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der Medizinischen Fakultät Mannheim
(Direktor: Prof.Dr.med.Bernhard Karl Krämer)

The role of nuclear factor of activated T cells 5 (NFAT5) in inflammation and the potential use of bifunctional enzyme triggered carbon monoxide releasing molecule in treatment of systemic inflammation

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ABRREVIATIONS

AKI	Acute kidney injury
Ap-1	Activated protein 1
APS	Ammonium persulfate
AR	Aldose reductase
BACH1	BTB Domain And CNC Homolog 1
BGT1	Sodium-chloride-betaine cotransporter
BMDM	Bone marrow derived macrophages
CCR2	C-C chemokine receptor type 2
ChIP	Chromatin immunoprecipitation
CO	Carbon monoxide
COHb	Carbon monoxide hemoglobin
CORMs	Carbon monoxide releasing molecules
COX-2	Cyclooxygenase-2
CYR61	Cysteine-rich angiogenic inducer 61
DAMP	Damage-associated molecular patterns
DEME	Dulbecco’s modified eagle’s medium
DMF	Dimethyl fumarate
DMSO	Dimethylsulfoxid
DN	Diabetic Nephropathy

DTT	Dithiothreitol
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked Immunosorbent Assay
ET-CORMs	Esterase triggered carbon monoxide releasing molecule
FCS	Fetal bovine serum
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
Hsp70	70 kilodalton heat shock proteins
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive care unit
IGEPAL	Octylphenoxypolyethoxyethanol
IKKβ	IκB kinase β
IL-1	Interleukin 1
IL-12β	Interleukin 12 beta
IL-6	Interleukin 6
iNOS	Nitric oxide synthases
MCP-1	Monocyte chemoattractant protein 1
MD2	Myeloid differentiation protein 2
miMCD	Mouse inner medullar collecting duct
MMF	Monomethyl fumarate
NFAT5	Nuclear factor of activated T cells 5

NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells)
NLR	Nucleotide binding oligomerization domain like receptor
NPAS2	Neuronal PAS domain protein 2
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PRR	pattern recognition receptors
RLR	Retinoic acid-inducible gene-I-like receptor
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SIRS	Systemic inflammatory response syndrome
SMIT	Sodium-myo-inositol cotransporter
SOFA	Sequential Organ Failure Assessment Score
TEMED	Tetramethylethylenediamine
TGF-β2	Transforming growth factor-beta 2
TLR	Toll-like receptors
TNFα	Tumor necrosis factor alpha
TonEBP	Tonicity response element binding protein

1 INTRODUCTION

1.1 Sepsis and Acute kidney Injury (AKI)

1.1.1 Sepsis and Septic Acute kidney injury (AKI)

With a yearly increasing incidence, sepsis is becoming a major public health concern affecting approximately one million of patients and causing more than 200,000 deaths per year in United States only [1]. It is now widely acknowledged that sepsis is caused by a dysregulated host response to infection, which ultimately leads to life-threatening organ dysfunction. The clinical criteria to identify patients with sepsis consist of suspected or documented infection and an acute increase of ≥ 2 SOFA (Sequential Organ Failure Assessment Score) points. This definition, proposed at a task force meeting convened by national societies of Critical Care Medicine, abandoned the use of systemic inflammatory response syndrome (SIRS) criteria to identify patients with sepsis.

The pathobiology of organ dysfunction in sepsis suggests a more complex sequel than infection plus accompanying inflammatory response alone and is consistent with the view that cellular defects underlie physiologic and biochemical abnormalities within specific organ systems [2]. Acute kidney injury (AKI) is a common and potentially fatal complication of sepsis that is associated with high morbidity and mortality in hospitalized patients, especially in the intensive care unit (ICU) [3]. Sepsis may cause AKI among critically ill patients in approximately 50% of cases [4]. Septic AKI is characterized by a rapid and often profound decline in renal function, usually evolving over hours to days after the onset of sepsis.

1.1.2 The pathophysiology of acute kidney injury (AKI)

The pathophysiology of AKI in sepsis is multi-factorial and consists of endothelial dysfunction, intrarenal hemodynamic changes, infiltration of inflammatory cells in the renal parenchyma, intraglomerular thrombosis, and obstruction of the tubules with necrotic cells and debris [5]. The innate immune response to infection triggers adaptive mechanisms affecting the kidney's tubular, vascular and glomerular functions. Essential for the innate immune response is the recognition of cellular

components or fragments of invading pathogens as well as components of damaged host cells or extracellular matrix generated during inflammatory stress. These so called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs) on immune cells [6]. Both PAMPs, e.g. lipopolysaccharide, lipoteichoic acid, peptidoglycan, lipopeptides, bacterial/fungal/parasitic DNA and viral RNA and DAMPs, e.g. heat shock proteins, fibrinogen, S 100 proteins are released in the circulation. As approximately one-fifth of the cardiac output is filtered by the kidney, renal cells in septic patients are continuously exposed to DAMPs and PAMPs, either via the blood or via its filtrate in the tubular lumen. Once PRRs on immune cells are triggered by PAMPs or DAMPs, the production of cytokines and chemokines is initiated evoking a systemic inflammatory response. In the kidney, and other affected organs, this is reflected by recruitment and mobilization of macrophages, natural killer cells and neutrophils (Figure 1).

The production of pro-inflammatory cytokines by immune cells, mostly TNFα, IL-6 and IL-1, to a large extent contribute to the progression of sepsis. This is amongst others appreciated by the fact that TNFα as a single factor can induce similar deleterious effects as endotoxin stimulation or sepsis itself [7]. TNFα causes renal damage by recruiting leukocytes, accelerating fibrin accumulation, promoting cell lysis and the release of vasoconstrictor substances [8]. Administration of anti-TNF monoclonal antibodies showed beneficial effects in numerous animal models of sepsis [9].

Also the local production of chemokines in the kidney plays a crucial role in the development and/or progression of AKI. Among the chemokines produced in renal tissue, monocyte chemoattractant protein 1 (MCP-1, also known as CCL2), seems to be particularly increased in mouse models of LPS induced septic AKI and in human AKI patients. In line with this, urinary MCP-1 protein levels have been suggested as early novel biomarker of AKI [10]. Apart from attracting monocyte and neutrophils into the renal parenchyma, MCP-1 perpetuates inflammation by increasing the expression of the IL-6 and intercellular adhesion molecule-1 (ICAM-1) through the activation of the transcription factors such as NF-kB and AP-1 in human tubular epithelial cells [11] .

Toll-like receptors (TLRs) are a family of single membrane-spanning receptors and belong to the so called PRR. TLRs are so far composed of 11 members in mammals

of which TLR4 seems to play an important role in septic AKI. TLR4 binds lipopolysaccharide (LPS) with high affinity and subsequent interacts with the myeloid differentiation protein 2 (MD2), which recognizes the Lipid A domain of LPS, to form an activated LPS-MD2-TLR4 homodimer. Once activated, the homodimer initiates an intracellular signaling cascades which result in the regulation of gene expression of a broad spectrum of genes involved in the defense against pathogens (e.g. TNFα, IL-6 and IL-1) [12]. In order to limit exaggerating cytokine release in the circulation, modulation of the MD2-TLR4 interaction has been considered as a novel therapeutic target in the treatment of septic AKI. Indeed eritoran, a synthetic lipid A antagonist that blocks LPS from binding to MD2-TLR4, has shown protective effects in mice and rats by decreasing excessive inflammatory responses [13] [14] [15]. However, eritoran failed to reduce mortality rate in prospective, randomized, double-blind, multicenter phase 2 and 3 clinical studies [16, 17]. Natural products such as curcuma longa, sulforaphane and iberin, behave in a similar manner as eritoran and prevent binding of LPS to the MDR2-TLR4 complex [18].

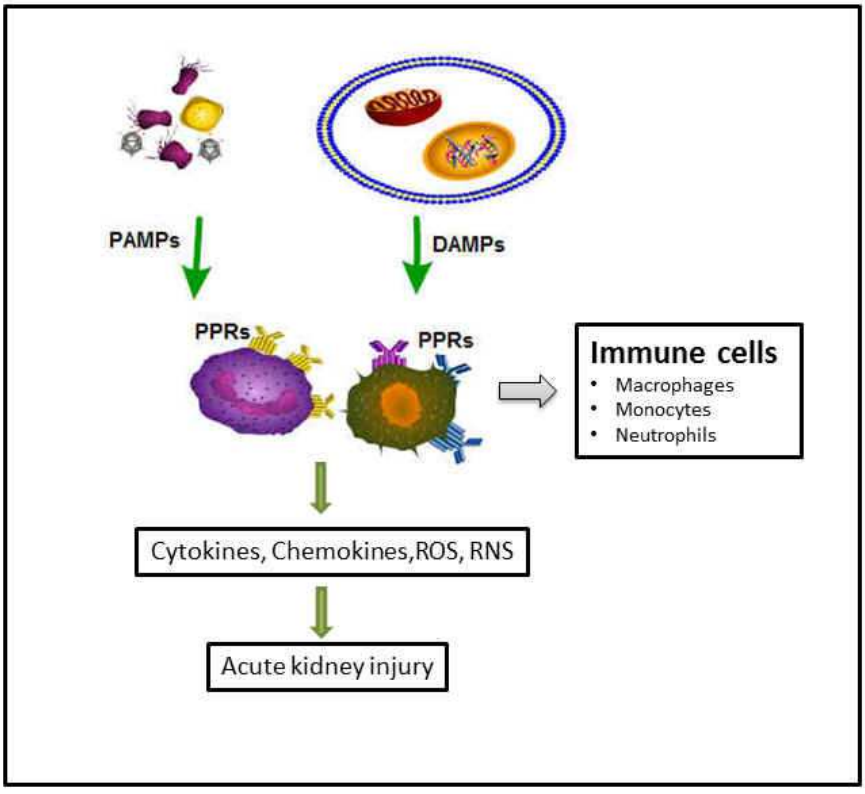


Figure 1 Sequel of events leading to septic acute kidney injury.

1.2 The role of tonicity response element binding protein (TonEBP/NFAT5) in inflammation

Nuclear factor of activated T cells 5 (NFAT5), also known as tonicity response element binding protein (TonEBP), is a transcription factor and member of the family of Rel-like domain-containing factors including NF-kB and NFAT1-4. The physiological function of TonEBP/NFAT5 was initially described as a protective factor against osmotic stress in the renal medulla as it transcriptionally regulates the expression of numerous osmoprotective genes, e.g. Hsp70 and aldose reductase (AR). Apart from this, TonEBP/NFAT5 promotes cellular accumulation of major organic osmolytes by regulating the expression of plasma membrane transporters such as the sodium-chloride-betaine cotransporter (BGT1) and the sodium-myo-inositol cotransporter (SMIT).

NF-kB and NFATc are essential transcription factors in the innate immune response and are activated through the interaction of specific DAMPs and different types of PPRs. The NF-kB signal pathway can be activated by cell surface receptors (TLR, TNFR, EGFR), cysotoslic receptors (retinoic acid induced gene 1 (RIG-I)-Like receptors (RLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs)) and by the c-type lectin receptor families. In addition it can be activated independent of receptors by phorbol esters, UV light, ionizing radiation and reactive oxygen species (ROS) [19]. NFATc cannot be activated by TLRs but respond to the calcium mobilization-coupled receptors Dectin 1 and CD14 [20]. Although TonEBP/NFAT5 has approximately 40% sequence homology in the DNA binding domain with NFATc, it does not contain a calcineurin-depended regulatory domain [21]. In recent years, a variety of studies uncovered that TonEBP/NFAT5 plays important roles in the initiation and progression of inflammatory responses associated with changes in local and systemic osmolality [22] . Nonetheless, despite extensive studies the exact role of TonEBP/NFAT5 with respect to inflammation, NFAT5 is still fragmentary as compared to that of the NF-κB and NFATc proteins.

Chronic inflammation and dysregulation of the immune system might partly underlie or perpetuate microvascular complications in diabetes. Hyperglycemia results in significant plasma hyperosmolarity in the range of 310-350 mOsm/kg H_2O in diabetic patients. Yang B et al. reported that exposure of human peripheral blood mononuclear cells (PBMC) to hyperosmotic conditions (330-410 mOsm/kg H_2O)

induce the expression of IL-1 and IL-8. Moreover, there were significant fold increases in DNA binding activities of TonEBP/NFAT5 to the tonicity elements OREB and OREC in PBMCs from patients with diabetic nephropathy compared to diabetic control subjects [23] . It also has been reported that hyperglycemia stimulates M1 polarization and migration of macrophages via upregulation of TonEBP/NFAT5, that TonEBP/NFAT5 haplo-deficiency in a mouse model of DN reduced the number of renal macrophages and renal inflammation and that genetic variants within TonEBP/NFAT5 are associated with renal function, blood pressure, and systemic inflammation, thus supporting a role for TonEBP/NFAT5 in diabetic microvascular complications [24]. Madonna.R et al. reported that the induction of COX-2 in endothelial cells is at large regulated by TonEBP/NFAT5 as its disruption prevents hypertonicity-mediated induction of COX-2 [25] .

Apart from being a transcription factor for hypertonicity regulated genes, several reports also indicate that TonEBP/NFAT5 may participate in the inflammatory response in the rheumatoid synovium independent of hypertonicity. TonEBP/NFAT5 is not only highly expressed in the synovium but in synoviocytes its activity is increased by proinflammatory cytokines, such as IL-6 and TNFα. Silencing of TonEBP/NFAT5 is associated with a reduced CYR61, CCR2, TGFβ2 and TNFRSF expression [26], likewise is the expression of iNOS, IL-6, COX-2, TNFα, IL-12β diminished in TonEBP/NFAT5$^{-/-}$ bone marrow derived macrophages (BMDMs) in response to LPS as compared to that of TonEBP/NFAT5$^{+/+}$ BMDMs [26]. Further analysis revealed that LPS-induced recruitment of TonEBP/NFAT5 to the iNOS promoter depends on inhibition of IκB kinase β (IKKβ) activity and de novo protein synthesis, and is sensitive to histone deacetylation. An in vivo study showed that TonEBP/NFAT5 is essential for the immunity against *Leishmannia major,* which need TLR and iNOS expression in macrophage [27]. Similar as shown for renal macrophage infiltration in a model of DN, Halterman J et al. reports that TonEBP/NFAT5 haplo-insufficiency reduced the formation of atherosclerotic lesion by 73%. In vitro studies revealed that TonEBP/NFAT5 is required for macrophage migration, which is an essential step in the progression of atherosclerosis [28]. Tellechea M et al. [65] studied the contribution of TonEBP/NFAT5 to macrophage functions in different polarization settings. They found that TonEBP/NFAT5 enhanced iNOS, IL-1β and IL-12β in M1 polarized macrophage, while it regulates markers of M2-polaized macrophages i.e. Arg1, CD163, YM-1 and Fizz-1. They concluded that

NFAT5 plays a predominant role in promoting proinflammatory macrophage functions.

1.3 Carbon monoxide

It is genuinely accepted that carbon monoxide (CO) is a poisonous gas at high concentration because of its strong affinity for haemoglobin and hemeproteins. In the last two decades this paradigm has been shifted as a large number of studies have demonstrated that CO, endogenously generated as a break-down product of heme, at low concentrations is a mediator of cell physiology and functions [29, 30]. Degradation of heme is catalyzed by heme oxygenases. Two isoforms (HO-1 and HO-2) are known which are encoded by two different genes (*Hmox1* and *Hmox2* respectively) [31]. The inducible isoform HO-1 is ubiquitously expressed while the constitutively expressed HO-2 is primarily found in the vasculature and testis. Heme oxygenases are the rate-limiting enzymes in the conversion of heme to biliverdin, releasing CO and Fe^{2+} as by-products. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase (figure 2).

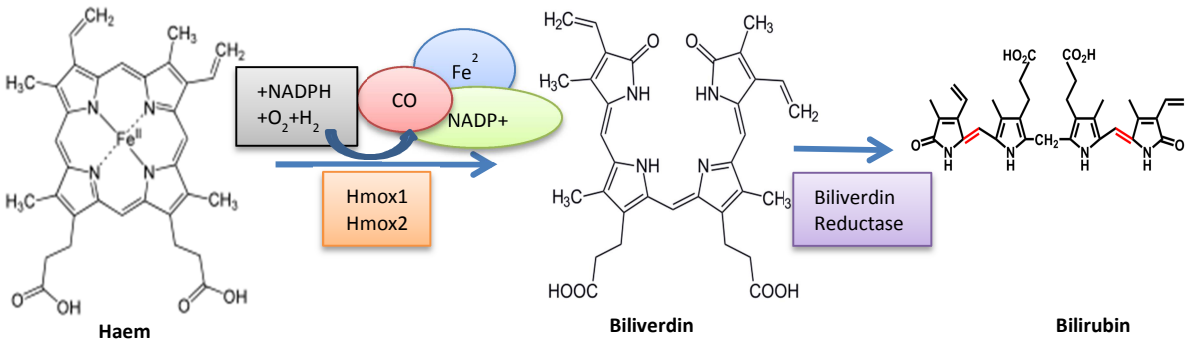


Figure 2 Endogenous Carbon Monoxide generation pathways.

Like other gasotransmitters, CO can diffuse freely through cell membranes and rapidly interact with heme containing proteins. This may lead to changes in signal transduction in various ways. By interacting with the soluble guanylyl cyclase (sGC)

CO can increase cyclic GMP concentrations directly or indirectly by interacting with NO synthase (NOS) to activate the NO-cGMP pathway. This in turn may stimulate potassium channels and change the cellular ion-homoeostasis. CO can also interact with more distal targets like the heme-containing transcriptional factors BACH1 and NPAS2. Finally CO can modulate the production of mitochondrial superoxide by interfering with oxidative phosphorylation and electron transport and thus influences the activity of redox dependent transcription factors.

Early studies on the biological properties of CO used CO in its gaseous form. As reported by Otterbein et al. CO inhalation (<250 ppm) improves survival rates in a model of LPS-induced endotoxemia in rats. This was accompanied by less severe lung alveolitis and reduced edema formation, and diminished iNOS expression in lung primary macrophages [32]. Improved survival rates for CO inhalation therapy have also been reported in a mouse model of cerebral malaria [33]. Even though CO inhalation seems to be an easy, straightforward and cost effective application, safety and practical issues constitute serious limitations. First of all, administration of CO in its gaseous form is problematic in clinical settings for outpatients. Moreover, no reliable methods have been established yet to achieve safe and effective tissue CO concentrations or to monitor CO toxicity. Despite the medical practice of monitoring CO intoxication by serum COHb, it is still not known if COHb constitutes the optimal measure for CO exposure and if COHb simply reflects CO intoxication. Experiments performed in dogs have revealed that dogs died with an average COHb level of 65% within an hour after administrated of 13% CO gas by inhalation. In contrast, severe anemic dogs that received a blood transfusion containing red blood cells with COHb levels of 80% survived indefinitely. Hence the authors postulated that for CO inhalation it is not the CO bound to hemoglobin that is toxic rather the fraction that escapes it [34]. This dissolved CO gas can subsequently penetrate the tissue and the cells and bind to intracellular targets, thus interfering with their biological activity.

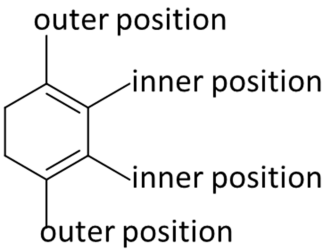
1.4 Carbon monoxide releasing molecules

Based on the avid affinity of CO to transition metals, Motterlini and colleagues envisioned the development of transition metal carbonyls as prototypic carbon monoxide-releasing molecules (CORMs) [35] [36]. Since CORMs contain a transition metal surrounded by a certain number of carbonyl groups as coordinating ligands they are able to carry and deliver CO to biological systems. The first published

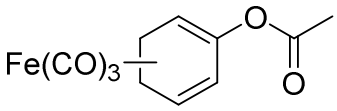
CORMs, using either manganese or ruthenium as transition metal, were only soluble in organic solvents (CORM1 and CORM2) [34]. Further optimization lead to CORM3 which is soluble in aqueous solution and releases CO under physiological conditions [35]. Although CORM2 and CORM3 have been widely used in numerous studies to uncover the biological properties of CO [37-40], there are some concerns related to the use of these CORMs as therapeutic compound.

Most of the published CORMs used in biological systems either spontaneously release CO when dissolved in aqueous solutions or require specific chemical or physical stimuli to favor CO dissociation from these complexes. Hence CO delivery is not target tissue specific as it can freely diffuse to other tissues. To avoid or to mitigate potential adverse effects of CO tissue specific CO delivery would be preferable. One way to meet this objective is allowing CO to be released only intracellularly through the action of cell specific enzymes. It should also be emphasized that for clinical applications ruthenium as transition metal for CORMs might not be an optimal choice since should be regarded as highly toxic [41]. To solve the problems related to ruthenium and other transition metals new approaches are on the verge. CORMs can be bound to macromolecular carriers, for example polymeric micelles, nanoparticles, copolymers and proteins, either by covalent binding or no covalent adhesion [42]. Although this to some extent solved the problem associated with the use the rare transition metals, these devices do not allow intracellular release of CO. With the development of acyloxybutadiene–$Fe(CO)_3$ complex the use of a transition metal common to the human body and intracellular delivery of CO was realized [39]. Acyloxybutadiene–$Fe(CO)_3$ complexes are stable under physiological conditions but once inside the cell the ester functionality is cleaved off, subsequently the labile dienol–iron carbonyl complex disintegrates under oxidative condition to release CO, iron and corresponding enones. Since CO release from Acyloxybutadiene–$Fe(CO)_3$ complexes are triggered by esterase activity, these complexes are also known as enzyme, or more precisely esterase triggered (ET)-CORMs. In close collaboration with the groups of Prof. Schmalz (Organic chemistry, University of Köln), the Vth medical department have performed various studies describing the behavior of ET-CORMs in various biological settings [40, 41]. ET-CORMs are capable to protect cells against cold inflicted injury, induce HO-1 expression and suppress TNFα-mediated VCAM-1 expression [43, 44]. The biological properties of ET-CORMs are highly related to the type and complexity of

the ester-functionality. Also, its position in the (η^4 - Diene) iron complex is pivotal for the kinetics of CO release. The simplest ET-CORMs are the 2-cyclohexenone derived ET-CORMs that contain an acetate as ester functionality either at the inner or outer position (figure 3). ET-CORMs seem to behave differently in endothelial as compared to epithelial cells, which might be explained by differences in the expression cell specific esterases.

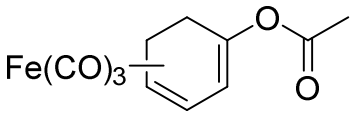


2-cyclohexenone derived, inner position



rac1

2-cyclohexenone derived, outer position



rac4

Figure 3 The chemical structure of ET-CORMs

Although ET-CORMs also have the disadvantage of not delivering CO in a strictly cell specific manner, these complexes paved the way for developing protease triggered CORM [45]. These CORMs are synthesized as tripartite compound consisting of a peptidase-specific (oligo-) peptide, a self-immolative linker attached to the cyclohexadiene-Fe$(CO)_3$ moiety (figure 4).

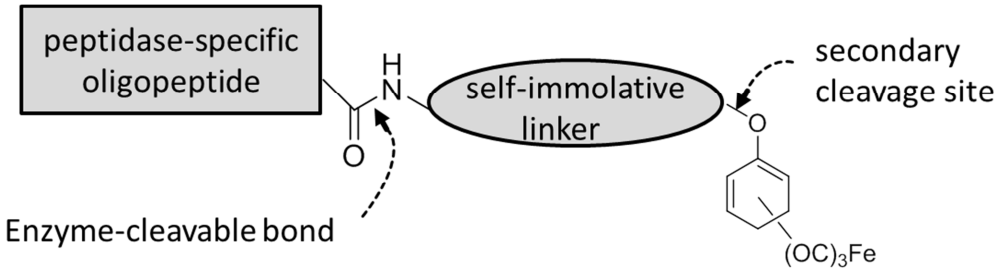
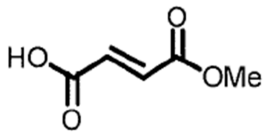


Figure 4 General design of protease-activated ET-CORMs

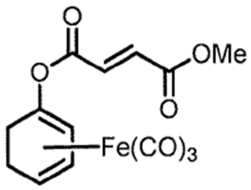
Apart from the design for specific cell targeting of CO release, ET-CORMs can also be synthesized as so called bi-functional molecules. Drugs that carry a free carboxyl functionality can easily be coupled in a similar fashion as done for acetate. Since CO down-regulates inflammation, bifunctional ET-CORMs may display synergistic effects on inhibiting inflammation when an anti-inflammatory compound like nonsteroidal anti-inflammatory drugs (NSAIDs). Since NSAID are known to display adverse effects, e.g. serious gastrointestinal, hepatic, cutaneous and renal adverse events, synergy between NSAID and CO may lead to reduced therapeutic NSAID concentrations.

In this doctoral thesis, we made use of a bifunctional ET-CORMs, consisting of monomethyl fumarate (MMF) as ester functionality. MMF-CORM can be synthesized with the ester either at the inner or outer position as described for other ET-CORMs (figure 5). Dimethyl fumarate was first licensed in Germany as oral therapy for psoriasis and later it was approved by US Food and Drug administration (FDA) and the European Medicines Agency (EMA) for treating relapsing multiple sclerosis. In

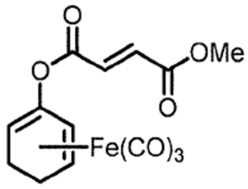
the small intestine dimethyl fumarate (DMF) is rapidly converted into its active metabolite, monomethyl fumarate (MMF) [46] [47]. Various studies have highlighted the therapeutical potential of DMF in a variety of animal models including Alzheimer's disease [48], traumatic brain injury [49], allergic encephalomyelitis [50], liver ischemia/reperfusion injury [51] and cardiomyopathy in type 1 diabetes [52]. Other in vitro studies have reported an anti-oxidative and anti-inflammatory effect of DMF.



Monomethyl fumarate



MMF-CORM-O



MMF-CORM-I

Figure 5 The chemical structures of monomethyl fumarate, MMF-CORM-O and MMF-CORM-I.

1.5 Aims of the study

As mentioned above, sepsis may lead to organ dysfunction such as AKI. AKI not only is characterized by a decreased glomerular filtration rate (GFR) it also impairs the urinary concentration ability [53, 54]. The latter depends on the expression of various transporters and channels in medullary nephron segments. It is believed that is caused by an LPS mediated increase in medullary nitric oxide production by iNOS induction, which in turn results in impairment of the transcriptional activity of TonEBP/NFAT5 by S-nitrosylation and ultimately to a reduced expression of TonEBP/NFAT5 target genes required for urinary concentration. Yet it remains to be assessed to what extent inflammatory mediators, e.g. LPS, TNFα or IL-1, can influence activation of this transcription factor. Our hypothesis is that these mediators cannot activate TonEBP/NFAT5 and thus cannot compensate for the reduced transcriptional activity of TonEBP/NFAT5 in renal medullar cells. Since CO mitigates inflammation and has been reported to inhibit iNOS expression, CORM holds the promise of preventing renal complications in the course of sepsis. Because DMF is

effective in preventing oxidative stress and inflammation in septic rats [55] this also provides a rational for the use of bifunctional CORMs, i.e. MMF-CORM, to obtain possible synergistic effects. To test the hypothesis the following questions were addressed in this thesis:

1. Is TonEBP/NFAT5 activated by mediators of inflammation, i.e. LPS and TNFα, in renal inner medullary collecting duct cells (miMCD cells). Does this result in regulation of TonEBP/NFAT5 target genes at the mRNA and protein level?
2. Is TonEBP/NFAT5 activation involved in regulation of inflammatory mediators (cytokines and chemokines) in miMCD cells, murine macrophage (Raw264.7 cell line) and isolated spleen cells from wild-type and TonEBP/NFAT5$^{-/-}$ mice?
3. Does CO regulate TonEBP/NFAT5 activation in Raw264.7 cells? Does it influence iNOS and HO-1 expression?
4. Is there synergy between CO and MMF in suppressing inflammation?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

2.1.1.1 Commercial chemicals

Table 1 Chemicals

Chemicals	Source
β-alanine	Sigma-Aldrich(germany)
Acrylamidmix 40%	Fluka(Swiss)
Agarose (PAGE)	Serva (Germany)
Albumin standard (BSA)	Thermo Scientific™ (USA)
Ampicillin	Bioline GmbH (Germany)
APS	Sigma-Aldrich (Germany)
CASY-ton	Roche (Germany)
Chemiluminescennce reagent Western Lightning™	Perkin Elmer (USA)
Collagen 0,1%	Sigma-Aldrich (Germany)
Collagenase (from Clostridium histolyticum) Type V	Sigma-Aldrich (Germany)
DMEM/F-12, GlutaMAXGibco®	Invitrogen (Germany)
DMSO	Sigma-Aldrich (Germany)
DNA ladder buffer 10 x	Invitrogen (Germany)
DNA ladder generuler 1kb	Invitrogen (Germany)
D-PBS 1 x Gibco®	Invitrogen (Germany)
DTT 0,1M	Invitrogen (Germany)
DTT 1M	Invitrogen (Germany)

Endothelial cell growth medium, advanced	Provitro (Germany)
Ethanol 100%	Sigma-Aldrich (Germany)
Gel red	Biotrend Germany
Formaldehyde 37%	Sigma-Aldrich (Germany)
FCS	Life Technologies (Germany)
Gelatin from bovine skin	Fluka (Swiss)
Glycerol (99%)	Sigma-Aldrich (Germany)
Glycine	Sigma-Aldrich (Germany)
Hydrogen chloride 1M	Sigma-Aldrich (Germany)
Isopropanol	Merck (Germany)
Lithium chloride	Merck (Germany)
Laemmli-buffer	Biorad
Lipopolysaccharides from E.coli O55:B5	Sigma aldrich
Magnesium chloride (MgCl2)	Fluka (Swiss)
2-β-Mercapto ethanol	Sigma-Aldrich (Germany)
Methanol	Carl Roth (Germany)
Milk powder	Fluka (Swiss)
NaCl (sodium chloride)	Merck (Germany)
dNTP-Mix(dATP,dTTP,dCTP,dGTP)	Carl Roth (Germany)
PageRuler Plus Prestained Protein ladder	Invitrogen (Germany)
PBS 10 x	Invitrogen (Germany)
Penicillin/streptomycin	Sigma-Aldrich (Germany)
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich (Germany)
Phorbol 12-myristate 13-acetate	Sigma-Aldrich (Germany)
Poly-D-lysine hydrobromide	Sigma-Aldrich (Germany)

Protease Inhibitor Cocktail Tablets	Sigma-Aldrich (Germany)
RNAse Inhibitor	Roche (Germany)
SDS	Carl Roth (Germany)
SiRNA NFAT5 EQ-009618	DHarmacon (USA)
TBE-buffer 10 x	Invitrogen (Germany)
TEMED	Sigma-Aldrich (Germany)
Ficoll-paque	GE healthcare life science(UK)

2.1.1.2 Chemicals preparations done in our laboratory

Table 2 Chemicals preparations done in our laboratory

Chemicals	Ingredient
ChIP cytosolic lysis buffer	Tris pH 8 50mM, EDTA pH8 2mM, NP40 0.1%, glycerol 10%
ChIP nuclear lysis buffer	SDS 1%, EDTA pH8 5mM, Tris pH8 50mM
Cytosolic extracts buffer A	100mM HEPES,PH 7,9,1M KCl, 1M $MgCl_2$,0,5M EDTA ,100mM DTT and 200mM PMSF
DB dilution buffer	NP40 0.5%, NaCl 200mM, EDTA 5mM, Tris pH8 50mM
EB-extraction buffer	TE 1x pH8, SDS2%
LiCl washing Buffer	SDS 0.1%, NP40 1%, EDTA 2mM, LiCl 500mM, Tris pH8 20mM
NaCl washing Buffer	SDS 0.1%, NP40 1%, EDTA 2mM, NaCl 500mM, Tris pH8 20mM
Nuclear extract buffer B	100mM HEPES, PH 7,9, 80% glycerol, 2M NaCl, 0.5M EDTA, 100mM DTT and 200mM PMSF,200mM Benzamidine and 5mg/ml leupeptin
PBMC isolation buffer	1 x PBS and 3 mM EDTA
TBS	50 mM Tris-Cl, pH 7.6; 150 mM NaCl
Transfer buffer	25mM Tris, 192mM glycine, 15% methanol (PH 8.4)

2.1.2 Antibodies

Table 3 Antibodies

Antibodies	Source	Dilution In WB	Company
NFAT5	mouse	1:200	Santa Cruz Sc-398171
Histone 3	mouse	1:4000	Enzo life sciences #61476
Beta actin	mouse	1:20000	Santa cruz
HO-1	rabbit	1:3000	Enzo life sciences ADI-SPA-895
iNOS	rabbit	1:200	Santa cruz Sc-651

2.1.3 Primers

Table 4 Primers

Primers	Sequence(5´→3´)	Tm [°C]	GC-content
NFAT5 Forward	5’- GTA ACC ATG ATT AGT CTT TTA GCT TTA TG -3’	59.6 oC	31%
NFAT5 Reverse	5’- GTT ACC ATG ATT AGT CTT TTA GCT TTA TG -3’	58.9 °C	43.5%
Cre Forward	5’- GCC AGC TAA ACA TGC TTC ATC -3’	57.9 °C	47.6%
Cre Reverse	5’- ATT GCC CCT GTT TCA CTA TCC -3’	57.9 °C	47.6%
TNFa Forward	Forward:5’- CCC AAC TCT CAA GCT GCT -3’	59.4°C	55%%
TNFa Reverse	Forward:5’- CCT CTG AAA GCT GGG TGC-3’	57.3°C	55%
CCL2 Forward	5’- ATC TGGA GCT CAC ATT CCA-3’	54.3 °C	47.4%
CCL2 Reverse	5’- TCC CTC TCA CTT CAC TCT GTCA-3’	60.3 °C	50%

2.1.4 Kits

Table 5 Kits used in experiments

Kits	Source
QIAquick Gel Extraction Kit	Qiagen (Germany)
GoTaq® DNA Polymerase Kit	Promega (Germany)
Genomic DNA isolation kit	Promega (Germany)
Coomassie(Bradford) Protein Assay kit	Thermo Scientific (Germany)

2.1.5 Consumables

Table 6 Plastic and other consumables

Consumables	Source
Cell culture flasks Falcon™ 25 cm2 / 75 cm2	BD Biosciences (USA)
Cell scraper Falcon™	BD Biosciences (USA)
Cryotubes Nunc™ 1.8 ml IVF CryoTube	Thermo Scientific™ (USA)
Disposable cuvettes	Eppendorf (Germany)
Disposable scalpel	Feather® (Japan)
Disposable syringes	BD Biosciences (USA)
Disposable pipette Falcon™ 1 ml/ 2 ml/ 5 ml/ 10 ml/ 25 ml	BD Biosciences (USA)
Ependorf Safe-lock Tubes 0.5 ml / 1.5 ml / 2 ml	Eppendorf (Germany)
Eppendorf epT.I.P.S 0.1-10 µl / 2-200 µl / 50-1000 µl	Eppendorf (Germany)

Falcon™ Tubes 15 ml / 50 ml	BD Biosciences (USA)
Flat bottom plates Falcon™ 6 / 12 / 24 well	BD Biosciences (USA)
Gel-Blotting-Paper (Filter paper)	Whatman® (England)
“Mr. Frosty” Freezing Container Nalgene™	Thermo Scientific™ (USA)
Parafilm® M	Bemis® (USA)
Petri dishes Falcon™	BD Biosciences (USA)
Polyethylen Film, Fisherbrand®	Fisher Scientific (Germany)
PVDF-Membrane	Roche (Germany)
Sterile filter (0.22 µm), Fisherbrand®	Fisher Scientific (Germany)
White 96-well-plates	NUNC Denmark (USA)
6-Tube Magnetic Separation Rack	Cell signaling Technology(USA)

2.1.6 Machines or software used for experiments

Table 7 Machines and software

Machines or software	Source
ABI step one plus Realtime PCR systems	Applied Biosystems® (USA)
Autoclave Typ ELVC 5075	Systec GmbH (Germany)
Bunsen burner Butane / Propan CV 470 Plus	Campinggaz® (Netherland)
Cell counting CASY®	Schärfe Systems (Germany)
CellF software	Olympus (Germany)
Centrifuge Biofuge Pico/ Fresco/ Primo R	Heraeus (Germany)
Centrifuge 5415/ 5417C	Eppendorf (Germany)
DNA concentration measurement (Infinite® 200 NanoQuant)	Tecan (Germany)
DNA Sequencing Analysis Software 5.2	Applied Biosystems

Electrophoresis horizontal chamber SUB-CELL®-GT	Bio-Rad Laboratories (USA)
Electrophoresis vertical chamber for western blot PAGE	Biometra (Germany)
ELISA Absorption measurement	Tecan (Germany)
MagellanTM Data Analysis Software	Tecan (Germany)
GraphPad Prism 5.0	GraphPad Software, Inc (USA)
Imaging system for blot	Biometra (Germany)
Immunofluorescence optical microscopy	Olympus (Germany)
Impulse Sealer	TEW(Taiwan)
Incubator for Cell culture HERAcell 150i	Heraeus GmbH (Germany)
Incubator and shaker Certomat® HK	B.Braun Biotech International (Germany)
Light microscope DMIL	Leica (Germany)
Microwave oven	Sharp (USA)
Mini-PROTEAN® Tetra handcast systems	Bio-Rad (Germany)
Multi-pipette plus	Eppendorf (Germany)
pH-Meter 538 Multical	WTW (Germany)
PowerPacTM HV High-Voltage Power Supply	Bio-Rad (Germany)
Pipette Research	Eppendorf (Germany)
Refrigerator 4 °C	Liebherr (Germany)
Refrigerator -20 °C	Liebherr (Germany)
Refrigerator -80 °C	Liebherr (Germany)
Shaker Unimax 210/ Rotamax 120/ Titramax 100	Heidolph (Germany)
Shaker Vortex-Genie 2	Scientific Industries (USA)
Shaker Vortex REAX 2000	Heidolph (Germany)
Spectro-photometer U-2000	Hitachi (Japan)
Sterile working bench for cell culture	Heraeus (Germany)

Thermo-cycler PCR 2720	Applied Biosystems (USA)
Thermoblock TechneDri-Block® DB-2D	Biostep GmbH (Germany)
‘‘The Imager Appligen‘‘ Software	OncorAppligene (USA)
Trans-Blot® TurboTMTransfer System	Bio-Rad (Germany)

2.2 Methods

2.2.1 Cell culture

The miMCD-3 cell line (ATCC) is a mouse cell line with characteristics of mice inner medulla collecting duct cells. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 4,5g/L glucose, supplemented with 5% fetal calf serum, L-glutamine and penicillin (100u/ml)/streptomycin(100ug/ml). Mouse RAW 264.7 macrophage cells were grown in RPMI-1640 (Invitrogen), supplemented with 10% heat-inactivated fetal calf serum and (100u/ml)/streptomycin(100ug/ml). Cells were cultured in a humidified atmosphere of 5% CO_2 and 93% O_2 at 37^{o}C. Cells were grown in 6-wells plates to confluency. Medium osmolarity was adjusted by addition of NaCl (table 8). Medium osmolarity was checked using an osmometer.

Table 8 Osmolality increased by addition of NaCl as endconcentration

NaCl (mM)	Osmolality (mOsm)
25	300
75	400
150	500

2.2.2 Monocyte isolation from Buffy Coat

2.2.2.1 Isolation of peripheral blood mononuclear cells (PBMCs) using Ficoll-Paque™

Fresh Buffy coat was got from German red cross blood bank.

Dilute cells with 4 x the volume of buffer PBS+(1 x PBS plus 3mM EDTA), carefully layer 30 ml of diluted cell suspension over 15 ml of Ficoll-Paque in a 50 mL falcon tube. Centrifuge at 400xg for 30-40minutes at 20^{o}C in a swing-out-bucket rotor without brake. Aspirate the upper layer leaving the mononuclear cell layer (lymphocytes, monocytes, and platelets undisturbed at the interphase.

Carefully transfer the mononuclear cell layer to a new 50ml falcon tube. Fill the tube with buffer until 50ml, mix and centrifuge at 300xg for 10 min at 20^{o}C. To remove the red blood cells, the red blood cells were resuspended in 10ml RBC lysate solution and incubated in Room temperature for 8 min, filled up with PBS to 50ml with PBS+ and centrifuged at 200xg for 10 min at RT. For removal of platelets, resuspended the cell pellet in 50ml of PBS (2mM EDTA) buffer and centrifuged at 200xg until the supernatant was clear after centrifugation. Resuspended cell pellet in 40 ml buffer and cell count was performed using CASY.

2.2.2.2 CD14^{+} monocytes were isolated from PBMC by positive selection using a MACS system (Miltenyi Biotech, Bergisch Gladbach)

CD14^{+} monocytes isolation was performed according to manufacturer instructions. PBS++ (2mM EDTA+0,5%BSA) was used throughout the whole procedure. Per 10^{7} cells 80 µl PBS++ and 20 µl CD14 microbeads were added and incubated for 15 min at 4°C.Cells were washed with 1ml PBS++ per 10^{7}cells, centrifuged at 300g for 10min and were resuspended in 500µl degassed PBS++ per 10^{7} cells. During magnetic separation degassed PBS++ was used. Magnetic separation was performed by rinsing a LS column with 3 ml PBS++, the cell suspension was applied to the column and washed three times with 3 ml of PBS++. The LS column was removed from the MACS Magnet and the CD14^{+} cells were immediately flushed out with 5ml PBS++. Enriched CD14^{+} monocytes were counted by using CASY and used in subsequent experiments.

2.2.3 Preparation of Nuclear and cytosolic extracts

The miMCD3 cells were cultured as described previously (2.2.1) and grown on T25 flask until confluent. Cells were scraped off the bottom and transferred to 10ml falcon tube, and then spun at 2000 rpm. Cell pellets were homogenized in lysis buffer A 100µl per 2×10^7 cells and incubated on ice 15min. Added 10% IGEPAL 12.5ul per 100µl buffer A and then vortex for 10 sec. The cell extract was immediately spun at 15,000 rpm for 1min, and the supernatant was collected as cytosolic extract. The pellet was resuspended in the buffer B and incubated on ice for 30 min while shaking, and then centrifuged at 15,000 rpm for 5 min at 4ºC. The supernatant was collected as nuclear extract and used in the subsequent experiment.

2.2.4 Westernblotting

Preparation of total protein, cytosolic and nuclear extract was performed as described at 2.2.3 and the concentration was determined using Coomossine (Bradford) protein assay kit. All standard and samples were triplicates. Pipetted 5µL of each standard and samples into the 96-well microplates, and then added 250 µL of the Coomassie Reagent to each well and incubated plate for 10 mins at room temperature. Measured all samples using TECAN infinite 200 with 595nm absorption wavelength. Prepare a standard curve by plotting the average blank-corrected 595nm measurement for standards versus its concentration in µg/mL. The three-parameter curve was employed to determine the protein concentration of each samples. Protein samples (15µg in 15µl), in the presence of laemmli buffer (1:4 diluted in laemmli buffer) were boiled for 10 min and cooled down on ice for another 10 min. Samples were shortly centrifuged down and then loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis polyacrylamide (SDS-PAGE) gel.
Stacking and separating gels for SDS-PAGE were made as below:

Table 9 Gels formula for Westernblotting

Ingredient	Separate gel (10%)	Stack gel
ddH2O	4.8ml	3.6ml
40 % Acrylamid	2.5ml	0.62ml
Tris-buffer	2.5ml (1.5mmol/L)	0.63ml

10 % SDS	0.1ml	0.05ml
TEMED	0.004ml	0.005ml
10%APS	0.1ml	0.05ml

All samples in the SDS-PAGE gel run at 200V for 50 min using Mini-PROTEAN® Tetra handcast systems and afterwards transferred to polyvinylidene fluoride (PVDF) membranes employing Trans-Blot® Turbo^{TM}Transfer System for 30 min. The membranes were then incubated for 1h in TBS containing 0.1% Tween-20 and 5% milk. The protein was then immunelabeled using specific primary antibodies which diluted in 5% milkpowder TBST (1%) solution at 4ºC overnight. The bound primary antibodies were detected using secondary antibodies diluted in 5% milkpowder TBST (1%) solution for 1 hour at room temperature after washing TBST buffer for 3 times. Detection of immune reactive bands was performed by enhance luminol reagent and visualized by chemiluminescence.

2.2.5 Real-Time Polymerase Chain Reaction (PCR) analysis

2.2.5.1 Total RNA Isolation with TRIzol™ reagent

The cells were cultured and grown on 6-well flask until confluent. Washed all the samples with 1×PBS for 3 times, added 1 ml TRIzol™ Reagent per well and the cells were homogenized by pipetting up and down. Homogenized solutions were incubated in room temperature for 10 min to permit complete dissociation of the nucleoprotein complex. Add 0.2mL of chloroform and shake samples vigorously for 15 sec. After incubation for 2 mins, centrifuge all samples for 15 mins at 12,000 × g at 4 °C. Transfer the aqueous phase containing the RNA to new tubes. Added 0.5mL of isopropanol to the aqueous phase and incubate for 10 mins. Afterwards, centrifuge for 10 minutes at 12,000 ×g at 4 °C. Total RNA precipitated forms a white gel-like pellet at the bottom of the tube. Discard the supernatant with a micropipettor. Resuspended the pellet in 1mL of 75% ethanol and mix by inversing. Centrifuge samples at 7500 ×g at 4°C. Discard the supernatant with a micropipette and air dry the RNA pellet for 5-10mins. Dilute the RNA Pellet in 20-30µL Nuclease free water. Concentration of RNA determined by measuring absorbance at 260nm using the formula A260 x dilution x 40 = µg RNA/ml.

2.2.5.2 cDNA Reverse Transcription

Used 1 µg total RNA per 20 µL reaction.

Prepared the 2 x RT master mix per 20µl reaction referring to the table below. All procedures performed on ice.

Component	Volume/Reaction(µL)
10 x RT Buffer	2.0
25 x dNTP Mix (100mM)	0.8
10 x RT Random Primers	2.0
MultiScrie ^{TM} Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2

Prepared the cDNA RT reactions by pipetting 10µL of 2X RT master mix into each well in to PCR tubes and 10 µL of RNA sample into each well subsequently by pipetting up and down two times to mix. Seal the tubes and briefly centrifuge the tubes to spin down the contents and to eliminate any air bubbles. Placed the tubes in PCR machine and started the thermal cyclers using the thermal conditions in table below:

Temperature(°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Store the cDNA products in -20 °C until next step experiments.

2.2.5.3 Taqman Assay

All procedures were performed on ice.

For each sample, pipet the following into a nuclease free 1.5-ml microcentrifuge tube, each samples in three replicates as well as endogenous controls and no template controls

PCR reaction mix component	Volume per 20μL reaction (μL) for 3 replicates
20x TaqMan® Gene Expression Assay	2.0
2x TaqMan® Gene Expression Master Mix	40.0
cDNA template	4.0
RNase-free water	34.0

Vortex the PCR reaction mix component for several times. Transferred 20 μL of PCR reaction mix into each well of 96-well plates. Seal the plates with the appropriate cover and centrifuged the plate briefly.
Loaded the plate into the ABI StepOne^{TM} plus RT-PCR machine and run the fast program showed in bellowed table.

Thermal cycling conditions		
Stage	Temperature (°C)	Time(mm:ss)
Hold	50	2:00
Hold	95	10:00
Cycle	95	0:15
(40 Cycles)	60	1:00

Table 10 Plate experiment parameters for TaqMan® Gene expression Assays
Measured Cycle threshold (Ct) values were normalized with beta-2-microglobulin and they were expressed as fold-over control samples.

2.2.6 Chromatin immunoprecipitation assay

2.2.6.1 Nuclei preparation and chromatin digestion

The miMCD cells were grown in T_{75} flasks until 70% confluent and then stimulated with LPS (1µg/mg) and NaCl (500mOsm) respectively. Fixation was performed at different time points, for example, 2h and 4h. Afterwards, 1% formaldehyde at room temperature for 10 mins and was quenched by TRIS 7.5 at final concentration of 125mM for 5 mins. After washing the cells with ice-cold PBS for 3 times, Collect the cells into a 15 ml falcon tube by scraping with PBS. Cell was spin down at 2000x rmp for 10 min and lysed in 600µL Buffer A (see in material) with protease and phosphatase inhibitors and 2 mM DTT for 5 min. centrifuge the samples at 3000xrmp for 5 min at 4°C. Collect the supernatant as cytoplasmic fraction and resuspend the pellet in 500 µL Buffer B (see in material) with protease and phosphatase inhibitors and 2 mM DTT. Furthermore, Cell lysates were sonicated (Bioruptor®Plus) for 30 cycles of 30s on plus 30s off.

Chromatin fragment sizes were always tested after each sonication as the followed procedures: 20 µL sonicate samples plus 280 µL DB buffer incubated at 65°C overnight while shaking at 200x rpm. The second day added 2 µL of 1mg/ml RNase A and incubate at 37°C for 1 hour and then add proteinase K (100 µL) and incubate at 50°C for 1 hour while shaking at 200x rpm. Isolate DNA using QIAquick Gel Extraction Kit and load 300ng purified DNA samples on a 1% agarose gel and then run the electrophoresis with a 100 bp DNA marker. Properly digested chromatin ranging from 150 to 500 base pairs (bp) showed in the bellowed figure (Figure 6).

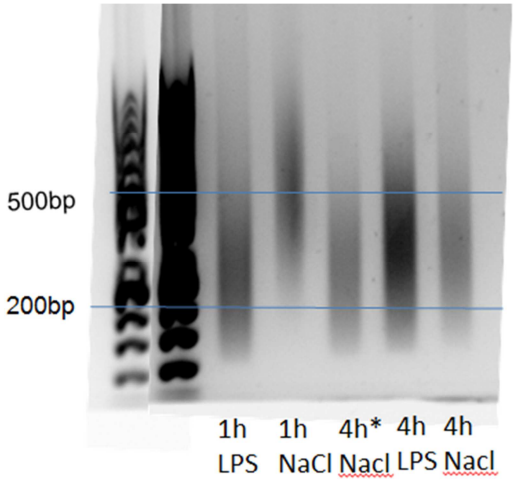


Figure 6 Properly digested chromatin migrates between 150-500 bp

2.2.6.2 Chromatin Immunoprecipitation and DNA purification

Each sample was diluted 10x in dilution buffer for immunoprecipitation. Pre-samples were incubated with 30 µL Chip-Grade Protein G magnetic beads per each IP reaction and incubate 2h at 4°C in rotation. Pellet protein G magnetic beads in each immunoprecipitation by placing the tubes in a Magnetic Separation Rack. Waited 1 min for solution to clear and then carefully transferred the supernatant to a new tube. Took 200 µL each sample as 10% Input and store at -80°C. Added 2µg antibody per IP and rotated samples overnight at 4°C. The second day added 30 µL protein-G magnetic beads per IP and rotated for 2 hours at 4°C. Pellet protein G magnetic beads by placing the tubes in a Magnetic Separation Rack and removed the supernatant carefully. Washed pellet protein G magnetic beads by adding 800 µL NaCl-washing buffer for 5 min on ice for 3 times. Washed pellet protein G magnetic beads with LiCl- washing buffer for 5 min on ice for 3 times. After the last washing procedure, aspirated the supernatant as much as possible and immediately added 100 µL EB-extraction buffer at room temperature for 30 min with gentle vortex (1,200 rmp). Pellet protein G magnetic beads by placing the tubes in a Magnetic Separation Rack and waited 1 min for solution to clear. Carefully transferred eluted chromatin supernatant to a new tube. To all samples, including 10% input samples, reversed cross-links by incubating at 65°C overnight while shaking at 200x rpm. The second day added 2 µL of 1mg/ml RNase A and incubate at 37°C for 1 hour and then added proteinase K (100 µL) and incubated at 50°C for 1 hour while shaking at 200x rpm. Isolate DNA using QIAquick Gel Extraction Kit and get 20 µL purified DNA in the end.

2.2.6.3 Quantification of DNA by SYBR-Green PCR:

Added 2 µL of the DNA samples to each tube.
Prepared the master reaction mix as described below. Added enough reagents for two extra reactions to account for loss of volume. Added 18 µL of reaction mix to each PCR reaction well.

Reagent	Volume for 1 PCR reaction
Nuclease-free H2O	6 µL
Primers	2 µL
SYBR-Green Reaction Mix	10 µL

Started the following PCR reaction Program:

a	Initial denaturation	95°C 3min
b	Denature	95°C 15sec
c	Anneal and extension:	60°C 60sec
d	Repeat steps b and c for a total of 40 cycles	

Analyze quantitative PCR results and aciculate the IP efficiency manually using the percent Input Method and the equation shown below.

Percent Input=10% x $2^{(CT\ 10\%\ Input\ Sample\ -CT\ IP\ Sample)}$

2.2.7 ELISA

Cell culture supernatant was harvest at different time points after onset of stimulation and was stored in -80°C until analysis. TNFα, IL-10 and CCL-2 protein in supernatant were measured by ELISA (R&D systems) kits. All reagents and samples were brought to room temperature before use. All samples, standards and controls be assayed in triplicate. The optical density was measured by microplate reader set to 450nm for absorption wavelength and 570nm for correction wave length. Megllan software were employed to analysis the readings and calculate the samples' concentrations.

2.2.8 Cre^{+} NFAT5 $^{flx/flx}$ conditional knockout mice and murine splenocytes isolation.

Cre^{+} NFAT5 $^{flx/flx}$ conditional knockout mice were described previously [56]. Mice was feed by tamoxifen diet for 4 weeks since 8-week-old and then been sacrificed using cervical dislocation method. One small piece of tail was dissected for the further genomic DNA isolation.

Mice were fixed on a clean dissection board and abdominal area was rinsed with 70% reagent alcohol. Abdominal cavity was incised and spleen was removed gently, which is located to the left side of the abdomen and inferior to the stomach, to the labeled 50ml falcon tube containing PBC with 2mM EDTA. Animal carcass was disposed in an appropriate freezer and stored at -20°C for disposal service pick up.

All procedures below were performed in the laminar flow hood. The isolated spleen was sliced into small pieces and places on the strainer attached to a petri dish. Remove the plunger form a 10-ml syringe and use the black rubber end to press the spleen. Firstly, press the spleen roughly through the strainer of larger mesh size (180nm) and wash the cells with the excess PBS, secondly, collect the filtration solution and press them through the strainer of small mesh size (50nm) and wash them again, thirdly collect the filtrated solution and transfer to the 50ml falcon tube. Centrifuge the cell suspension at 300g for 5 minutes and aspirate the supernatant. Suspend the cell pellet in cell culture medium (RIPM 1640 +10% FCS+P/S) for the further experiments.

2.2.9 Statistics

Quantitative data are depicted as mean ± SEM. One way ANOVA was used to compare differences between control and treatment and Two-way ANOVA was used to compare differences between groups. Significance was defined according to a $p < 0.01$. Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad software, Inc. La Jolla. California, USA).

3 RESULTS

3.1 Inflammatory conditions fail to induce NFAT5 nuclear accumulation in mouse inner medulla collecting duct cells (miMCD cells)

Although a variety of studies have shown activation of NFAT5 by pro-inflammatory stimuli in immune cells, it is currently not clear to what extent NFAT5 becomes activated in the inner medulla collecting duct under inflammatory conditions. We therefore investigated if LPS and TNFα, either alone or under hypertonic conditions, promote nuclear accumulation of NFAT5 in miMCD cells. As depicted in Figure 7, nuclear accumulation of NFAT5 was already evident 2 hrs after subjecting miMCD cells to hypertonic conditions. While 300 mOsm/kg H_20 alone or in combination with LPS, did not result in nuclear NFAT5 accumulation, it was clearly detected in westernblot at 500 mOsm/kg H_20 (figure 7A). To exclude that LPS concentrations were too low for NFAT5 activation, also 1 and 5 µg/ml of LPS were tested but likewise failed to increase nuclear NFAT5 expression (figure 7B). Under hypertonic conditions nuclear NFAT5 expression strongly increased at 24 hrs, without synergistic effects of LPS. The addition of TNFα also did not result in nuclear NFAT5 accumulation, when applied alone or in combination with 500 mOsm/kg H_2O (figure 7C). This was also found for early time points when using higher TNFα concentrations (ranging from 12.5 to 100 ng/ml) (figure 7D).

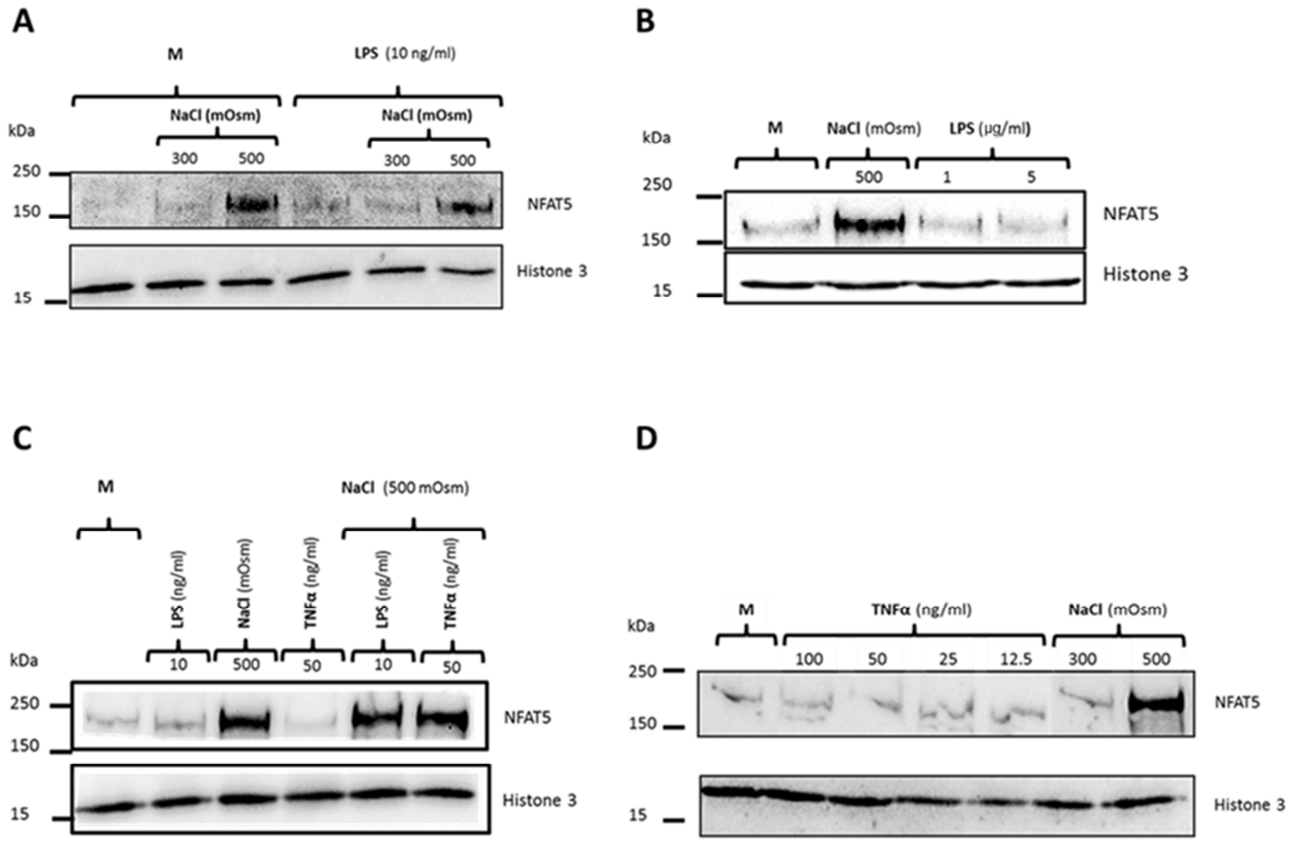


Figure 7: **Nuclear NFAT5 accumulation in response to hypertonic conditions and inflammatory stimuli in miMCD cells.** (A) miMCD cells were stimulated for 2 hrs with different concentrations of NaCl (25 and 100 mM) resulting in isotonic or hypertonic conditions (300 and 500 mOsm/Kg H_2O). Similar as described above miMCD cells were also stimulated with NaCl in the presence of LPS (10 ng/ml). Nuclear extracts were prepared as described in the methods and subjected to SDS-Page and Westernblotting. (B) NFAT5 expression was also tested in Westernblot using nuclear extracts obtained from miMCD cells stimulated for 12 hrs using higher concentrations of LPS (1 and 5 µg/ml). (C) Similar to LPS, TNFα was not able to activate NFAT5. No synergy between hyperosmotic conditions and TNFα or LPS stimulation were noticed. (D) TNFα stimulation also did not result in nuclear NFAT5 accumulation assessed 12 hrs after addition of TNFα.

3.2 Inflammatory conditions fail to increase NFAT5 mRNA transcription.

The NFAT5 protein regulates gene expression by binding to TonEBP consensus sequences in promoter regions. Since the promoter of NFAT5 also contains such sequences, NFAT5 transcriptional activity is expected to increase under hypertonic conditions [57]. There is however only limited data available as to whether inflammatory mediators have the propensity to regulated NFAT5 mRNA transcription.

Indeed we observed that NFAT5 transcription is upregulated by hypertonicity in a time- and dose-dependent manner (figure 8 A and B). In contrast, NFAT5 mRNA transcription was neither affected by LPS (figure 8C) nor by TNFα (data not shown). We also did not observe synergy between hypertonicity and LPS (figure 8D).

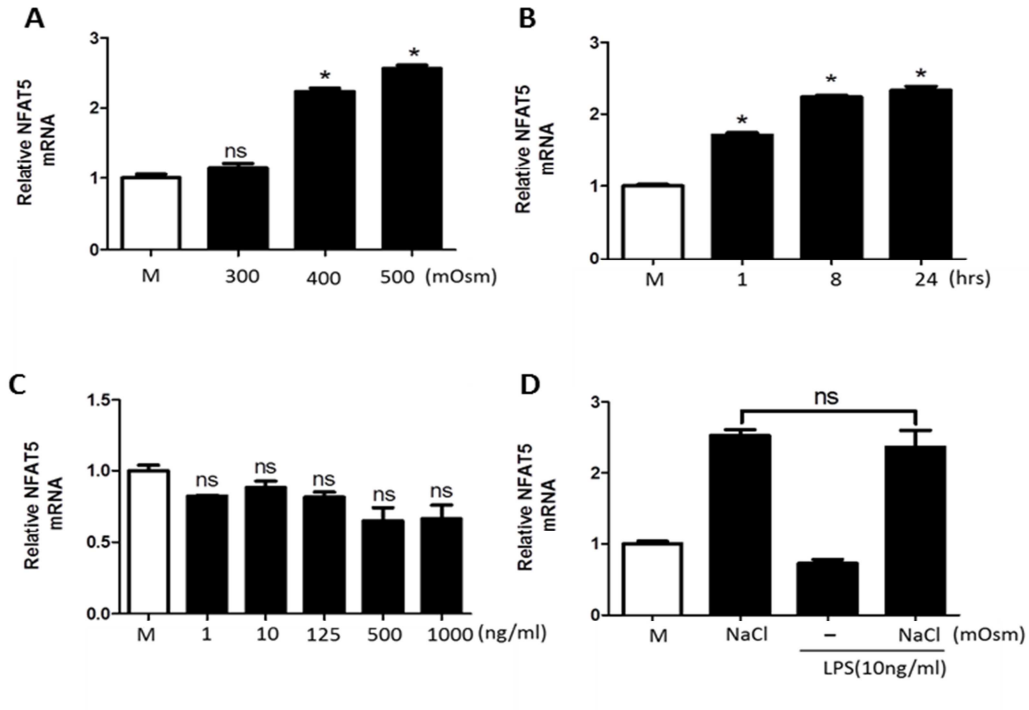


Figure 8: NFAT5 mRNA expression in miMCD cells in response to hypertonicity and LPS stimulations. (A) miMCD cells were stimulated for 8 hours with isotonic - (300 mOsm/kg·H_2O) or hypertonic (400 or 500 mOsm/kg·H_2O) NaCl concentrations (filled bars). miMCD cells cultured in normal medium served as control (open bar). (B) miMCD cells were stimulated with a hypertonic (500 mOsm/kg·H_2O) NaCl concentration for different time periods (filled bars). miMCD cells cultured in normal medium served as control (open bar). (C) miMCD cells were stimulated for 8 hours with different concentrations of LPS (1 - 1000 ng/ml) (filled bars). miMCD cells cultured in normal medium served as control (open bar). (D) miMCD cells were stimulated for 8 hours with hypertonic (500 mOsm/kg·H_2O) NaCl concentrations alone or in combination with 10 ng/ml of LPS (filled bars). miMCD cells cultured in normal medium or stimulated with LPS alone served as controls (open bars). For A-D total RNA was isolated to assess NFAT5 mRNA by qPCR analysis as described in the methods. Data are normalized to ß$_2$-microglobulin and expressed as mean NFAT5 mRNA level ± SEM relative to control unstimulated cells. *: P<0.01 (by one-way ANOVA), ns: not significant.

3.3 Hypertonic NaCl concentration increase the expression of pro-inflammatory cytokines in an NFAT5 dependent manner

Both the CCL2 and the TNFα gene contain TonEBP concensus sequences [58], we therefore assessed if miMCD cells exposed to hypertonicity (400 and 500 mOsm/Kg·H_2O, 75 and 100 mM NaCl respectively) induce CCL2 or TNFα mRNA

expression as compared to cells that remained under isotonic (300 mOsmo/kg·H_2O, 25 mM NaCL) conditions. As illustrated in figure 9, hypertonicity increased CCL2 and TNFα mRNA expression in a dose- (figure 9 upper panels) and time-dependent manner (figure 9 lower panels). For both, mRNA expression increased already at early time point reaching a peak value 8 hrs after stimulation and gradually declined at 24 hrs.

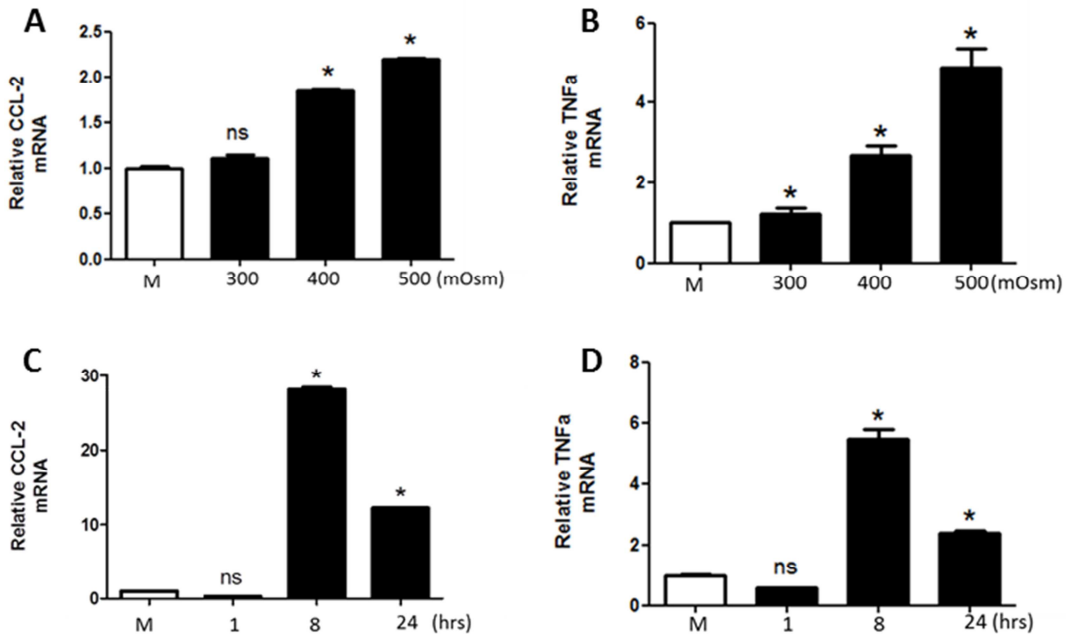


Figure 9: Hypertonic NaCl concentration increase the expression of pro-inflammatory Upper panels**:** miMCD3 cells exposed for 8 hrs to different hypertonic (300 – 500 mOsm/kg H_2O) NaCl concentrations (25 – 100 mM NaCl) (filled bars). Lower panels: miMCD3 cells exposed to hypertonic (500 mOsm/kg H_2O) conditions for different time periods (filled bars). mRNA expression of CCL2 (panels to the left) and TNFα (panels to the right) were assessed by qPCR analysis as described in the methods. Data are normalized to ß$_2$-microglobulin and expressed as mean CCL2 or TNFα mRNA level ± SEM relative to control unstimulated cells (open bars). *: P<0.01 (by one-way ANOVA), ns: not significant

To formally demonstrate that NFAT5, CCL2 and TNFα were transcriptionally regulated by NFAT5, miMCD cells were transfected with NFAT5 or scrambled siRNA and subsequently exposed for 8 hours to hypertonic (500 mOsm/Kg H2O) NaCl concentrations (figure 10). While in control (scrambled) siRNA transfected cells hypertonicity resulted in upregulation of NFAT5, TNFα and CCL2 mRNA expression, this was significantly blunted in NFAT5 siRNA transfected cells (figure 10 panels to

the left). In line with this TNFα and CCL2 concentrations in supernatants of these cells were significantly decreased (figure 10, panels to the right).

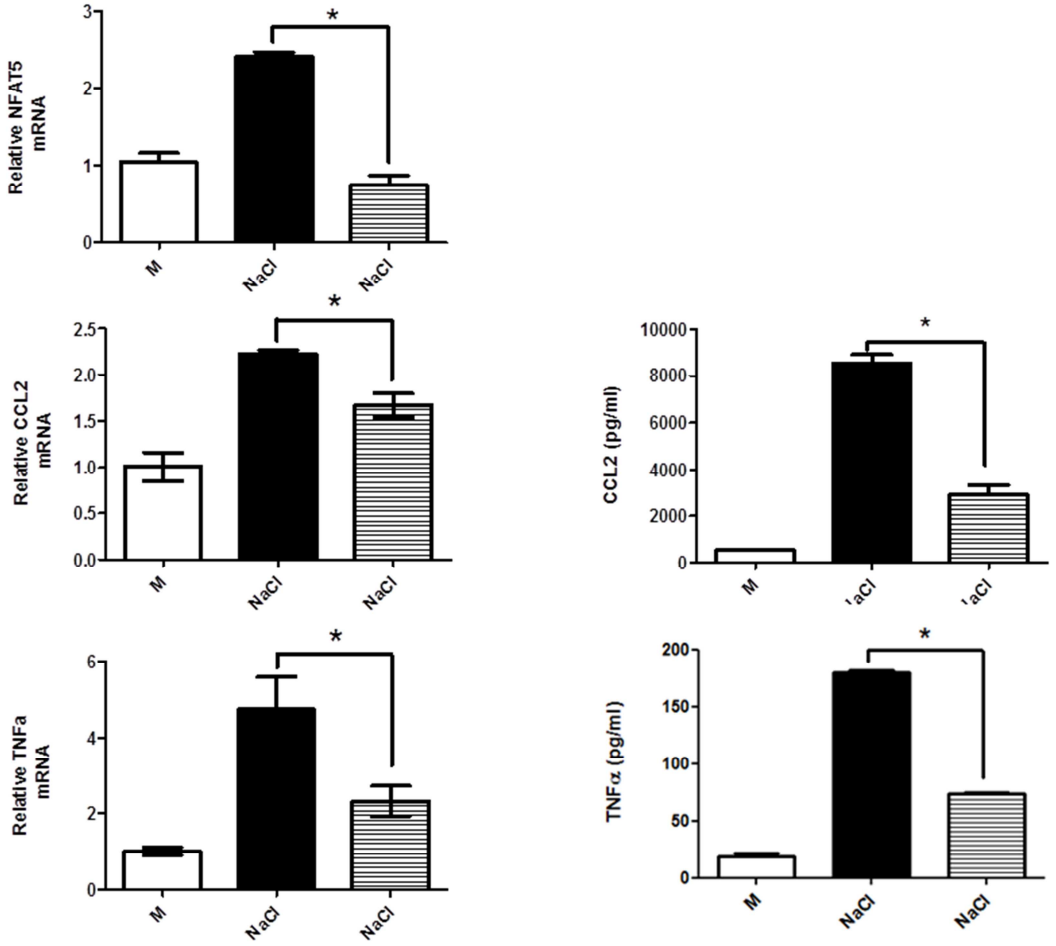


Figure 10: NFAT5 is required for the expression of CCL2 and TNFα in response to hypertonic NaCl concentrations. miMCD cells were transfected with NFAT5 (hatched bars) or scrambled siRNA (filled bars) and subsequently exposed for 8 hours to hypertonic (500 mOsm/Kg H2O) NaCl concentrations. mRNA expression of NFAT5, CCL2 and TNFα was measured by Taqman qPCR (panels to the left). Data are normalized to $ß_2$-microglobulin and expressed as mean NFAT5, CCL2 or TNFα mRNA level ± SEM relative to control unstimulated cells (open bars). CCL2 and TNFα concentrations were also assessed in the supernatants of the cells by ELISA (panels to the right) *: P<0.01 (by one-way ANOVA).

3.4 LPS-induced pro-inflammatory cytokines expression in miMCD cells is mediated by NFAT5

Although LPS did not result in nuclear NFAT5 accumulation as measured by westernblotting, it cannot be ruled out that small amounts of nuclear NFAT5 regulate TNFα and CCL2 expression in a concerted action with other transcription factors (e.g. NF-κB). We therefore first performed dose and time response experiments with

LPS to assess the optimal conditions (time and dose) for LPS stimulation in subsequent experiments using NFAT5 siRNA transfected cells. As shown in figure 11, CCL2 and TNFα mRNA transcription was detected at concentrations equal to or above 10 ng/ml of LPS (figure 11 upper panels). CCL2 and TNFα mRNA transcription displayed a similar kinetic as described for NaCl stimulation (figure 11 lower panels).

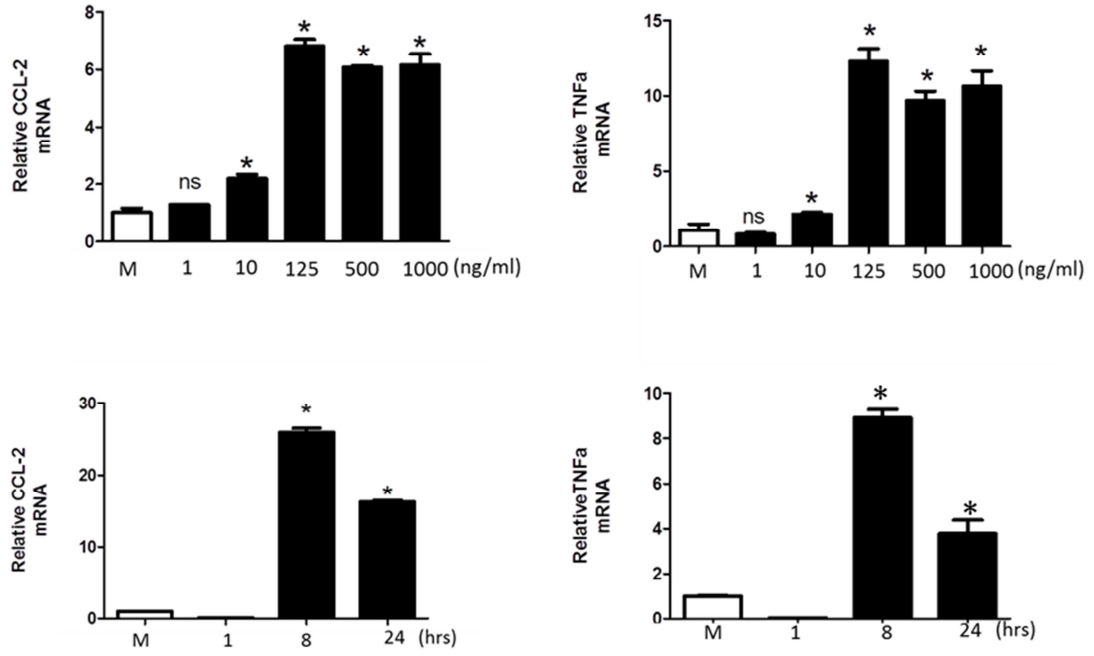


Figure 11: TNFα and CCL2 expressions in IMDC3 cells in response to LPS stimulation. Upper panels**:** miMCD3 cells were stimulated for 8 hours to different concentrations of LPS (1 -1000 ng/ml) (filled bars). Lower panels: miMCD3 cells were stimulated for different time periods with 10 ng/ml of LPS (filled bars). mRNA expression of CCL2 (panels to the left) and TNFα (panels to the right) were assessed by qPCR analysis as described in the methods. Data are normalized to ß$_2$-microglobulin and expressed as mean CCL2 or TNFα mRNA level ± SEM relative to control unstimulated cells (open bars). *: P<0.01 (by one-way ANOVA), ns: not significant.

Next we assessed to what extent NFAT5 is involved in the regulation of CCL2 and TNFα. To this end, we stimulated NFAT5 or scrambled siRNA transfected cells for 8 hours with 10 ng/ml of LPS. As depicted in figure 12, NFAT5 mRNA expression was not influenced by LPS in control siRNA transfected cells, but was slightly inhibited by LPS in NFAT5 siRNA transfected cells. In contrast, CCL2 and TNFα mRNA expression strongly increased upon LPS stimulation in control (scrambled) siRNA transfected cells, which was significantly blunted in NFAT5 siRNA transfected cells

(figure 12 panels to the left). CCL2 and TNFα concentrations in the supernatants of these cells decreased in parallel (figure 12, panels to the right).

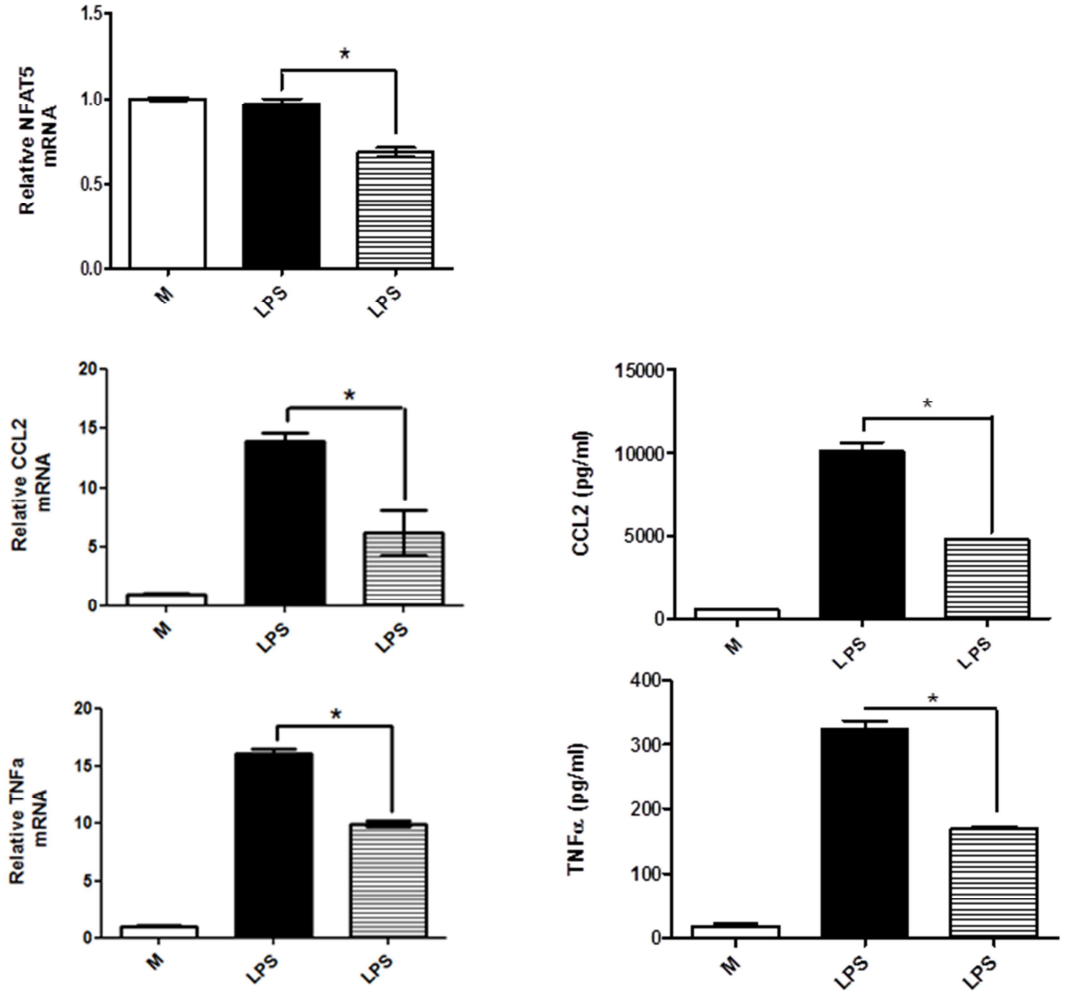


Figure 12: NFAT5 is required for the expression of CCL2 and TNFα in response to LPS. miMCD cells were transfected with NFAT5 (hatched bars) or scrambled siRNA (filled bars) and subsequently stimulated for 8 hrs with 10 ng/ml of LPS. mRNA expression of NFAT5, CCL2 and TNFα was measured by Taqman qPCR (panels to the left). Data are normalized to ß2-microglobulin and expressed as mean NFAT5, CCL2 or TNFα mRNA level ± SEM relative to control unstimulated cells (open bars). CCL2 and TNFα concentrations were also assessed in the supernatants of the cells by ELISA (panels to the right). *: P<0.01 (by one-way ANOVA), ns: not significant.

3.5 Synergy between LPS and hypertonic NaCl concentration on CCL2 and TNFα expression

Since LPS is known to activate the transcription factor NFκB while hypertonicity leads to activation of NFAT5 we assessed if there was synergy in the regulation of CCL2 and TNFα when cells were stimulated with LPS under hypertonic conditions. As depicted in figure 13, synergy was not observed for CCL2 expression, while TNFα

was significantly increased by the combination of LPS and hypertonicity as compared to either of these conditions alone. This was found for both TNFα mRNA and protein expression.

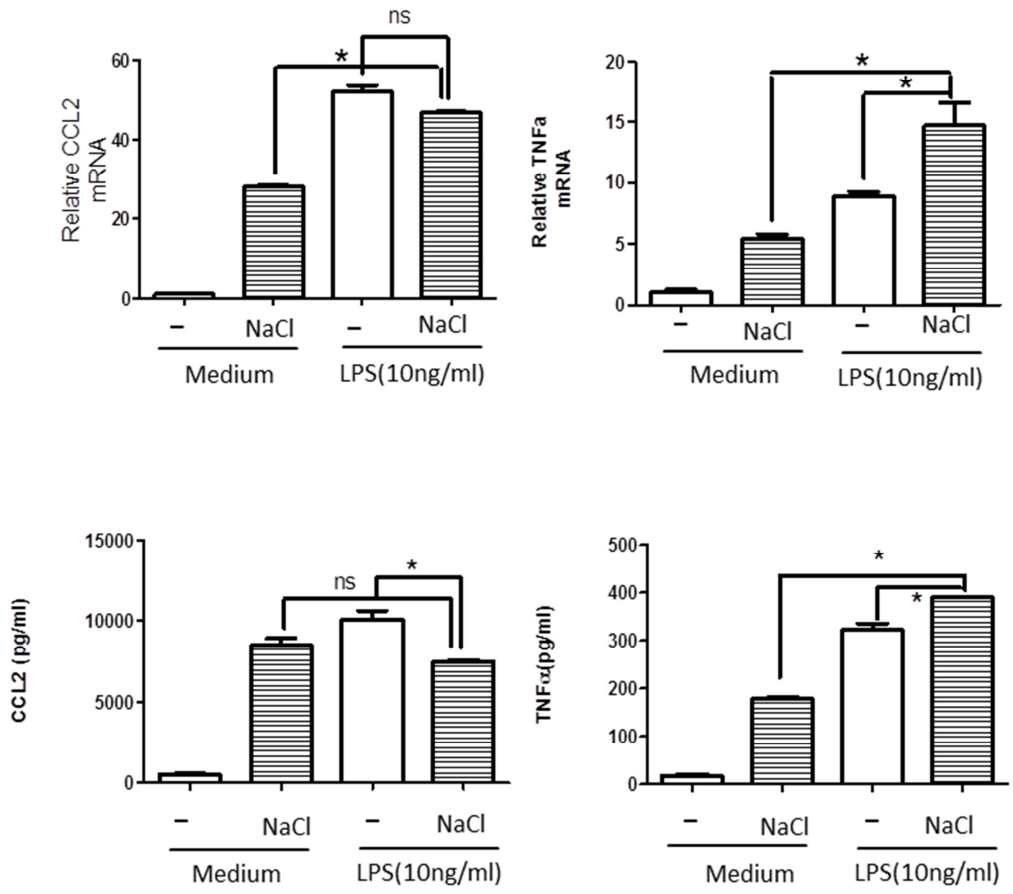


Figure 13: LPS and hypertonicity display synergy in the regulation of TNFα but not CCL2. miMCD cells were stimulated for 8 hrs with hypertonic (500 mOsm/kg H_2O) NaCl concentrations (hatched bars), LPS (10ng/m) or both. mRNA expression of CCL2 and TNFα was measured by Taqman qPCR (upper panels). Data are normalized to ß2-microglobulin and expressed as mean CCL2 or TNFα mRNA level ± SEM relative to control unstimulated cells. In the lower panels CCL2 and TNFα production in the supernatants of the cells is depicted. *: P<0.01 (by one-way ANOVA), ns: not significant.

3.6 NFAT5 is bound in the promoter of CCL2 and TNFα after stimulation

We performed ChIP-analysis to assess if NFAT5 is present at the regulatory sites of the CCL2 and TNFα promoter when miMCD cells were challenged with LPS for 2 or 4 hours. Quantification was performed by SYBR® green real-time PCR and expressed as percentage of the total not precipitated DNA and corrected for the non-specific

precipitation using rabbit IgG. Although, there were only able to pull-down a minimal amount of specific CCL2 or TNFα DNA, i.e. less than 1% of the input, we obtained reproducible assessment for CCL2 after NaCl stimulation and to a much lesser extent for TNFα after NaCl or LPS stimulation. Importantly, we were not able to pull-down NFAT5 at the CCL2 or TNFα promoter in cells that were not stimulated. (Figure 14)

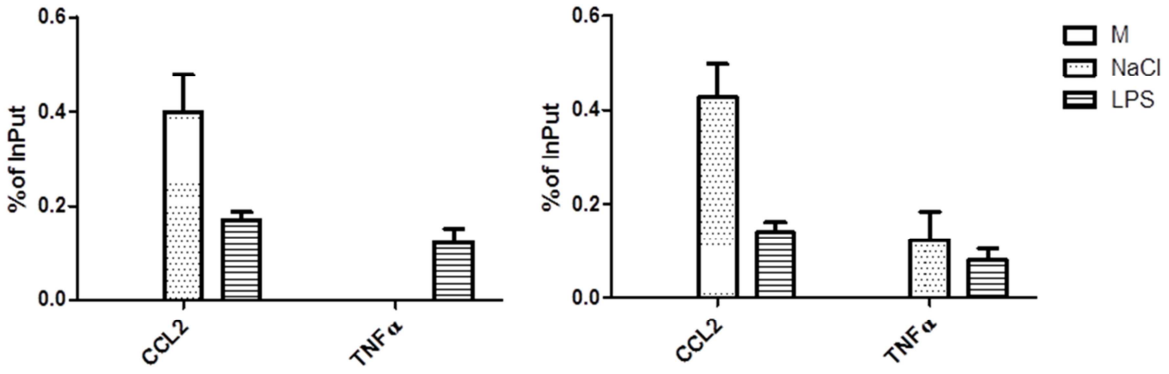


Figure 14: NFAT5 is associated with the CCL2 and TNFα promoter upon stimulation with LPS and hypertonic NaCl concentrations. miMCD cells were stimulated for 2 - (graph to the left) or 4 hours (graph to the right) with hypertonic (500mOSmo/Kg·H_2O) NaCl concentrations or LPS (1µg/ml). ChIP analysis was performed using a polyclonal NFAT5 antibody as described in the method section. The results of three independent experiments are expressed as mean % of the total input DNA ± SEM corrected for unspecific precipitation using normal Rabbit IgG.

3.7 NFAT5 partly mediates CCL2 and TNFα expression in LPS stimulated murine splenocytes.

Acute renal failure (ARF) in septic patients is frequently associated with polyuria and urine concentration defects [53]. It believed that endotoxemia decreases vasopressin V2 receptor and aquaporin-2 (AQP2) expression in vivo, which may account for the decrease in urine osmolality [59]. Moreover, endotoxemia may inactivate TonEBP/NFAT5 in a nitric oxide-dependent manner, causing down-regulation of renal medullary solute transport proteins and AQP2. We tested to what extent lack of NFAT5 affects the expression of pro-inflammatory cytokines in immune cells. To this

end, Cre^{+} $NFAT5^{flx/flx}$ mice were kept on a tamoxifen containing diet to obtain $NFAT5^{-/-}$ mice. NFAT5 was successfully deleted only in mice positive for Cre and harboring the floxed NFAT5 gene (Figure 15).

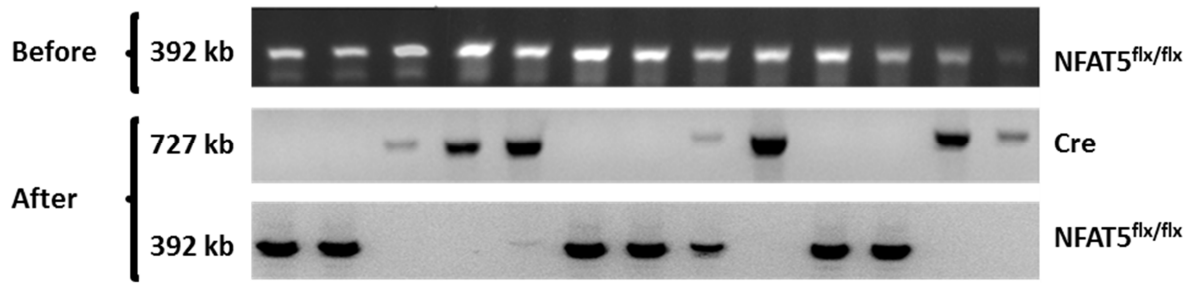


Figure 15: Tamoxifen treatment of Cre^{+} and Cre^{-} $NFAT5^{flx/flx}$ mice. Cre^{+} and Cre^{-} $NFAT5^{flx/flx}$ mice received a tamoxifin containing diet for 4 weeks. Genomic DNA was isolated from tail cuts before tamoxifin, and from splenocytes after tamoxifin treatment. PCR analysis was performed for Cre and $NFAT5^{flx/flx}$ as described in the methods. Note that NFAT5 was only detected in Cre^{-} mice but not Cre^{+} mice in genomic DNA isolated from splenocytes after 4 weeks of tamoxifin treatment.

Splenocytes were isolated from of which after diet NFAT5 is knockout (n=6) and Cre^{+} $NFAT5^{flx/flx}$ (n=6) and from control Cre^{-} $NFAT5^{flx/flx}$ (n=6) mice respectively. The cells were stimulated with LPS (1µg/ml) for 12 hours and CCL2 and TNFα concentrations were assessed in the supernatants by ELISA. As depicted in figure 16, both TNFα, and to a larger extent CCL2 production was significantly lower in splenocytes obtained from $NFAT5^{-/-}$ mice.

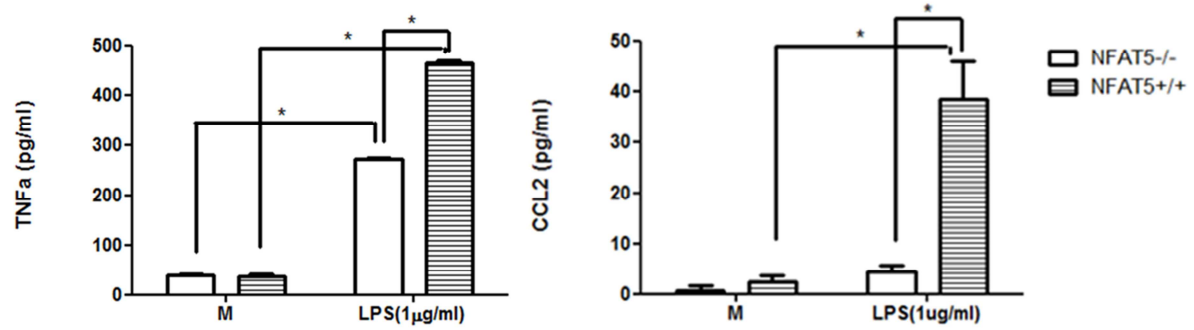


Figure 16: LPS mediated CCL2 and TNFα expression is impaired in murine splenocytes of $NFAT5^{-/-}$ mice. Cre^{+} and Cre^{-} $NFAT5^{flx/flx}$ mice (n=6 for both groups) were fed for 4 weeks with a tamoxifen containing diet. Splenocytes were isolated and stimulated with LPS (1µg/ml) for 12 hours. Supernatants were harvested and assessed for production by ELISA. Results are expressed as mean CCL2 or TNFα production ± SEM from at least three independent experiments (*P<0.01).

3.8 Nuclear NFAT5 accumulation, CCL2 and TNFα expression in LPS stimulated murine macrophage cells (RAW264.7).

Since we observed that CCL2 and TNFα production was impaired in NFAT5$^{-/-}$ mice, we studied nuclear NFAT5 accumulation and CCL2 and TNFα expression in more detail by making use of the murine macrophage cell line RAW 264.7. As depicted in figure 17, LPS led to a time dependent nuclear NFAT5 expression, which became evident 12 hrs after LPS stimulation (figure 17 upper panels). This was paralleled by an increased mRNA expression for CCL2, IL-1β and TNFα (figure 17 panels in the middle). To assess if cytokine production was partly mediated by NFAT5, siRNA experiments were carried out. Knock-down of NFAT5 resulted in a decreased CCL2 and TNFα production in supernatants of LPS stimulated RAW 264.7 cells (figure 17, lower panels).

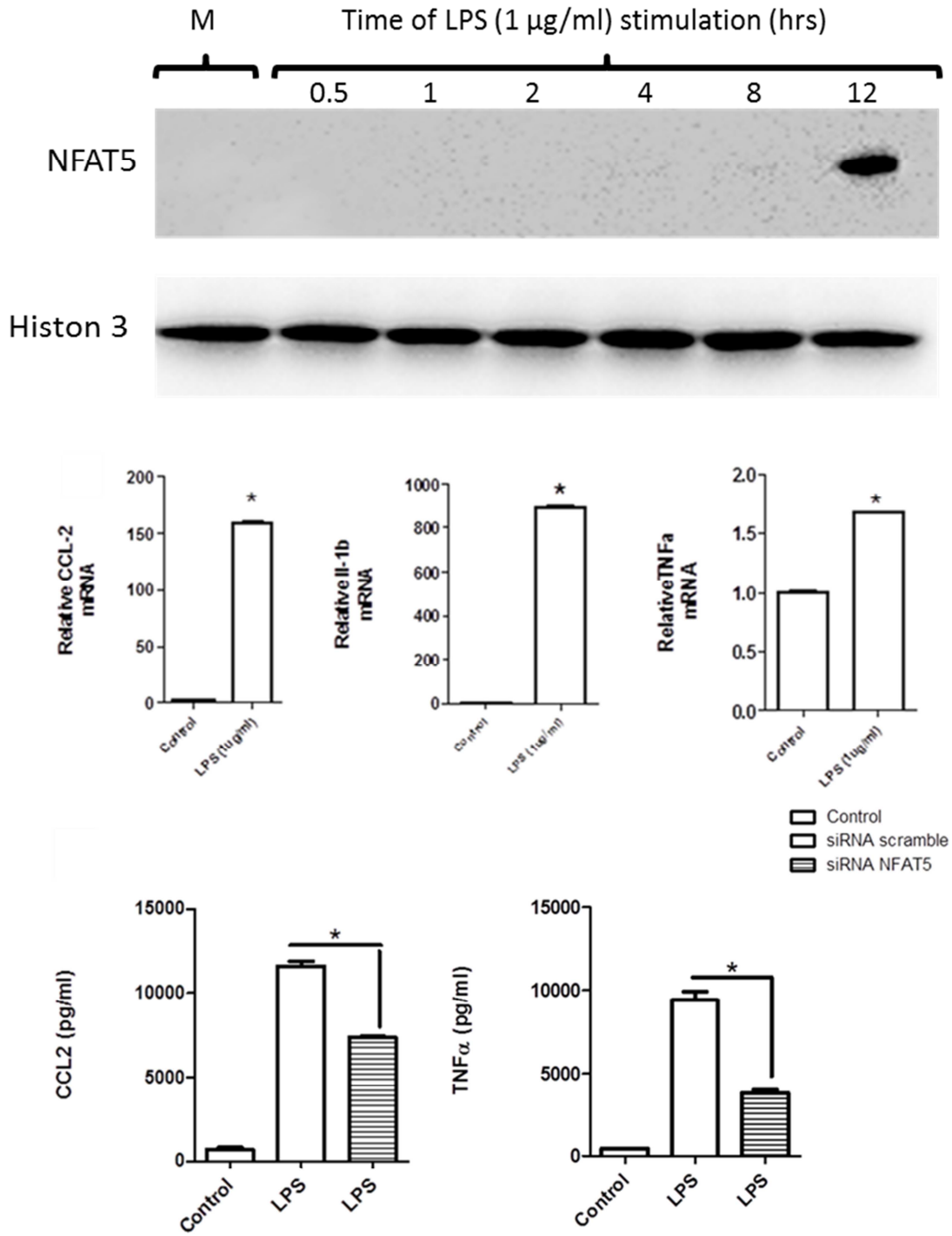


Figure 17: Nuclear NFAT5 accumulation, CCL2 and TNFα expression in LPS stimulated murine macrophage cells (RAW264.7) (Upper panels): Westernblot for nuclear NFAT5 expression in LPS (1µg/ml) stimulated RAW264.7 (Panels in the middle): mRNA expression of CCL2, IL-1ß, and TNFα was measured by Taqman qPCR. Data are normalized to ß2-microglobulin and expressed as mean CCL2, IL-1ß or TNFα mRNA level ± SEM relative to control unstimulated cells (Lower panels): TNFα and CCl2 production in culture supernatants obtained from NFAT5- and control siRNA transfected RAW264.7 12 hours after LPS (1µg/ml) stimulation were measured by ELISA. Results are expressed as mean CCL2 or TNFα production ± SEM. *: P<0.01 (by one-way ANOVA).

3.9 Influence of carbon monoxide (CO) on inflammatory mediators in LPS-stimulated RAW264.7.

There is ample evidence that carbon monoxide (CO) released from carbon monoxide releasing molecules (CORMs) is able to suppress inflammatory characteristics of murine macrophages [38, 60]. This is in part mediated via upregulation of HO-1 and inhibition of iNOS. To assess if these mediators are also influenced by CORMs that release CO directly intracellular, we made use of so-called esterase triggered (ET)-CORMs. These ET-CORMs either contained acetate (rac4) or Monomethyl fumarate (MMF-CORM) as esterfunctionality. The latter was included in this study to create a so called bi-functional CORM, not only releasing CO but also the drug Monomethyl fumarate. As depicted in figure 18, LPS mediated iNOS expression was completely inhibited by MMF-CORM but not by MMF. HO-1 was strongly induced by MMF-CORM and to a lesser extent by LPS, MMF or the combination of both.

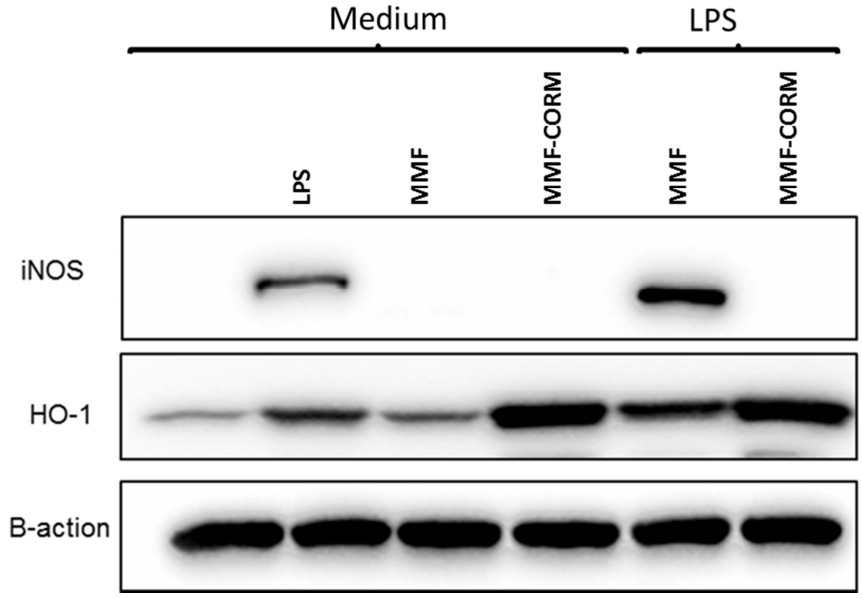


Figure 18: HO-1 and iNOS expression in murine macrophage cells (RAW264.7) Cells were cultured overnight in normal medium containing either LPS (1 µg/ml), MMF (12.5 µM) or MMF-CORM (12.5 µM). This was also carried out for the latter two conditions in the presence of LPS (1 µg/ml). Westernblot analysis was performed for iNOS, HO-1 and ß-actin to control for equal loading. The results a representative experiment from a total of 3 are shown.

We next compared rac4, which only releases CO, MMF and MMF-CORM in a dose dependent manner, to test if MMF-CORM was superior to MMF or CO alone. As

depicted in figure 19, MMF-CORM was more superior compared to rac4 or MMF in inhibiting iNOS and inducing HO-1.

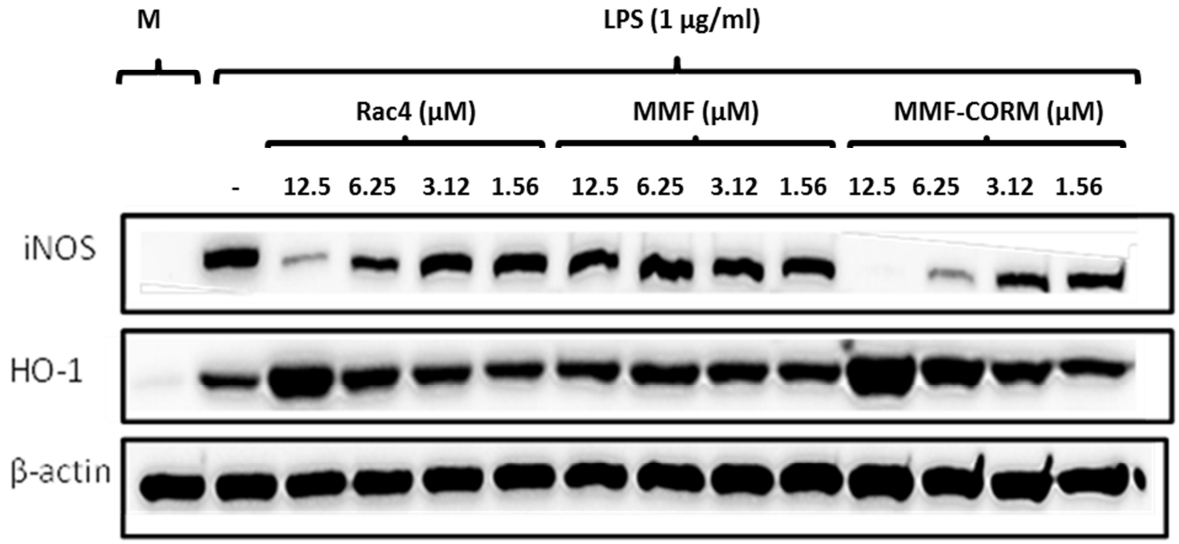


Figure 19: Efficacy of rac4, MMF and MMF-CORM to inhibit iNOS and induce HO-1 expression in murine macrophage cells (RAW264.7). Cells were stimulated overnight by LPS (1 µg/ml) in the presence of different concentrations (12.5-1.56 µM) of each rac4, MMF or MMF-CORM. Cells that were cultured overnight in normal medium served as control. Westernblot analysis was performed for iNOS, HO-1 and ß-actin to control for equal loading. The results a representative experiment from a total of 3 are shown.

Next we assessed the influence of CO, released either from a simple ET-CORM (rac4) or from a bifunctional ET-CORM (MMF-CORM), on the expression of CCL-2 and IL-1β mRNA in RAW264.7 cell stimulated by LPS for 12 hours (figure 20, upper panels). Likewise we tested if CCL2 and TNFα protein expression was influenced by these compounds. As demonstrated in figure 20, MMF-CORM strongly inhibited the mRNA expression of CCL-2 and IL-1β, suggesting a strong synergy between CO release and MMF. While this was also observed for CCL2 production in supernatants of RAW264.7 cells the inhibitory properties on TNFα production were less but still significant (figure 20, lower panels).

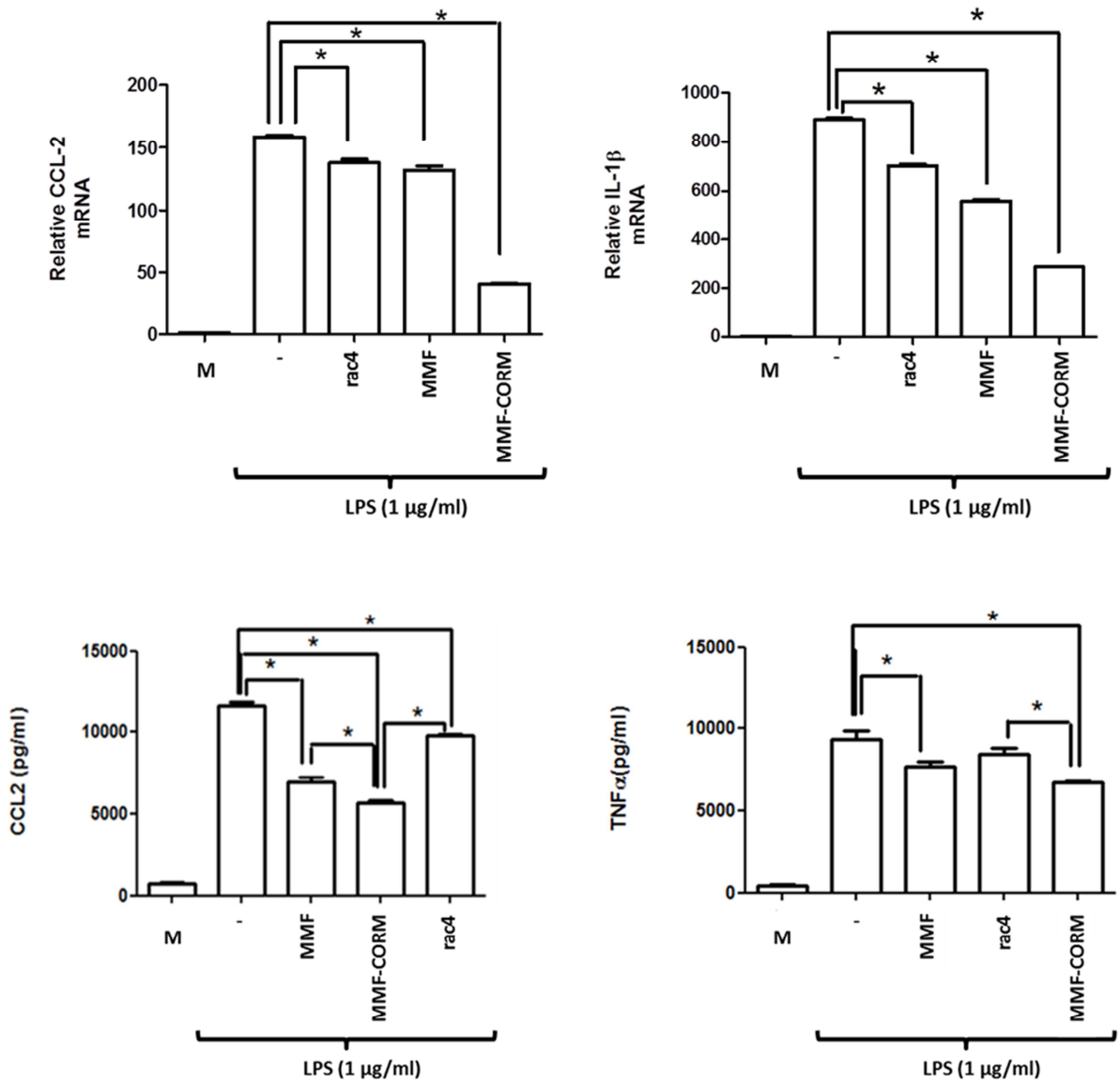


Figure 20: Efficacy of rac4, MMF and MMF-CORM to inhibit the expression of pro-inflammatory cytokines in murine macrophage cells (RAW264.7). Cells were stimulated for 12 hours with LPS (1 µg/ml) in the presence of rac4, MMF or MMF-CORM (all at 12.5 µM). Cells that were cultured in normal medium (M) or stimulated with LPS alone (-) served as control. Upper panels: mRNA expression of CCL2 and IL-1ß, TNFα was measured by Taqman qPCR. Data are normalized to ß2-microglobulin and expressed as mean CCL2, or IL-1ß mRNA level ± SEM relative to control unstimulated cells. The results a representative experiment from a total of 3 are shown. Lower panels: CCl2 and TNFα production in culture supernatants were measured by ELISA. Results are expressed as mean CCL2 or TNFα production ± SEM. *: P<0.01 (by one-way ANOVA).

3.10 Nuclear NFAT5 accumulation is inhibited by MMF-CORM in murine macrophage cells (RAW264.7)

Since it has previously been reported that NFAT5 is essential for LPS mediated induction of iNOS, we investigated if CORMs prevent nuclear NFAT5 accumulation. While nuclear NFAT5 expression slightly increased after LPS stimulation, it was strongly suppressed by MMF-CORM but not rac4 or MMF (figure 21).

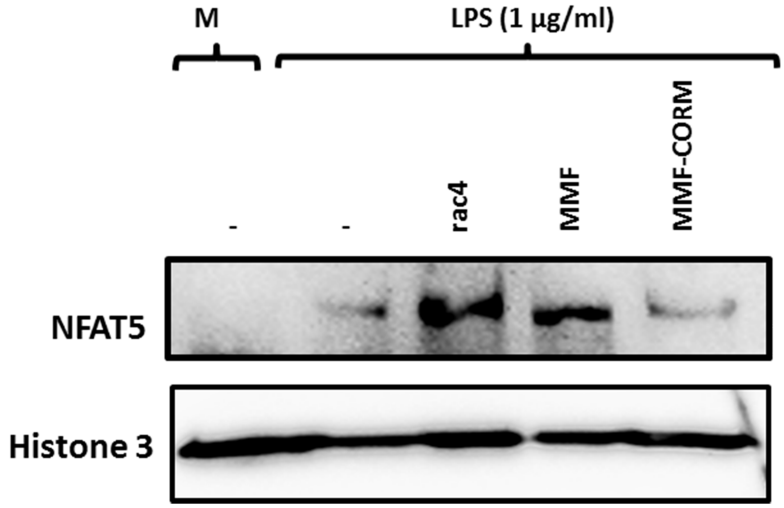


Figure 21: Nuclear NFAT5 expression is inhibited by MMF-CORM in murine macrophage cells (RAW264.7). Westernblot for nuclear NFAT5 expression in LPS stimulated RAW264.7. Cells were stimulated for 12 hours with LPS (1 µg/ml) in the presence of rac4, MMF or MMF-CORM (all at 12.5 µM). Cells that were cultured in normal medium (M) or stimulated with LPS alone (-) served as control.

3.11 MMF-CORM inhibits TNFα and IL-10 production in human monocytes

Finally we studied if MMF-CORM is able to inhibit the expression of LPS stimulated monocytes. Because we have previously demonstrated [44], that release of CO from ET-CORM is partly depending on the position of the ester functionality within the iron tricarbonyl complex, in the present study we tested if this also holds true for the position of the methylfumarate ester. To this end, we used MMF-CORM-I (innerposition) and MMF-CORM-O (outerposition) (for both see Fig 5. in the introduction of this thesis) and compared the results with MMF alone. While MMF-CORM-O strongly inhibited the production of TNFα and IL-10 in a dose dependent

manner, only IL-10 was inhibited and to a lesser extent as compared to MMF-CORM-I. No influence of MMF alone was noted at equimolar concentration (figure 22).

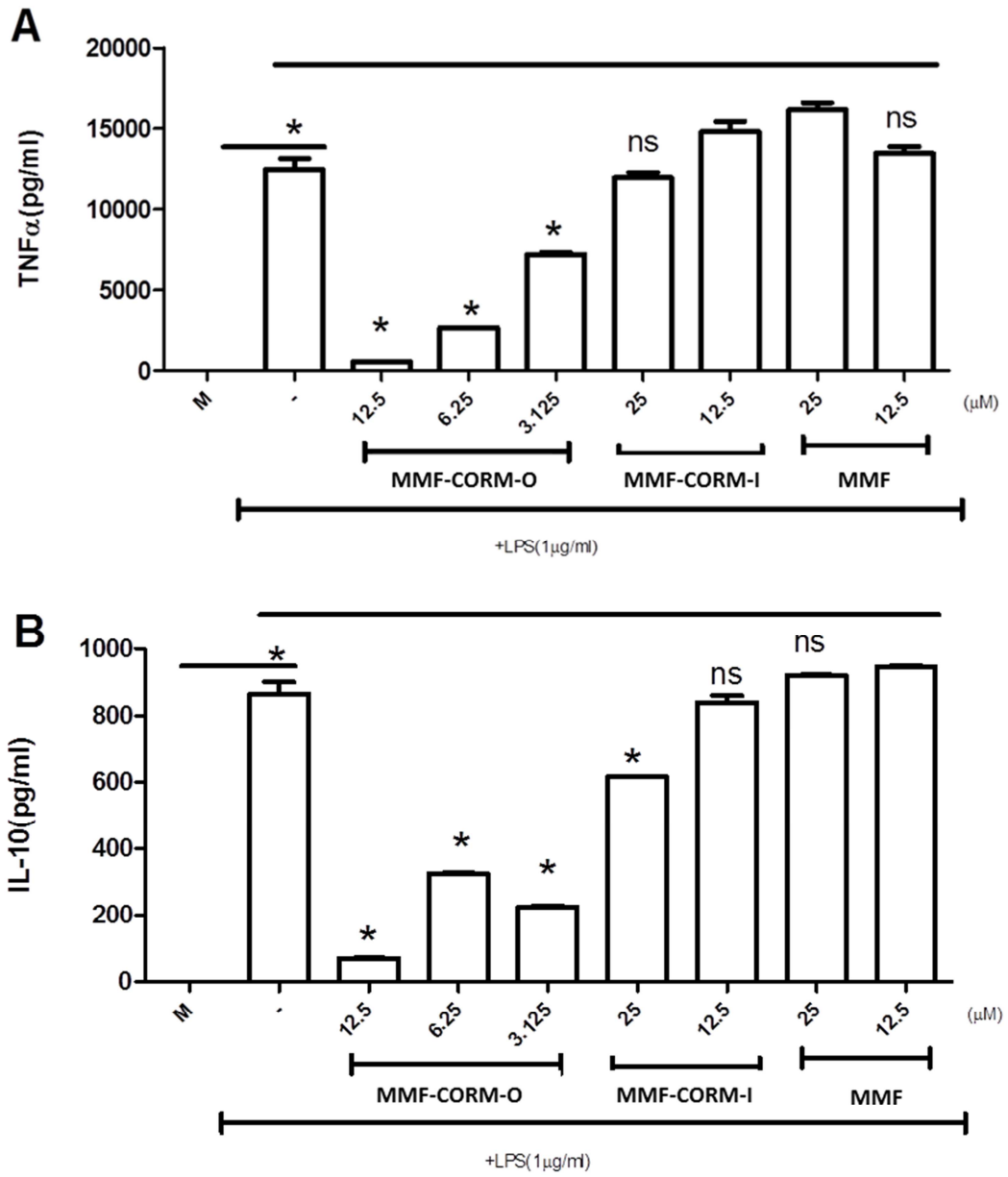


Figure 22: Influence of ester position in MMF-CORM on LPS mediated TNFα and IL-10 production in human monocytes. Human monocytes were isolated from buffy coat and stimulated for 12 hours with LPS (1 µg/ml) in the presence of MMF-CORM-O (12.5 - 3.125 µM), MMF-CORM-I (25 and 12.5 µM) or MMF (25 and 12.5 µM). Cells that were cultured in normal medium (M) or stimulated with LPS alone (-) served as control. TNFα (A) and IL-10 (B) production in supernatants was assessed by ELISA. Results are expressed as mean TNFα or IL-10 production ± SEM. *: P<0.01 (by one-way ANOVA).

4 DISCUSSION

4.1 The role of NFAT5 in inflammation induced by hypertonicity stress

The renal interstitial fluid contains high NaCl and urea concentrations to provide an osmotic gradient required for the concentration of urine. Hence, renal medullary cells are embedded in a hyperosmolar milieu that may cause damage to these cells. Under some conditions the interstitial fluid may reach osmolality up to 1200 mOsmol/kg at the tip of the inner medulla. To avoid significant shriveling and shrinking that leads to cell death, cells elicit a genetic program of osmoregulation that gradually replaces electrolytes like Na^+, Cl^-, and K^+ by small, uncharged organic osmolytes like sorbitol, betaine, and myo-inositol. This response is to a large extent mediated by the transcription factor NFAT5/TonEBP [61]. However NFAT5/TonEBP seems also to regulate the expression of pro-inflammatory cytokines such as CCL2 and TNFα. Indeed our in vitro experiments clearly indicate that murine inner medullary collecting duct (iMCD) cells exposed to hyperosmolar stress (NaCl 100 mM) upregulate mRNA expression of CCL2 and TNFα. The involvement of NFAT5/TonEBP in the regulation of CCL2 and TNFα was supported by the finding that NFAT5/TonEBP siRNA abrogated hyperosmolar stress induced expression of CCL2 and TNFα. Our results are in agreement with the findings of Kojima R et al. that hypertonicity also initiates a pro-inflammatory response in the rat epithelial NRK-52E cells as reflected by the upregulation of CCL2 [58]. Moreover they demonstrate that the 5'-flanking region of the CCL2 gene indeed contains a hypertonicity-sensitive cis-acting element, i.e. a NFAT5/TonEBP binding site. Our data also demonstrate that NFAT5/TonEBP is recruited at the CCL2 promoter during hyperosmolar stress as evidenced by ChIP.

It has been suggested that NFAT5/TonEBP and NF-κB act in concert to upregulate the expression of CCL2 and TNFα during hyperosmolar stress. Roth I et al [62] demonstrated a tonicity-dependent interaction between NFAT5 and NF-κB p65 that significantly enhances the transcription of NF-kB-regulated genes. Although a physical or functional interaction between both transcription factors was not investigated in our study, our data are in concordance to previous finding that hypertonicity indeed can induce inflammation in a NFAT5/TonEBP dependent manner. Hence it seems that apart from protecting cells against hyperosmolar stress

NFAT5/TonEBP may jeopardize these cells through the induction of pro-inflammatory cytokines.

4.2 **Activation of NFAT5/TonEBP by pro-inflammatory mediators**

In recent years evidence has accumulated suggesting that NFAT5/TonEBP not only is involved in osmoregulation but, independently of hypertonicity, also regulates inflammation. As reported by Lee.HH et al., NFAT5/TonEBP is contributing to the formation of the LPS-induced NF-kB enhancesome and as such is required for the recruitment of p300 in macrophages [63]. However, whether NFAT5/TonEBP is activated by inflammatory cytokines in renal iMCD cells is not well studied.

Although our data indicate that nuclear NFAT5/TonEBP accumulation, as assessed by westernblot, does not occur in LPS or TNFα stimulated iMCD cells, NFAT5/TonEBP siRNA transfection significantly decreased CCL2 and TNFα expression upon LPS stimulation as compared to cells transfected with control siRNA. Moreover, ChIP revealed recruitment of NFAT5/TonEBP at the CCL2 and TNFα promoters upon LPS stimulation of iMCD cells, albeit to a lesser extent as compared to hypertonicity. It does seem that only minimal amounts of NFAT5/TonEBP are recruited, not sufficient enough to become detected by westernblot, yet functionally active in the regulation of CCL2 and TNFα. These data are consistent with the findings by Roth I et al. that LPS cannot induce nuclear NFAT5/TonEBP accumulation nor does it upregulate NFAT5/TonEBP mRNA transcription. Nonetheless there are some discrepancies between our findings and those of Roth I et al. while in the latter study it was found that NFAT5/TonEBP siRNA only downregulated TNFα but not CCL2 mRNA, our own study shows that both TNFα and CCL2 expression is affected in NFAT5/TonEBP siRNA transfected cells. This incongruity might be explained by differences between rat and murine collecting duct cells. It should however be emphasized that the data presented by Roth et al. also showed a reduced CCL2 expression but this did not reach statistical significance. Altogether, our finding demonstrated that LPS mediated TNFα and CCL2 expression in murine iMCD cells is partly regulated by NFAT5/TonEBP. Interestingly we did observe that concurrent stimulation of these cells with LPS and hyperosmolar concentrations of NaCl synergistically increased TNFα but not CCL2 expression. Thus, our data are compatible with the assumption that there is a cooperative action between NFAT5/TonEBP and NF-kB in response to inflammatory cytokines.

4.3 Splenocytes obtained from conditional NFAT5/TonEBP knockout mice display reduced cytokine responses upon LPS stimulation

As conventional NFAT5/TonEBP knockout mice show high perinatal lethality due to impaired renal [64] and heart development [65] as well as impaired immune response [66], we studied the role of NFAT5/TonEBP in inflammatory responses by making use of conditional NFAT5/TonEBP knockout mice. In these mice the 4th NFAT5/TonEBP exon flanked by loxP sites allowing cre-mediated recombination of this allele [56]. NFAT5/TonEBP$^{flx/flx}$ mice were crossed with mice expressing the cre-recombinase gene behind a tamoxifen inducible ubiquitin promoter. Our data showed that NFAT5/TonEBP was successfully deleted after 4 weeks of feeding a tamoxifen containing chow. Splenocytes isolated from these mice produced significantly lower amounts of pro-inflammatory cytokines after LPS stimulation as compared to NFAT5/TonEBP$^{+/+}$ mice. These findings are in agreement with studies from Buxade M et al. [27] demonstrating that TNFα and CCL2 expression is down regulated in LPS stimulated NFAT5/TonEBP$^{-/-}$ BMDM. Likewise, Lee H [63] et al. showed that myeloid-specific deletion of NFAT5/TonEBP decreases systemic inflammation in sepsis. In *in vivo* experiments, LPS stimulation increased nuclear NFAT5/TonEBP expression in wild type macrophage, while in “haplo-insufficient” NFAT5/TonEBP$^{+/-}$ These and our own data collectively indicate that NFAT5/TonEBP plays an essential role in regulating cytokines responses upon LPS stimulation.

Buxade M et al. postulated that in BDMDs NFAT5/TonEBP is constitutively bound to the TNF gene promoter regardless of TLR stimulation. This suggests that TNF might be an early primary response gene which is already associated with certain transcription factors and chromatin remodeling complexes under resting conditions [67, 68]. This pattern of constitutive NFAT5/TonEBP binding was also observed in other primary response target genes, i.e.IL1a, Traf1 and CCL2 [27]. Our own ChIP data showed that in murine iMCD cells NFAT5/TonEBP is not associated with the TNFα and CCL2 promoters under basal conditions. Hence it seems that constitutive NFAT5/TonEBP binding to promoters of pro-inflammatory genes might differ between tissues, while in response to LPS stimulation NFAT5/TonEBP seems to be recruited to these promoters irrespective of the cell-type.

Apart from its role in inflammation, recent studies have also suggested a role in Coxsackievirus B3 (CVB3) induced myocarditis [69] and muscle regeneration [70].

4.4 **Synergy between carbon monoxide (CO) and methyl fumarate**

A number of studies are currently performed to assess the potential clinical use of a variety of bifunctional gasotransmitters-based complexes. Naproxcinod is the first in a new class of anti-inflammatory agents known as CINODs (Cyclooxygenase-Inhibiting Nitric Oxide Donators) in development [71]. It is a nitrosylated naproxen derivative which can release nitric oxide and naproxen. As such, Naproxcinod retains the classic therapeutic profile of NSAID but prevents or reduces the adverse effect of NSAID, e.g. gastrointestinal bleeding [72] and increased blood pressure [73]. Similar to NO, in rodent models H_2S also causes less NSAID mediated gastrointestinal damage and exerts various beneficial effects on cardiovascular and metabolic disorders [74-78].

In the present study we tested the biological efficacy of bifunctional esterase triggered CO releasing molecule (ET-CORM) with monomethyl fumarate (MMF) as ester functionality (MMF-CORM). The mechanism by which MMF-CORM releases carbon monoxide and fumarate consist of two steps. Firstly, intracellular esterases recognize the ester bond and hydrolyze the complex to release monomethyl fumarate and the remaining enol complex. This complex is oxidative labile and disintegrates by oxidation thereby releasing carbon monoxide, ferric iron and 2-cyclohexenone in a ratio of 3:1:1. Previously we demonstrated that the position of the ester moiety in ET-CORMs determine the rate of CO release and thus affects it biological efficacy. In case the ester functionality is at the outer position of the cyclohexanone moiety the biological efficacy is in general increased. In analogy to this, we tested the efficacy of MMF-CORM with MMF either at the inner (MMF-CORM-I) or outer (MMF-CORM-O) position. In line with previous data using different ET-CORMs [43, 44], we also could show that MMF-CORM-O is more efficacious to inhibit IL-10 and TNFα expression in human monocytes in response to LPS stimulation. Importantly, our data showed that monomethyl fumarate itself did not suppress IL-10 and TNFα production, indicating that the anti-inflammatory effect of MMF-CORM is either due to the release of CO or CO is acting in concert with MMF to inhibit cytokine production. To explore these possibilities further, we compared the biological efficacies of a simple ET-CORM, i.e. *rac*-**4**, with MMF-CORM and MMF alone. Like *rac*-**4**, MMF-CORM-O has an ester functionality at the outer position but instead of MMF the ester being used as acetate. While *rac*-**4** also inhibited TNFα, CCL2 and IL-1ß in LPS stimulated RAW264.7 cells, MMF-CORM-O was clearly more efficacious as compared to MMF alone or *rac*-**4.**

There are three types of nitric oxide synthase (NOS), i.e. endothelial - (eNOS), neuronal - (nNOS), and inducible nitric oxide synthase (iNOS). Endothelial nitric oxide synthase exists mainly in vascular endothelial cells while iNOS exists mainly in the cytoplasm of some inflammatory cells, e.g. macrophages. NO is produced mainly by eNOS catalysis, and also by the upregulation of iNOS expressions during inflammation. It has been reported that iNOS is induced to produce large amounts of NO by lipopolysaccharide, interleukin-1 (IL-1), and tumor necrosis factor (TNF), which play a role in the pathophysiological process of some diseases and in many inflammation and immune reactions [79]. Although it also acts as an effector molecule in innate immunity, overproduction of NO might be harmful and have been linked to many chronic inflammatory diseases. Based on animal experiments, selective inhibition of iNOS appears to be a promising means for the treatment of inflammatory diseases, although iNOS inhibitors have not yet shown to be beneficial in clinical trials [80, 81] [82].

Since it has been postulated that urinary concentration problems in septic patients might be due to overproduction of NO that subsequently leads to nitrosylation of NFAT5/TonEBP, we explored if a bifunctional CO releasing molecule may have a potential clinical benefit by inhibiting both iNOS expression and reducing the production of pro-inflammatory cytokines. MMF in conjunction with CO was studied to this end, since both compounds have been reported to inhibit iNOS and to reduce inflammation. Indeed our results show that iNOS expression was markedly inhibited by MMF-CORMs in LPS-stimulated RAW264.7.

A variety of studies have revealed that the anti-inflammatory properties of carbon monoxide and CORMs are mediated via various signal transduction pathways. Lee.D et al. reported that CORM3 negatively regulates NLRP3 inflammasome activation in macrophages in a glycolysis-dependent manner [83]. Qin S et al. demonstrated that the nuclear factor-erythroid 2-related factor-2 (Nrf-2) is essential for the anti-inflammatory effect of CORM2 as it could no longer reduce cytokine expression in septic Nrf-2 knock-out mice [38]. Choi E et al. showed that CORM3 suppresses the production of NO and IL-1β in LPS-activated murine macrophages via HO-1 induction and inhibition of the NF-kB and STAT1 pathways [84].

As mentioned above our data indicate that MMF-CORMs can suppress iNOS expression. Preliminary ongoing experiments suggest that MMF-CORM inhibits nuclear accumulation of NFAT5 in RAW264.7 cells. This assumption is supported by

the studies from Buxade M et al.[27] in which it has been found that NFAT5/TonEBP is a IKKβ-dependent regulator of iNOS expression. However, because *rac*-**4** also inhibits iNOS expression but not nuclear NFAT5/TonEBP accumulation further experiment are required to address if indeed NFAT5/TonEBP is a genuine target of CO.

Like already mentioned for cytokine expression, MMF-CORM were far more effective in inhibiting iNOS as compared to MMF or *rac*-**4** alone. This was also found for the induction of HO-1. It thus seems that CO and MMF act synergistically to inhibit cytokine and iNOS expression and to induce the expression of HO-1.

Although HO-1 is generally considered as a protective mediator due to its an anti-oxidative and anti-inflammatory properties [85-87], recent studies have suggested that HO-1 might be harmful in triggering LPS-induced cardiac dysfunction in vivo [88]. This is based on the fact that HO-1 can interact with iNOS, thereby preventing lysosomal degradation of the iNOS protein and thus more NO-production leading to reactive nitrate species.

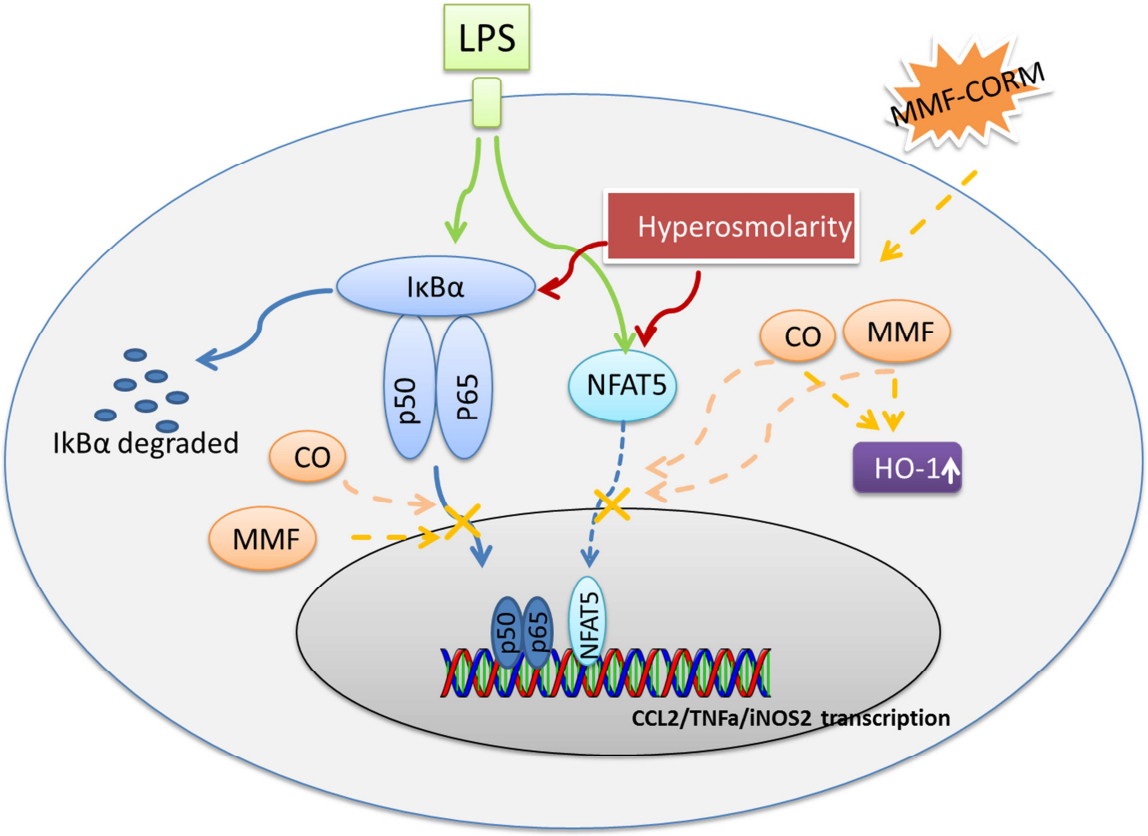


Figure 23 Putative signaling network of hyperosmolarity and LPS challenges and effect of MMF-CORMs treatment based on the results of the present study and previous findings. (1) Hypertonicity and LPS activate NF-kB and NFAT5 translocation to the nucleus and initiates CCL2/TNFα transcription in the inner medullar collecting duct cell. (2) MMF-CORM may inhibit nuclear NFAT5 accumulation and thereby downregulating cytokines and iNOS expression in macrophages. Also the upregulation of HO-1 may contribute to the anti-inflammatory properties.

5 CONCLUSION

Tonicity-Responsive Enhancer Binding Protein (TonEBP), also known as nuclear factor of activated T cells 5 (NFAT5), is a ubiquitously expressed transcription factor involved in osmoregulation. As such TonEBP/NFAT5 regulates the expression of various osmoprotective genes including transporters and synthetic enzymes mediating the intracellular accumulation of small, compatible organic osmolytes. More recently studies have revealed that TonEBP/NFAT5 is also implicated in diabetic microvascular disease in the kidney, inflammatory bowel diseases, and hypertension. In addition to its osmoprotective and homeostatic roles, TonEBP/NFAT5 is also involved in regulating the expression of a variety of pro-inflammatory genes in immune cells.

Our data presented here reveal that TonEBP/NFAT5 not only is essential for CCL2 and TNFα expression in miMCD cells subjected to hyperosmolar conditions, but also contributes to CCL2 and TNFα expression in response to LPS stimulation. With regard to hypertonicity, we observed that nuclear TonEBP/NFAT5 accumulation occurred in a time and dose dependent manner and was associated with increased CCL2 and TNFα mRNA and protein expression. In TonEBP/NFAT5 siRNA transfected miMCD cells CCL2 and TNFα mRNA and protein expression were significantly blunted after hyperosmolar stress. ChIP analysis indeed revealed TonEBP/NFAT5 binding to the CCL2 and TNFα promoters after stimulation. Like hyperosmolar stress also in LPS stimulated miMCD3 TonEBP/NFAT5 regulated CCL2 and TNFα expression, albeit that nuclear TonEBP/NFAT5 accumulation was not detected by westernblot analysis. Nonetheless both ChIP analysis and siRNA experiments revealed that small but functionally active amounts of TonEBP/NFAT5 were recruited to the CCL2 and TNFα promoters and partly required for the expression of these cytokines

In line with previous publications on immune cells we also demonstrated that, TonEBP/NFAT5 is important for the production of systemically released cytokines, e.g. TNFα and CCL2, in the course of sepsis. While LPS stimulation of splenocytes obtained from wild type mice resulted in profound expression of CCL2 and TNFα, in splenocytes obtained from conditionally TonEBP/NFAT5$^{-/-}$ mice this response was significantly diminished.

Since sepsis-induced urinary concentration defects are believed to be related to nitric oxide-dependent inactivation of TonEBP/NFAT5, we also explored the potential of carbon monoxide (CO) and monomethyl fumarate (MMF) to inhibit iNOS expression in murine macrophages. Both CO and MMF have been reported to inhibit iNOS expression and inhibit inflammatory mediators in vivo and vitro. CO (rac-4: esterase triggered (ET) CO releasing molecule (CORM)) and MMF were either applied alone or as so called bifunctional MMF-CORM. With respect to iNOS inhibition and HO-1 induction we found that administration of MMF-CORM exert stronger effects than either application of ET-CORM (rac-4) or monomethyl fumarate (MMF) alone. As NFAT5 is the critical mediator of iNOS expression in macrophage, we further studied if inhibition of iNOS could be attributed to impaired nuclear TonEBP/NFAT5 translocation. Preliminary ongoing experiments indeed suggest that MMF-CORM inhibits nuclear accumulation of NFAT5 in RAW264.7 cells. This finding is supported by the studies from Buxade M et al. in which it has been found that NFAT5/TonEBP is a IKKβ-dependent regulator of iNOS expression. However, because rac-4 also inhibits iNOS expression but not nuclear NFAT5/TonEBP accumulation further experiment are required to address if indeed NFAT5/TonEBP is a genuine target of CO.

In conclusion, this study revealed that NFAT5 is an essential mediator of inflammation in medullar collecting duct cells that are either challenged by hyperosmolar stress or LPS. Bifunctional MMF-CORM may be of clinical relevance in the treatment of sepsis induced acute kidney injury as it not only strongly diminishes the expression of systemically released pro-inflammatory cytokines, but also inhibits iNOS expression. The latter might be important for preventing sepsis induced urinary concentration defects.

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7 CURRICULUM VITAE

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