such as systemic lupus erythematosus, in which the principal pathology is autoantibody production, tends to flare up during pregnancy, whereas rheumatoid arthritis is ameliorated in the maternal host [17].

The pregnancy hormone human chorionic gonadotropin (hCG) exerts endocrine as well as immunosuppressive functions [18]. Recently, we demonstrated that hCG induces dendritic cells to differentiate towards a tolerogenic phenotype [19]. In addition, hCG was observed to decrease IFN-γ production and prevent the onset of autoimmune diabetes in nonobese diabetic mice [20]. Remarkably, the anti-diabetic effect was not due to native hCG, but resided in a 400-2000 Da peptide fraction that likely originated from proteolytic degradation of loop 2 of β-hCG [21]. On the basis of preferential cleavage sites within loop 2 of β-hCG, we developed and tested the activity of synthetic oligopeptides in experimental inflammation [22-24]. These studies demonstrated that the oligopeptides LQGV and AQGV (alanine substitution) reduced proinflammatory cytokine levels, reduced adhesion molecule expression, and diminished granulocytic infiltration into the liver following hemorrhagic shock and the kidney following ischemia & reperfusion, [22-23]. In addition, the oligopeptide VLPALP reduced lipopolysaccharide (LPS)-induced mortality as effectively as did the 400-2000 Da hCG-derived peptide fraction [25].

To gain more insight into the effects of hCG-related oligopeptides on innate immune responses during infection with live bacteria, we tested the extent to which hCG-related oligopeptides altered host defenses in experimental *L. monocytogenes* infection in mice. Our data suggest that treatment with hCG-related oligopeptides impairs innate immune responses against *L. monocytogenes* by inhibiting chemokine responses and subsequent cellular recruitment to infected organs. This results in delayed bacterial clearance and more aggressive bacterial propagation. These data underscore the importance of early activation of innate immune responses following *L. monocytogenes* infection.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 mice (Jackson Laboratory and Harlan) were 8–16 wk of age when used in experiments and allowed food and water *ad libitum*. Experiments were approved by the local animal care and use committees.

Bacteria

Inocula of live *L. monocytogenes* (strain EGD) and heat killed *L. monocytogenes* (HKLM) were prepared as described elsewhere [26].

L. monocytogenes infection model

Mice were infected by intravenous injection of 2.0–5.5 $\log_{10}$ colony-forming units (CFU) wild-type *L. monocytogenes* or 7.0 $\log_{10}$ CFU of the *L. monocytogenes Δhly* mutant DP-L2161 [27]. The hCG-related oligopeptides VLPALP, LQGV, and AQGV (GL Biochem Shanghai) were dissolved in PBS and intraperitoneally injected (50 mg/kg body weight) starting 24 h before infection and continued every 24 h thereafter. PBS-treated mice served as controls. Mice were euthanized at fixed time points.

Tissue collection and evaluation of bacterial CFUs

Peritoneal lavage was performed with ice-cold PBS. Liver, spleen and bone marrow were isolated aseptically at necropsy. Blood was collected from the submandibular vein into EDTA-containing tubes (Greiner, Bio-one) and plasma was obtained by centrifugation (3000 rpm; 10 min), immediately frozen and stored at -80°C until assayed. Liver and spleen were divided into sections for evaluation of bacterial CFUs or for flow cytometric analysis. Sections were weighed, homogenized and bacterial CFUs were quantified as described elsewhere [26]. CFUs bacteria in blood, peritoneal washings and bone marrow cell suspensions were determined similarly. Results were expressed as mean (± standard error of the mean) $\log_{10}$ CFUs bacteria per gram tissue, per mL blood, per mL peritoneal fluid, or per femur.

In vitro stimulation of splenocytes

Uninfected mice were injected intraperitoneally with PBS or LQGV (50 mg/kg); after 18 h, splenocytes were isolated and cultured at a density of 10^6 cells/mL in Roswell Park Memorial Institute medium containing antibiotics and 5% fetal calf serum. Cells were stimulated with HKLM (10^3-10^8) or LPS (100 ng/mL) and supernatants were collected after overnight incubation for cytokine level measurements.

Evaluation of cytokines and chemokines in plasma and culture supernatant

Levels of TNF-α, IL-6, IL-12p70, IL-10 and CCL2/MCP-1 were determined in plasma by means of cytometric bead array (BD Biosystems) as described elsewhere [22]. CXCL1/KC in plasma, TNF-α and IL-6 levels in culture supernatant were determined by means of enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems Europe). Results are expressed as mean (± standard error of the mean) pg/mL.

Flow cytometric analysis

Flow cytometric analysis was performed on blood, peritoneal lavage, bone marrow and spleen cell suspensions, as described elsewhere [26]. Briefly, cells were incubated with monoclonal antibodies directed against Ly6C (ER-MP20 [28]), Ly6G (1A8), and CD11b (M1/70) (BD PharMingen) for 30 min, washed 3 times with PBS/0.5%BSA/20mM sodium-azide, and fixed with 1% paraformaldehyde (weight per volume). Monocytes are defined as CD11b-positive, Ly6C-positive and Ly6G-negative. Neutrophils were defined as CD11b-positive, Ly6C-positive and Ly6G-positive. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed using Flow-Jo software (version 7.5.4; TreeStar).

Statistical analysis

Statistical analysis was performed using SPSS software (version 15; SPSS). Differences between groups were analyzed with Kruskal-Wallis statistical test. If this resulted in $p < 0.05$, a Dunn's Multiple Comparison post-test was performed and a $p < 0.05$ was considered statistically significant. Correlation coefficients were determined with Pearson's correlation analyses with significance set at a $p < 0.05$.

RESULTS

Association between hCG-related oligopeptide treatment and increased bacterial numbers after lethal infection

To assess the effects of LQGV, VLPALP and AQGV during *L. monocytogenes* infection, oligopeptide-treated and control mice were infected with 4 $\log_{10}$ CFUs bacteria (1-3 median lethal dose) and then bacterial loads in target organs were measured.

In the spleens of PBS-treated mice, the mean bacterial load increased from 4.2 $\log_{10}$ CFUs of bacteria at 6 h after infection to 7.4 $\log_{10}$ at 72 h after infection. By comparison, for each oligopeptide, CFUs of bacteria were statistically significantly higher in oligopeptide-treated mice than those in control mice at 6 h after infection. Moreover, at each time point, splenic CFUs were statistically significantly higher in the LQGV-treated

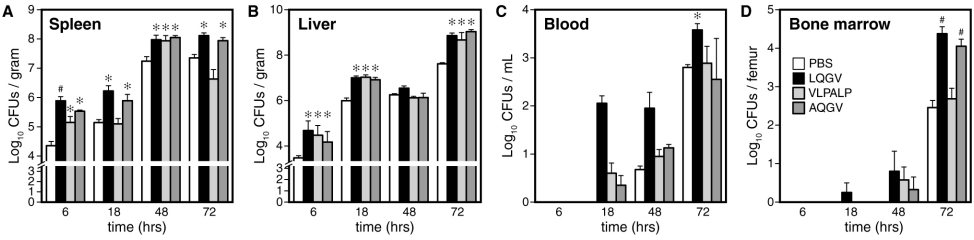

Figure 1. Increased bacterial loads as a result of hCG-related oligopeptide treatment after lethal *Listeria monocytogenes* challenge.

Phosphate-buffered saline (PBS; control), or one of the oligopeptides (VLPALP, LQGV, or AQGV) was administered intraperitoneally daily starting 24 h before infection with 4 $\log_{10}$ colony-forming units (CFUs) of bacteria. Mice were killed at the indicated time and CFUs of bacteria in tissues were quantified. Results are presented as CFUs of bacteria in (A) spleen ($\log_{10}$/g), (B) liver ($\log_{10}$/g), (C) blood ($\log_{10}$/mL), and (D) bone marrow ($\log_{10}$/femur) from the indicated treatment groups at 6, 18, 48, 72h after infection. Results shown are from one representative experiment out of three (n = 4 mice/group). * $p < 0.05$, # $p < 0.01$ compared with PBS-treated mice.

mice ($p < 0.01$) and AQGV-treated mice ($p < 0.05$) compared with PBS-treated mice (Figure 1A).

There was a mean of 3.8 $\log_{10}$ CFUs detected in the livers of PBS-treated mice at 6 h after infection, which increased to a mean of 7.6 $\log_{10}$ CFUs at 72h after infection. As also found in the spleen, CFUs of bacteria in the liver were statistically significantly higher in oligopeptide-treated ($p < 0.05$) mice at 6, 18, and 72h after infection than in PBS-treated mice (Figure 1B).

Bacteria were not detected in blood and bone marrow of PBS-treated mice until 48h and 72h after infection, respectively. In contrast, bacteremia was noted by 18h after infection in oligopeptide-treated mice, with LQGV-treated mice always showing the highest bacterial load in the blood (Figure 1C). In bone marrow, bacteria were present at 18 h after infection in LQGV-treated mice, and by 48h after infection in VLPALP-treated mice and AQGV-treated mice. LQGV-treated mice and AQGV-treated mice had the highest bacterial load in bone marrow at 72 h after infection (Figure 1D).

Effect of LQGV treatment during sublethal challenge with wild-type *L. monocytogenes* and infection with avirulent *L. monocytogenes* mutants

Because LQGV treatment consistently produced the highest bacterial loads during lethal wild-type infection, subsequent experiments tested the effect of this oligopeptide in 2 different models of sublethal infection. First, mice were infected with 2.0 $\log_{10}$ CFUs wild-type *L. monocytogenes* (0.01 median lethal dose) and bacterial loads in organs were quantified as before. As also found in lethal infection, LQGV treatment resulted in statistically significantly increased bacterial loads in the spleen ($p < 0.01$) and liver

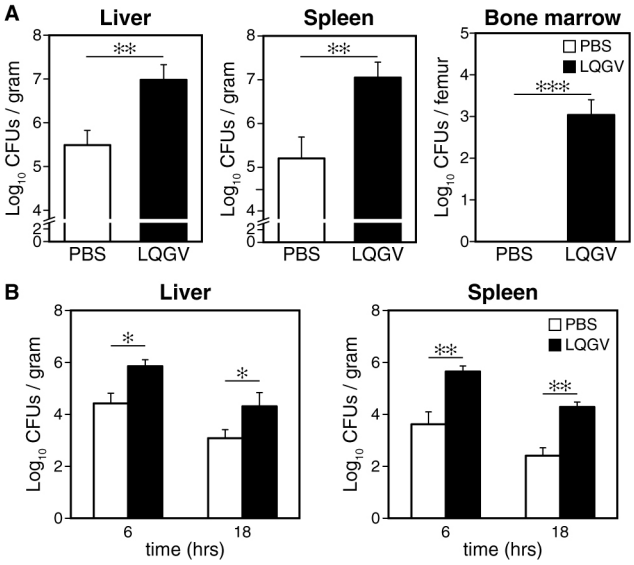

Figure 2. Increased bacterial loads as a result of LQGV treatment following a sub-lethal *L. monocytogenes* challenge or infection with avirulent Δ*hly L. monocytogenes* mutants.
(A) Results for infection with wild-type *L. monocytogenes.* Phosphate-buffered saline (PBS) or LQGV was administered intraperitoneally daily starting 24h before infection with 2 $\log_{10}$ colony-forming units (CFUs) of wild type *L. monocytogenes.* CFUs of bacteria in spleen, liver and bone marrow were quantified at 72h after infection (B) Results for infection with Δ*hly L. monocytogenes* mutants. PBS or LQGV were administered intraperitoneally daily starting 24h before infection with 7 $\log_{10}$ Δ*hly L. monocytogenes* mutants. CFUs of bacteria in spleen and liver were measured at 6 and 18 h after infection (n = 4 mice/group). * $p < 0.05$, ** $p < 0.01$ compared with PBS-treated mice.

($p < 0.01$) at 72 h after infection compared to PBS treatment. Furthermore, 3.0 $\log_{10}$ CFUs of bacteria were detected in bone marrow from LQGV-treated mice, whereas bone marrow from PBS-treated mice remained sterile (Figure 2A). In a second set of experiments, mice were infected with 7.0 $\log_{10}$ CFU of avirulent *Δhly L. monocytogenes* mutants that do not produce listeriolysin O or escape phagosomes and are eliminated rapidly. As found before, there were greater numbers of bacteria in spleens and livers of LQGV-treated mice at 6 and 18 h after infection compared to PBS treated mice (Figure 2B). Collectively, these data suggest that LQGV impairs host defenses against bacterial infection in general, rather than acting specifically against mechanisms triggered by bacteria that access the cytosol.

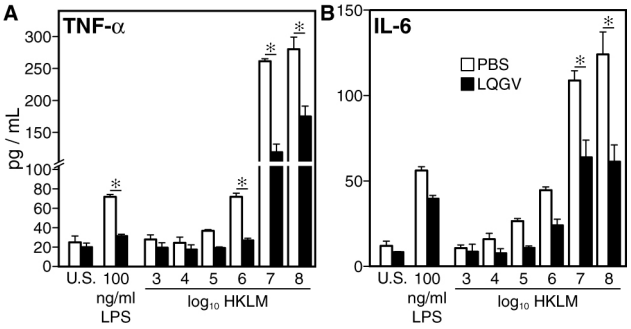

Figure 3. Impaired production of proinflammatory cytokines displayed by splenocytes from LQGV-treated mice.
Uninfected mice were treated with phosphate-buffered saline (PBS) or LQGV. After 18 h splenocytes were isolated and subsequently cultured *in vitro* overnight with increasing amounts of heat killed *Listeria monocytogenes* (HKLM) or 100 ng/ml lipopolysaccharide (LPS). Concentrations of tumor necrosis factor (TNF)-α (A) and interleukin (IL)-6 (B) in culture supernatants were measured by enzyme-linked immunosorbent assay. Data depicted are from one representative experiment out of three (n = 4 mice/group). U.S., unstimulated. * $p < 0.05$ LQGV-treated cells compared with PBS-treated cells.

Decrease of *in vitro* cellular responsiveness to HKLM and LPS by LQGV treatment in uninfected mice

Next, we tested the extent to which LQGV treatment impaired the ability of cells to generate proinflammatory cytokines. For this, uninfected mice were treated with PBS or LQGV. After 18 h splenocytes were isolated and stimulated *in vitro* overnight with varying amounts of HKLM or 100 ng/mL LPS. The results showed that HKLM induced dose-dependent production of TNF-α and IL-6 by splenocytes from both groups of mice (Figure 3). However, splenocytes from LQGV-treated mice produced statistically significantly less TNF-α and IL-6 than did cells from control mice ($p < 0.05$). Similar results were obtained when splenocytes were stimulated *in vitro* with LPS (Figure 3).

Inhibition of early neutrophil accumulation during *L. monocytogenes* infection by oligopeptide treatment

Subsequent experiments tested the degree to which treatment with LQGV, VLPALP, or AQGV impaired cellular recruitment following infection. For this, we analyzed neutrophil and monocyte numbers in spleen, blood, and peritoneal cavity in PBS-treated or oligopeptide-treated mice at steady state and after infection. The percentages of neutrophils in blood, spleen, and peritoneal cavity were increased over steady state at 6 h after infection in PBS-treated mice. Interestingly, treatment with each oligopeptide resulted in lower neutrophil counts in each compartment at 6 h after infection as well as

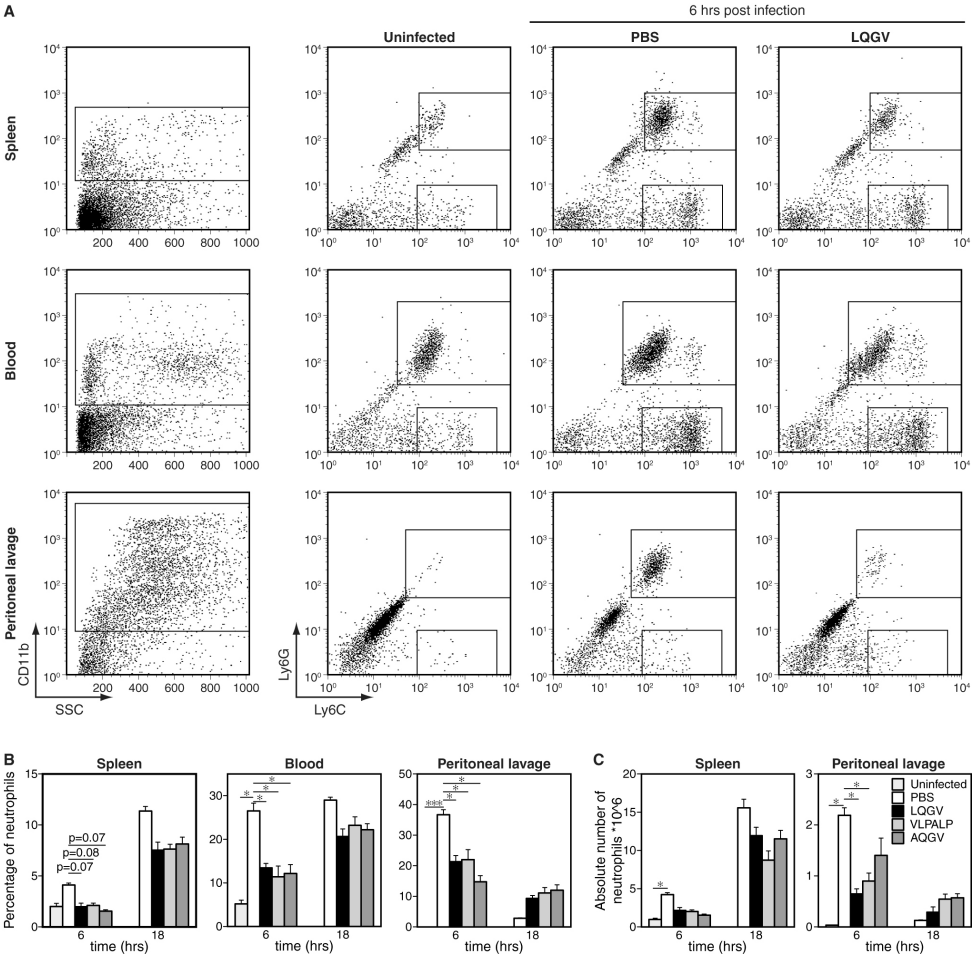

Figure 4. Inhibition of the initial mobilization of neutrophils by synthetic hCG-related oligopeptides during *L. monocytogenes* infection.

(A) Dot plots showing representative flow cytometric profiles from the spleen, blood and peritoneal cavity from mice 6 h after infection with 4 $\log_{10}$ CFUs of *L. monocytogenes* and from uninfected mice. CD11b positive cells were selected, and monocytes and neutrophils were analyzed on plots of Ly6C vs. Ly6G. Monocytes were defined as CD11b-positive, Ly6C-positive, and Ly6G-negatieve. Neutrophils were defined as CD11b-positive, Ly6C-positive, Ly6G-positive. Neutrophil (upper box) and monocyte (lower box) windows are shown. (B) Quantification of percentages of neutrophils in spleen, blood, and peritoneal lavage. (C) Neutrophil numbers in spleen and peritoneal cavity. Data are given as mean ± SEM from four mice per group from one representative experiment out of three with similar findings. * $p < 0.05$ oligopeptide-treated groups compared with PBS-treated control, or PBS-treated compared with uninfected mice.

at 18 h after infection, with the exception of the peritoneal cavity (Figure 4A and B). At 18 h after infection, the percentage and absolute numbers of neutrophils in the peritoneal lavage decreased in all mice compared to 6 h after infection, but this decrease was not as marked in oligopeptide-treated mice as in PBS-treated mice (Figure 4B and C). In contrast to the results with neutrophils, monocyte populations were not altered in any of the oligopeptide treated groups (data not shown). These data suggest that the previously demonstrated increase in bacterial loads in target organs could be due to inhibition of neutrophil accumulation.

Alteration of systemic cytokine and chemokine levels upon hCG-related oligopeptide treatment during *L. monocytogenes* infection

Next, we tested whether reduced mobilization of inflammatory cells *in vivo* correlated with inhibition of systemic proinflammatory chemokine and cytokine levels. At 6 h after infection, plasma concentrations of CCL2/MCP-1 and CXCL1/KC increased significantly in PBS-treated mice compared to uninfected mice (CCL2/MCP-1: 148 pg/mL vs 20 pg/mL, respectively; $p < 0.05$) and (CXCL1/KC: 1086 pg/mL vs 215 pg/mL, respectively; $p < 0.05$ (Figure 5)). Although oligopeptide-treated mice also showed increased CCL2/MCP-1 levels at 6 h after infection, both CCL2/MCP-1 and CXCL1/KC levels were statistically significantly lower in oligopeptide-treated mice than the levels found in PBS-treated mice ($p < 0.05$). Increased concentrations of TNF-α, IL-12p70, IL-6, and IFN-γ were first detected in plasma at 18 h after infection in all mice and were statistically significantly higher in oligopeptide-treated ($p < 0.05$) mice than in PBS-treated mice (Figure 5). At 18h after infection, CCL2/MCP-1 and CXCL1/KC levels further increased in all mice to comparable levels. These data demonstrate that elevation of systemic levels of chemokines precedes the elevation of systemic levels of pro-inflammatory cytokines and that administration of LQGV, VLPALP or AQGV initially inhibits the early chemokine response.

Determination of chemokine and cytokine plasma levels by bacterial load

To explain the apparent contradiction between depression of early chemokine levels but enhanced proinflammatory cytokine levels following oligopeptide treatment, we hypothesized that increased levels of proinflammatory cytokines at 18 h after infection were related to increased bacterial loads in target organs at this time point. To test this, mice were infected with increasing amounts of bacteria (4 $\log_{10}$, 5 $\log_{10}$, 5,5 $\log_{10}$ CFUs) and cytokine and chemokine plasma levels were measured at 18 h after infection. The results revealed a statistically significant, positive correlation between increasing amounts of infecting bacteria with higher plasma levels of TNF-α, IL-12p70, IL-6, IFN-γ, CCL2/MCP-1, and CXCL1/KC (Figure 6A) as well as greater bacterial loads *in vivo* (Figure 6B). These data support a causal relationship between bacterial load and the

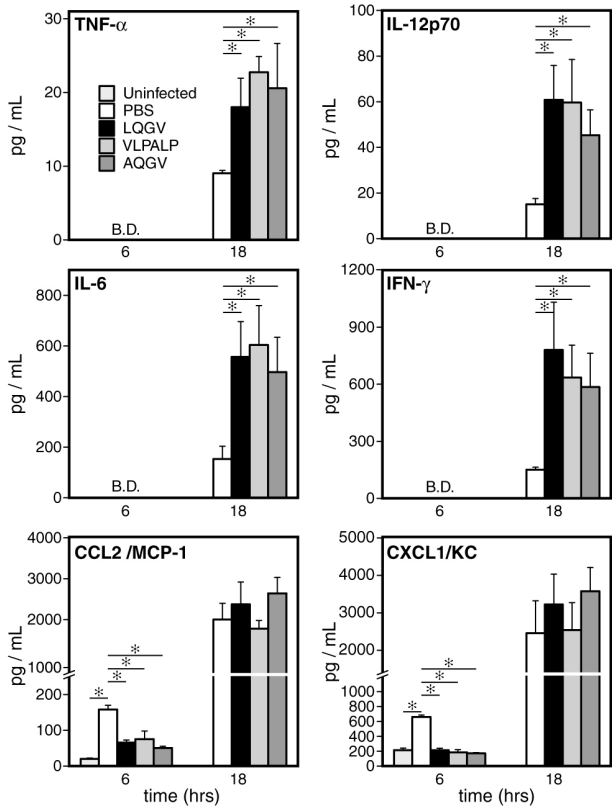

Figure 5. Massively increased levels of cytokines as a result of treatment with LQGV, VLPALP, or AQGV following lethal challenge with *L. monocytogenes.*
Phosphate-buffered saline (PBS), VLPALP, LQGV, or AQGV was administered intraperitoneally daily starting 24 h before infection with 4 $\log_{10}$ CFUs of *L. monocytogenes*. Mice were killed at fixed time points. Plasma levels of tumor necrosis factor α (TNF)-α, interleukin 12 (IL-12p70), interleukin 6 (IL-6), interferon γ (IFN-γ), CCL2/MCP-1 and CXCL1/KC in different experimental groups were determined at 6 and 18 h after infection. Data depicted are from one representative experiment out of three with similar findings (n = 4 mice/group). B.D., below detection limit. * $p < 0.05$ oligopeptide-treated groups compared with PBS-treated controls, or PBS-treated compared with uninfected mice.

concentration of inflammatory mediators in plasma. Moreover, statistically significant correlations between TNF-α, IL-12p70, IL-6, and IFN-γ plasma levels with splenic CFUs were observed at 18 h after infection in LQGV-treated mice, VLPALP-treated mice, and AQGV-treated mice. In contrast, this correlation did not hold for CCL2/MCP-1 and CXCL1/KC (Figure 6C). These data indicate that the increased cytokine levels in plasma at 18 h after infection in LQGV-treated mice, VLPALP-treated mice and AQGV-treated mice are caused by the increased bacterial load at this time point.

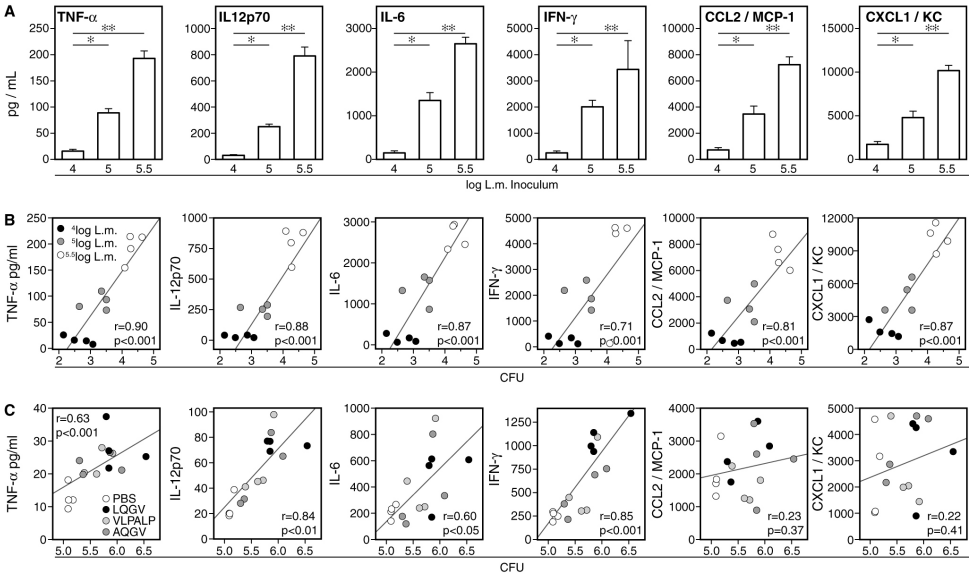

Figure 6. Positive correlation between systemic cytokine and chemokine levels and bacterial load.

Mice were infected with 4 $\log_{10}$, 5 $\log_{10}$, or 5.5 $\log_{10}$ colony-forming units (CFUs) of wild type *L. monocytogenes.* Mice were killed at 18h after infection and plasma levels of tumor necrosis factor α (TNF)-α, interleukin 12 (IL-12p70), interleukin 6 (IL-6), interferon γ (IFN-γ), CCL2/MCP-1 and CXCL1/KC in different experimental groups were determined. (A) Mean ± (standard error of the mean) plasma concentrations of TNF-α, IL-12p70, IL-6, IFN-γ, CCL2/MCP-1 and CXCL1/KC determined from groups of 4 mice. Statistical differences between groups of mice are shown (* $p < 0.05$, ** $p < 0.01$). (B) Correlation analysis between plasma cytokine/chemokine concentrations and CFUs of bacteria in spleens of mice infected with different inocula. Statistical significance was determined by Pearson's correlation analyses. (C) Correlation analysis between plasma levels and splenic CFUs in different experimental groups determined at 18h after infection. Data depicted are from one representative experiment out of three (n = 4 mice/group).

DISCUSSION

Innate immune responses against *L. monocytogenes* are characterized by the early mobilization of neutrophils and monocytes, as well as the production of proinflammatory cytokines and chemokines [29]. In this study we show that hCG-related oligopeptides inhibit innate immune activation during *L. monocytogenes* infection, which results in overwhelming bacterial propagation and excessive inflammation. In addition, we demonstrate that a systemic chemokine response manifested by increased plasma concentrations of CCL2/MCP-1 and CXCL1/KC precedes similar elevations of

proinflammatory cytokines. These observations are in line with other studies showing MyD88-independent production of CCL2/MCP-1 in the spleen within 6 h of infection [30]. Inhibition of this early response leads to a more aggressive infection as was also found in our studies [30]. These data underscore the importance of the magnitude and rapidity with which innate immune activation follows *L. monocytogenes* infection.

Neutrophil migration to infected foci is important for controlling bacterial growth locally and for preventing bacterial dissemination [31]. In this study we show that treatment with hCG-related oligopeptides reduced neutrophil accumulation in blood, spleen, and peritoneal cavity after *L. monocytogenes* infection. Reduced neutrophil recruitment is likely related to diminished CXCL1/KC production, as reflected by decreased plasma levels and splenic mRNA levels (data not shown) at 6 h after infection. Consequently, bacterial numbers increased more rapidly in spleen and liver, and were detected earlier in blood and bone marrow than in control mice. These data are in line with previous studies demonstrating that both reduced neutrophil migration and neutrophil depletion lead to overwhelming bacterial propagation in *L. monocytogenes* infection and in cecal ligation and puncture models [13,32-33]. CCL2/MCP-1 regulates monocyte recruitment during the initial stage of inflammation [34]. We observed decreased CCL2/MCP-1 levels upon treatment with hCG-related oligopeptides, but we found no significant effect on monocyte recruitment. This may be explained by preserved expression of other monocyte-attracting chemokines, such as CCL7/MCP-3 [35].

Neutrophil recruitment to infected organs, IFN-γ production by NK cells, and TNF-α and IL-6 production by monocytes, macrophages, and TNF-α and inducible nitric oxide synthase producing dendritic cells are all essential features of the innate immune response against *L. monocytogenes* [29]. In this study we found that LQGV treatment of uninfected mice impaired the ability of splenocytes to produce TNF-α and IL-6 *in vitro* in response to HKLM or LPS stimulation. This suggests that LQGV interferes with innate immune receptor-mediated activation, although the mechanisms involved are not yet clear. In contrast to decreased cytokine production *in vitro*, IFN-γ, TNF-α, IL-6, and IL-12p70 plasma levels increased dramatically when mice were treated with hCG-related oligopeptides prior to *L. monocytogenes* infection. These increased plasma levels correlated statistically significantly with splenic bacterial numbers, suggesting a causal relationship between bacterial load in target organs and the magnitude of the systemic inflammatory response. This notion was confirmed experimentally by infecting mice with increasing doses of *L. monocytogenes* as well as by *in vitro* data from HKLM stimulation of splenocytes. LQGV clearly impaired the ability of splenocytes to produce TNF-α and IL-6 upon HKLM stimulation. Nevertheless both TNF-α and IL-6 increased markedly when cells were stimulated with greater amounts of HKLM, and they exceeded cytokine levels produced by splenocytes from PBS-treated mice stimulated with a lower dose of HKLM. This mimics the *in vivo* situation of oligopeptide-treated mice having a higher

bacterial burden than PBS-treated mice and supports the notion that the increased cytokine levels detected *in vivo* in oligopeptide-treated mice are due to greater bacterial loads. In sublethal *L. monocytogenes* infection, LQGV treatment also was associated with increased bacterial numbers in spleen and liver, as well as greater bacterial numbers in the bone marrow. The latter finding is significant because the bone marrow was sterile in control mice. This underscores the strong immunosuppressive capacity of LQGV in mice. These data show that the early innate immune activation is important for the initial infiltration of phagocytes at sites of infection to control *L. monocytogenes* growth locally, a response that is fully active within the first 6 h after infection [36]. This rapid response also is essential for limiting bacterial dissemination to other organs or compartments, such as the bone marrow.

An alternative explanation for increased bacterial numbers following oligopeptide treatment could be a reduced capacity of resident effector cells to kill bacteria immediately following infection. Although we cannot fully exclude this possibility, we consider it to be less likely, because after cellular invasion, virulent bacteria escape from phagosomes in a listeriolysin O-dependent fashion, then replicate intracellularly and infect neighboring cells [37-38]. *L. monocytogenes Δhly* mutants lack listeriolysin O and are easily eradicated by the immune system [27]. Our results showed decreasing CFUs of *Δhly* mutants in spleen and liver of LQGV-treated mice similar to PBS-treated mice, but with delayed kinetics. This finding suggests that LQGV treatment does not significantly impair bacterial internalization and killing by effector cells. More rapid bacterial replication following treatment with hCG-related oligopeptides therefore is likely due to decreased chemokine production and subsequent reduced neutrophil recruitment, although formally we cannot exclude that LQGV interferes with bactericidal activities. Our data are in line with other studies demonstrating that treatment with hCG preparation also reduces chemokine production during thioglycolate-induced inflammation [39].

Pregnant women are prone to placental infection with *L. monocytogenes,* which typically occurs during later stages of pregnancy [1,29,40]. During the third pregnancy trimester, increased nicked β-hCG and β-core fragment levels occurr in urine and plasma, as compared to the first trimester [41]. At present we are unable to detect hCG-derived oligopeptides in blood or urine. However, the relative amounts of nicked β-hCG and β-core fragments can be detected [42], providing an indirect estimate for the concentration of hCG-derived oligopeptides. We postulate that high amounts of smaller fragments, including LQGV and VLPALP, may be liberated from the loop 2 region during the third trimester of pregnancy. This could cause not only local immune suppression at the placenta, which is needed to prevent fetal allograft rejection, but also could make the host more prone to placental *L. monocytogenes* infection later in pregnancy. Interestingly, placentas of humans and guinea pigs are much more

susceptible to infection with *L. monocytogenes* compared with mouse placentas [43-45]. The explanation for differential susceptibility to *L. monocytogenes* infection is unclear but has been related to structural similarities between human and guinea pig placentas, whereas the mouse placenta is more different [43,46-47]. Alternatively, a placental immunological environment in humans and guinea pigs that is more permissive for growth of *L. monocytogenes* than in mice could explain this differential susceptibility. Interestingly, βCG-derived oligopeptides could contribute to such an immunological environment in humans and guinea pigs, since both species produce the βCG subunit and share 63% homology, whereas mice lack the βCG gene and thus do not produce βCG. Clearly, more studies on animal models of pregnancy with *L. monocytogenes* infection are needed to unveil the precise role of CG-related oligopeptides during pregnancy.

Elsewhere, we reported that LQGV and AQGV attenuated life-threatening inflammation and organ damage associated with hemorrhagic shock and renal ischemia & reperfusion [22-23]. In addition, we show that VLPALP treatment reduced mortality upon LPS injection [25]. Although the exact mechanism of action of LQGV, VLPALP, and AQGV is (are) not yet known, our current study suggests that suppressive effects on innate immune responses have contributed to beneficial outcomes found in these models. These data show that hCG-related oligopeptides have strong immune suppressive activity, which provides the underlying foundation for phase I trials as currently performed in humans.

In summary, we demonstrate that a chemokine response precedes the pro-inflammatory cytokine and cellular response in *L. monocytogenes* infection. This chemokine response is essential for rapid cellular recruitment to prevent further progression of infection. Furthermore, this study reveals that the synthetic hCG-related oligopeptides LQGV, VLPALP, and AQGV inhibit early innate immune responses with subsequent increased bacterial propagation. Our data underscore the importance of early innate immune activation following bacterial infection and suggest that hCG-derived oligopeptides at the placenta could increase host susceptibility to certain infections, e.g. *L. monocytogenes*.

ACKNOWLEDGMENTS

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III

THE EFFECT OF LQGV ON PERIPHERAL BLOOD NEUTROPHILS IN MICE: A PILOT STUDY

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ABSTRACT

Neutrophils are the first cells to respond to an injury by migrating from the circulation into the inflammatory site. Previously, we showed that LQGV impaired neutrophil recruitment during a *Listeria monocytogenes* infection in mice. In other studies LQGV administration was found to reduce hepatic and renal E-selectin expression during hemorrhagic shock and renal ischemia reperfusion injury, respectively. This may account for the reduced neutrophil accumulation at the site of inflammation due to the LQGV treatment. However, the mechanisms of action that contributed to this reduced neutrophil accumulation were not studied. Therefore, in the present study we explored the effect of LQGV on neutrophil viability and neutrophil-endothelial cell interactions *in vivo* and *in vitro*. LQGV administration at a dose of 50 mg/kg body weight to naive C57BL/6 mice was associated with a transient increase in the number of apoptotic neutrophils in the blood. These apoptotic neutrophils reduced the heat-killed *L. monocytogenes* driven TNF-α and IL-6 production by splenocytes from untreated C57BL/6 mice *in vitro.* LQGV also reduced O_2^- activity by PMA activated neutrophils as well as in the cell free cytochrome C assay. Collectively these data suggest that LQGV induces a transient reduction in the number of circulating neutrophils by apoptosis, possibly by reducing their O_2^- activity. This may have contributed to the inhibition of innate immune activation by LQGV as described in our *L. monocytogenes* infection study.

INTRODUCTION

Neutrophils play a central role in the early antibacterial host defense system [1]. *Listeria monocytogenes* is an intracellular bacterium that can cause invasive infection in individuals with compromised host defenses [2]. Upon *L. monocytogenes* infection, activated neutrophils transmigrate from the circulation into vital organs where they produce and secrete pro-inflammatory mediators, reactive oxygen species (ROS; including O_2^-, OH^- and H_2O_2) and proteases in order to eliminate the invading pathogen [3,4]. However, excessive ROS production results in damage to cellular structures, including lipids, proteins, and DNA [5,6]. Also neutrophil-derived proteases, such as elastase and matrix metalloproteinases, cause tissue damage [7,8]. These processes are in part responsible for the loss of organ integrity and contribute to multiple organ failure during a severe *L. monocytogenes* infection [3,9,10].

Previously, we found that oligopeptides related to the primary structure of human chorionic gonadotropin (hCG) reduced the inflammatory response upon lipopolysaccharide (LPS) injection, ischemia-reperfusion injury, and *L. monocytogenes* infection [11-14]. In these models administration of hCG-related oligopeptides was

found to be associated with reduced adhesion molecule expression and neutrophil accumulation at the site of inflammation [11-13]. These data suggest that these oligopeptides may have inhibited neutrophil accumulation by means of reducing adhesion molecule expression. We can, however, not exclude the possibility that other mechanisms, such as redistribution to other organs or apoptosis-induced depletion, also contributed to the reduction of neutrophil accumulation at inflammatory sites [11,12]. Therefore, in this study we explored the effect of LQGV on neutrophil viability and neutrophil-endothelial cell interactions in naive mice.

Here we demonstrate that LQGV induces apoptosis of circulating blood neutrophils. Furthermore, LQGV can reduce O_2^- activity, which may have accounted for the pro-apoptotic effect. Such LQGV-induced apoptotic neutrophils impaired the capacity of splenocytes from untreated mice to produce cytokines upon overnight stimulation with heat-killed *L. monocytogenes* (HKLM). Collectively, these data suggest that LQGV-induced neutrophil apoptosis may have contributed to the early inhibition of innate immune activation as found in our study on severe *L. monocytogenes* infection [14].

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 male mice (Harlan, Horst, The Netherlands), aged 8–12 wk, were used in experiments that were approved by the local Animal Experiments Committee.

Tissue collection, *in vitro* splenocyte stimulation and cytokine analysis

Mice were intra-peritoneally (ip) injected with 200 μL LQGV (50 mg/kg BW; GL Biochem Shanghai, China) or PBS and euthanized at the indicated time points. Blood was obtained by cardiac puncture and collected in EDTA-tubes (Greiner, Bio-one, Alphen aan den Rijn, The Netherlands). Spleen and lungs were isolated and collected in PBS. Plasma was obtained by centrifugation (3000 rpm; 10 min), immediately frozen and stored at -20°C until assayed. Splenocytes were isolated and cultured (10^6 cells/ml) overnight in the presence or absence of apoptotic neutrophils in a 1:1 cell ratio in RPMI medium containing 5% FCS, antibiotics, and with or without $7\log_{10}$ HKLM [14]. Culture supernatants were collected and TNF-α and IL-6 levels were determined by ELISA (R&D Systems Europe, Abingdon, UK).

Flow cytometric analysis

Flow cytometric immunophenotyping was performed on leukocytes as described previously [15]. Briefly, cells were incubated with 5% normal mouse serum (Sanquin

Reagents, The Netherlands). Neutrophils were visualized with anti-Ly6C (ER-MP20 [16]) and anti-Ly6G (1A8) mAbs. T-cell subsets were detected using anti-CD3 (145-2C11), anti-CD4 (RM4-5), and anti-CD8α (53-6.7) mAbs. B-cells with anti-CD19 (ID3), and anti-IgM (R6-60.2) (BD PharMingen, Breda, The Netherlands). Neutrophils (Ly6G-pos, Ly6C-pos) were subsequently labeled with Annexin V and 7-AAD, according to the manufacturers protocol (BD PharMingen, Breda, The Netherlands). Early apoptotic neutrophils (Annexin V positive and 7AAD negative) and late apoptotic neutrophils (Annexin V positive and 7AAD positive) were analyzed on a FACSCanto (BD Biosciences, San Jose, CA, USA). Data were analyzed using Flow-jo software (TreeStar, Ashland, OR, USA).

Neutrophil isolation

Neutrophils were isolated as described [17]. Briefly, neutrophils were isolated through ficoll centrifugation (lymphocyte separation media; ICN Biomedicals, Zoetermeer, The Netherlands) followed by hypotonic lysis of erythrocytes. The neutrophil-rich sample was analyzed for cell number by a Coulter counter (Beckman Coulter, Woerden, The Netherlands). From naive mice, neutrophils were isolated and subsequently cultured overnight to generate apoptotic neutrophils. From LQGV-treated mice, neutrophils were isolated at one hour after LQGV administration. Both isolated neutrophil populations were co-cultured as described above. Apoptosis was confirmed by flow cytometric staining for Annexin V and 7-AAD, according to manufacturers protocol (BD PharMingen, Breda, The Netherlands).

Isolation of pulmonary endothelial cells

Pulmonary endothelial cells were isolated as described [18]. Briefly, lungs were cut with two scalpels into smaller sections, which were subjected to collagenase III digestion (Sigma-Aldrich, Zwijdrecht, The Netherlands). The obtained cell suspension was washed three times with PBS and cultured in a culture flask in M199 medium containing FCS, endothelial growth supplement, heparin, and antibiotics [19]. Every other day the medium was replaced. Endothelial cells were used up to maximum passage six.

Adhesion assay

Mouse pulmonary endothelial cells (passage 2 - 6) were plated into 96 wells plates followed by two to three days incubation at 37°C, after which a monolayer was present as confirmed by light microscopy, as described elsewhere [17,19]. One hour after PBS or LQGV administration, neutrophils (1×10^6 cells/ml) were isolated and labelled with calcein-AM (Molecular Probes, Leiden, The Netherlands). The labelled neutrophils (100µl) were added to the endothelial cell monolayer. Thereafter, plates were centrifuged

for 1 minute at 80 × g, incubated for 1 hour at 37°C, and washed twice with medium to remove non-adherent neutrophils. The remaining fluorescence per well was measured on a plate reader using 485 nm excitation and a 530 nm emission filter. Neutrophil adherence was expressed as the percentage increase in cell number as compared with the wells where no neutrophils were added.

Lactate dehydrogenase (LDH) analysis

LDH levels were determined at the Erasmus MC clinical chemistry diagnostic facility according to standard procedures.

Superoxide anion detection in a cell free system

Superoxide anion (O_2^-) production was measured with the cytochrome C reduction assay as described [20]. Briefly, a cytochrome C (Sigma-Aldrich, Zwijdrecht, The Netherlands) solution was prepared in Hanks' Balanced Salt Solution (HBSS; Invitrogen, Molecular Probes, Carlsbad, CA). This cytochrome C solution was added to a cuvette, to which either superoxide dismutase (SOD; positive control), HBSS (negative control) or different dosages of LQGV were added. Subsequently, xanthine-oxidase was added after which the optical density was measured in time for a total of 90 seconds. Hereafter, hypoxanthine was added to the cuvette to initiate the reaction between xanthine-oxidase and hypoxanthine that leads to O_2^- production. The cuvette was measured in time for a total of 30 measurements, and each measurement lasted for 30 seconds. All buffers were pH 7.4 during the reaction. The cuvette was measured on a spectrophotometer (Shimadzu, Tokyo, Japan) at 550 nm and results are expressed as extinctions.

Extracellular superoxide anion detection

Extracellular O_2^- production was measured using lucigenin (bis-N-methylacridinium; Sigma-Aldrich, Zwijdrecht, The Netherlands), which yields a chemiluminescent signal in the presence of superoxide anion. SOD (50 U/ml) was used as control for O_2^- neutralization. Neutrophils were suspended in HBSS containing Mg^{2+} and Ca^{2+} (Invitrogen, Molecular Probes, Carlsbad, CA) and seeded in triplo into 96 wells U-bottom plates (GreinerBio, Alphen a/d Rijn, The Netherlands) that already contained HBSS with different dosages of LQGV. Subsequently, cells were stimulated with 500 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Zwijdrecht, The Netherlands). After adding lucigenin and PMA to the cells, chemiluminescence was immediately measured using a 96 wells luminometer (LUMIstar Galaxy, BMG, LABTECH, Offenburg, Germany). Each well was measured in time for a total of 70 measurements, and each measurement lasted for 10 seconds. Results are expressed as O_2^- production (average counts per minute (cpm)).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad software, CA, USA). Intergroup differences were analyzed with Mann-Whitney statistical test and $p < 0.05$ was considered statistically significant. For survival analysis, a Kaplan-Meier analysis followed by a log rank test was performed.

RESULTS

LQGV treatment decreases the blood neutrophil numbers

In order to investigate the effect of a single LQGV injection on circulating leukocyte populations, C57BL/6 mice were treated with LQGV (50 mg/kg BW) and flow cytometric analysis was performed on blood. To this end, we quantified T-cells (Figure 1A), B-cells (Figure 1B), neutrophils (Figure 1C), and monocytes (Figure 1D). LQGV treatment caused a significant reduction of neutrophil numbers as compared to PBS treatment at one hour ($3.4x10^8$ cells/L vs $6.2 x10^8$ cells/L; $p < 0.05$) and at three hours ($2.5x10^8$ cells/L vs $5.6x10^8$ cells/L; $p < 0.05$) after treatment. T-cell, B-cell, and monocyte numbers were not affected by LQGV.

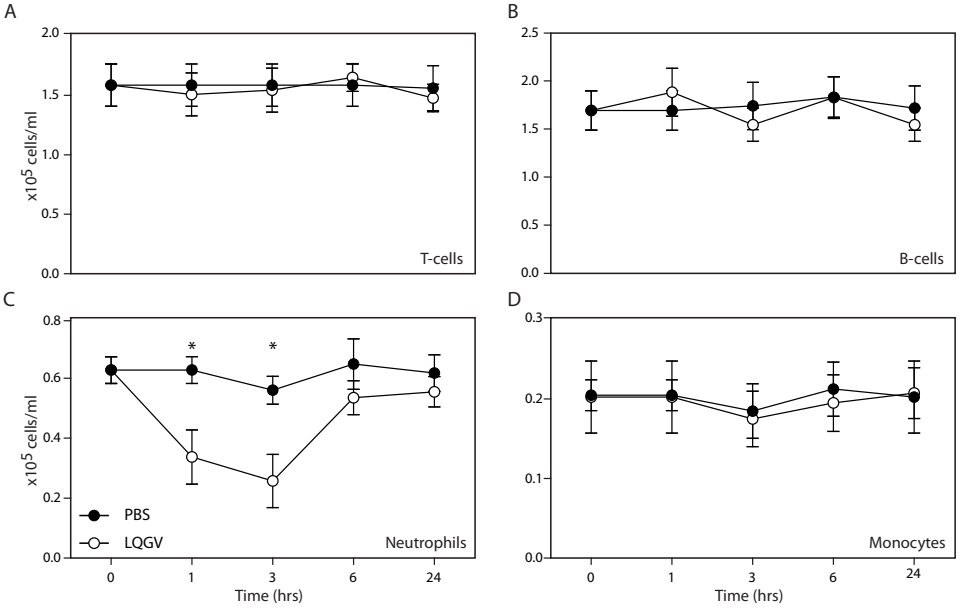

Figure 1. LQGV treatment transiently decreases blood neutrophil numbers.
Mice were treated with LQGV (50mg/kg BW) or PBS and at 1, 3, 6, and 24 hours after treatment the numbers of (A) T-cells, (B) B-cells, (C) neutrophils and (D) monocytes in blood were determined. Data depicted are from 8 mice per group. * means $p < 0.05$.

LQGV does not affect the adhesive capacity of neutrophils to endothelial cells

The reduced neutrophil numbers that were observed upon LQGV administration (50 mg/kg BW) might be due to increased adhesion to endothelial cells. Therefore, we analyzed neutrophil adhesion to endothelial cells *in vitro*. Blood neutrophils, isolated at 30 and 60 minutes after *in vivo* LQGV administration, were co-cultured with primary pulmonary endothelial cells. This revealed no significant differences between the adherent capacity of neutrophils from LQGV-treated and PBS-treated mice (Figure 2).

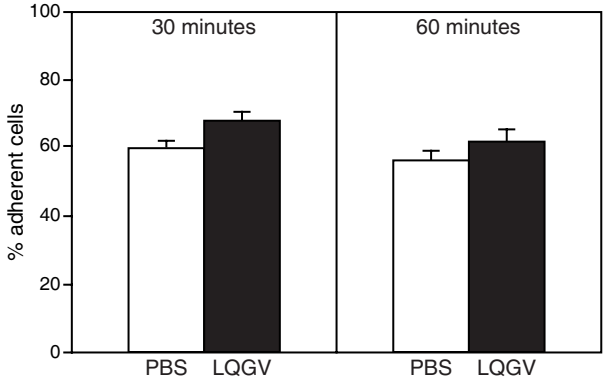

Figure 2. LQGV treatment does not affect the adherent capacity of neutrophils to endothelial cells.
Neutrophils were isolated from the blood of mice at 30 and 60 minutes after PBS or LQGV administration and subsequently used in the endothelial cell adhesion assay.

LQGV treatment is associated with increased systemic LDH levels and neutrophil apoptosis

Since LQGV did not affect the adherent capacity of neutrophils to endothelial cells, we next examined neutrophil apoptosis. First, we determined LDH levels in plasma, as a marker for cell damage. In untreated mice, the LDH plasma levels were approximately 34 ± 4 IU/L. Upon LQGV (50 mg/kg BW) treatment, the LDH plasma levels increased significantly as compared to PBS treatment at 40 minutes (75 IU/L vs 42 IU/L) and at 60 minutes (98 IU/L vs 48 IU/L; $p < 0.05$) (Figure 3A). Next, we performed flow cytometic analysis on the blood neutrophils at 30 and 60 minutes after either PBS or LQGV administration. The populations of annexin-V and 7-AAD positive neutrophils from LQGV-treated mice were significantly larger ($p < 0.05$) than those from PBS-treated mice and naive mice at 60 minutes after LQGV-administration (Figure 3B). This indicates increased neutrophil apoptosis upon LQGV treatment.

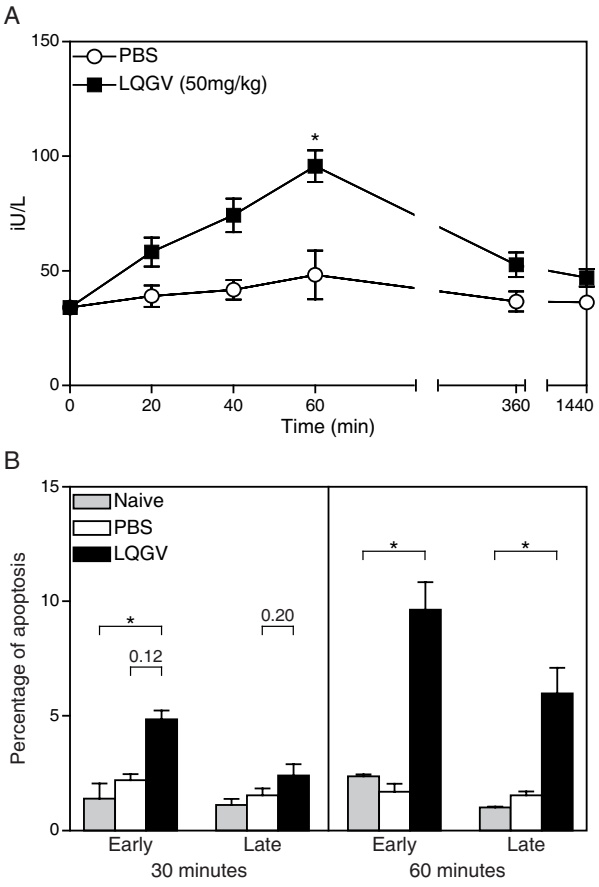

Figure 3. LQGV treatment is associated with increased systemic LDH levels and increased numbers of apoptotic neutrophils.
(A) LQGV treatment is associated with increased LDH levels in plasma. (B) Quantification of apoptotic blood neutrophil numbers after LQGV treatment. Early apoptotic neutrophils were defined as Annexin V positive and 7-AAD negative. Late apoptotic neutrophils were defined as Annexin V positive and 7-AAD positive. Data depicted are from 4 mice per group. * means $p < 0.05$.

LQGV decreases superoxide anion production

Neutrophil viability is regulated by several mechanisms, one of which is the regulation of intracellular ROS levels, such as O_2^- [21]. We investigated the effect of LQGV on PMA-stimulated neutrophils. This revealed that LQGV dose dependently reduced extracellular O_2^- levels upon PMA stimulation (Figure 4A). Also in a cell free system, O_2^- levels were reduced when LQGV was added to the assay, again in dose dependently (Figure 4B).

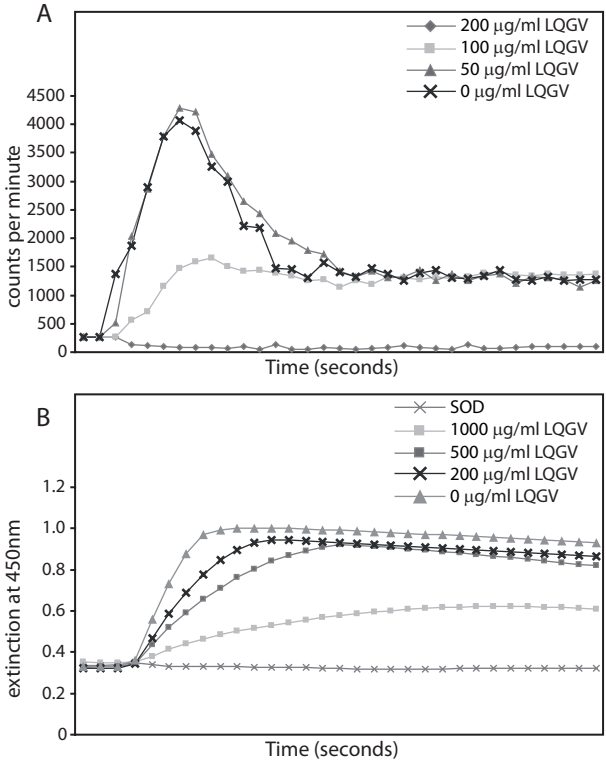

Figure 4. LQGV reduces O_2^- levels.
(A) Blood neutrophils stimulated with PMA in the presence of LQGV showed a dose dependent reduction of extracellular O_2^- formation. (B) Adding LQGV to a cytochrome C reduction assay leads to a dose dependent inhibition of hypoxanthine/oxidase-induced cytochrome C reduction. Superoxide dysmutase (SOD).

LQGV-induced apoptotic neutrophils are anti-inflammatory.

It has been recognized that apoptotic neutrophils exert anti-inflammatory activity upon TLR activation [22,23]. We examined whether apoptotic neutrophils obtained one hour after *in vivo* LQGV administration (50 mg/kg BW) or obtained after overnight culture *in vitro* reduced the responsiveness of splenocytes from naïve mice to HKLM stimulation. This revealed that LQGV-induced apoptotic neutrophils as well as apoptotic neutrophils generated by overnight culture impaired the capacity of splenocytes from untreated mice to produce TNF-α (Figure 5A) and IL-6 (Figure 5B) upon HKLM stimulation.

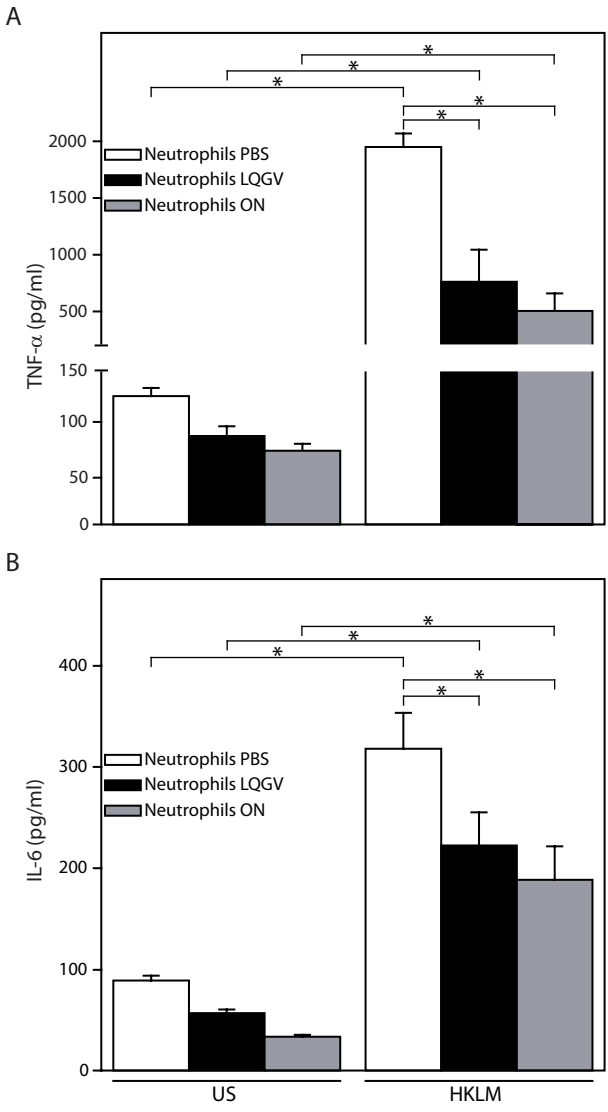

Figure 5. LQGV-induced apoptotic neutrophils reduce cytokine secretion.
Splenocytes from naïve mice were unstimulated or stimulated with HKLM in the presence of neutrophils from LQGV-treated mice or overnight (ON) cultured neutrophils. (A) TNF-α and (B) IL-6 levels were determined in culture supernatant by ELISA. US, unstimulated. * means $p < 0.05$.

DISCUSSION

Multiple organ dysfunction syndrome (MODS) is an often fatal complication of SIRS. Although the exact mechanisms involved are not completely known yet, studies in which neutrophils were depleted prior to the onset of SIRS showed a reduced MODS [24-26], indicating that neutrophils are a contributing factor. However, in case of an infection neutrophil depletion leads to an enhanced susceptibility to the infectious agent, due to the hosts inability to clear the pathogen [27]. Previously, we found that LQGV (50 mg/kg BW) enhanced the susceptibility of mice to a *L. monocytogenes* infection [14]. In this model, neutrophils play an important role. In our current study, we describe that high dose (50 mg/kg BW) LQGV-treatment of C57BL/6 mice specifically induces neutrophil apoptosis, probably through reducing O_2^- activity. In addition, we show that these apoptotic neutrophils exert anti-inflammatory activity when co-cultured with naive splenocytes and stimulated overnight with HKLM.

Neutrophil viability is tightly regulated by intracellular ROS levels [28]. Is has been demonstrated that SOD injection reduces intracellular O_2^- levels in neutrophils, leading to caspase-3 activation and subsequent apoptosis [21,29]. We found that LQGV has the ability to reduce O_2^- activity and such a process may have contributed to the LQGV-induced neutrophil apoptosis. LQGV, however, did not affect the viability of monocytes, probably because intracellular ROS levels do not regulate monocyte viability [30,31].

Neutrophils are the first cells to be recruited to the inflammatory foci [2]. This extravasation process can be divided into five sequential stages: (a) selectin-mediated leukocyte sticking; (b) integrin-mediated intravascular crawling; (c) firm arrest of leukocytes to the endothelium; (d) chemokine-induced leukocyte activation and migration over the endothelium; and finally (e) transendothelial migration through a chemokine gradient [32,33]. Previously, we found that LQGV treatment reduced E-selectin expression in the liver, which was associated with reduced hepatic neutrophil accumulation [12]. Although our previous study may suggest a decreased migration of neutrophils into the liver [12], our current data show that high dose (50 mg/kg BW) LQGV-treatment induces a transient neutropenia, which may also have contributed to this reduced accumulation. We cannot exclude that other granulocytes, for instance eosinophils and mast cells, of which the viability is also tightly regulated by intracellular ROS levels, are also transiently reduced by apoptosis [34].

The LQGV-induced apoptotic neutrophils reduced the capacity of splenocytes from untreated mice to secrete TNF-α and IL-6 to HKLM stimulation. Such effects of apoptotic neutrophils may have contributed to the decline in inflammatory cytokines (e.g. TNF-α and IL-6) that we found in mice infected with *L. monocytogenes* [14].

In conclusion, we show that high dose (50 mg/kg BW) LQGV-treatment of C57BL/6

mice induces neutrophil apoptosis, possibly by neutralization of intracellular O_2^- levels. Both the transient depletion of neutrophils and the anti-inflammatory activity of apoptotic neutrophils may have contributed to the inhibition of early innate immunity during a *L. monocytogenes* infection in mice [14]. More studies are needed to investigate these effects of LQGV on neutrophil viability, ROS production and inflammation.

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IV

THE BETA-hCG-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 gonadotropin β-chain related tetrapeptide LQGV reduces hemorrhagic and LPS-induced SIRS, while 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 pro-survival 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 vitro* 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 and luteinizing hormone receptors as well as of 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 adrenocorticotropic hormone-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, while 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 gonadotropin (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 and third pregnancy trimester [10]. It was 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 [9,13,14].

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 pro-inflammatory mediators, such as IL-1, IL-6, TNF-α, CCL2, CXCL1, and CXCL2 [16,17]. High levels of these pro-inflammatory 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 *L. 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 and third pregnancy trimester, the phase that 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 specific 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 wk, were used in the experiments that were approved by the local Animal Experiments Committee.

Lipopolysaccharide-induced shock

Mice were injected intraperitoneally (ip) either with 200 µl LQGV (50 mg/kg BW dissolved in PBS; purity 99,2%; GL Biochem Shanghai Ltd., China) or PBS (as

control), directly followed by a second ip 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 a lipopolysaccharide (LPS) injection (30 mg/kg BW of *Escherichia coli* strain 0111:B4; Sigma Aldrich, Zwijndrecht, The Netherlands). The survival was scored every 12 hours for three days.

Adrenalectomy

Adrenalectomy (ADX) was performed as described [26], after which mice acclimatized for seven days before start of the experiment. All mice postoperatively received 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 medium containing antibiotics, 5% FCS, and LQGV (50, 5, 0.5 µg/ml) or PBS was added to the adrenal gland culture for six hours. The cAMP-blocker H-89 (10 µM; Biomol, Plymouth, USA), the ACTH-blocker corticotropin-inhibiting peptide CIP (10^{-6} mM, 10^{-7} mM, 10^{-8} mM or 10^{-9} mM; Phoenix Pharmaceutics INC, Phoenix, USA) and the luteinizing hormone (LH)-receptor antagonist Antide (100 µM; Sigma Aldrich, Zwijndrecht, The Netherlands) were added 5 minutes before LQGV stimulation. Culture supernatants and adrenal glands were collected and stored at -80 ºC until assayed.

***In vitro* stimulation of LHR-Y1-cells**

The murine LH-receptor-transfected Y1-adrenal cells (LHR-Y1-cells; kindly provided by Dr. Bill Moyle; University of Medicine and Dentistry of New Jersey) were cultured as described [29]. The cAMP-blocker H-89 (10 µM), ACTH-blocker CIP (10^{-6} mM), and LH-receptor antagonist Antide (100µM), were added 5 minutes before addition of recombinant hCG (300 U/ml; Sigma Aldrich, Zwijndrecht, The Netherlands) 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 ip injected with 200 µL LQGV (50 mg/kg BW) or PBS. Thirty minutes later 500 µl of the GR antagonist mifepristone or vehicle was administrated ip. 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 neither received 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 7 $\log_{10}$ 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, UK).

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, Zwijndrecht, The Netherlands). *CYP11B1* (encoding 11-β-hydroxylase) gene expression levels were determined by RQ-PCR using an Applied Biosystems 7900 PCR machine (Foster City, CA, USA). 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, Ill). Intergroup differences were analyzed using Mann-Whitney statistical 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 a $p < 0.05$.

RESULTS

LQGV reduces LPS-induced mortality via GR activation

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

***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. Hereto

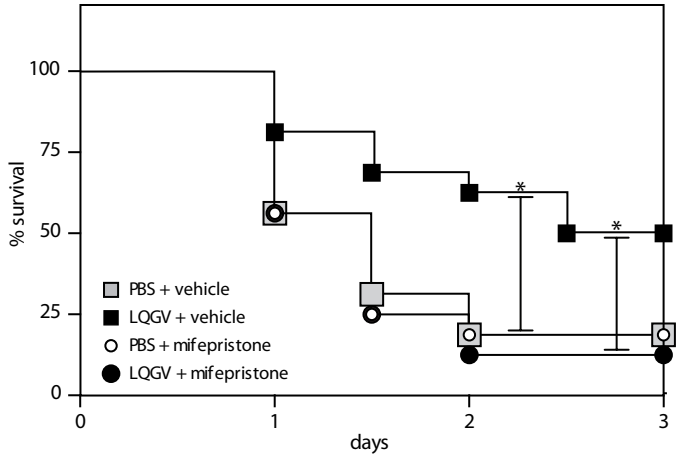

Figure 1. Mifepristone injection abolishes the LQGV pro-survival effect.
Mice were ip injected with either 200 µl LQGV (50 mg/kg BW) or PBS, directly followed by a second ip injection with 500 µl mifepristone (10 mg/kg BW) or vehicle. The next day, mice were challenged with an LPS injection. Survival was scored every 12 hours. Data depicted are from 19 mice per group. * $p < 0.05$ for LQGV + vehicle treatment compared with LQGV + mifepristone treatment, or PBS + vehicle.

splenocytes from untreated mice were 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 (Figure 2A and B). In contrast, splenocytes obtained from mice 18h 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 splenocytes from PBS-treated mice (Figure 2C and D). This suppressive effect of LQGV was completely reversed when LQGV-treated mice also received mifepristone (Figure 2C and D). These data demonstrate that LQGV does not directly stimulate GR activity in splenocytes nor that it directly interferes 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.

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. Hereto, plasma was obtained from mice 18 hours following PBS or LQGV injection. Plasma from both LQGV- and PBS-treated mice inhibited HKLM-induced TNF-α production by naïve splenocytes in

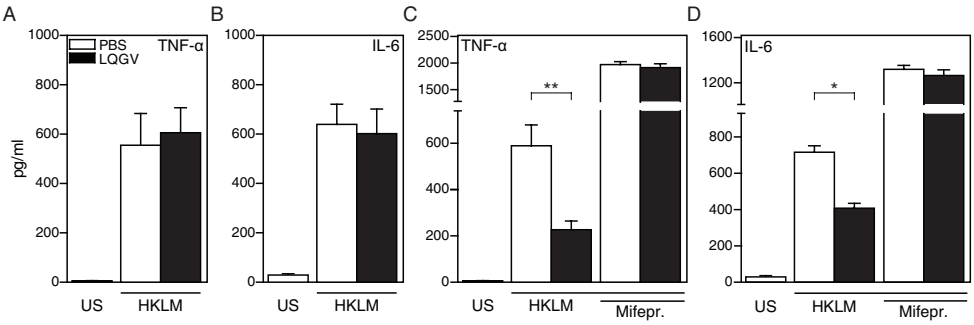

Figure 2. *In vivo* LQGV administration reduces HKLM-induced TNF-α and IL-6 production by splenocytes.
Splenocytes from untreated mice were stimulated overnight with $7\log_{10}$ HKLM in the presence of 50 µg/ml LQGV whereafter TNF-α (A) and IL-6 (B) levels in culture supernatants were determined by ELISA. In other experiment, mice were treated with LQGV (50 mg/kg BW) or PBS, in combination with or without mifepristone, 18h later splenocytes were isolated and cultured overnight with $7\log_{10}$ HKLM, whereafter TNF-α (C) and IL-6 (D) levels in culture supernatants were determined by ELISA. Data depicted are from 5 mice per group. US, unstimulated. * means $p < 0.05$ and ** means $p < 0.01$.

a dose dependent manner (Figure 3A). 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 (Figure 3B and C). Addition of mifepristone to the cultures completely reversed the immunosuppressive effect of plasma from LQGV-treated mice (Figure 3B and 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 (Figure 4A), as reflected by the TNF-α and IL-6 levels detected in 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 (Figure 4B). These data demonstrate that the adrenal glands are involved in establishing the immunosuppressive effect of LQGV.

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,

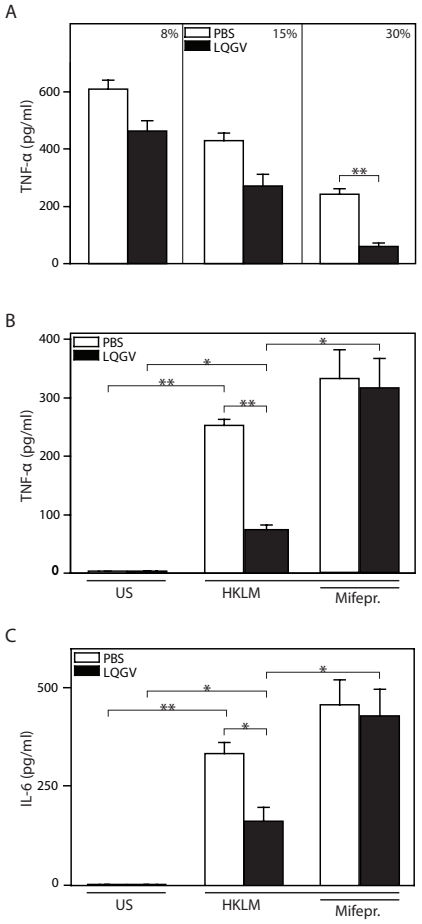

Figure 3. LQGV induces the release of an immunosuppressive mediator in plasma.
Mice were treated with LQGV (50 mg/kg BW) or PBS, and 18h later plasma was obtained and used in increasing amounts (v/v) in overnight cultures of splenocytes from untreated mice stimulated with $7\log_{10}$ HKLM. Thereafter 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 18h 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 7 mice per group. US, unstimulated. * means $p < 0.05$ and ** means $p < 0.01$.

we determined plasma corticosterone levels at different time points following LQGV administration. Corticosterone plasma levels were significantly higher at one hour (0.265 µMol/L vs 0.074 µMol/L; $p < 0.05$), at six hours (0.664 µMol/L vs 0.055 µMol/L; $p < 0.05$), and at twenty-four hours (0.406 µMol/L vs 0.050 µMol/L; $p < 0.05$) in LQGV-

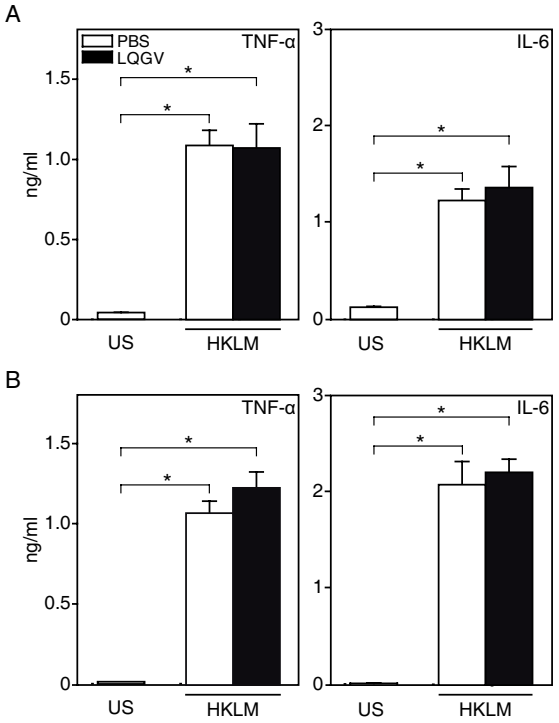

Figure 4. Adrenalectomy abolishes the immunosuppressive effect of LQGV.
ADX mice were treated with LQGV (50 mg/kg BW) or PBS, and 18h later splenocytes were obtained and cultured overnight with $7\log_{10}$ HKLM. TNF-α and IL-6 levels in culture supernatants were determined by ELISA (A). In other experiments, ADX mice were treated with LQGV or PBS, 18h 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 5 mice per group. * means $p < 0.05$.

treated mice than in PBS-treated mice (Figure 5A). *Ex vivo* stimulation of complete adrenal glands revealed that LQGV dose dependently and significantly ($p < 0.05$) induced corticosterone secretion (Figure 5B). 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 (Figure 5C). When a total of 8% (v/v) adrenal gland culture medium was added to the

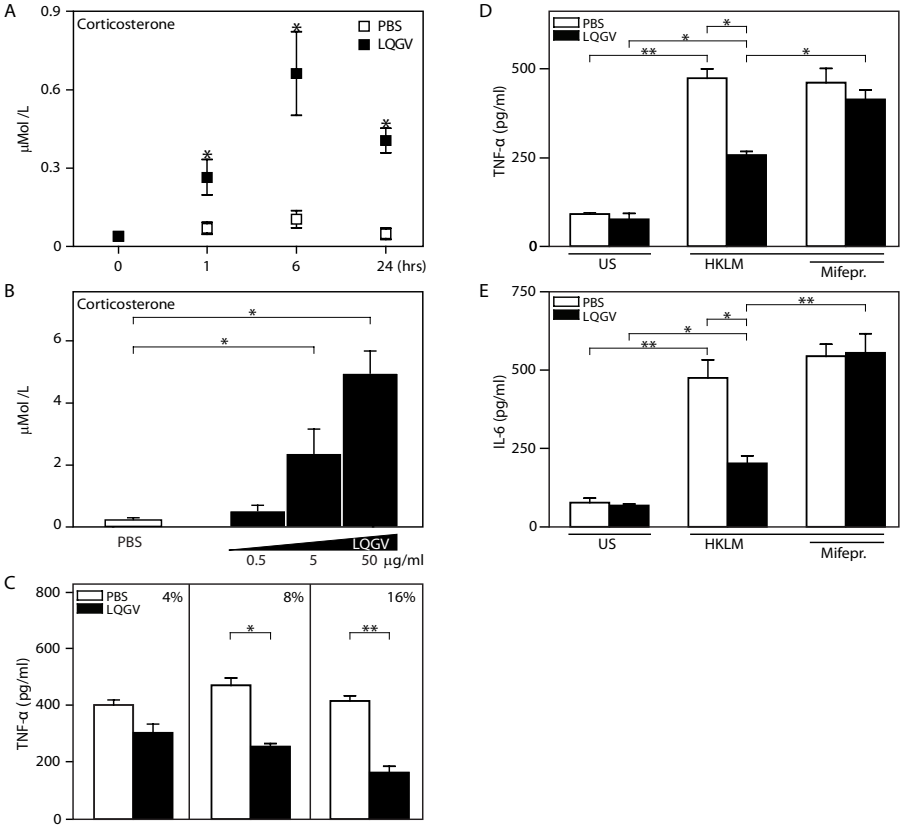

Figure 5. LQGV activates the adrenal glands to secrete corticosterone.
Mice were treated with LQGV (50 mg/kg BW) or PBS and plasma corticosterone levels were determined at 0, 1, 6 and 24 hours after treatment. Plasma corticosterone levels in LQGV-treated mice increased and peaked at 6h after which the 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. * means $p < 0.05$ (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 4 mice per group. * means $p < 0.05$ and ** means $p < 0.01$ (C). Culture supernatant (8% (v/v)) was added to an overnight stimulation of splenocytes from untreated mice with $7\log_{10}$ HKLM and TNF-α (D) and IL-6 (E) concentrations in culture supernatants were measured by ELISA. Data depicted are from 7 mice per group. US, unstimulated. * means $p < 0.05$ and ** means $p < 0.01$.

culture, supernatant from LQGV-stimulated adrenal glands reduced the HKLM-induced TNF-α ($p < 0.05$) (Figure 5D) and IL-6 ($p < 0.05$) (Figure 5E) production to significantly lower levels than observed with supernatant from PBS-stimulated adrenal glands. This effect was completely abolished when mifepristone was co-added to the cultures (Figure 5D and E). These data suggest that LQGV activates the adrenal glands to synthesize and secrete corticosterone, which reduces the *in vitro* responsiveness of splenocytes to HKLM.

LQGV activates the adrenal ACTH-receptor

Next, we determined the mRNA expression levels of 11-β-hydroxylase, the enzyme that converts deoxycorticosterone to corticosterone. LQGV dose dependently enhanced the mRNA expression levels of 11-β-hydroxylase (Figure 6A), which correlated positively and significantly ($r = 0.426$; $p < 0.05$) with the corticosterone levels detected in the culture media (Figure 6B). The cAMP-blocker H-89 and ACTH-receptor blocker CIP completely abolished the LQGV-induced corticosterone release, while this was not observed when the LH-receptor antagonist Antide was added to the organ culture (Figure 6C). Moreover, CIP inhibited the LQGV-induced increase of 11-β-hydroxylase mRNA expression in a dose dependent manner (Figure 6D). H-89, LH-receptor antagonist, and CIP alone did not affect the basal adrenal 11-β-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.

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 and LH receptor activation by using the adrenal cell line LHR-Y1 (in which the LH-receptor has been cloned [29]). These cells were stimulated with recombinant hCG or LQGV in the presence or absence of H-89, CIP or LH receptor antagonist. In line with previous observations [31,32], recombinant hCG increased the corticosterone levels and the 11-β-hydroxylase mRNA expression levels. This increase was inhibited by H-89 and the LH receptor antagonist (Figure 7A and B). LQGV dose dependently increased the corticosterone levels and the 11-β-hydroxylase mRNA expression levels, which were reduced by H-89 and CIP, but not by the LH receptor antagonist Antide (Figure 7A and B). In addition, mRNA expression levels of 11-β-hydroxylase correlated positively and significantly with corticosterone levels detected in supernatants (Figure 7C). The irrelevant tetrapeptide EPPE did not increase the 11-β-hydroxylase mRNA expression (data not shown). These results demonstrate that LQGV induces 11-β-hydroxylase mRNA expression through ACTH-receptor induced cAMP signaling.

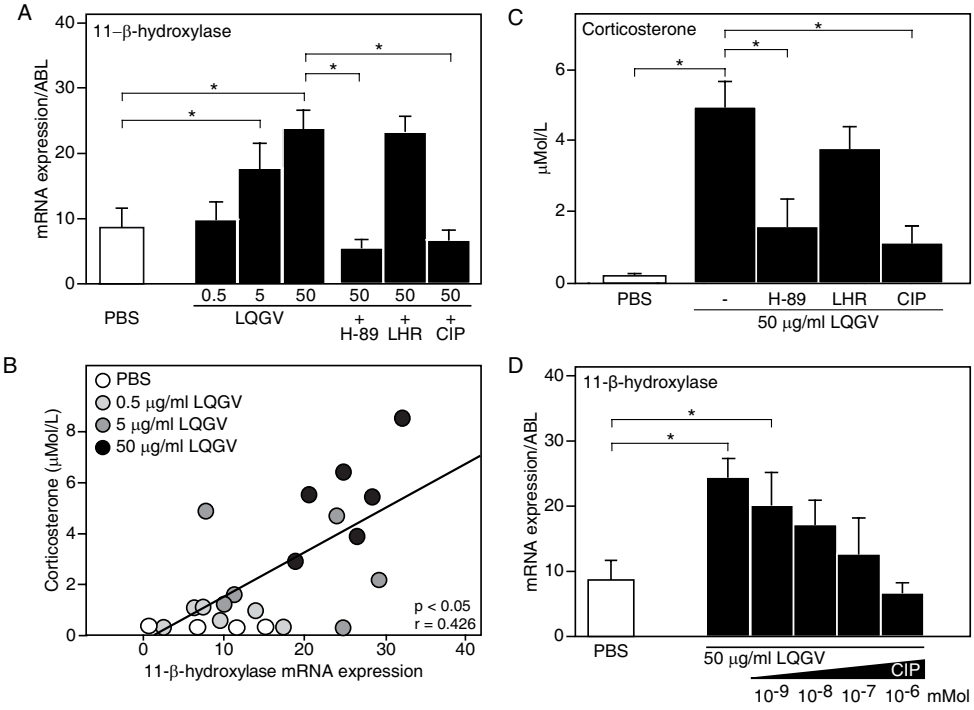

Figure 6. LQGV activates the adrenal ACTH-receptor.
Adrenal glands were *ex vivo* stimulated with either 0.5, 5, or 50 µg/ml LQGV or with a combination of 50 µg/ml LQGV with either the cAMP-blocker (H-89), LH-receptor antagonist (LHR) or ACTH-receptor antagonist (CIP) and 11-β-hydroxylase mRNA expression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

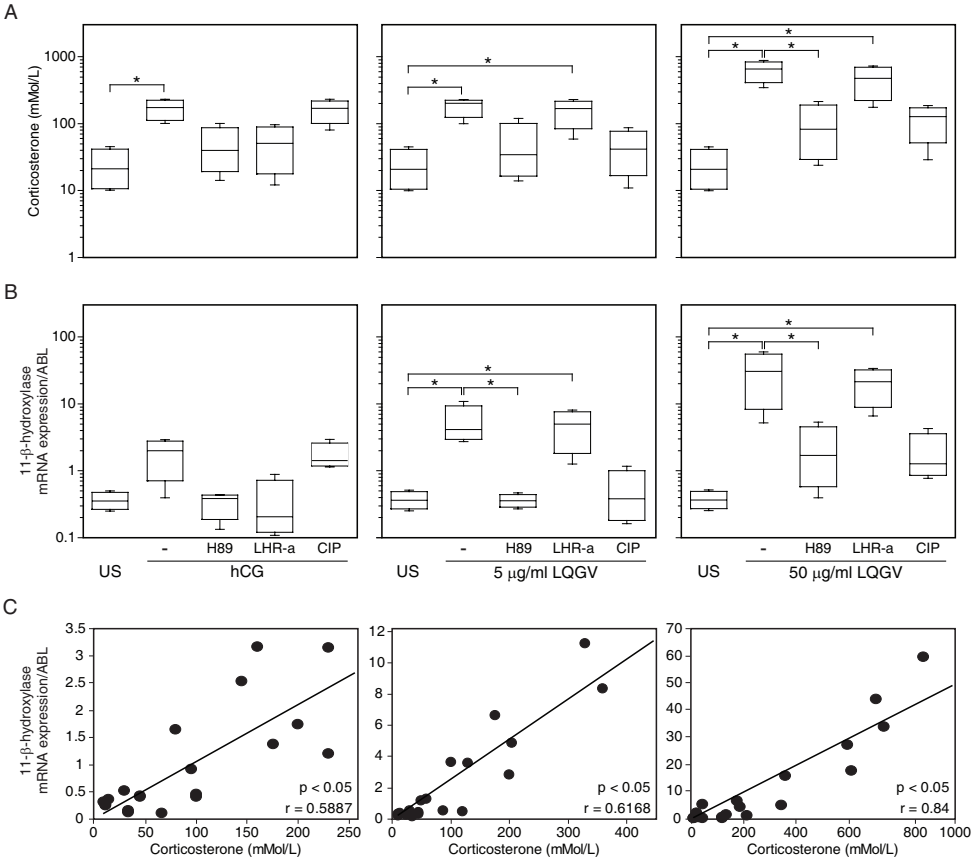

Figure 7. LQGV activates the ACTH-receptor on the murine adrenal cell line LHR-Y1.
LHR-Y1-cells were stimulated with either 300U recombinant hCG, 5, or 50 µg/ml LQGV in combination with either the cAMP-blocker (H-89), LH-receptor antagonist (LHR), or ACTH receptor antagonist (CIP). Subsequently, corticosterone levels in supernatant (A) and 11-β-hydroxylase mRNA expression levels (B) were determined in the cultured cells**.** Correlation analysis between 11-β-hydroxylase mRNA expression levels and corticosterone levels after 6 hours stimulation with either 300U recombinant hCG, 5µg/ml LQGV or 50 µg/ml LQGV in combination with either the cAMP-blocker (H-89), LH-receptor antagonist (LHR) or ACTH-receptor antagonist (CIP) (C) * means $p < 0.05$, ** means $p < 0.01$. Data depicted are from four single experiments * means $p < 0.05$.

DISCUSSION

Endogenous glucocorticosteroid production and function are crucial in the control and resolution of inflammatory responses [22,25,33]. In this study we demonstrate for the first time that the hCG-related tetrapeptide LQGV, which reduces immune activation in response to hemorrhagic shock and resuscitation (HS/R), *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 into SIRS and finally multiple organ dysfunction syndrome (MODS), which is a major cause of in hospital deaths worldwide [35]. LPS challenge of mice is an TLR-4 driven *in vivo* SIRS model that can result in MODS and eventually death [35]. In line with our 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 (50 mg/kg BW) exerts its *in vivo* pro-survival 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 co-administrated 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 progesterone [38,39]. Therefore, we believe that corticosterone is the major contributing anti-inflammatory glucocorticosteroid induced by high dose (50 mg/kg BW) LQGV-treatment in our studies.

Glucocorticosteroid production is regulated by 11-β-hydroxylase within the zona fascilata of the adrenal cortex [40]. In rodents, 11-β-hydroxylase converts deoxycorticosterone to corticosterone, while in humans it converts deoxycortisol to cortisol [40]. The expression of 11-β-hydroxylase is dependent on ACTH-induced ACTH-receptor activation and subsequent cAMP-signaling [40-42]. LQGV increased 11-β-hydroxylase mRNA expression levels and stimulated corticosterone production in *ex vivo* adrenal gland cultures. These effects were abolished when cAMP-signaling or ACTH-receptor activation were blocked. While LQGV is part of the amino acid sequence of loop 2 of β-hCG [13], it exerts its effects independent of the LH/βCG-receptor as is evident from our LH/βCG-receptor blocking studies in adrenal gland cultures and LHR-Y1-cells. This notion is strengthened by our observation that LQGV did not directly inhibit the response of splenocytes to HKLM, despite the fact that immune cells can express the LH/βCG-receptor and do respond to recombinant hCG [8,9,43,44]. 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 one hour up to 24 hours following 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 minutes [45]. Also, the *ex vivo* LQGV-induced 11-β-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 following LQGV administration could be due to an increased level of corticosteroid-binding globulin (CBG), which upon binding enhances the corticosterone half-life from 30-60 minutes to approximately 5 days [46]. We regard this unlikely as CBG is produced in the liver and therefore could not have influenced the increase in corticosterone levels detected in our adrenal gland culture supernatants [47]. Furthermore, glucocorticoids bound to CBG are biologically inactive [48], while 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 LPS-induced septic shock, while 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 [25,45,49], while synthetic glucocorticosteroids are protective in these models [50,51]. High corticosterone levels also render mice more susceptible to *L. monocytogenes* infection [45,52,53]. The effects of increased endogenous corticosterone levels on a *L. monocytogenes* infection was presented in a recent study [54]. These authors found that activation of the HPA axis by influenza virus leads to a prolonged increase of corticosterone levels that enhanced the susceptibility to *L. monocytogenes* infection [54]. 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].

In conclusion, the data presented indicate 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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V

SYNTHETIC OLIGOPEPTIDES RELATED TO THE β-SUBUNIT OF HUMAN CHORIONIC GONADOTROPIN ATTENUATE INFLAMMATION AND LIVER DAMAGE AFTER (TRAUMA) HEMORRHAGIC SHOCK AND RESUSCITATION

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ABSTRACT

Severe hemorrhagic shock (HS) followed by resuscitation induces a massive inflammatory response, which may culminate into systemic inflammatory response syndrome, multiple organ dysfunction syndrome and finally death. Treatments that effectively prevent this inflammation are limited so far. In a previous study, we demonstrated that synthetic oligopeptides related to the primary structure of human chorionic gonadotropin (hCG) can inhibit the inflammatory response and mortality that follow high-dose LPS-induced inflammation. Considering this powerful anti-inflammatory effect, we investigated whether administration of similar synthetic hCG-related oligopeptides (LQGV, AQGV, LAGV), during HS, were able to attenuate the inflammatory response associated with this condition. Hemorrhagic shock was induced in rats for 60 minutes by blood withdrawal until a MAP of 40 mmHg was reached. Rats received a single injection with one of the hCG-related oligopeptides (LQGV, AQGV or LAGV) or 0.9% NaCl solution as control 30 minutes after induction of HS. Treatment with LQGV, AQGV or LAGV prevented systemic release of TNF-α and IL-6 and was associated with reduced TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. LQGV treatment prevented neutrophil infiltration into the liver and was associated with reduced liver damage. Our data suggest that hCG-related oligopeptides, in particular LQGV, have therapeutic potential by attenuating the life threatening inflammation and organ damage that is associated with (trauma) hemorrhagic shock and resuscitation.

INTRODUCTION

Severe hemorrhagic shock (HS) is caused by massive blood loss that cannot be compensated for by the body without treatment. The primary treatment of HS is focused on controlling bleeding and restoring intravascular volume to improve tissue perfusion. Many patients with severe HS who are successfully resuscitated develop an inflammatory response, which may culminate into systemic inflammatory response syndrome (SIRS) and finally multiple organ dysfunction syndrome (MODS) [1]. In addition, approximately 40% of patients with hemorrhagic shock develop sepsis as a result of increased gut permeability and development of compensatory anti-inflammatory syndrome [1-2]. Sepsis and MODS are the leading causes of death in critically ill patients on the intensive care unit all over the world with approximately 50% mortality [3].

The inflammatory response after HS and resuscitation is characterized by increased expression of adhesion molecules, such as E-selectin and intracellular adhesion molecule-1 (ICAM-1), on endothelial cells and hepatocytes [4]. Up-regulation of these adhesion molecules facilitates tissue infiltration by neutrophils, resulting in cell-mediated

organ injury [5]. Furthermore, increased levels of cytokines, such as TNF-α, IL-1β, IL-6 and IL-10, are found systemically and locally in liver, lungs and intestine [6-8]. These cytokines, mainly produced by immune cells, affect organ integrity directly or indirectly through induction of secondary mediators, such as thromboxanes, leukotrienes, and complement [9-10].

The last decade, research has focused on reducing systemic and local inflammatory responses with therapeutic agents that neutralize cytokine activity or inhibit inflammatory mediator production. However, in the case of HS such treatments require initiation before the onset of shock to achieve an effect [11-13]. Clearly, this is impossible in most clinical settings. Therefore, therapies that efficiently inhibit the inflammatory response when initiated after hemorrhage-induced shock are more relevant. Studies on such treatments are limited [14], but are highly needed.

During pregnancy, the maternal immune system tolerates the fetus by reducing cell-mediated immune responses while retaining normal humoral immunity. In addition, clinical symptoms of cell-mediated autoimmune diseases regress in many patients during pregnancy [15]. Most likely a specific hormonal environment is responsible for modulating the immune system during pregnancy [15]. The hormone human chorionic gonadotropin (hCG) is secreted by placental syncytiotrophoblasts during human pregnancy. Human chorionic gonadotropin preparations exhibit not only endocrine effects, but also immunosuppressive activity [16]. We found that hCG preparations inhibited the onset of autoimmune type-I diabetes in nonobese diabetic mice [17]. This anti-diabetic/anti-inflammatory activity was not due to the heterodimeric hCG nor to its α- or β-subunits, but resided in a peptide fraction of 400-2000 Dalton, which likely originates from proteolytic cleavage of loop 2 of the hCG β-subunit [17-19]. Subsequently, we successfully demonstrated that synthetic oligopeptides, related to the primary sequence of loop 2 of the hCG β-subunit, inhibit inflammation, disease severity, and mortality in high-dose LPS-induced SIRS [18-19]. Considering this powerful effect of synthetic hCG-related oligopeptides on inflammation, we hypothesized that the administration of such oligopeptides after induction of HS could inhibit the inflammatory response associated with this condition. To this end, we used LQGV, which is part of the primary structure of loop 2 of the β-subunit of hCG, and two alanine replacement variants, namely AQGV and LAGV.

Using a rat model of (trauma) HS and resuscitation, we demonstrate that either LQGV, AQGV or LAGV, administered after the induction of HS, significantly prevented TNF-α and IL-6 release into the plasma and attenuated the increase in TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. In addition, LQGV treatment significantly prevented neutrophil accumulation in the liver, which correlated with decreased organ damage as reflected by reduced lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) plasma levels.

MATERIALS AND METHODS

Animals

Adult male specific pathogen-free Wistar rats (Harlan CPB, Zeist, The Netherlands), weighing 350–400g were used. Rats were housed under barrier conditions at 25°C with a twelve-hour light/dark cycle, and were allowed food and water *ad libitum*. 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.

hCG-related synthetic oligopeptides

The hCG-related oligopeptides (LQGV, AQGV and LAGV) were synthesized by Ansynth Service B.V. (Roosendaal, The Netherlands) and dissolved in 0.9% NaCl at a concentration of 5 mg/ml.

Surgical procedures

Rats were deprived of food overnight before the start of the experiment, but were allowed water *ad libitum*. Rats were anesthetized using a mixture of N_2O/O_2/isoflurane (Pharmachemie B.V., Haarlem, The Netherlands). Body temperature was continuously maintained at 37.5°C by placing the rats on a thermo controlled 'half-pipe' (UNO, Rotterdam, The Netherlands). Endotracheal intubation was performed, and rats were ventilated at 60 breaths per minute with a mixture of N_2O/O_2/isoflurane. Polyethylene tubes (PE-50, Becton Dickinson; St. Michielsgestel, The Netherlands) were flushed with heparin and placed via the right carotid artery in the aorta and in the right internal jugular vein. A 5 cm midline laparotomy was performed and a supra pubic catheter was inserted to monitor urine production.

Experimental procedures

After an acclimatization period of 15 minutes, the rats were randomized into five different groups (eight rats per group): 1) sham, 2) HS, 3) HS with LQGV treatment (HS/LQGV), 4) HS with AQGV treatment (HS/AQGV) and 5) HS with LAGV treatment (HS/LAGV). Hemorrhagic shock was induced by blood withdrawal, reducing the circulating blood volume until a MAP of 40 mmHg was reached. This level of hypotension was maintained for 60 minutes. Rats received a single intravenous bolus injection of 5 mg/kg body weight of either LQGV, AQGV, LAGV or 0.9% NaCl solution 30 minutes after the induction of HS. The peptides and dosage were based on previous studies, in which we performed dose-escalation experiments [38]. Sixty minutes after induction of hemorrhagic shock, rats were resuscitated by four times their shed blood volume over a

period of 30 minutes to normalize the MAP, and monitored for another 120 minutes after which they were sacrificed (Figure 1A). The rats received no heparin before or during the experiment. Sham animals underwent the same surgical procedure as the hemorrhagic shock animals, but without blood withdrawal and administration of oligopeptide.

Measurements of mean arterial pressure

During the experiments, MAP was continuously measured using transducers (Becton Dickinson, Breda, The Netherlands) that were connected in line to an electronic recorder (Hewlett Packard, 78354-A, Cheshire, USA).

Plasma collection and storage

Arterial blood was obtained 15 minutes before and 30, 60, 90, 120, 150 and 180 minutes after onset of hemorrhage (Figure 1A). After blood withdrawal, leukocyte numbers were determined using a coulter counter (Beckman Coulter, Mijdrecht, The Netherlands) and corrected for the hematocryte. Approximately, 0.3 ml of blood was placed into mini collect tubes (Greiner, Bio-one, Alphen aan den Rijn, The Netherlands); plasma was obtained by centrifugation (1500 rpm; 5 min), immediately frozen, and stored at -80°C until assayed.

Tissue collection and storage

Liver, lungs, ileum and sigmoid were surgically removed at 180 minutes after hemorrhagic shock induction, snap-frozen, and stored at -80°C until assayed.

Evaluation of cytokines in plasma

TNF-α, IL-6 and IL-10 plasma levels were determined by enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

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

RNA was isolated using the Qiagen RNeasy kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. *TNFA* (encoding TNF-α), *IL6* (encoding IL-6), *IL10* (encoding IL-10), *SELE* (encoding E-selectin), and *ICAM1* (encoding ICAM-1) gene expression levels were determined by RQ-PCR using an Applied Biosystems 7700 polymerase chain reaction machine (Foster City, CA, USA). The expression levels of these genes were quantified by normalization against the mRNA levels of the household gene *GAPDH*. Primers and probes used are available upon request.

Immunohistochemical analysis

Cryo-sections (6 μm) were fixed in acetone/0.05%H_2O_2 for five minutes and subsequently air dried for 10 minutes. Neutrophils were visualized by staining for

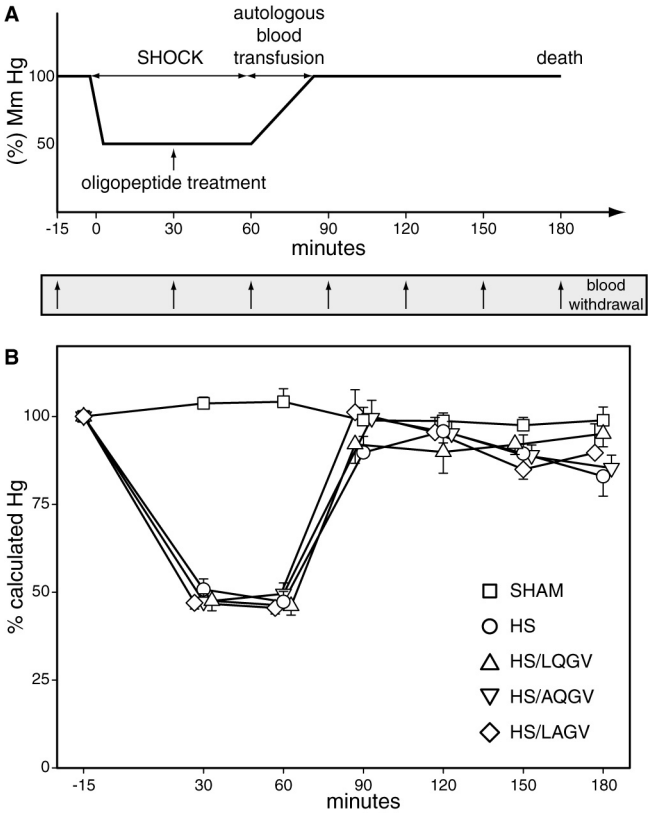

Figure 1.
(A) Schematic representation of the experimental design of induction of hemorrhagic shock in rats. (B) The measured blood pressure in mmHg was recalculated in percentages to standardize the experiment and to compensate for animal differences.

myeloperoxidase (MPO). Hereto, sections were incubated overnight at 4°C with a mouse-anti-rat MPO monoclonal antibody (Hbt, Uden, The Netherlands). Subsequently, sections were incubated for 60 minutes at room temperature with a goat-anti-mouse-Horse Radish Peroxidase (HRP)-labeled monoclonal antibody (Dako B.V., Glostrup, Denmark). For visualization of HRP activity, 3-amino-9-ethylcarbazole substrate (Sigma Co., St Quentin Fallavier, France) dissolved in 50mM sodium acetate/0.02% hydroxyperoxide was used. Sections were embedded in Kaisers Gycerol/gelatin (Boom B.V., Meppel, The Netherlands). Numbers of MPO positive cells were counted per high power field (HPF) at a magnification of 200x. Per section a total of 15 HPF were

analyzed. Per organ a total of three consecutive sections, each separated 18μm from each other, were analyzed.

Blood biochemical analysis

Plasma alanine aminotransferase (ALT), AST and LDH were determined at the Erasmus MC diagnostic facility according to standard procedures.

Statistical analysis

Data are presented as the mean values ± standard deviation (SD) of the eight rats per group. Statistical analysis was performed using SPSS version 11 software (SPSS Inc., Chicago,Ill). Intergroup differences were analyzed with Kruskal-Wallis statistical test. If Kruskal-Wallis statistical testing resulted in a $p < 0.05$, a Dunn's Multiple Comparison test was performed and a $p < 0.05$ was considered statistically significant.

RESULTS

Induction of HS

Rats were rapidly bled, within 10 minutes, to a MAP of 40 mmHg, which was successfully maintained for 60 minutes in all four experimental groups (Figure 1B). No change in MAP was observed in sham treated rats (Figure 1B). Sixty minutes after hemorrhagic shock, rats were resuscitated to induce organ reperfusion, which was associated with a normalization of urine production (data not shown). These data indicate that shock was induced equally in all four experimental groups and was followed by successful organ reperfusion. Heart rates in all four experimental groups increased immediately after induction of hemorrhagic shock and returned to normal after resuscitation. Over time, heart rate slowly increased in all four experimental HS groups (data not shown).

Oligopeptide treatment prevents the release of pro-inflammatory cytokines into plasma

Before induction of hemorrhagic shock, TNF-α plasma levels were comparable in all five groups (~15-24 pg/ml; Figure 2). In the HS group, TNF-α levels started to increase 30 minutes after induction of hemorrhagic shock. These levels were significantly increased after 60 minutes as compared to the sham group (331 pg/ml vs 29 pg/ml; $p < 0.01$). TNF-α levels reached a maximum of 384 pg/ml after 90 minutes in the HS group, after which levels declined again but continued to remain increased compared to the sham group (Figure 2). In contrast, none of the oligopeptide-treated HS groups showed an increase in TNF-α plasma levels during the experiment (Figure 2). In this model of

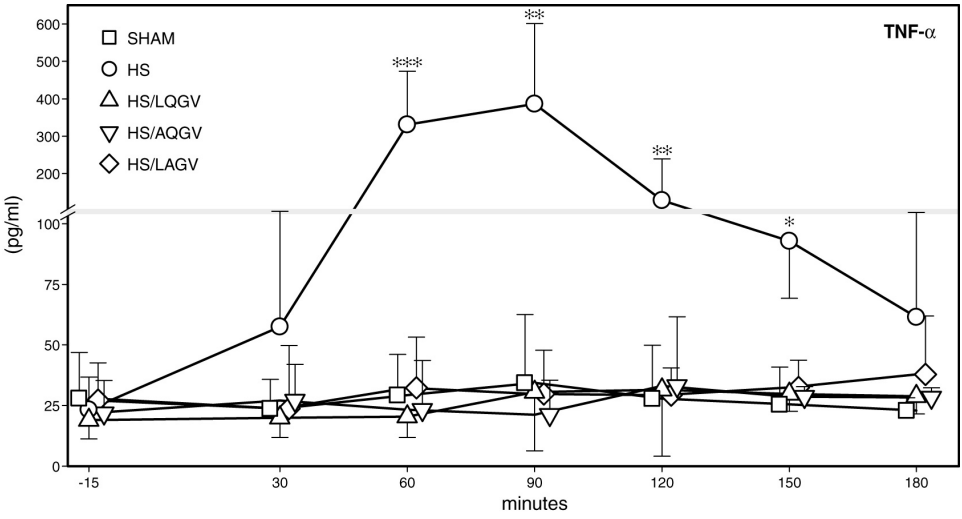

Figure 2.
TNF-α plasma levels in different experimental groups determined at 15 minutes before and 30, 60, 90, 120, 150 and 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD. * means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

hemorrhagic shock, IL-6 levels are known to increase at a later time-point than TNF-α [20]. Therefore, we determined IL-6 levels in blood samples collected 120, 150 and 180 minutes after the onset of hemorrhagic shock. In the HS group, IL-6 plasma levels were significantly increased as compared to the sham group at 120 minutes (2003 pg/ml vs 331 pg/ml; $p < 0.001$), at 150 minutes (2444 pg/ml vs 333 pg/ml; $p < 0.001$) and at 180 minutes (2940 pg/ml vs 343 pg/ml; $p < 0.001$) (Figure 3). Although, IL-6 levels tended to increase in the HS/oligopeptides-treated rats as compared to sham-treated rats, this never reached significance. Treatment with oligopeptides significantly diminished the release of IL-6 into plasma as compared to the non-treated hemorrhagic shock group ($p < 0.05$; Figure 3). IL-10 was undetectable in plasma of all groups throughout the experiment (data not shown). These data demonstrate that treatment with a single dose of either LQGV, AQGV or LAGV, after induction of hemorrhagic shock, significantly attenuated the increase in TNF-α and IL-6 into plasma.

Oligopeptide treatment is associated with a decrease in TNF-α and IL-6 mRNA transcript levels in the liver

We also analyzed the TNF-α and IL-6 mRNA transcript levels in liver, lungs, ileum and sigmoid tissues at 180 minutes after the onset of hemorrhagic shock. In the liver,

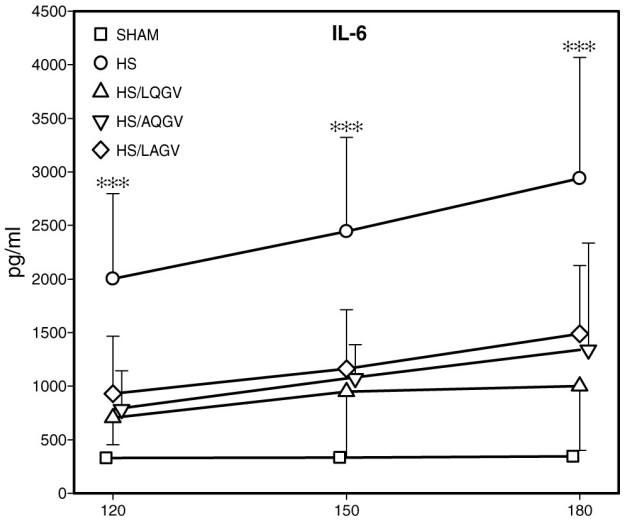

Figure 3.
IL-6 plasma levels in different experimental groups determined at 120, 150 and 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD. *** means $p < 0.001$.

TNF-α transcript levels were significantly increased in the HS group as compared to the sham group ($p < 0.001$). Oligopeptide treatment was associated with reduced TNF-α transcript levels in the liver as compared to non-treated HS rats, with only HS/LQGV showing a significant reduction ($p < 0.05$; Figure 4A). In the HS group, IL-6 transcript levels in the liver were increased ~83 times as compared to the sham group ($p < 0.001$; Figure 4B). None of the oligopeptide treated groups showed a significant increase in IL-6 transcript levels as compared to the sham group. LQGV and AQGV treatment was associated with significantly lower IL-6 transcript levels as compared to the HS group ($p < 0.05$; Figure 4B). Although, IL-10 was undetectable in plasma, IL-10 transcript levels were increased in the livers of the HS group as compared to the sham group, which approached significance ($p = 0.08$). Although not significant, LQGV treatment was associated with decreased IL-10 transcript levels as compared to the non-treated HS group (data not shown). In lungs, ileum and sigmoid tissues no differences could be detected between the various groups for TNF-α, IL-6 and IL-10 transcript levels (data not shown). These data imply that oligopeptide treatment after shock induction significantly attenuated the increase in TNF-α and IL-6 transcript levels in the liver.

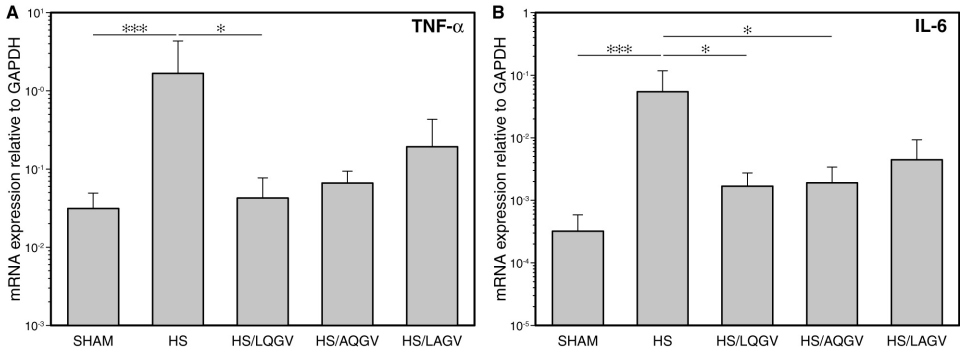

Figure 4.
Relative expression of TNF-α (A) and IL-6 (B) mRNA transcripts in the liver, 180 minutes after the onset of hemorrhagic shock. Transcript levels are normalized to the expression level of GAPDH. Data are presented as the mean of eight rats per group ± SD. * means $p < 0.05$, *** means $p < 0.001$.

Oligopeptide treatment is associated with a decrease in E-selectin mRNA transcript levels in the liver

In the HS group, the ICAM-1 transcript level was significantly increased in the liver as compared to the sham group ($p < 0.001$; Figure 5A). ICAM-1 transcript levels in the liver tended to decrease in the oligopeptide treated groups as compared to the non-treated HS group. The E-selectin transcript level in the liver of the HS group was significantly increased as compared to the sham group ($p < 0.001$). LQGV and AQGV treatment was associated with significantly lower E-selectin transcript levels in the livers as compared to the non-treated HS-group ($p < 0.05$; Figure 5B). These data demonstrate that LQGV and AQGV treatment after shock induction significantly attenuated the increase in E-selectin transcript levels in the liver, while ICAM-1 transcript levels were down-regulated to a lesser extent.

LQGV treatment prevents neutrophil accumulation in the liver

In the HS group, the number of neutrophils in the liver was significantly increased as compared to the sham group ($p < 0.05$; Figure 6A). LQGV treatment significantly ($p < 0.05$) prevented this neutrophil accumulation, while AQGV and LAGV treatment did not prevent neutrophil accumulation in the liver ($p < 0.05$; Figure 6).

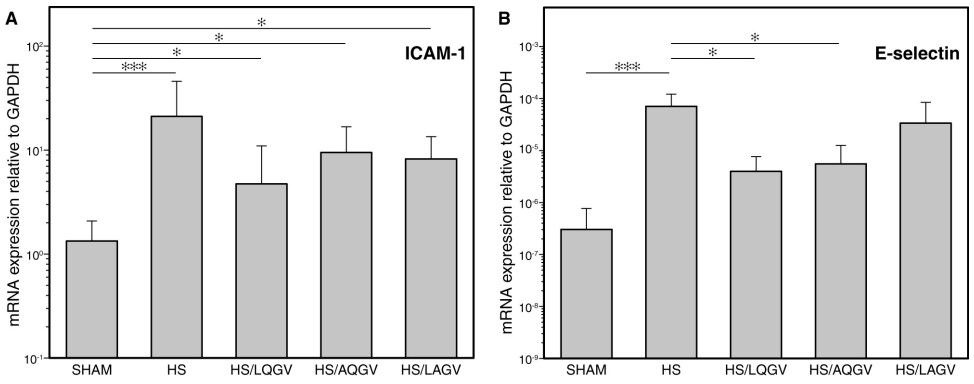

Figure 5.
Relative expression of ICAM-1 (A) and E-selectin (B) mRNA transcripts in the liver, 180 minutes after the onset of hemorrhagic shock. Transcript levels are normalized to the expression level of GAPDH. Data are presented as the mean of eight rats per group ± SD. * means $p < 0.05$, *** means $p < 0.001$.

LQGV treatment attenuates organ damage

Alanine aminotransferase, AST and LDH plasma levels were significantly increased in the HS group as compared to the sham group (ALT; $p < 0.01$; Figure 7A), (AST; $p < 0.01$; Figure 7B) and (LDH; $p < 0.01$; Figure 7C), while LQGV treatment significantly ($p < 0.05$) attenuated this rise in AST and LDH. AQGV and LAGV treatment did not affect ALT, AST and LDH plasma levels as compared to the untreated HS group.

DISCUSSION

In this study we used a rat model of (trauma) hemorrhagic shock to test the therapeutic capacity of three synthetic hCG-related oligopeptides (LQGV, AQGV or LAGV). We demonstrate that a single administration of either LQGV, AQGV or LAGV, 30 minutes after shock induction, markedly prevents TNF-α and IL-6 release into plasma and diminishes the increase of TNF-α, IL-6 and E-selectin mRNA transcript levels in the liver. In addition, LQGV treatment significantly prevented neutrophil accumulation into the liver, which coincided with lower AST and LDH plasma levels.

Hemorrhagic shock followed by resuscitation is characterized by a massive production of pro-inflammatory cytokines, such as TNF-α and IL-6, by immune cells [10]. Despite improvement in treatment strategies, (trauma) hemorrhage patients may still develop an inflammatory response that can lead to sepsis, MODS and finally

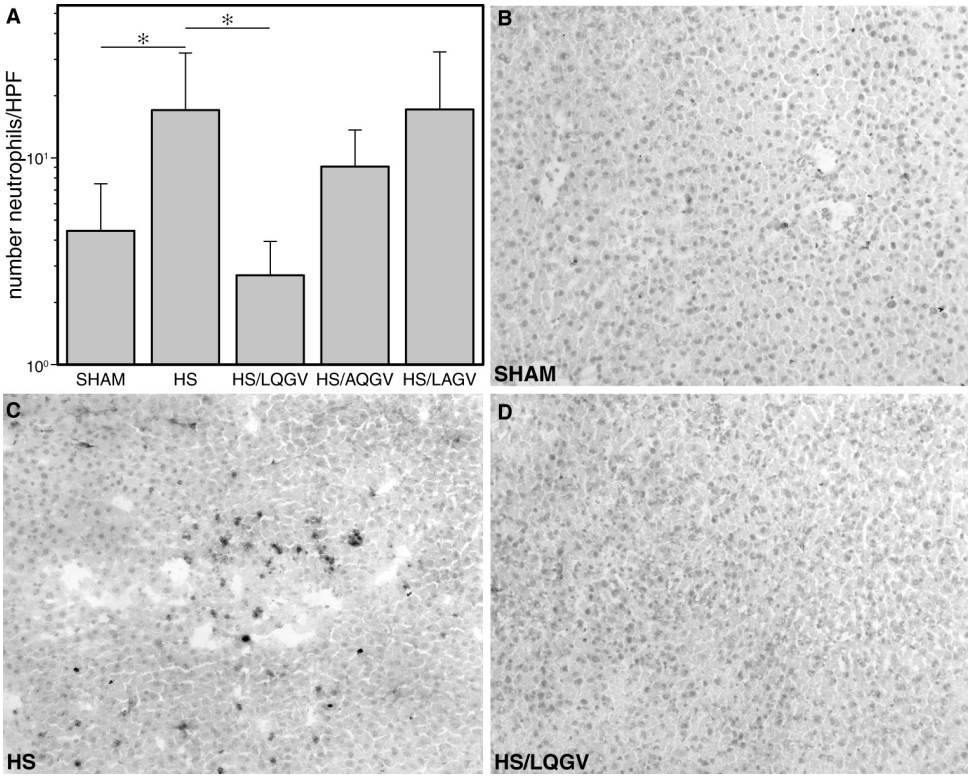

Figure 6.
(A) Number of neutrophils per High Power Field (HPF; magnification 200x) in the liver 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD. Representative examples of livers from sham (B), HS (C) and HS/LQGV (D), 180 minutes after the onset of hemorrhagic shock. * means $p < 0.05$.

death. In our model of HS and resuscitation, we observed an inflammatory response, as reflected by significantly increased levels of TNF-α and IL-6 in plasma. TNF-α is a key mediator of the innate immune system that is crucial for the generation of a local protective immune response against infectious or non-infectious agents [21]. However, uncontrolled TNF-α production is lethal, as it induces tissue damage and promotes the production of secondary pro-inflammatory mediators, such as IL-6 [22].

Experimental treatment strategies aimed at neutralizing bioactive cytokines, especially monoclonal antibodies against TNF-α, have been successfully applied in several inflammatory disorders, including Crohn's disease and rheumatoid arthritis [23-24]. However, clinical studies using monoclonal antibodies against TNF-α showed

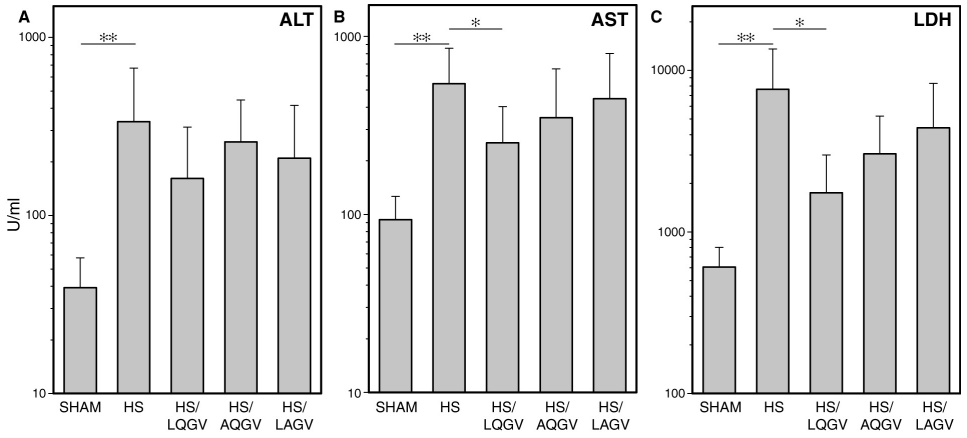

Figure 7.
Plasma levels of ALT (A), AST (B) and LDH (C) 180 minutes after the onset of hemorrhagic shock. Data are presented as the mean of eight rats per group ± SD. * means $p < 0.05$, ** means $p < 0.01$.

no improvement in trauma-patients [25]. IL-6 is a highly pluripotent cytokine, which facilitates neutrophil infiltration into organs, thereby contributing to cell-mediated organ damage [26]. In our model of (trauma) HS and resuscitation, oligopeptide treatment was associated with significantly decreased levels of TNF-α and IL-6 in plasma. Reducing TNF-α and IL-6 plasma levels is of clinical importance, because high systemic levels of TNF-α and IL-6 correlate with poor outcome and decreased survival in patients with severe trauma and infection [27]. We found local TNF-α and IL-6 production in the liver after hemorrhagic shock and resuscitation, which was reduced upon oligopeptide treatment, in particular with LQGV. In lungs, ileum and sigmoid, we found no effect of HS on the transcript levels of TNF-α and IL-6. Trauma-hemorrhage has been recognized to induce acute lung injury/inflammation in humans and animals [28-29]. In our model we detected no increase of TNF-α, IL-6, E-selectin and ICAM-1 transcript levels in the lungs three hours after hemorrhagic shock, suggesting that a pulmonary inflammatory response was not (yet) evident. We have found that our oligopeptides efficiently inhibited SIRS and mortality that was induced upon LPS administration, which is an inflammatory model characterized by involvement of several organ systems, including the lungs [18-19]. Therefore, although we cannot conclude it from the current study, we expect that our oligopeptides do prevent hemorrhagic shock-induced pulmonary inflammation.

IL-10 is an anti-inflammatory cytokine that reduces cell-mediated immune responses and pro-inflammatory cytokine production following hemorrhagic shock [8]. We were

unable to detect IL-10 in plasma during the time frame of the experiments. However, local IL-10 production in the liver was detected, since IL-10 transcripts increased in the HS group. LQGV treatment was associated with decreased IL-10 mRNA levels as compared to the non-treated HS group. Although, this did not reach statistical significance, we propose that this may be of biological relevance since high IL-10 levels are associated with a high incidence of infection, MODS and increased mortality in (trauma) hemorrhage patients [30].

Neutrophils induce organ damage and enhance inflammation by the release of oxygen radicals, proteolytic enzymes and cytokines [31-32]. Neutrophil infiltration into organs is an early event of HS and resuscitation, and neutrophil depletion has been shown to prevent HS-induced inflammation and organ damage [5,33]. These data indicate a central role for neutrophils in the pathophysiology of HS and resuscitation. Leukocyte migration from blood into organs requires the consecutive events of rolling and sticking to activated endothelial cells, followed by diapedesis and chemotaxis [34]. Among these processes, selectin-mediated rolling is indispensable for initiation of leukocyte transmigration and inflammation [35]. In line with this, L- or E- selectin blockage, using monoclonal antibodies, reduced liver infiltration by neutrophils as well as inflammation and organ damage following hemorrhagic shock [36]. In our experiments, treatment with LQGV or AQGV significantly decreased E-selectin transcript levels in the liver. Furthermore, LQGV treatment prevented neutrophil accumulation in the liver and was associated with lower AST and LDH plasma levels after hemorrhagic shock and resuscitation. These data suggest that oligopeptide treatment, in particular LQGV, after hemorrhagic shock and resuscitation diminishes the expression of adhesion molecules, thereby inhibiting tissue infiltration by neutrophils and subsequent organ damage and systemic inflammation.

In this model, LQGV, originating from the primary sequence of loop 2 of the β-subunit of hCG, was the most effective oligopeptide for every parameter determined. Alanine replacement in this sequence reduced the biological activity. We cannot exclude that the decreased TNF-α and IL-6 mRNA levels, upon oligopeptide treatment, that we observed in the liver are the result of a diminished cellular infiltrate. However, the decrease in E-selectin mRNA, which is only expressed by endothelial cells, indicates that the tested oligopeptides also interfere with mechanisms that regulate expression/activation of genes involved in inflammation and immunity. So far it is unclear what the underlying mechanism is by which these oligopeptides exert their effects. It is possible that they use yet unidentified receptors. However, we cannot exclude the possibility that, due to their small size and molecular weight, they penetrate the cell membrane [37] and exert their action either by interfering with signaling cascades or the transcriptional machinery. We do not exclude that different oligopeptides have different modes of action. Studies are in progress to reveal how these hCG-related oligopeptides exert their action.

In summary, we demonstrated that administration of a synthetic hCG-related oligopeptide (LQGV, AQGV or LAGV) after the induction of severe hemorrhagic shock significantly attenuated the pro-inflammatory response both systemically and locally in the liver. Treatment with LQGV prevented neutrophil infiltration into the liver and subsequent liver damage. These data suggest that these oligopeptides, in particular LQGV, have therapeutic potential and may reduce the morbidity and mortality associated with hemorrhagic shock and resuscitation.

ACKNOWLEDGMENTS

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VI

THE BETA-hCG-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 (hCG) 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 (CLP)-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 CLP-induced mortality and inflammation C57BL/6 mice were exposed to a moderate CLP procedure (40% ligation and double puncture) with a mortality of ~80% within 5 days in control mice. In addition, to examine whether LQGV was of additive value to standard sepsis care (antibiotics and fluid resuscitation) a more severe CLP-procedure was used (80% ligation and double puncture), yielding ~100% mortality within 12 days in control mice. LQGV (5 mg/kg body weight), PBS (as control), or dexamethasone (2.5 mg/kg body weight) were 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 following moderate CLP. 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 NF-κB activation and pulmonary damage. In the severe CLP-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 following CLP. This is likely established by a modest reduction of the acute inflammatory response through a NF-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 (MODS) 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 (e.g.

interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α)) and chemokines (e.g. chemokine (C-C motif) ligand 2 (CCL2) and macrophage inflammatory protein-1α (MIP-1α)) by inflammatory cells and endothelial cells [4]. This pro-inflammatory 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 (e.g. IL-10 and transforming growth factor-β (TGF-β)) as well as anergy and apoptosis of immune effector cells (e.g. B cells, T cells, and dendritic cells) [5-8]. Septic patients may die during the early hyperinflammatory phase of sepsis, but mostly succumb during 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 auto-immune diseases, such as rheumatoid arthritis and multiple sclerosis, improve during pregnancy while symptoms of systemic lupus erythematosus, in which the principal pathology is auto-antibody driven, tend to flare up [11]. The pregnancy-associated hormonal environment 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 auto-immune diabetes, hemorrhagic shock, and LPS-induced shock [15-17]. Moreover, *in vivo* administration of LQGV reduced the capacity of splenocytes to produce IL-6 and TNF-α upon lipopolysaccharide (LPS) 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, appeared to be non-effective 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, as fluid resuscitation and antibiotics can be administered to evaluate the potential of new anti-inflammatory agents [25,26].

In order 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, in order 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 ~70 - 80% mortality during the acute phase of sepsis (first five 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 hours 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 ~100% 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 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 ~50% during the chronic phase of sepsis mice received s.c. injections of Tienam (25 mg/kg body weight (BW); Merck Sharp & Dohme, Haarlem, The Netherlands,) dissolved in 1 ml 0.9% NaCl starting 2 hours after surgery followed by subsequent injections every 12 hours 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 minutes before and 20 minutes 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 minutes 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 minutes), 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 B.V., Woerden, The Netherlands). The first 2 ml peritoneal wash was centrifuged (1500 rpm; 10 minutes), 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) 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 hours at 37ºC. 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).

Histologic analysis

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

RNA isolation and real-time quantitative PCR

RNA was isolated (RNeasy Micro Kit; Qiagen, Hilden, Germany) from lung tissue obtained 6 hours 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 AppliedBiosystems 7900 PCR machine (Foster City, CA, USA) and quantified by normalization against ABL [29].

Electrophoretic mobility shift assay (EMSA)

NF-κB activity was evaluated in nuclear extracts from lung tissue of mice sacrificed 6 hours after CLP. Lung tissue was grinded and resuspended in ice-cold lysisbuffer 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 (Roche, mini protease inhibitor, ethylenediaminetetraacetic acid (EDTA) free). After 20 minutes incubation on ice followed by centrifugation, nuclei were lysed in ice cold lysisbuffer 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 (Roche, mini protease inhibitor, EDTA free). EMSA 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). 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 in order 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, or indicated otherwise. A p-value <0.05 was considered as 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 (Figure 1). LQGV treatment significantly ($p < 0.05$) improved survival up to 50% during the acute phase of sepsis (Figure 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 (Figure 1).

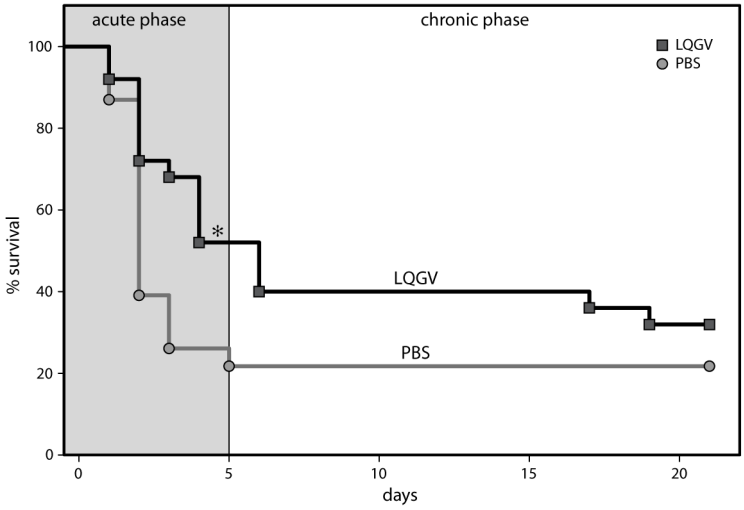

Figure 1. LQGV treatment improved survival.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). Survival was monitored for 21 days after CLP. LQGV treatment improved survival during the acute phase of sepsis (≤ day 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 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 hours (Figure 2). In the LQGV-treated mice plasma levels were consistently lower, although this never reached significance, as ANOVA-analysis showed that the profiles of the mean cytokine levels did not significantly differ between treatment groups (Figure 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.

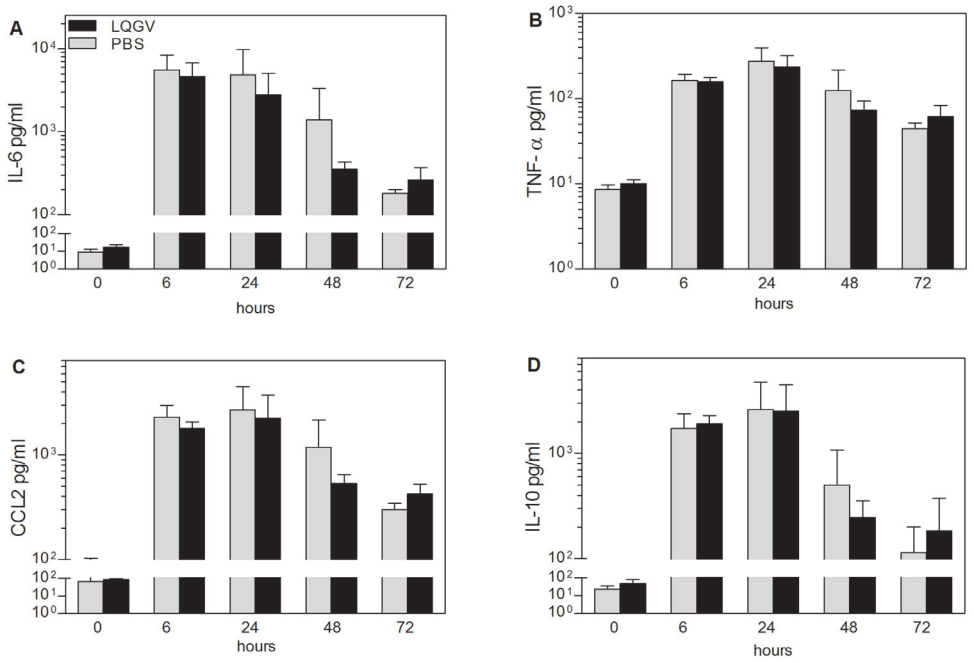

Figure 2. LQGV treatment moderately reduced plasma cytokine levels.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). CLP induced an increase of plasma IL-6 (A), TNF-α (B), CCL2 (C), and IL-10 (D) in both PBS and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

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 hours 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 hours after CLP, in both groups (Figure 3). However, at 6 hours after CLP, IL-6 and IL-10 levels were significantly ($p < 0.05$) lower in LQGV-treated mice (Figure 3), whereas TNF-α levels were not affected (Figure 3B).

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 hours and 24 hours following CLP. In both groups peritoneal cell numbers were significantly ($p < 0.05$) higher at 24 hours post CLP than 6 hours post CLP (Figure 4A). LQGV treatment did not affect the total number of peritoneal cells (Figure 4A), or the cellular composition

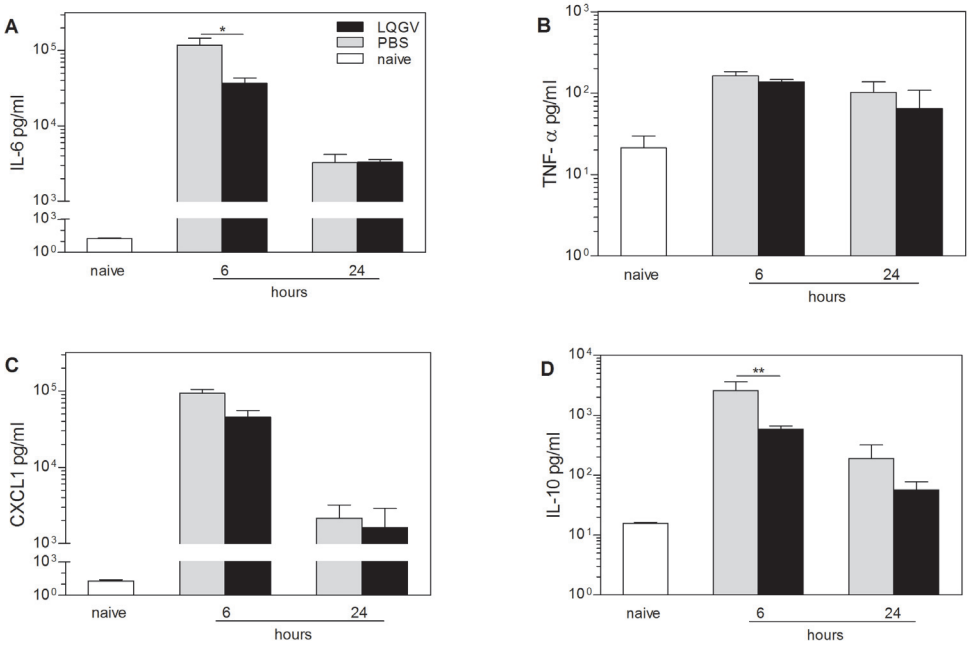

Figure 3. LQGV treatment reduced peritoneal cytokine levels.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). Peritoneal washes were performed at 6 hours and 24 hours after CLP and cytokine levels were determined. 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. * $p < 0.05$, ** $p < 0.01$.

which mainly consisted of neutrophils at 6 hours post CLP and at 24 hours 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 hours after CLP (Figure 4E). In PBS-treated mice, peritoneal cells obtained 24 hours after CLP produced significantly ($p < 0.05$) more IL-10 than cells obtained 6 hours post CLP (Figure 4E). No such increase in IL-10 production was observed for peritoneal cells from LQGV-treated mice. In LQGV-treated mice, peritoneal cells obtained 24 hours after CLP produced significantly ($p < 0.05$) less CXCL1 than peritoneal cells obtained 6 hours after CLP. This decrease did not occur in PBS-treated mice (Figure 4D).

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

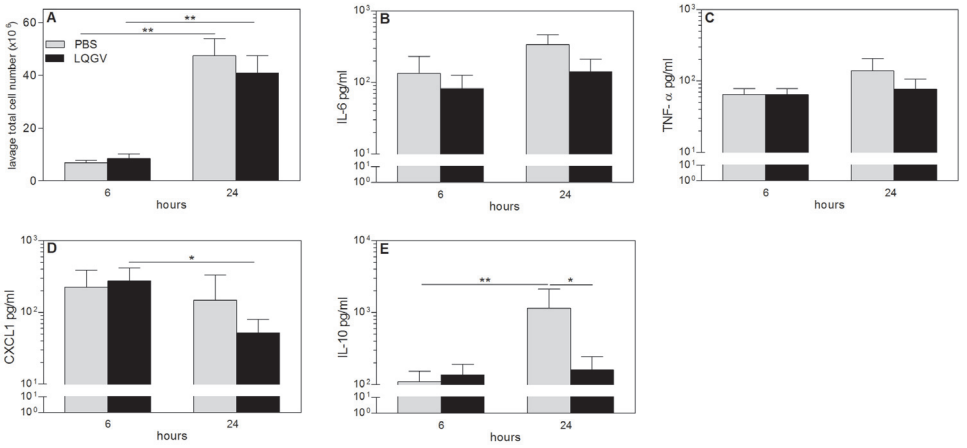

Figure 4. LQGV treatment reduced cytokine production by peritoneal cells.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). Peritoneal wash was performed at 6 hours and 24 hours following CLP and cell numbers were determined. (A) CLP resulted in an increase of total cell numbers in both groups, which peaked at 24 hours. 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 hours. CXCL1 production levels (D) decreased significantly between 6 and 24 hours in the LQGV-treated mice, while this was not observed in the PBS-treated mice. IL-10 production (E) increased significantly between 6 and 24 hours 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 hours post CLP. *n* = 8 mice/ group. * $p < 0.05$, ** $p < 0.01$.

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.

E-selectin mRNA levels at 6 hours 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 (Figure 5A). LQGV treatment significantly ($p < 0.05$) reduced IL-6, CXCL1, and E-selectin mRNA levels in the lungs (Figure 5A). Also IL-10 mRNA levels were reduced upon LQGV treatment, although not statistically significant (Figure 5A). LQGV treatment did not affect the increase of pulmonary TNF-α mRNA (Figure 5A).

The transcription factor NF-κB regulates the production of many cytokines and initial experiments demonstrated clear pulmonary NF-κB activity at 6 hours following CLP (data not shown). Therefore, we determined whether LQGV treatment affected the pulmonary NF-κB activity at 6 hours after CLP. Pulmonary NF-κB activation was lower in 75% of the LQGV-treated mice as compared with PBS-treated mice (Figure 5B). 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 hours post CLP had thickened alveolar septa, mainly due to edema (Figure 5C). LQGV treatment reduced these histopathologic alterations (Figure 5C).

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 hours after CLP the number of CFU in blood and peritoneal lavage fluid did not differ between both groups (Figure 6).

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 (Figure 7A). Dexamethasone treatment did however significantly ($p < 0.05$) reduce plasma IL-6 and CCL2 levels as well as peritoneal IL-6 and CCL2

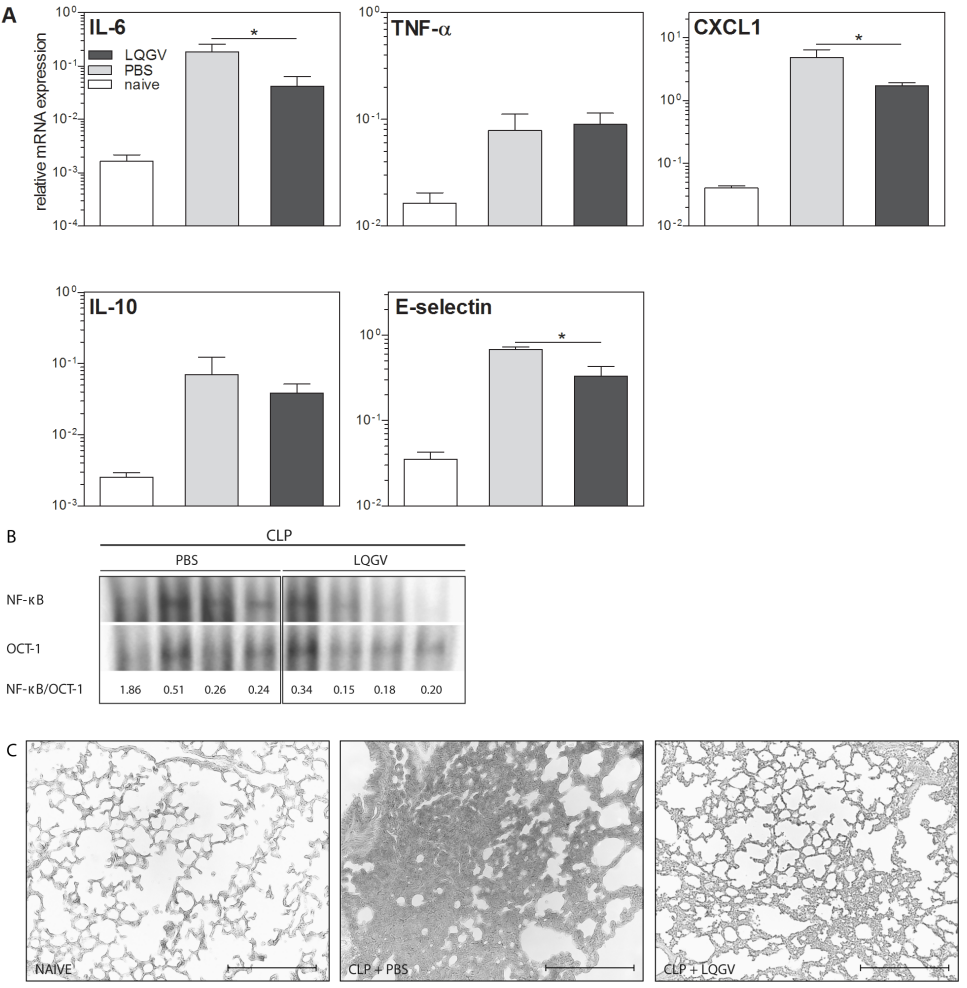

Figure 5. LQGV treatment reduced pulmonary inflammation.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). Lung tissue was obtained at 6 hours post CLP. (A) LQGV treatment was associated with reduced IL-6, CXCL1, 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, * means $p < 0.05$. Bars, 50 μm.

levels at 6 hours after CLP (Figure 7B). Dexamethasone treatment also significantly ($p < 0.05$) reduced pulmonary IL-6 mRNA levels, while TNF-α remained unaffected (Figure 7B).

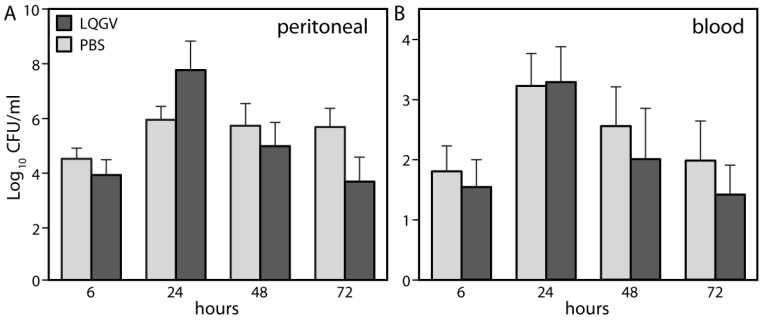

Figure 6. LQGV treatment did not alter bacterial load.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP) and bacterial load was determined in blood and peritoneal fluid obtained at different time points after CLP. 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.

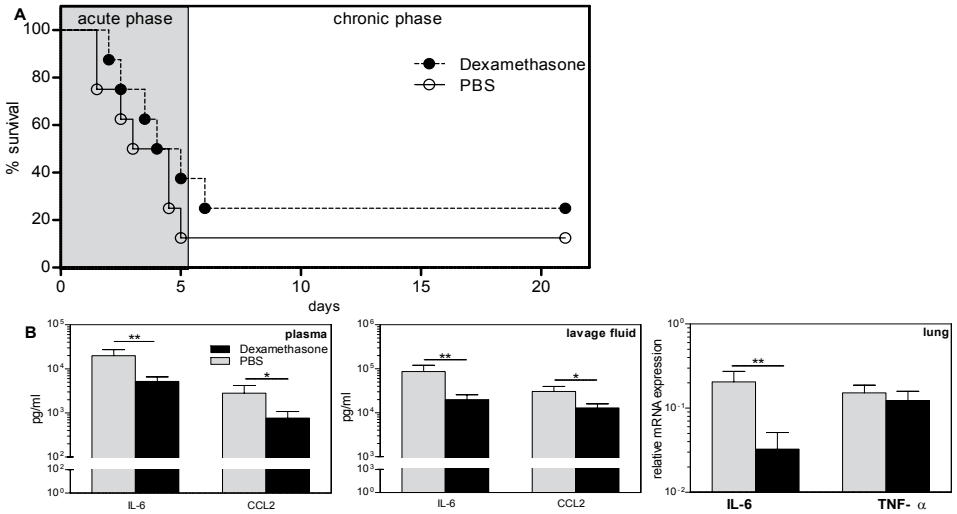

Figure 7. Dexamethasone treatment did not alter mortality and inflammation.
Dexamethasone was administered (2.5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after low-grade cecal ligation and puncture (CLP). (A) Survival was monitored for 21 days after CLP. Dexamethasone treatment did not affect mortality 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 hours post CLP. Relative IL-6 and TNF-α mRNA expression levels were determined in lung tissue obtained at 6 hours post CLP. Dexamethasone reduced the IL-6 and CCL2 plasma levels as well as the peritoneal IL-6 and CCL2 level and the IL-6 mRNA expression level in the lung. *n* = 6 mice/group, * means $p < 0.05$, ** means $p < 0.01$.

LQGV as addition to standard sepsis treatment improves survival following severe 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 (Figure 8). In this severe CLP-model treatment with LQGV plus postoperative fluid resuscitation alone did not improve survival (Figure 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 (Figure 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 (Figure 8).

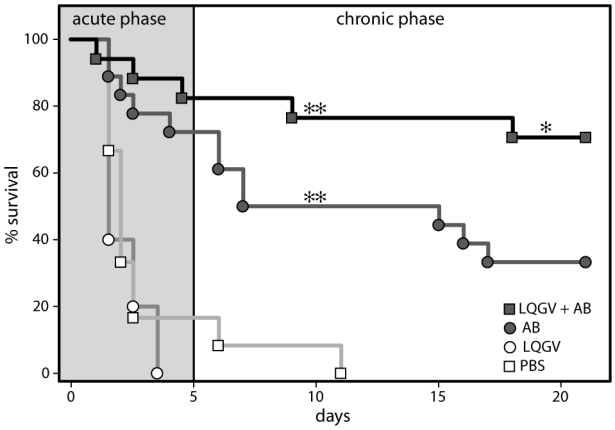

Figure 8. LQGV treatment in combination with standard care improved survival.
LQGV was administered (5 mg/kg body weight) intravenously 5 minutes before and 20 minutes after high-grade cecal ligation and puncture (CLP), followed by 5 day treatment with fluid resuscitation and antibiotics or fluid resuscitation alone. Survival was monitored for 21 days. PBS or LQGV treatment in combination with fluid resuscitation alone (LQGV) did not affect mortality. 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-treated mice 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, MODS, 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 succumb from CLP-induced sepsis during the early acute hyperinflammatory septic phase (< 5 days following 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 while 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 due to 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 following *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 as 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 following 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 following 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 (van der Zee, van den Berg et al., manuscript submitted). 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 [49,50]. Here, we found that dexamethasone significantly, and more effectively than LQGV, reduced the CLP-induced inflammatory response. However, dexamethasone was not associated with survival improvement, which is in line with previous observations [49]. Therefore, our results suggest that extensive downregulation of the inflammatory response during polymicrobial sepsis (as established by dexamethasone) does not improve survival, while 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, while this survival benefit was not observed when a higher dosage of IL-6 neutralizing antibody was used [51] 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 is likely due to 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 due to 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 [8,52], 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 due to 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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VII

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 (DEX) treatment in varying doses on cecal ligation and puncture (CLP)-induced inflammation and mortality.

Design: Animal study.

Setting: University research laboratory.

Subjects: Male C57BL/6 mice.

Interventions: Mice were subjected to CLP, DEX 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 minutes postoperatively. Mice receiving PBS served as controls. Survival was recorded up to 21 days and inflammatory markers were determined in plasma, lung, liver, and kidney at 6 hours following CLP.

Measurements and main results: L/DEX treatment significantly improved survival compared with control mice whereas treatment with higher concentrations of DEX (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 hours post-CLP. In addition, M/DEX or H/DEX powerfully reduced cytokine mRNA expression in the lung, liver, and kidney. In contrast, treatment with L/DEX was associated with a mild, but non-significant, reduction of cytokine plasma levels. In addition, L/DEX only moderately reduced cytokine mRNA expression in lung, liver, and kidney.

Conclusions: A mild downregulation of the early sepsis associated inflammatory response improves survival in a murine CLP model. Apparently, mild anti-inflammatory therapy allows the immune system to battle the invading pathogens sufficiently. 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 of which 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. Treatment of sepsis patients has essentially remained unchanged during the last decades. Overall, the sepsis-related inflammatory response may develop into septic shock and multiple organ dysfunction syndrome (MODS), which are leading causes of morbidity and mortality in critically ill patients [9,10]. This emphasizes the need for therapeutic strategies with immunoregulatory properties.

During the last decades the application of general anti-inflammatory agents, such as corticosteroids, has been investigated in severe sepsis and septic shock. However, contradictory results have been obtained. 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 TNF-α specific monoclonal antibodies, have also been investigated. Although such studies showed a remarkable survival improvement in lipopolysaccharide (LPS)-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 has a beneficial effect on short-term mortality in patients with septic shock, in contrast to high-dose corticosteroid treatment [22]. This 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 and colleagues [22] led us to hypothesize that mild immunosuppression is beneficial during a septic event whereas extensive immunosuppression is not.

In order to examine this hypothesis we investigated the effect of three different DEX dosages on acute inflammation and mortality induced by CLP in mice. Our study demonstrates that low-dose 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, lung, liver, and kidney. In contrast, treatment with a medium-dose DEX (0.25 mg/kg BW) or high-dose DEX (2.5 mg/kg BW) did significantly reduce the inflammatory response, but had no beneficial effect on survival. We propose 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.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice with an average weight of 25 gram were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 hour light/dark cycle and were allowed food and water *ad libitum*. Mice were used in the experiments at the age of 8 to 12 weeks. 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 five 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 hours 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 ~85% 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 dosages of DEX 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 (up to 21 days) 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 hours after surgery followed by subsequent injections every 12 hours 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.

Dexamethasone treatment

Mice received a single intravenous injection of DEX (Sigma Aldrich, Zwijndrecht, The Netherlands; dissolved in PBS) 20 minutes after the CLP procedure. This time-

point was based on previous studies [25]. Treatment groups were: low-dose DEX (L/DEX; 0.05 mg/kg BW), medium-dose DEX (M/DEX; 0.25 mg/kg BW), or high-dose DEX (H/DEX; 2.5 mg/kg BW). Control mice received an injection with PBS.

Blood

Blood was obtained 6 hours following CLP by retro-orbital plexus puncture 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 minutes), immediately frozen, and stored at -80ºC until assayed.

Cytokine measurement

Plasma levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, the chemokines CCL2, CXCL1, CXCL2, and eotaxin, as well as the anti-inflammatory cytokines and soluble cytokine receptors IL-1Ra, IL-10, sTNF-R1, and sTNF-R2 were determined using a sequential enzyme-linked immunosorbent assay (ELISA) 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 hours 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 ICAM-1 mRNA levels were determined by real-time quantitative PCR (RQ-PCR) using an Applied Biosystems 7900 PCR machine (Foster City, CA, USA) and quantified by normalization against the household gene ABL [28].

Statistical analysis

Data are presented as the mean values ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad software, CA, USA) and SPSS version 15 (SPSS Inc., Chicago, IL). Statistical significance was determined by the non-parametric Mann-Whitney U test and log-rank survival analysis. Log–rank survival analyses were performed (until day 21) and corrected for population stratification of different experiments. 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 DEX had no effect on survival; 39% and 33% survival, respectively. L/DEX treatment significantly improved survival up to 75% at day 21 (L/DEX vs. PBS $p < 0.05$; L/DEX vs. H/DEX $p < 0.05$) (Figure 1). L/DEX treatment also reduced the severity of clinical manifestations of sepsis, such as lethargy, body weight loss, and diarrhoea during the acute phase of sepsis. This was not observed for both higher DEX dosages (data not shown).

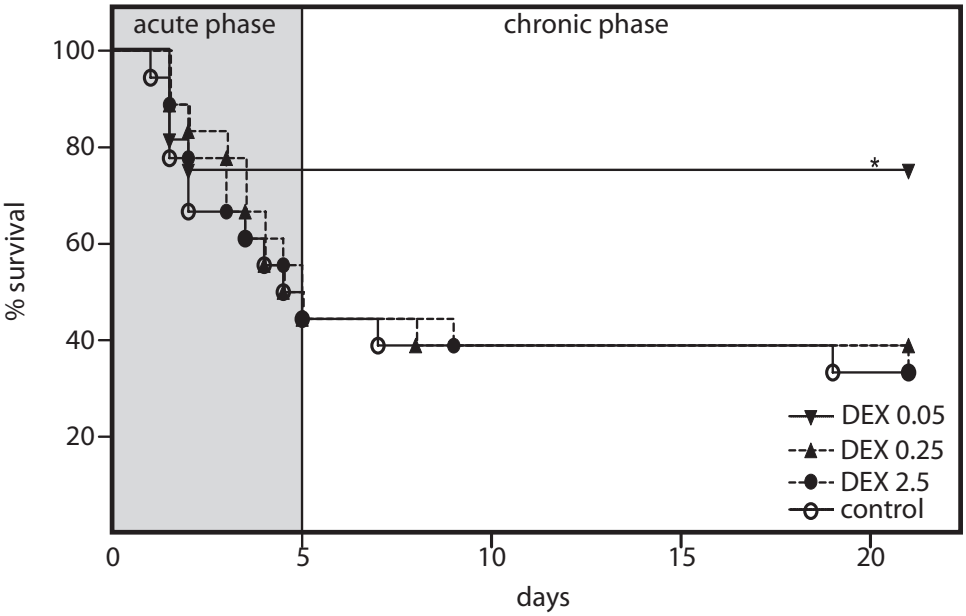

Figure 1. Effect of dexamethasone treatment on survival.
Moderate polymicrobial sepsis was induced in C57BL/6 mice by ligation of the cecum 1 cm from the distal end followed by a double puncture with an 18-G needle. DEX was administered intravenously 20 minutes following CLP. Survival was monitored for 21 days after CLP. L/DEX (0.05 mg/kg BW) treatment was associated with improved survival as compared with treatment with PBS, M/DEX (0.25 mg/kg BW), or H/DEX (2.5 mg/kg BW). Presented results were obtained in three identical independent experiments and corrected by stratification. *n* = 16 - 18 mice/group. * means $p < 0.05$.

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 hours 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 (Figure 2). In the L/DEX treated mice plasma levels of CCL2, CXCL1, CXCL2, eotaxin, IL-1ra, IL-6, IL-10, sTNF-R1, and sTNF-R2 were consistently lower than in control mice, although

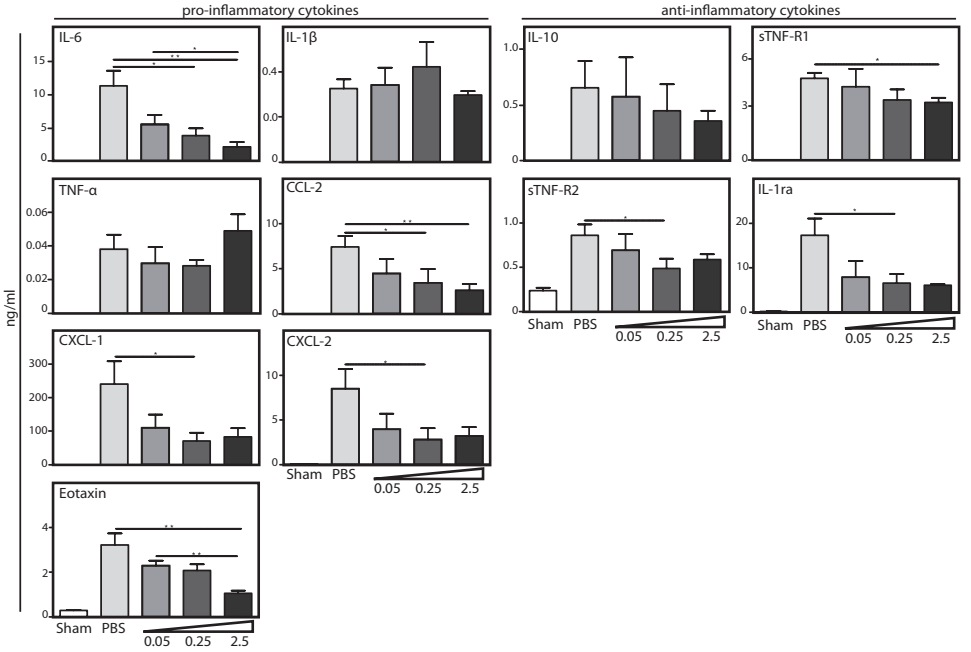

Figure 2. Effect of dexamethasone treatment on systemic inflammatory response.
Moderate polymicrobial sepsis was induced in C57BL/6 mice by ligation of the cecum 1 cm from the distal end followed by a double puncture with an 18-G needle. DEX was administered intravenously 20 minutes following CLP. Plasma cytokine levels were determined at 6 hours following CLP. L/DEX (0.05 mg/kg BW) treatment was associated with reduced plasma levels of pro-inflammatory cytokines and anti-inflammatory cytokines. M/DEX (0.25 mg/kg BW) and H/DEX (2.5 mg/kg BW) treatment was associated with significantly reduced IL-6, CCL2, CXCL1, CXCL2, eotaxin, TNF-sr1, TNF-sr2, and IL-1ra levels as compared with PBS treated mice. *n = 6* mice/group. * means $p < 0.05$, ** means $p < 0.01$.

this was never significant (Figure 2). The tested DEX concentrations did not affect the CLP-induced increase of plasma IL-1β and TNF-α (Figure 2).

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

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

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

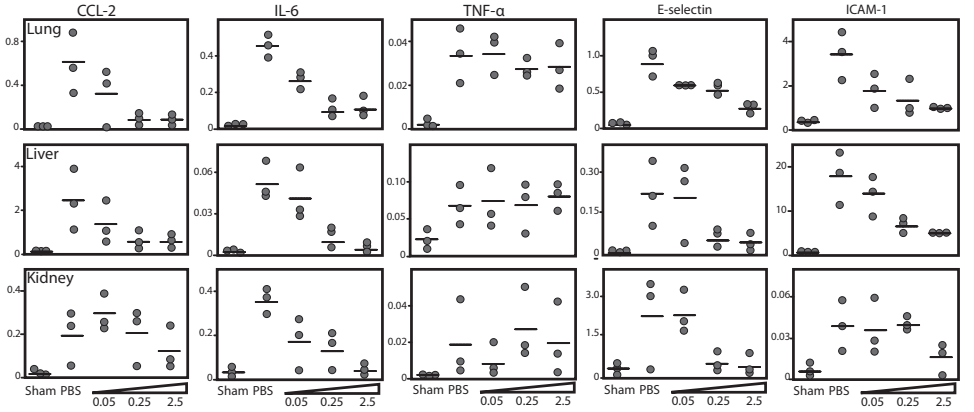

Figure 3. Effect of dexamethasone treatment on pulmonary, hepatic, and renal inflammation. Moderate polymicrobial sepsis was induced in C57BL/6 mice by ligation of the cecum 1 cm from the distal end followed by a double puncture with an 18-G needle. DEX was administered intravenously 20 minutes following CLP. Lung, liver, and kidney tissue was obtained at 6 hours following CLP. Relative IL-6, CCL2, TNF-α, E-selectin, and ICAM-1 mRNA expression levels were determined in lung, liver, and kidney tissue. L/DEX (0.05 mg/kg BW), M/DEX (0.25 mg/kg BW), and H/DEX (2.5 mg/kg BW) treatment was associated with reduced IL-6, CCL2, E-selectin, and ICAM-1 mRNA expression levels compared with PBS treated mice. *n* = 3 mice/group.

severe CLP-induced sepsis model. In control mice that only received fluid resuscitation the overall survival at day 21 was 17% (Figure 4). In mice that received treatment with fluid resuscitation and antibiotics, survival significantly increased to 50% ($p < 0.05$) at 21 days (Figure 4). 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 (Figure 4). L/DEX treatment in combination with fluid resuscitation and antibiotics increased survival up to 92%, which was significantly ($p < 0.05$) higher than with fluid resuscitation and antibiotics alone (Figure 4).

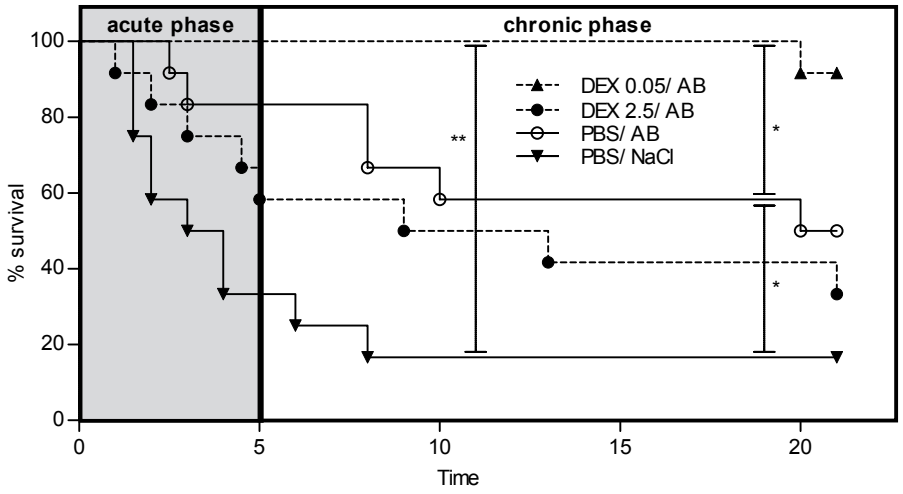

Figure 4. Effect of dexamethasone treatment in combination with standard sepsis care on survival.
Severe polymicrobial sepsis was induced in C57BL/6 mice by ligation of the cecum just below the ileocecal valve followed by a double puncture with an 18-G needle. DEX, or PBS as control, was administered intravenously 20 minutes after cecal ligation and puncture, followed by 5 day treatment with antibiotics and fluid resuscitation (AB) or fluid resuscitation alone (NaCl). Survival was monitored for 21 days after cecal ligation and puncture. Control treatment in combination with fluid resuscitation alone did not affect mortality. Control treatment followed by postoperative treatment with antibiotics and fluid resuscitation was associated with improved survival. H/DEX (2.5 mg/kg BW) treatment in combination with antibiotics and fluid resuscitation did not affect survival compared with control treatment. L/DEX (0.05 mg/kg BW) treatment in combination with antibiotics and fluid resuscitation did improve survival compared with control treatment in combination with antibiotics and fluid resuscitation. 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. * means $p < 0.05$, ** means $p < 0.01$.

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 pro-inflammatory 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 pro-survival 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 downregulation of the inflammatory response. Our data demonstrates 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, conflicting results have been obtained with corticosteroid treatment. Some studies described that high-dose corticosteroid treatment (30 mg/kg methylprednisolone) resulted in survival improvement [37], 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 minutes after the CLP procedure improved survival while M/DEX and H/DEX did not. Remarkably, in septic patients it also appears that only low-dose corticosteroid treatment, defined as a total daily dosage of ≤ 300 mg hydrocortisone (or equivalent), has a beneficial effect on short-term mortality [22]. 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 [38], which suggests that DEX dosages currently administered to septic patients [22] may be reduced to gain further survival improvement.

Pro-inflammatory 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, non-significant, 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. In contrast, M/DEX and

H/DEX treatment resulted in significant reduction of the inflammatory response. This illustrates that mild downregulation of the pro-inflammatory response is associated with survival improvement while strong immunosuppression is not. In line with this, marked suppression of the innate immune system prior to infection favors excessive bacterial replication [42,43]. Deletion of signaling molecules, such as MyD88, that orchestrate cytokine responses upon a septic stimulus, also enhances vulnerability to infection [44]. Strong immunosuppressive therapy therefore likely 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 the observation that, although 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]. This indicates that IL-6 is involved in the protective mechanisms upon a septic insult. Altogether, these data suggest the existence of a specific range in which the sepsis related inflammatory response can be pharmacologically reduced 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. Therefore, mild reduction of the inflammatory response, as established by L/DEX, further improves the therapeutic potential of the current standard sepsis care.

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 minutes) 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 range in which anti-inflammatory drugs can be of benefit in a septic setting.

CONCLUSION

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. 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 (Figure 5). As the septic inflammatory response is complicated and differs among patients [29] insight into the immunological status of the individual patient will aid to maximize the chance of treatment success.

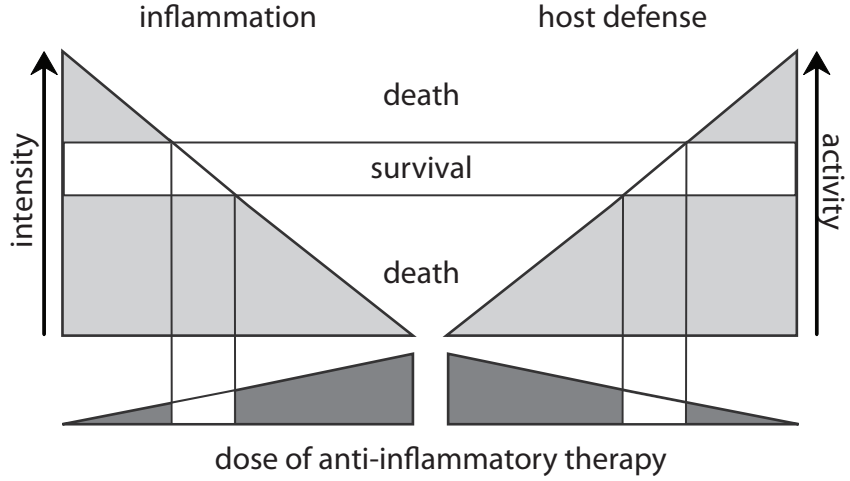

Figure 5. Model explaining how anti-inflammatory therapy can be successfully applied in a septic setting.
The figure represents two pillars of the sepsis-related inflammatory response, i.e. intensity of the inflammation (upper left triangle) and host defense (upper right triangle). Success of anti-inflammatory therapies in a septic setting fundamentally depends on establishing a critical level of immunosuppression that prevents inflammation-induced pathology but still allows an adequate host defense against invading pathogens. Slight or no downregulation of the inflammatory response increases the risk of death. In addition, robust anti-inflammatory therapy inhibits the protective functions of the inflammatory response (left triangle) as reflected by diminished host defense mechanisms (right triangle), also increasing the risk of death. However, mild anti-inflammatory therapy ameliorates the detrimental effects of SIRS and preserves the protective effect of the inflammatory response, enhancing the chance of adequate host defense and subsequent survival. This suggests the existence of a specific range of anti-inflammatory treatment in which the sepsis-related inflammatory response can be modulated in such a way that survival improvement is obtained.

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VIII

CONCLUSIONS AND GENERAL DISCUSSION

CONCLUSIONS AND GENERAL DISCUSSION

1. Main findings

The research described in this thesis was initiated to increase the knowledge on the effects and the mechanistic aspects of hCG-related oligopeptides in experimental animal models that are associated with SIRS. In this context also different doses of dexamethasone were investigated.

Chapter II describes that high dose LQGV, VLPALP, and AQGV (50 mg/kg BW) enhance the susceptibility of mice to infection with *L. monocytogenes*. This is established by weakening innate immune activation through inhibition of early chemokine responses, impairing cellular recruitment and blunting the responsiveness of immune cells to *L. monocytogenes* antigens.

Chapter III demonstrates that LQGV (50 mg/kg BW) stimulates neutrophil apoptosis and reduces O_2^- activity. Possibly there is a relationship between these two observations.

Chapter IV describes that LQGV (5 and 50 mg/kg BW) dose dependently activates the murine adrenal gland to produce and secrete the anti-inflammatory hormone corticosterone, and that activation of glucocorticoid receptor signaling is involved in the anti-inflammatory effects of high doses (50 mg/kg BW) of LQGV in LPS-induced SIRS.

Chapter V describes that a low dose of LQGV, AQGV and LAGV (5 mg/kg BW) attenuate the systemic and hepatic inflammatory response that follows hemorrhagic shock and fluid resuscitation.

Chapter VI describes the application of the same low dose of LQGV (5 mg/kg BW) in a CLP model of sepsis in mice. This study shows that this dose of LQGV improves survival following CLP-induced sepsis and that this is associated with a modest reduction of the systemic, pulmonary and peritoneal inflammation. This dose of LQGV also acts as a useful addition to standard sepsis care with antibiotics and fluid resuscitation in a more severe CLP model.

Chapter VII describes that a low dose of the synthetic glucocorticoid dexamethasone (0.05 mg/kg BW) improves survival following CLP-induced sepsis in mice while higher doses of dexamethasone do not. This observation resulted in a model that states that survival improvement by anti-inflammatory therapy in a septic setting crucially depends on the level of immunosuppression. The optimum level of immunosuppression in a septic setting still allows appropriate activation of host defense mechanisms against the invading pathogen(s).

In this chapter the observations of chapters II - VII will be discussed in the context of literature dealing with inflammation, shock, glucocorticosteroids, and pregnancy. In addition, directions for future research will be discussed.

2. Modulation of the inflammatory response during infectious and noninfectious insults

Inflammation is a response against an infectious or noninfectious insult with the aim to restore homeostasis [1]. PRR activation upon infection (PAMP and DAMP) or tissue damage (DAMP) activates transcription factors, such as NF-κB and AP-1, which subsequently drive the production of pro-inflammatory mediators, for instance chemokines and cytokines [2,3]. These mediators activate and orchestrate host defense mechanisms against the insult and contribute to repair mechanisms [1,4-6]. Mice that lack key chemokines, cytokines, their cytokine/chemokine receptors or downstream signaling proteins show a diminished inflammatory response upon an insult [7-11]. This leads to an increased susceptibility to infection, for instance with *L. monocytogenes*, and reduced pathology during a sterile inflammation, as may result from LPS injection or ischemia reperfusion injury [7-11].

In recent years, a lot of effort has been made to investigate the inflammatory response that is associated with sepsis by using LPS infusion into mice [12,13]. Although this model clearly provides a way to examine a hyper-inflammatory response that results from TLR-activation, it does neither represent a true bacterial septic condition nor a true septic inflammatory response [14,15]. One of the most important differences between LPS infusion and bacterial sepsis is that LPS is a bacterial product that is cleared from the body by immune defense mechanisms, while during sepsis multiplication of the invading pathogen occurs that increases the load of toxins and antigens derived from the pathogen. Also the cytokine response is more rapid and stronger upon LPS injection [16], while the clearance of systemic cytokines is delayed in a bacterial sepsis as compared to LPS infusion [17,18].

The SIRS-associated uncontrolled production of pro-inflammatory mediators can lead to MODS and death [19,20]. Therefore, attempts to control this response may limit organ damage and death. Inhibition of cytokine production or cytokine bioactivity, for instance with monoclonal antibodies directed against TNF-α or IL-6, as well as treatment with high doses of glucocorticosteroids, improves survival upon LPS infusion [15,21,22]. However, inhibiting TNF-α activity with neutralizing antibodies does not protect against CLP-induced septic shock [23]. This suggests that TNF-α alone is not an important cytokine in a septic condition, while TNF-α is important in sterile conditions, for instance after ischemia reperfusion injury and after LPS injection [24].

IL-6 is a cytokine that has a broad range of activities and is involved in maintaining the inflammatory response upon LPS and TNF-α injection [25]. In CLP mice, IL-6 plasma levels are considered as a predictor of death [26,27]. High doses of IL-6 neutralizing antibodies do not improve survival following CLP in mice, while a lower dose of IL-6 neutralizing antibody does improve survival [28]. Also a complete lack of IL-6 does not improve survival following CLP in mice [29]. In contrast, a complete lack of IL-6 does

reduce pathology after renal ischemia reperfusion and LPS infusion [30,31]. These observations suggest that IL-6 exerts important physiological functions during sepsis that need to be maintained during therapeutic intervention in sepsis.

In this thesis it was found that the success of an anti-inflammatory therapy with dexamethasone or LQGV, with regard to survival improvement in CLP-induced sepsis, most likely depends on finding an optimal level of immunosuppression. In line with this a recent literature based study suggested that low-dose corticosteroid treatment has a beneficial effect on short-term mortality in patients with septic shock, in contrast to high-dose corticosteroid treatment [32]. Therefore, it can be speculated that a specific level of immunosuppression that reduces SIRS-induced pathology, but still allows an adequate host defense against invading pathogens, is crucial to the success of anti-inflammatory therapies. As the septic inflammatory response is complicated and differs among patients, insight into the immunological status (activated or suppressed) of the individual patient will aid to optimize immunosuppressive treatment and thus will influence therapy success [14].

Administration of LQGV and VLPALP two hours after LPS infusion improved survival in mice, while no survival benefit was observed when LQGV and VLPALP where administrated 24 hours after LPS injection [13,33]. However, administration of MTR, MTRV, VLPALPQ, and VVC did improve survival when administrated 24 hours after LPS infusion [33]. AQGV, the alanine replacement variant of LQGV, was effective when administrated either two or 24 hours after LPS injection in mice [33]. In a hemorrhagic shock and fluid resuscitation model in rats LQGV, AQGV and LAGV attenuated the inflammatory response when administrated shortly after bleeding **(Chapter V)**. As both the LPS injection and the hemorrhagic shock/fluid resuscitation models represent a non-infectious SIRS, the beneficial effects on inflammation and survival cannot be simply translated to sepsis. Therefore, we investigated the anti-inflammatory effects of hCG-related oligopeptides in two different infection models: *L. monocytogenes* infection and CLP-induced sepsis in mice.

In case of an *L. monocytogenes* infection in mice, a high dose of LQGV, AQGV or VLPALP (50 mg/kg BW) inhibited innate immune activation, leading to bacterial overgrowth **(Chapter II)**. Mice that lack innate immune activators, such as TNF-α, IL-6, and IL-12, have an increased susceptibility to *L. monocytogenes* infection [34,35]. *L. monocytogenes* activates the extracellular receptor TLR-2, which results in the production of cytokines and chemokines [36]. LQGV treatment *in vivo* impaired the ability of splenocytes to produce TNF-α and IL-6 upon HKLM and LPS stimulation *in vitro*, but these responses were not completely blocked **(Chapter II)**. In addition, LQGV reduced the initial chemokine response *in vivo* **(Chapter II).** These data suggest that LQGV administration to mice results in decreased PRR activation by bacterial antigens. In line with this, PRR gene mutations or polymorphisms in humans, for instance for

TLR, are linked to altered PAMP recognition and innate immune activation that have been linked to an increased susceptibility to severe infections [37]. Thus, PRR are key components in initiating the inflammatory response by microbes, and LQGV likely inhibits this activation.

In case of CLP-induced sepsis, we employed a lower dose of 5 mg/kg BW of LQGV. This dose did not completely reduce the cytokine response, but rather showed a minor to minimal reduction of the systemic, pulmonary and peritoneal inflammatory response, as compared to *L. monocytogenes* infection **(Chapters II and VI).** As hCG-related oligopeptides were found to act in a dose dependent manner in experimental animal *in vivo* models, such as LPS infusion, renal ischemia reperfusion injury and transplantation of Lewis lung carcinoma [38,39], the differences observed between CLP- and *L. monocytogenes*-induced sepsis could be related to dose differences, as 50 mg/kg BW was used during *L. monocytogenes* infection while only 5 mg/kg BW was used during CLP.

In summary, LQGV can exert anti-inflammatory activity upon infection and tissue injury. The successful use of LQGV as an anti-inflammatory therapeutic in an infectious condition most likely depends on finding the optimal level of immunosuppression that is needed for treatment success, just like in dexamethasone-induced immunosuppression **(Chapter VII)**.

3. Mode of action of hCG-related oligopeptides

3.1 Adrenal gland activation

Addition of LQGV to cell cultures, for instance of splenocytes, did not reduce HKLM or LPS induced TNF-α and IL-6 production **(Chapter IV).** LQGV also did not reduce LPS and TNF-α induced E-selectin expression on endothelial cells in *in vitro* culture systems (unpublished data). These data suggest that LQGV by itself does not inhibit TLR-driven TNF-α, IL-6 and E-selectin production in these *in vitro* culture systems. However, the *in vivo* models clearly revealed that LQGV has anti-inflammatory activity, including suppression of TLR-driven TNF-α, IL-6 and E-selectin production.

Glucocorticosteroid secretion by the adrenal gland is under the control of the HPA-axis. Glucocorticosteroids are involved in maintaining homeostasis by regulating lipid and protein metabolism and in modulation of the cardiovascular system, the central nervous system and the immune system [40]. Synthetic glucocorticosteroids are widely and successfully applied for the treatment of pathological conditions associated with severe inflammation [40]. Almost all cells within the body express the cytoplasmic GR [40,41]. Upon ligand binding GR translocate into the nucleus, where they interact with glucocorticoid responsive elements in the promoter region of target genes and thereby regulate gene expression [40]. The activated GR can also regulate gene expression

through direct interaction with transcription factors, including AP-1 and NF-κB [42].

In this thesis we show that LQGV can inhibit innate immune activation in mice through activation of the GR **(Chapter IV)**. As LQGV did not inhibit pro-inflammatory mediator production *in vitro*, it is unlikely that LQGV by itself activated GR signaling. This suggests that the immunosuppressive and anti-inflammatory effects of LQGV are established through an *in vivo* released secondary mediator that stimulates GR activity. The experiments described in **Chapter IV** demonstrate that in mice high dose (50 mg/kg BW) LQGV-treatment can stimulate ACTH receptor driven adrenal gland activation, leading to a prolonged increase of corticosterone plasma levels. High endogenous and exogenous glucocorticosteroid levels impair the inflammatory response, which increases the susceptibility to *L. monocytogenes* [43-45]. An elegant example of the effects of increased endogenous corticosterone levels on an *L. monocytogenes* infection was presented in a recent study [46]. The authors found that activation of the HPA axis by influenza virus leads to a prolonged increase of corticosterone levels that enhance the susceptibility to *L. monocytogenes* infection [46]. In contrast, inflammatory responses that follow hemorrhagic shock/fluid resuscitation and LPS infusion were reduced by high endogenous and exogenous glucocorticosteroid levels, resulting in less organ damage [22,47]. Furthermore, blockage of GR signaling increases the vulnerability to LPS and hemorrhagic shock [48,49]. Therefore, it might be that the protective effect of LQGV on LPS and hemorrhagic shock also involves the stimulation of adrenal gland corticosterone production and subsequent GR activation. The role of the adrenal glands in the protective effect of LQGV *in vivo* is supported by the observation that LQGV had no protective effect against LPS-induced mortality in mice that underwent ADX (Figure 1). Also plasma from LQGV-treated ADX mice did not inhibit HKLM induced cytokine production by splenocytes from naive mice, while plasma from LQGV-treated control mice did inhibit this response **(Chapter IV)**.

Adrenal glucocorticoid secretion is linked to pituitary ACTH release [40]. However, nature tends to incorporate some redundancy into vitally important organ systems and ACTH-independent pathways for adrenal gland activation have been demonstrated as well [50]. This was illustrated for the first time in corticotropin-releasing hormone (CRH)-knockout mice that do not secrete ACTH, but still produced high levels of corticosterone [51]. Also, the pentapeptide VPDPR that is derived from endogenous cleavage of pro-colipase, exerts its anti-analgesic effect through adrenal gland activation and subsequent GR signaling in neurons [52].

Previously, it was shown that LQGV did not protect against renal ischemia reperfusion injury, while LQGV did reduce inflammation and organ damage during hemorrhagic shock and fluid resuscitation [39]. Although GR levels are equally expressed in liver and kidney the corticosterone binding capacity for GR is higher in the liver than in the kidneys under both normal and stress conditions [53,54]. This might explain why LQGV

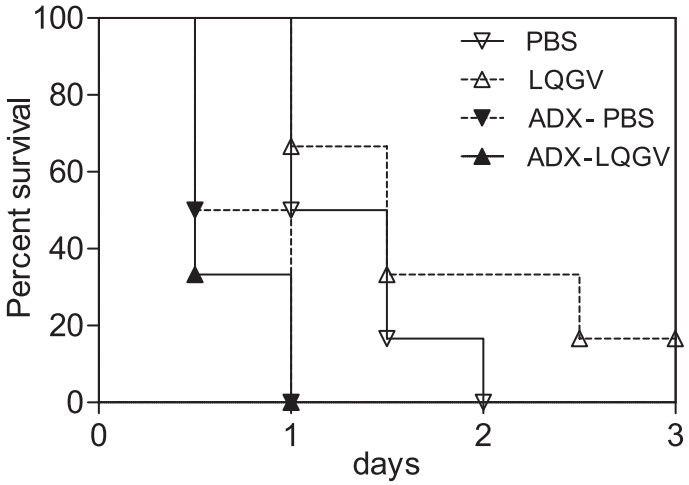

Figure 1. ADX abolishes the LQGV pro-survival effect after LPS infusion.
Non-ADX and ADX C57BL/6 mice were treated with either 200 µl LQGV (50 mg/kg BW) or PBS. The next day, the mice were challenged with an LPS injection. Survival was scored every 12 hours. Data depicted are from 5 mice per group.

was effective during hemorrhagic shock/fluid resuscitation, but not in renal ischemia reperfusion injury.

In conclusion, adrenal gland activation is a natural mechanism that keeps the pro-inflammatory response at bay. High dose (50 mg/kg BW) LQGV-treatment of mice can stimulate adrenal gland activation and account for a prolonged increase of corticosterone plasma levels with subsequent GR activation. May be next to this effect, LQGV can exert its anti-inflammatory effect also along other cellular and molecular processes. This would require further studies.

3.2 Neutrophil accumulation

Neutrophil extravasation is an early event of the inflammatory response [55]. The extravasation process requires a multistep cascade of adhesive and migratory events that are mediated by three classes of adhesion molecules, the selectins, integrins and adhesion receptors of the immunoglobulin superfamily, as well as chemokines. The extravasation process of neutrophils can be divided into five sequential stages: (a) selectin-mediated sticking to the endothelium; (b) integrin-mediated crawling over the endothelium; (c) firm arrest to the endothelium; (d) chemokine-induced activation; and finally (e) transendothelial migration [5,56]. Subsequently, through a chemokine gradient, neutrophils migrate to the inflamed loci, where they release their contents

that affect local cell and tissue function [56-58]. Depending on the intensity of the inflammatory response, the tissue can be either completely shut down or attempts are made to restore homeostasis, during which some pathological changes can occur [59].

Neutrophil accumulation in tissues is an important contributor to tissue damage and tissue dysfunction that follows hemorrhagic shock and fluid resuscitation [60,61]. LQGV reduced hepatic neutrophil accumulation following hemorrhagic shock and fluid resuscitation, while AQGV and LAGV did not **(Chapter V).** Also following an *L. monocytogenes* infection LQGV reduced neutrophil accumulation. The reduced neutrophil accumulation in hemorrhagic shock was found to be associated with reduced mRNA expression of the endothelial specific adhesion molecule E-selectin **(Chapter V)**, suggesting that *in vivo* LQGV can influence the E-selectin gene expression. E-selectin blockage has been found to protect against ischemia/fluid resuscitation and endotoxin-induced damage [62,63]. Glucocorticosteroids can prevent the migration of immune cells from the circulation to an inflammatory site by reducing E-selectin and chemokine production upon injury [64-66]. LQGV treatment *in vivo* also reduced the production of the neutrophil attracting chemokines CXCL1/KC and CCL2/MCP1 upon an *L. monocytogenes* infection **(Chapter II).** Although LQGV did reduce CCL2/MCP-1 plasma levels during an *L. monocytogenes* infection, no effect on monocyte recruitment/accumulation into *L. monocytogenes* infected organs was observed. The latter is in line with the observation that LQGV also did not reduce monocyte recruitment into the lungs and liver following hemorrhagic shock and fluid resuscitation (Figure 2). This may indicate that LQGV more or less specifically interferes with neutrophil recruitment. The mechanism behind

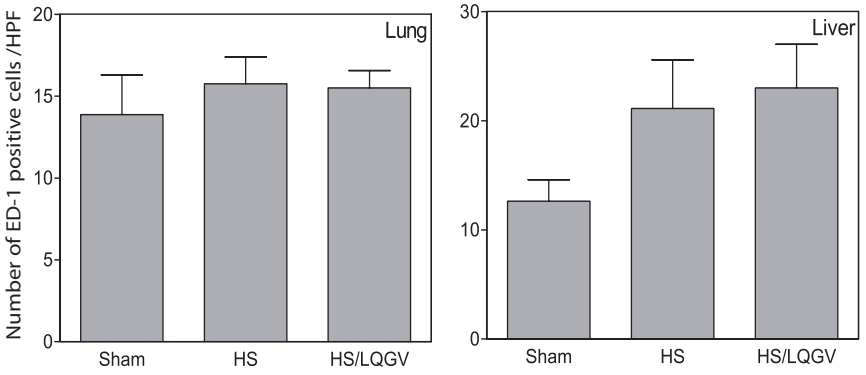

Figure 2. Monocyte infiltration into lung and liver.
Number of ED1 positive cells (monocytes) per high power field (HPF) in the lungs and liver at 3 hours after the onset of hemorrhagic shock. Data are presented as the mean ± S.E.M. of eight rats per group.

this observation is unclear so far, but the possibility should be considered that specific induction of neutrophil apoptosis contributes to this observation **(Chapter III)**. This would be in line with the observation that neutrophil depletion protects against LPS-induced shock and hemorrhagic shock followed by fluid resuscitation, while neutrophil depletion enhances *L. monocytogenes* susceptibility [67,68].

3.3 ROS activity and neutrophil apoptosis

Free radicals can be defined as molecules containing one or more unpaired electron(s) in atomic or molecular orbitals [69-71]. This unpaired electron usually gives a considerable degree of reactivity to the free radical. Molecular oxygen (dioxygen) has a unique electron configuration and is itself a radical [69-71]. The addition of one electron to dioxygen forms the superoxide anion radical (O_2^-). Superoxide anion, arising either through metabolic processes or following oxygen activation by physical irradiation, is considered the primary reactive oxygen species (ROS) and can further interact with other molecules to generate secondary ROS [72]. The production of superoxide anion occurs mostly within the mitochondria of a cell. The mitochondrial electron transport chain is the main source of adenosine triphosphate (ATP) in the cell and thus essential for life [72,73].

ROS generation is relatively low during an ischemic event, however following fluid reperfusion a massive burst of ROS occurs that can induce tissue damage [74]. This burst is derived from several cell types but predominantly from neutrophils [58,75]. ROS activity can be neutralized by superoxide dismutase (SOD), which has been shown to reduce tissue damage and inflammation after hemorrhagic shock and fluid resuscitation [76,77]. Here we show hat LQGV as well as other hCG-related oligopeptides reduced superoxide anion activity (Figure 3) and that LQGV stimulated neutrophil apoptosis **(Chapter III)**. Remarkably, SOD can also induce neutrophil apoptosis when excessively present [77]. Preliminary data using FITC-labeled LQGV revealed an uptake by phagocytic cells, mainly neutrophils and some monocytes, while lymphocytes remained FITC negative (unpublished data). This suggests that the uptake of LQGV-FITC by phagocytes is an active process, rather than the result of passive diffusion. Possibly, intracellular LQGV reduces O_2^- activity thereby stimulating neutrophil apoptosis.

In conclusion, these studies show that adrenal glands can be involved in the LQGV-induced immunosuppression. The neutralization of ROS by hCG-related oligopeptides and the transient apoptosis-induced neutrophil depletion may have contributed to the immunosuppressive effect observed in the tested *in vivo* models. These observations are visualized in a model that is depicted in Figure 4.

4. hCG-derived oligopeptides; implications for the biology of pregnancy?

Pregnancy remains to some extent an immunological enigma as the fetus inherits

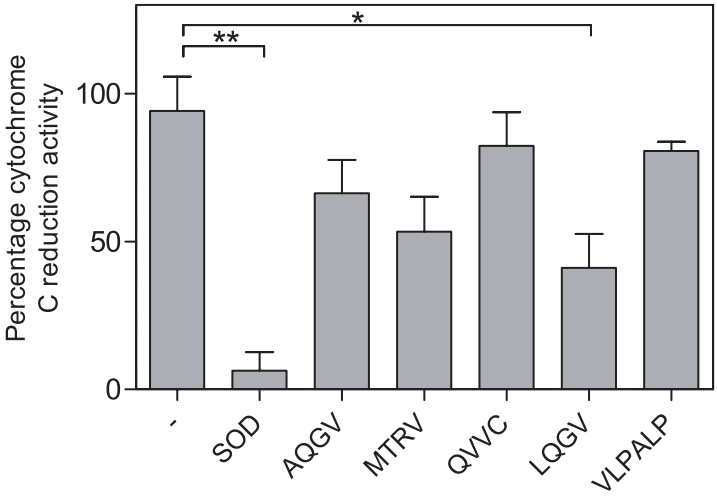

Figure 3. Cytochrome C reduction activity.
Adding hypoxanthine to a pH neutral cytochrome C, xanthine oxidase, and oligopeptide solution (1000 μg/ml) initiated the reduction of cytochrome C reaction. At the end of each experiment the level of cytochrome C reduction was calculated to percentage. Data are presented as the mean ± S.E.M. of three separate experiments, in which each of the tested oligopeptides was measured in sixfold. * $p < 0.05$; ** $p < 0.01$

paternal antigens and yet coexists within the mother's uterus in harmony throughout pregnancy. Characteristic for human pregnancy is the production of hCG, which is a member of the glycoprotein hormone family to which also luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) belong [78]. Hormones from this family consist of similar heterodimeric structures sharing an identical α-subunit, but each of these hormones have a unique β-subunit that confers the hormonal biological specificity [79]. It has been demonstrated that hCG preparations, besides their endocrine functions, also influence the functionality of the immune system [80-83].

Besides the intact hCG hormone several other isoforms of hCG exist, especially during the second and third trimester of pregnancy [84]. In this phase of pregnancy loop 2 of β-hCG is extensively nicked by leukocyte elastase-like proteases, which generates hCG β-core and several nicked β-hCG forms [84]. These molecules can be detected in serum and urine in the second and third pregnancy trimester. Besides the generation of hCG β-core and nicked β-hCG, also small breakdown products of three to seven amino acids long, that originate from loop 2 of β-hCG, are likely generated [85]. It has been demonstrated that such small peptides can exert anti-inflammatory effects [13,38,83,85 and this thesis]. The hCG-related oligopeptides used in our studies have not been detected in pregnancy urine yet, but the relative amount of hCG β-core and

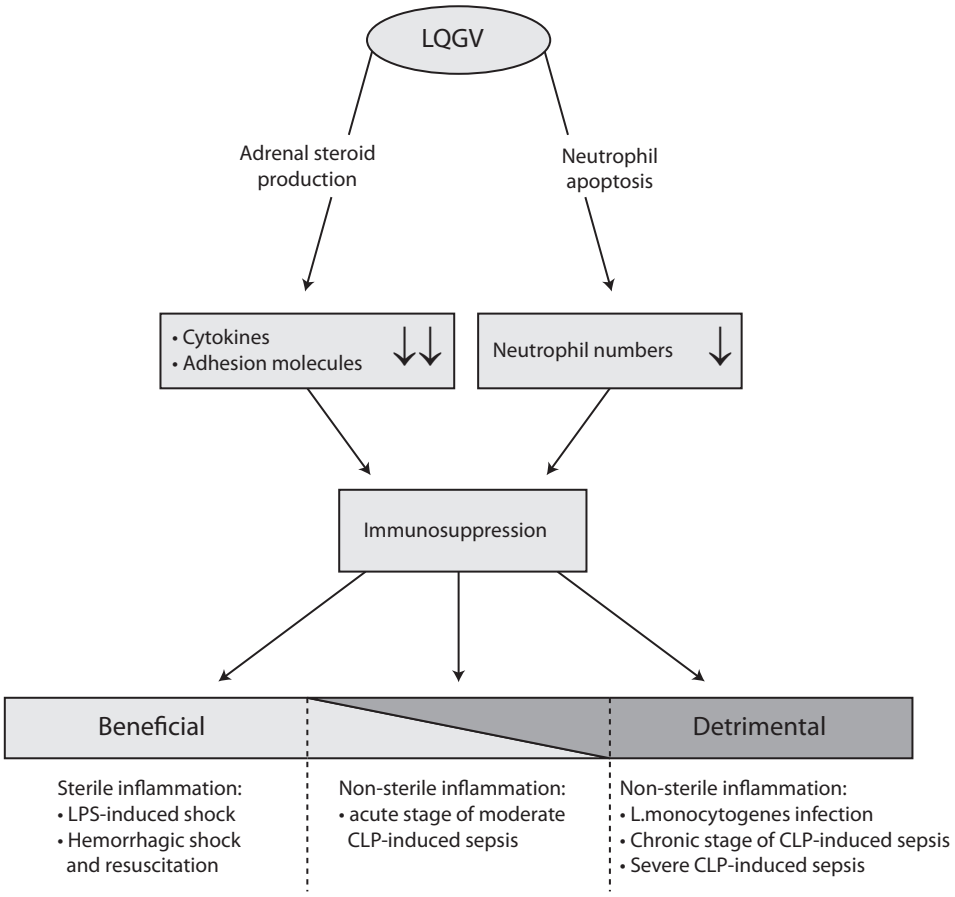

Figure 4. Proposed mode of action of LQGV.
LQGV activates the adrenal gland to secrete glucocorticosteroids. Simultaneously LQGV induces a transient neutropenia. These two processes impaired the inflammatory response, which in a sterile condition prevents morbidity and mortality, while in case of infection this can increase the susceptibility to the pathogen.

nicked β-hCG detected provide an estimate of the expected release of hCG-derived oligopeptides.

During the second and third pregnancy trimester the HPA axis is activated, leading to enhanced glucocorticoid production [86]. Elevated glucocorticosteroid levels influence the maternal immune system as to facilitate fetal allograft maintaince [87]. Placental and hypothalamic derived CRH activate the anterior pituitary to produce ACTH,

which stimulates the adrenal cortex to produce glucocorticoids [40]. Adrenal steroid production may also be stimulated in a CRH/ACTH independent manner, however, the mechanisms involved herein are poorly understood so far [88]. Although, positive correlations between hCG and cortisol (the human analogue of rodent corticosterone) have been described [89,90], hCG administration to women does not result in cortisol production [91]. Because LQGV likely occurs in high amounts during the second and third pregnancy trimester (the moment at which cortisol levels rise as well [86]) and because of the observation that LQGV in mice stimulates corticosterone production, it can be hypothesized that LQGV and similar hCG-derived oligopeptides contribute to the pregnancy-associated adrenal glucocorticoid production.

During pregnancy, neutrophil numbers in the peripheral blood steadily rise and may even lead to a neutrophilia [92]. Excessive increase in neutrophil numbers occurs during pre-eclampsia [92]. Besides the increase in numbers, neutrophils also increase their CD11b, CD14 and CD64 expression and intracellular ROS levels [93]. This activated phenotype is almost identical to the one seen in septic patients [93]. However, neutrophils at the maternal-fetal interface do not gain an activated phenotype [94]. This suggests that neutrophil activity is negatively regulated at this interface. The mechanism that prevents neutrophils at the maternal-fetal interface from gaining an activated phenotype is poorly understood, but a role for syncytiotrophoblasts herein has been suggested [95]. It could well be that hCG-derived oligopeptides, like LQGV, play a central role in this phenomenon.

To summarize, hCG-derived oligopeptides, in particular LQGV, may well be involved in the control of the maternal immune system to facilitate pregnancy success. This pregnancy-associated immune regulation possibly involves the stimulation of adrenal gland activation and inhibition of neutrophil activity at the maternal-fetal interface.

5. Future research directions

The studies described in this thesis demonstrate that the tested hCG-related oligopeptides exert anti-inflammatory activity in experimental animal models that are associated with SIRS. Although the picture of the mechanisms of action is largely incomplete for most hCG-related oligopeptides, some conclusions can be drawn, for instance about the dose response effect, the role of the adrenal glands and increased corticosterone levels, and the transient neutrophil depletion. Still these studies should be further expanded to provide a more detailed insight into the mechanisms of action.

Approximately 40% of patients with hemorrhagic shock develop sepsis as a result of increased gut permeability and development of immune paralysis [16,96]. Therefore, it would be of clinical interest to evaluate the effects of hCG-related oligopeptides, in particular LQGV, in a double hit model of hemorrhagic shock/fluid resuscitation and subsequent CLP-induced septic shock. It is well known that the inflammatory response

that results from trauma and sepsis differs considerably between individuals. Therefore, the incorporation of genetic variability, as additional parameter, may be of clinical importance. This means that the use of outbred mouse models for future CLP-studies is of interest.

The studies in this thesis showed that LQGV (5 mg/kg BW) or dexamethasone (0.05 mg/kg BW) administration before or around the CLP procedure reduced mortality. These administration schemes can be considered preventive rather than therapeutic. Thus studies should be performed to determine the therapeutic time-window of LQGV and low dose dexamethasone administration, as later anti-inflammatory treatment is of more interest for sepsis patients.

LQGV activates the ACTH receptor, however, the nature of the molecular interaction between LQGV and the ACTH-receptor is still unknown. Experiments with mutant cell lines that lack specific domains of the ACTH receptor can give more insight into the LQGV-ACTH receptor interaction.

From an immunological point of view the existence of various oligopeptides in cells and tissues makes sense, considering that discriminating between self and non-self relies on MHC class I and II peptide presentation. It has been demonstrated that MHC class I presented peptides represent a comprehensive and balanced summary of the endogenous proteomic content of the cell [97]. Although peptides presented by MHC class I are typically 8-10 amino acids in size [98] it is likely that smaller peptides with regulatory activity are formed as well during the proteolytic processing. Therefore, screening peptide libraries for small oligopeptides with biological activities and originating from other proteins than hCG may be of interest. Many peptide components have been identified in tissues/organs and body fluids from different species, as well as in blood cells and tissue cultures [99]. The concept has emerged that tissue specific proteins are endogenously degraded by peptidases and thereby generate a large group of oligopeptides, ranging from 2 to 35 amino acids long. These peptides represent the tissue specific peptide pool defined as peptidome. This peptidome might present a new class of bioregulators that regulate important processes in life (inflammation and regeneration) [99]. Under pathological conditions, the tissue specific peptide pool will undergo dynamic changes that probably are involved in restoring tissue homeostasis. Also some of these oligopeptides provide a powerful tool for diagnostic purposes [99]. More research will possibly lead to the identification of many novel (endogenous) regulatory peptides that may originate from a wide variety of proteins.

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SUMMARY / SAMENVATTING

SUMMARY

Inflammation is the body's way of informing itself of changes in homeostasis. Depending on the triggering event and anatomical location, the inflammatory response has different physiological purposes and pathological consequences. The production and secretion of inflammatory mediators can alter tissue functionality such that the tissue adapts to the harmful insult and restores tissue homeostasis with some pathological changes.

The systemic inflammatory response syndrome (SIRS) is an excessive inflammatory response inflicted by a variety of clinical insults, such as severe hemorrhage, sepsis, and pancreatitis. Uncontrolled production of pro-inflammatory mediators can lead to multiple organ dysfunction syndrome (MODS) and death. Therefore, controlling the inflammatory response might limit tissue damage and death. However, despite improvement in our knowledge of the pathology of SIRS and the availability of novel drugs it appears to be difficult for controlling the inflammatory response during a severe infection.

The research described in this thesis was initiated to increase the knowledge on the effects and the mechanistic aspects of hCG-related oligopeptides in experimental animal models that are associated with SIRS. In this context also different doses of dexamethasone were investigated.

To explore the effects of hCG-related oligopeptides during infectious SIRS, we used two different models: (a) *L. monocytogenes* infection model in mice (**Chapter II)** and (b) CLP-induced sepsis model in mice **(Chapter VI)**. These studies show that the hCG-related oligopeptides LQGV, VLPALP and AQGV blunt innate immune activation. In case of *L. monocytogenes* infection this impairment increases the susceptibility of mice to the invading pathogen. In a CLP-induced sepsis model this impairment did not increase the susceptibility of mice, but rather decreased mortality. Furthermore, in combination with the standard sepsis care with antibiotics and fluid resuscitation, LQGV enhanced survival compared with antibiotics and fluid resuscitation treatment or LQGV treatment alone. As hCG-related oligopeptides were found to act in a dose dependent manner in experimental *in vivo* models, such as LPS infusion, renal ischemia reperfusion injury and transplantation of Lewis lung carcinoma, the differences observed between CLP- and *L. monocytogenes*-induced sepsis could be related to dose differences, as 50 mg/kg BW was used during *L. monocytogenes* infection while only 5 mg/kg BW was used during CLP.

To examine the effects of LQGV, AQGV and LAGV during a non-infectious SIRS, we used a hypovolemic shock and resuscitation model in rats **(Chapter V)**. This study also shows that the tested oligopeptides exert anti-inflammatory activity, as they attenuate the systemic and hepatic inflammatory responses that typically follow hemorrhagic shock and fluid resuscitation.

Mechanistic studies on the mode of action of high dose (50 mg/kg BW) LQGV-treatment revealed that this oligopeptide dose dependently stimulated ACTH receptor driven adrenal gland activation, leading to a prolonged increased plasma level of the anti-inflammatory hormone corticosterone, with subsequent GR activation **(Chapter IV)**. High endogenous and exogenous glucocorticosteroid levels impair the inflammatory response and increase the susceptibility to *L. monocytogenes*. However, glucocorticosteroids reduce the inflammatory response pathology that follows hemorrhagic shock/fluid resuscitation and LPS infusion. This explains the effects observed in **Chapters II and IV**. Also it was found that LQGV reduced O_2^- activity and stimulated neutrophil apoptosis **(Chapter III)**. The latter may also have contributed to the reduced neutrophil accumulation observed in the different SIRS models. We do not exclude that LQGV and other hCG-related oligopeptides also have other effects that contribute to their anti-inflammatory effects.

During the last decades the treatment with synthetic glucocorticoids, such as dexamethasone, has been investigated in severe sepsis. However, contradictory results have been obtained. Some studies demonstrated survival improvement after high-dose corticosteroid treatment while others did not. Therefore, to investigate the effects of different dosages of dexamethasone during an infectious SIRS, we investigated the effects of high, median, and low dose dexamethasone treatment on mortality and inflammation in a murine CLP model in mice **(Chapter VII)**. In this study it was demonstrated that an extreme low dose dexamethasone 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. In this thesis 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 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 range will not result in survival improvement

In conclusion, the studies described in this thesis show that (a) hCG-related oligopeptides can exert anti-inflammatory activity; (b) high dose LQGV-treatment can exert its anti-inflammatory effect by stimulating adrenal gland activation, leading to increased and prolonged corticosterone production and subsequent GR signaling; (c) hCG-related oligopeptides reduce O_2^- activity and decrease blood neutrophil numbers, which may also contribute to the anti-inflammatory activity of the tested oligopeptides; (d) in a septic setting the survival improvement by dexamethasone and probably other anti-inflammatory therapeutics crucially depends on the level of immunosuppression that still allows appropriate activation of host defense mechanisms against the invading pathogen(s) but reduces SIRS-induced pathology.

SAMENVATTING

Een ontsteking is een natuurlijke reaktie op een verandering in weefsel homeostase. Deze ontstekingsreactie is afhankelijk van de ontstekingsinitiator en de locatie in het weefsel. Een ontstekingsreactie kan verschillende pathologische gevolgen hebben.

Een te krachtige locale ontstekingsreactie kan leiden tot een systemische ontstekingsreactie die vervolgens andere organen kan aantasten. In sommige gevallen kan de ongecontroleerde ontstekingsreactie organen uitschakelen; dit proces wordt orgaanfalen genoemd. In ziekenhuizen is orgaanfalen een groot medisch probleem, en verantwoordelijk voor een groot aantal sterfgevallen op de afdeling 'Intensive Care'. Door de heftige ontstekingsreactie onder controle te brengen door behandeling met medicijnen, zou het aantal sterfgevallen mogelijk kunnen worden verminderd. In de laatste jaren is de medische kennis toegenomen met betrekking tot deze systemische ontstekingsreacties, maar men krijgt nog steeds geen grip op de behandeling van deze problematiek. In dit proefschrift is een aantal experimentele therapieën beschreven die mogelijk de systemische ontstekingsreactie onder controle kunnen brengen. Voor dit onderzoek zijn diermodellen gebruikt, zoals een hemorragische shock model in ratten en twee bacteriële infectiemodellen in muizen. De potentieel therapeutische stoffen die door ons zijn getest, zijn synthetische steroiden en oligopeptiden. De aminozuursequentie van deze oligopeptiden zijn gerelateerd aan het zwangerschapshormoon humaan choriogonadotropine (hCG).

De hCG-gerelateerde oligopeptiden zijn getest in twee bacteriële infectiemodellen, namelijk een *Listeria monocytogenes* model **(Hoofdstuk II)** en een muizenmodel voor blindedarm ontsteking (CLP model) **(Hoodfstuk VI)**. In deze studies is aangetoond dat de geteste hCG-gerelateerde oligopeptiden een remmende werking hebben op de ontstekingsreactie. Bij behandeling met een hoge dosis oligopeptide worden *L. monocytogenes* geïnfecteerde muizen gevoeliger voor de bacterie, terwijl bij een veel lagere dosis oligopeptide de CLP-geinduceerde sepsis muizen een overlevingsvoordeel hebben. Tevens hebben we een combinatietherapie bestudeerd van het hCG-gerelateerde oligopeptide LQGV met antibiotica behandeling. Dit liet zien dat deze combinatietherapie een nog gunstiger effect heeft op de overleving van de muizen dan antibiotica en LQGV afzonderlijk.

De hCG-gerelateerde oligopeptiden LQGV, AQGV en LAGV zijn ook getest in steriele ontstekingsmodellen, zoals een hemorragische shock in ratten **(hoofdstuk V)**. In dit model bleken lage doses (5 mg/kg BW) van de geteste oligopeptiden in staat de systemische ontstekingsreactie te remmen. Deze remming heeft mogelijk tevens een gunstig effect op de overleving van de ratten, maar dit is niet door ons getest.

Ons onderzoek naar het werkingsmechanisme van LQGV laat zien dat LQGV de bijnieren kan activeren via de adrenocorticotroop hormoon (ACTH) receptor.

Deze bijnier activiteit leidt vervolgens tot verhoogde corticosterone waarden in het bloed waardoor geactiveerde immuuncellen minder ontstekingsfactoren kunnen produceren **(Hoofdstuk IV)**. Deze observatie is belangrijk omdat in het verleden is aangetoond dat door een verhoogde bijnieractiviteit de gevoeligheid voor een *L. monocytogenes* infectie toeneemt in muizen, maar ook bij vrouwen tijdens de zwangerschap. Tevens is aangetoond dat het hCG-gerelateerde oligopeptide LQGV de activiteit van zuurstofradicalen vermindert en celdood van neutrofielen induceert. Vermoedelijk gaan de neutrofielen dood omdat de vitaliteit van deze cellen afhankelijk is van de activiteit van zuurstofradicalen **(Hoofdstuk III)**. De celdood van neutrofielen kan verklaren waarom er minder van deze cellen werden gevonden in de organen tijdens een systemische ontstekingsreactie. Mogelijk zijn er ook nog andere processen die worden beïnvloed door deze oligopeptiden, maar dit moet in de toekomst verder worden onderzocht.

Tot op heden lijkt de overleving van septische patiënten niet te verbeteren door behandeling met anti-inflammatoire therapieën. Echter, op basis van eerdere studies, waarin gebruik is gemaakt van IL-6 knock-out muizen en IL-6 neutraliserende antistoffen veronderstellen wij dat een matige remming van de systemische ontstekingsreactie, zoals deze optreedt in muizen na CLP geïnduceerde sepsis, resulteert in een verbeterde overleving, terwijl sterke downregulatie van de ontstekingsreactie geen gunstig effect heeft. In **hoofdstuk VII** wordt geïllustreerd dat slechts een matige remming van de met sepsis geassocieerde ontstekingsreactie door corticosteroïden resulteert in een verbeterende overleving van CLP-muizen. Dit impliceert dat er een range is waarbinnen de remming van de ontstekingsreactie een gunstig effect heeft. Voorwaarde is evenwel dat de capaciteit van het immuunsysteem om de sepsisgerelateerde micro-organismen te bestrijden, gewaarborgd blijft. Deze balans van voldoende remming van de ontstekingsreactie met behoud van voldoende antimicrobiële afweer is mogelijk van cruciaal belang voor het slagen van anti-inflammatoire therapie bij patiënten met sepsis en septische shock.

ABBREVIATIONS

AB	antibiotics
ACTH	adrenocorticotropin hormone
ADX	adrenalectomy
ALT	aminotransferase
APC	antigen presenting cells
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BW	body weight
cAMP	cyclic adenosin monophoshate
CARS	compensatory anti-inflammatory response syndrome
CBG	corticosteroid-binding globulin
C/EBP-δ	CCAAT/enhancer-binding protein-δ
CFU	colony forming units
CLP	cecal ligation and puncture
CpG	cystine purine repeat
CRH	corticotropin-releasing hormone
CRP	C-reactive protein
CTL	cytotoxic T lymphocytes
DAMP	danger-associated molecular patterns
DC	dendritic cells
DEX	dexamethasone
ELISA	enzyme-linked immunosorbent assay
FDC	follicular dendritic cells
FSH	follicle stimulating hormone
GR	glucocorticoid receptor
HBSS	Hanks' balanced salt solution
hCG	human chorionic gonadotropin
HCMV	human cytomegalovirus
H/DEX	high-dose DEX (2.5 mg/kg BW dexamethasone)
HKLM	heat killed listeria monocytogenes
HPA	hypothalamic pituitary adrenal
HPF	high power field
HRP	Horse Radish Peroxidase
HS	hemorrhagic shock
HSP	heat shock protein
ICAM-1	intracellular adhesion molecule-1
ICE	IL-1β-converting enzyme

ICU	intensive care unit
IFN	interferon
IL	interleukin
IP	intraperitoneal
IPAF	IL-1β-converting enzyme (ICE)-protease activating factor
IRF	interferon-regulatory factor
IV	intravenous
LBP	LPS-binding protein
LD	lethal dose
L/DEX	low-dose DEX (0.05 mg/kg BW dexamethasone)
LDH	lactate dehydrogenase
LH	luteinizing hormone
LLO	listeriolysin O
LPS	lipopolysaccharide
MAL	MyD88 adapter-like
MAP	mean arterial pressure
MDA5	melanoma differentiation-associated gene 5
M/DEX	medium-dose DEX (0.25 mg/kg BW dexamethasone)
MHC	major histocompatibility complex
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
MyD88	myeloid differentiation factor 88
NAIP	neuronal apoptosis inhibitor factors
NALP	NLR-domain leucine rich repeat and pyrin domain-like receptors
NF	nuclear factor
NK	natural killer
NLR	(NOD)-domain-like receptor
NOD	nonobese diabetic
NOD	nucleotide oligomerization-binding
ON	overnight
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PCT	procalcitonin
PG	peptidoglycan
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptors
RIG-1	retinoic acid inducible gene protein 1
RLR	RNA helicase family

RNI	radical nitrogen intermediates
ROI	radical oxygen intermediates
ROS	reactive oxygen species
RQ-PCR	real-time quantitative polymerase chain reaction
RUNX	runt-related transcription factor
SD	standard deviation
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
SR	soluble receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
TNF	tumor necrosis factor
TRIF	TIR domain-containing adapter-inducing IFN-β
TSH	thyroid stimulating hormone

DANKWOORD

En dan is het zover, het boekje is af! Gelukkig heb ik al het werk niet alleen hoeven doen; daarom hartelijk dank aan iedereen die een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift. Ik wil graag een aantal mensen persoonlijk bedanken.

Als eerste wil ik natuurlijk Wim bedanken voor de directe begeleiding en ondersteuning tijdens mijn promotieonderzoek. Beste Wim, het is alweer een tijdje geleden dat jij werkgroepleider van de IRD groep werd, met als extra taak de “tijdelijke” begeleiding van het peptidenonderzoek. Ik wilde echter graag dat jij langer bij het peptidenonderzoek betrokken zou blijven. Om die reden heb ik je gelijk in jouw eerste week als werkgroepleider gevraagd of jij mijn copromotor wilde worden. Als ik nu door dit boekje blader, zie ik dat dit zijn vruchten heeft afgeworpen. Tijdens mijn promotieonderzoek heb jij mij ALTIJD met raad en daad ter zijde gestaan. Jouw gedachten en gedrevenheid hebben een belangrijke bijdrage geleverd aan mijn wetenschappelijke ontwikkeling.

Beste professor Benner, beste Rob, jij was als mijn promotor en één van de initiators van het peptidenonderzoek op de afdeling enorm betrokken bij dit onderzoek. Na mijn eerste sollicitatiegesprek zag jij het zitten om met mij in zee te gaan. Ik wil je graag bedanken voor je begeleiding en voor de nuttige discussies die wij over het onderzoek hebben gehad.

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De fibroblast is een belangrijke cel voor de inductie en controle van een immuunrespons, althans dat hoor ik de laatste twee jaar van Leendert. Beste Leendert, succes met het afronden van je promotieonderzoek en met je verdere opleiding tot chirurg (of toch oogarts ☺).

Beste Kim, halverwege jouw promotieonderzoek kwam jij in onze werkgroep terecht en dat is succesvol geweest. Op dit moment ben jij druk bezig met het afronden van jouw onderzoek. Ik wens je veel succes daarmee. Ik heb enorm genoten van de TREC verhalen, al kan ik ze niet meer navertellen.

Jan Willem, mijn medepromovendus in het sepsis- en ontstekingsonderzoek. Samen op congres gaan was altijd een feest, ik bedoel een wetenschappelijke ervaring.

In Keulen moesten wij voor het eerst ons onderzoek presenteren tijdens een internationaal congres; wat waren wij zenuwachtig! Achteraf was dit erg leerzaam en leuk. Onze overige congresbezoeken zal ik ook nooit vergeten en misschien komen er tijdens mijn feestje wel enkele foto's voorbij die wij niet (of toch wel) snel zullen vergeten. Ook jij staat straks naast mij om mij te helpen bij de verdediging van dit boekje.

Beste Pieter, de schakel tussen Rotterdam en Oklahoma. Zonder jou was de Listeria studie niet mogelijk geweest.

Tessa, Gemma, Petra, Tim, Ivar, Rogier, Ron en Nisar, jullie ook bedankt voor de plezierige samenwerking!

Iedereen op de afdeling Immunologie wil ik bedanken voor de gezellige labuitjes en feestjes op de afdeling. Tijdens mijn promotieonderzoek heb ik enorm veel geleerd van een ieder van jullie. Geertje, Wendy, Erna, Marcia en Daniëlle, bedankt voor de ondersteuning en voor het verzorgen van de lay-out van mijn proefschrift.

Tijdens mijn promotieonderzoek heb ik de mogelijkheid gekregen om meerdere werkbezoeken af te leggen aan het lab van prof.dr. D.A. Drevets aan de Oklahoma University in Oklahoma City. Dear Doug, thank you for the visits to your lab. These visits to Oklahoma were important for my personal development as a scientist. With pleasure, I think back on the time that was spend in the O'Donoghue Research Building (ORB). The experiments were enormous, but in the end we have an excellent publication in the Journal of Infectious Diseases. Marylin, thank you for your assistance with the infections; without your help I would probably have spent more time at the ORB and hardly have any time left to visit the Oklahoma attractions. You made this possible, thank you. Dear Jennifer, although your name is not on the listeria paper, you still had an important contribution to this work. Your assistance with preparing the lab at the ORB helped a lot and I really appreciate it. Prof.dr. Brackett, dear Dan, thank you for offering lab space to us to perform all the experiments. We enjoyed our time in the ORB, especially the thanksgiving lunches; thanks everybody!

Na werktijd blijft het onderzoek door je hoofd spoken. Ik kan me voorstellen dat dit tot frustraties heeft geleid bij mijn familie. Lieve memke en Klaas, ik wil jullie bedanken voor de steun die jullie mij hebben gegeven tijdens mijn promotieonderzoek.

De laatste alinea is voor jou Yolanda. Lieve Yolanda, promoveren is al bijzonder, maar samen vijf jaar lang een promotietraject doorgaan is héél bijzonder. Wij hebben elkaar gesteund en door moeilijke tijden geholpen. Ook zijn wij samen naar Oklahoma geweest en hebben wij daar onderzoek gedaan. De experimenten in het ORB en dan ook nog de nachtelijke testen van het tornadowaarschuwingssysteem in het gebouw maakte ons leven toch nog een beetje avontuurlijk. Ik houd van jou.

Marlen

CURRICULUM VITAE

Marten van der Zee was born on July 4th, 1979 in Leeuwarden where his parents (Anne en Baukje) raised him together with his brother (Klaas). He attended secondary school at the Delta and Friesland College in Leeuwarden, and proceeded to study zoology at the Higher laboratory education (HLO) in Utrecht. During this study he conducted research on the characterization of constitutively active Bruton's tyrosine kinase on B-cell development in mice (Dr. R.W. Hendriks, Department of Immunology, Erasmus MC, Rotterdam). In 2003 he obtained his bachelor of Applied Sciences degree and started to study Biology at the Free University in Amsterdam. During this study he investigated the role of the extracellular matrix molecule ER-RT7 in secondary lymphoid organs (Prof.Dr. R.E. Mebius, Department of Molecular Cell Biology and Immunology, Free University, Amsterdam) and the role of the adaptor molecule TIR domain-containing adapter-inducing IFN-β (TRIF) in endothelial cells upon TLR signaling (Dr. C. van 't Veer, Department of Experimental Medicine, AMC). In 2005 he obtained his Master of Science Degree. In the same year he started his PhD project at the Department of Immunology at the Erasmus MC, under the supervision of Prof.Dr. R. Benner and Dr. W.A. Dik. In June 2010 he started his postdoc project entitled "*Cross-talk of sex hormones and Wnt/β-catenin signaling in endometrial cancer*" at the Department of Obstetrics and Gynecology at the Erasmus MC (Dr. L. Blok and Prof Dr. R Fodde).

LIST OF PUBLICATIONS

Wang, Y., **M. van der Zee**, R. Fodde and L. Blok. Endometrial cancer: unbalanced sexhormone signaling and wnt/β-catenin pathway activation. Oncotarget (to be submitted).

van den Berg, J.W., M. van der Zee, C. van Holten-Neelen, J.N.M. IJzermans, R. Benner, R.W.F. de Bruin and W.A. Dik. Mild versus strong anti-inflammatory therapy during early sepsis; a matter of life and death (resubmission).

van der Zee, M., **J.W. van den Berg**, C. van Holten-Neelen and W.A. Dik (2010). The beta hCG-related oligopeptide LQGV exerts anti-inflammatory effects through activation of the adrenal gland and glucocorticoid receptor in C57BL/6 mice. J Immunol (in press).

van den Berg, J.W., W.A. Dik, **M. van der Zee**, F. Bonthuis, C. van Holten-Neelen, G.M. Dingjan, R. Benner, J.N.M. IJzermans, N.A. Khan, R.W. de Bruin (2010). The beta-hCG-related oligopeptide LQGV reduces mortality and inflammation in a murine polymicrobial sepsis model. Crit Care Med (in press).

Kersseboom, R., L. Kil, R. Flierman, **M. van der Zee**, G.M. Dingjan, S. Middendorp, A. Maas and R.W. Hendriks (2010). Constitutive activation of Bruton's tyrosine kinase induces the formation of autoreactive IgM plasma cells. Eur J Immunol 40: 2643-2654.

van der Zee, M., W.A. Dik, Y.S. Kap, M.J. Dillon, R. Benner, P.J. Leenen, N.A. Khan and D.A. Drevets (2010). Synthetic human chorionic gonadotropin-related oligopeptides impair early innate immune responses to *Listeria monocytogenes* in mice. J Infect Dis 201: 1072-1080.

van den Berg, H.R., N.A. Khan, M. van der Zee, F. Bonthuis, J.N.M. IJzermans, W.A. Dik, R.W. de Bruin and R. Benner (2009). Synthetic oligopeptides related to the β-subunit of human chorionic gonadotropin attenuate inflammation and liver damage after (trauma) hemorrhagic shock and resuscitation. Shock 31: 285-291.

PHD PORTFOLIO SUMMARY

Summary of PhD training activities

'Regulation of inflammatory responses in shock related syndromes by synthetic oligopeptides and steroids', October 13th, 2010

Name PhD student: Marten van der Zee
Erasmus MC Department: Immunology
Research School: Molecular Medicine

PhD period: 1 dec 2005 – 1 juni 2010
Promotor(s): Prof.Dr. R. Benner
Copromotor: Dr. W.A. Dik

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Academic writing in English for PhD-students (EUR)	2008	4 weeks
- NOW talentendag: Onderhandelen en subsidieaanvragen	2006	8 hours
- Management course for PhD students (NIBI)	2008	2 days
- Data analysis ESP03 (NIHES)	2006	1 week
- Biomedical research techniques (Molmed)	2005	1 week
- Small nuclear polymorphisms and human diseases (Molmed)	2005	1 week
- Basic and translational oncology (Molmed)	2005	1 week
- In vivo imaging (Molmed)	2005	1 week
In-depth courses		
- Classical methods for data-analysis (NIHES)	2008	5.7 ECTS
- Molecular immunology	2006	7 days
Seminars and workshops		
- Weekly Monday morning department meetings	2005-2010	
- Monthly department seminars	2005-2010	

Oral presentations

- Small oligopeptides liberated from human chorionic gonadotropin (hCG) have potent anti-inflammatory activity - Queen Mary University, London, UK — 2010
- The synthetic hCG-related oligopeptide LQGV impair early innate responses to *Listeria monocytogenes* in mice through activation of the adrenal glands - Jaarcongres NVVI 2009, Noordwijkerhout, The Netherlands — 2009
- Synthetic hCG-related oligopeptides impair early innate responses to *Listeria monocytogenes* in mice - European Shock Society Meeting 2009, Lisbon, Portugal — 2009
- Tetrapeptide LQGV inhibits inflammation and organ damage after hemorrhagic shock and resuscitation - Department of Surgery, Oklahoma University, Oklahoma City, Oklahoma, USA — 2008
- Small oligopeptides liberated from human chorionic gonadotropin (hCG) have potent anti-inflammatory activity - Department of Surgery, Oklahoma University, Oklahoma City, Oklahoma, USA — 2007

Posters

- Synthetic hCG-related oligopeptides impair early innate responses to *Listeria monocytogenes* in mice - Molecular Medicine Day, Rotterdam, The Netherlands — 2010
- Synthetic oligopeptides related to the β-subunit of hCG attenuate inflammation and liver damage after (trauma-) hemorrhagic shock and resuscitation - Molecular Medicine Day, Rotterdam, The Netherlands — 2008
- Synthetic oligopeptides related to the β-subunit of hCG attenuate inflammation and liver damage after (trauma-) hemorrhagic shock and resuscitation - Jaarcongres NVVI 2007, Noordwijkerhout, The Netherlands — 2007

(Inter)national conferences

- Trauma, Shock, Inflammation, and Sepsis, 8th World Congress, Münich, Germany	2010	5 days
- Molecular Medicine Day, Rotterdam, The Netherlands	2010	1 day
- European Shock Society Meeting, Lisbon, Portugal	2009	3 days
- Annual meeting NVVI, Noordwijkerhout, The Netherlands	2009	2 days
- International Shock Meeting, Cologne, Germany	2008	5 days
- Annual meeting NVVI, Lunteren, The Netherlands	2008	2 days
- Annual meeting NVVI, Noordwijkerhout, The Netherlands	2007	2 days
- Basic transcriptional and translational biology, Cold Spring Harbor, New York, USA	2007	2 days
- Molecular Medicine Day, Rotterdam, The Netherlands	2007	1 day
- Annual meeting NVVI, Noordwijkerhout, The Netherlands	2006	2 days
- Annual meeting NVVI, Lunteren, The Netherlands	2006	2 days
- Molecular Medicine Day, Rotterdam, The Netherlands	2006	1 day
- Nieuwe ontwikkelingen in de immunologie, Rotterdam, The Netherlands	2005	1 day

Working visits

- Oklahoma University Health Science Center, Oklahoma City, USA	2008	6 weeks
	2007	6 weeks
	2006	4 weeks

Didactic skils

- Basic training didactics ‘ Teach the teacher’	2008	16 hours

2. Teaching

	Year	Workload (Hours/ECTS)
Supervising practicals and excursions, tutoring		
- Immunology practicals for medical students	2005-2010	
Supervising bachelor theses		
- Ivar Noordstra: Effecten van hCG-gerelateerde oligopeptiden op celmigratie *in vitro*	2009	5 months
- Tim Lage Venterink: De effecten van hCG gerelateerde oligopeptiden op LPS en TNF-α geactiveerde endotheelcellen	2007	5 months

3. Grants

- TRUST fund Erasmus University: travel grant	2010
- European Shock Society Meeting 2009 in Lisbon: travel grant	2009
- TRUST fund Erasmus University: travel grant	2008
- NVVI: travel grant for PhD-students	2007