Chemokines (CCL3, CCL4, CCL5) inhibit ATP-induced release of IL-1β by monocytic cells

Inaugural-Dissertation
for attaining the title of Doctor of Medicine
from the Faculty of Medicine
of the Justus Liebig University Giessen

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Giessen 2018

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Date of Doctoral Defense: 13.03.2019

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IV. List of abbreviations

AA	arachidonic acid
ACh	acetylcholine
ADP	adenosine diphosphate
al.	alii
AP-1	activator protein 1
APS	ammonium persulfate
ASC	apoptosis-associated speck-like protein containing a CARD
ATK	arachidonyl trifluoromethyl ketone
ATP	adenosine triphosphate
BEL	bromoenol lactone
bp	base pair
BSA	bovine serum albumine
BzATP	2'(3')-O-(4-Benzoylbenzoyl)adenosine-5'-triphosphate
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CAPS	cryopyrin-associated autoinflammatory syndrome
CARD	C-terminal caspase activation and recruitment
CARS	compensatory anti-inflammatory response
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyltrasferase
COPD	chronic obstructive pulmonary disease
cPLA2	cytosolic phospholipase A2
DAMP	damage associated molecular pattern
DC	dendritic cell
dH_2O	aqua desillata
DIRA	deficiency of the interleukin-1 receptor antagonist
DNA	double-stranded deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPPC	dipalmitoylphosphatidylcholine
EAE	experimental autoimmune encephalomyelitis

EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
G-PC	glycerophosphocholine
GAG	glycosaminoglycan
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine-5'-triphosphate
h	hour(s)
HCl	hydrochloric acid
HF	high molecular mass fraction
HIV	human immunodeficiency virus
HMBS	hydroxymethylbilane synthase
HMGB1	high-mobility group box 1
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HSP	heat shock protein
Ig	immunoglobulin
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
iPLA2β	calcium-independent phospholipase A2β
Jak	Janus kinase
K^+	potassium
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
kDa	kilodalton
LDH	lactate dehydrogenase
LPA	lysophosphatidic acid
LPC	lysophasphatidylcholine
LPS	lypopolysaccharid
mA	milliampere
mAChR	muscarinic acetylcholine receptor
MAPK	mitogen-activated protein kinase

MCP	monocyte chemotactic protein
Mec	mecamylamine hydrochloride
$MgCl_2$	magnesium chloride
min	minutes(s)
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
Na^+	sodium
nAChR	nicotinic acetylcholine receptor
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen phosphate
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLR	NOD-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
OD	optical density
P_2X_7R	P_2X_7 receptor
P_2YR	P_2Y receptor
PAMP	pathogen associated molecular pattern
PBGD	porphobilinogen deaminase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween
PC	phosphocholine
PCR	polymerase chain reaction (PCR) Nucleotide Mix (dNTPs)
PGD_2	prostaglandin D_2
PLA2	phospholipase A2
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
PYD	pyrin domain
RA	rheumatoid arthritis

RANTES	regulated on activation, normal T cell expressed and secreted
RNase	ribonuclease
ROS	reactive oxygen species
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
s	second
SDS	sodium dodecylsulfate
siRNA	small interfering ribonucleic acid
SIRS	systemic inflammatory response syndrome
sPLA2	secretory phospholipase A2
STAT	signal transducer and activator of transcription
Stry	strychnine
TAE	tris-acetate EDTA
TEMED	tetramethylethylenediamine
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
TNRF	tumor necrosis factor receptor
Treg	regulatory T cell
UDP	uridine diphosphate
UF	ultrafiltrate
UTP	uridine-5'-triphosphate
UV	ultraviolet
V	volt
α-Bun	α-bungarotoxin
β-NAD	β-nicotinamide adenine dinucleotide

1 Introduction

1.1 Insights into sterile and non-sterile inflammation

Tissue damage, either as a result of trauma or infection, triggers a complex inflammatory host response that is ultimately responsible for injury resolution and repair and thus for patient survival [34, 237]. A prerequisite for this favorable outcome is a well-regulated, self-resolving inflammation. The initial host response comprises simultaneously occurring inflammatory and anti-inflammatory events that only when balanced result in restoration of immune system homeostasis [196, 199, 264]. Tipping this balance in either direction results in an inadequate response for the host's biological needs in form of either insufficient or overwhelming inflammation [110, 196, 199]. An overwhelming systemic inflammatory response syndrome (SIRS) can progress to shock, multiorgan dysfunction and death [135, 149, 154]. An exuberant compensatory anti-inflammatory response (CARS) causes immunosuppression with high susceptibility to opportunistic infection and subsequent sepsis [35, 196, 199, 264].

Until recently, surviving infection and trauma was solely dependent on the host immune response, whose intertwined network of mediators and cells was optimized by millions of years of evolutionary pressure. Mortality was high, with pandemics throughout history known to have decimated up to a third of the world's population [104]. Modern medical therapy though, has made it possible to overcome the early stages of severe infection and trauma, allowing the inflammatory process to further unfold. This on the other hand gave way to the new challenge of dealing with the consequences of more frequently occurring syndromes of immune dysregulation [110, 188, 199]. Despite numerous efforts and extensive research including studies on animal models [31, 105, 203] and clinical trials [59, 76, 230], there is currently no available therapy through which immune homeostasis can effectively be achieved. This is why we continue to observe an elevated mortality rate due to severe sepsis and trauma complicated with multiorgan dysfunction, which in intensive therapy units exceeds 50% [268].

The main difficulties reside in the complexity of the immune response as well as in the failure to translate the so far acquired basic knowledge into therapeutic strategies. Due to the complexity of inflammation, explaining the pathway from danger recognition to a competent immune reaction or to organ damage has taken a piece-meal approach.

Defining mechanisms of inflammation that unify the large dataset generated in preclinical and clinical studies remains challenging despite available computational models [188, 231, 291, 292]. Furthermore, therapeutic success in animal experiments fail to be translated into clinical setting because of the difficulties in generating appropriate animal models that reflect the complexity of a patient cohort with accompanying variations in age, sex, genetic background and comorbidities [188, 232, 244].

1.2 Inflammatory response initiation at the site of injury

The capacity of the immune system to detect invasion by pathogens relies on the initial interaction of so-called pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) on the surface of immune cells such as macrophages and polymorphonuclear cells [32, 202, 237]. This accounts for the first stage of the innate immune response that is immediate but non-specific [175]. PAMPs are compounds that are produced by groups of related pathogens and that are essential for the pathogen's viability, such as lipopolysaccharide (LPS), lipotheicoic acid or peptidoglycans [32, 116, 175]. Introducing the danger theory in 1994, Matzinger proposed an analogy to the PAMP-PPR interaction to explain inflammation induced by sterile injury [172]. According to this, endogenous analogues to PAMPs, termed damage-associated molecular patterns (DAMPs) bind to the same PPRs initiating the inflammatory process in response to trauma [172, 173]. DAMPs are intracellular molecules, such as chromatin-associated high-mobility group box 1 (HMGB1) [81, 248], heat shock proteins (HSPs) [170], mitochondrial DNA [314] and purine metabolites like uric acid, adenosine triphosphate (ATP) and adenosine [236] that are released into the extracellular milieu by damaged cells.

Toll-like receptor 4 (TLR4) was the first PRR to be described, identified as the main driver in inflammation regardless of the nature of the stimuli as it can be activated by both LPS and HMGB1, the prototypical PAMP and DAMP, respectively [130, 181, 206, 264]. Although both ligands are known to bind to TLR4, the inflammatory responses they elicit differ in certain respects [41, 130]. Upon LPS recognition, TLR4 triggers intracellular signaling pathways resulting in activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) [146, 198, 205]. This ultimately leads to dendritic cell (DC) maturation and pro-inflammatory cytokine production. The fully activated DCs provide the link to an adaptive immune response by delivering all necessary signals for naive T-cell activation [41, 237, 286].

HMGB1 on the other hand not only acts as a PRR agonist but also as a PRR co-receptor and chemotactic agent for leukocytes [41, 248]. As a non-histonic chromatin protein HMGB1 is passively released by necrotic cells into the extracellular space, where it is oxidized. The oxidized HMGB1 can then bind to TLR4 leading to down-stream NF-κB activation [72, 92, 130]. The resulting inflammatory response, triggered by cell death in the absence of a pathogenic invasion is less potent, and there is little evidence of adaptive immunity activation [41, 308].

In both sterile and pathogen-associated tissue damage, PRR-mediated activation of intracellular signaling pathways in immune cells leads to secretion of cytokines and chemokines that propagate the inflammatory process by recruiting further immune cells to the site of injury [237, 264, 286]. Already at this stage, the immune response integrates positive and negative feedback elements, combining pro- and anti-inflammatory activity that will ultimately influence the clinical course of the patient [214].

1.3 Chemokines and their role in inflammation

1.3.1 Chemokines and their receptors: an intricate system

The chemokine system coordinates leukocyte trafficking both under homeostatic and altered immune conditions. Local recruitment and timely activation of specific immune cell subsets upon pathogen invasion or tissue injury are dependent on the chemokine network that till date comprises 50 ligands and their corresponding receptors [18, 36, 95, 222]. Chemokines are small peptides of 8-12 kDa, that are classified into two major subfamilies (CC and CXC) and two minor subfamilies (CX3C and XC) based on the positioning of the N-terminal cysteine residues (C represents cysteine and X/X3 one or three non-cysteine amino acids) [13, 177, 265, 289]. 20 chemokine receptors that are part of the G protein-coupled receptor (GPCR) family are responsible for mediating the downstream effects of chemokines and are also classified in 4 subfamilies according to the ligands they bind: CCR1-10, CXCR1-6, CX3CR1 and XCR1 [18, 289]. Additionally, there are three known scavenger receptors, that reduce chemokine potency by internalizing upon ligand binding without further downstream signaling [192, 282].

As expected of their central chemotactic role, chemokine receptors are found mainly on the surface of bone marrow-derived cells. Accounting for their other known roles in haematopoesis, apoptosis, angiogenesis, extracellular matrix remodeling, etc., chemokine receptors are also expressed on the surface of endothelia, smooth muscle cells, stromal

cells, neurons and epithelial cells [87, 265].

Based on their function, chemokines can also arbitrarily be classified into inducible and constitutive chemokines [87, 289]. Inducible chemokines are produced under inflammatory conditions by activated immune cells and are responsible for leukocyte recruitment, whereas constitutive chemokines maintain basal leukocyte trafficking responsible for immune surveillance and participate in spatial organization of secondary lymphoid organs [13, 87, 289].

Different types of leukocytes express different patterns of chemokine receptors, enabling selective immune cell recruitment on account of the chemokine gradients generated by the affected tissue. Despite this apparent selectivity, the chemokine system was generally perceived as redundant, since the same chemokine receptor can be expressed by different types of leukocytes and can be stimulated by different ligands, that in turn can bind to different receptors [18, 163, 270]. This theoretically supports the experimental observation that knock-out mice for most of the chemokine receptors have no significant change in phenotype [13, 219]. However, more recent data revealed that, although the common function of chemotaxis is induced by overlapping chemokine-receptor combinations, each ligand receptor pair activates distinct intracellular signaling pathways that deliver different functional outcomes, nuancing the known chemotactic effect [249]. For example, chemokines CXCL9, -10 and -11 each induce T-cell chemotaxis when binding to the same receptor: CXCR3. CXCL11 though, exerts an anti-inflammatory effect by inducing the differentiation of regulatory T cells in contrast to CXCL9 and -10 whose effects are pro-inflammatory [220, 319]. Explaining this dichotomic functional outcome caused by CXCR3 activation are differences in ligand affinity and receptor binding sites, receptor internalization and downstream signaling cascades. This attribute of chemokines to preferentially activate one of several available downstream pathways when binding to the same receptor is known as functional selectivity or biased signaling [14, 224, 225]. Steen et al. summarized the known examples of biased signaling in the chemokine system, defining three different categories: ligand bias, receptor bias and tissue or cell bias, referring to signal variation with the ligand, with the receptor and with the tissue or cell-type, respectively [14, 225, 262, 320]. Growing evidence supporting the importance of selective signaling is gradually replacing the appearance of system redundancy while emphasizing context-defined chemokine receptor interactions.

1.3.2 Downstream chemokine-receptor signaling

Chemokine receptors change the conformation of their 7 transmembrane-spanning domains upon binding to their cognate ligand, activating further signal transduction through either a heterotrimeric (αβγ) G protein or arrestin intermediary [18, 289]. Signaling through G proteins implies transition of the Gα subunit from an inactive guanosine diphosphate (GDP)-bound to an active guanosine-5'-triphosphate (GTP)-bound complex that dissociates from the receptor and from the Gβγ heterodimer [15, 177]. There are four known Gα protein classes mediating signal transduction either through phospholipase C activation [120] or regulation of cyclic adenosine monophosphate (cAMP) production [186]. The Gβγ heterodimer can act either as an inhibitor of the Gα subunit or participate as an effector in signaling cascades leading to ion channel regulation or phosphorylation of extracellular signal-regulated kinases (ERK) [224, 289].

Initially discovered to be involved in receptor desensitization, β-arrestins are now attributed a wide range of subsequent effector pathways involving ERK phosphorylation, mitogen-activated protein kinase (MAPK) regulation, etc. that ultimately influence chemotaxis, apoptotic and anti-apoptotic signaling and receptor trafficking [224, 265].

The mechanism through which these molecular signaling pathways effectively translate into directional cell migration, allowing leukocytes to follow a chemotactic signal is yet to be fully elucidated. Several observations using time-lapse videomicroscopy have clearly shown a polarization of certain leukocytes, including lymphocytes, monocytes, natural killer (NK) cells, DCs and granulocytes, that develop distinct morphological and functional poles, rendering them capable of moving along the extracellular chemokine gradient [71, 193, 246]. These functional poles, termed filopodia on the leading edge of the cell and uropod at the rear of the cell, are the result of complex interactions between membrane proteins, the actin cytoskeleton and internal signaling pathways [71, 193, 207]. As chemokine receptors are evenly distributed across the cellular membrane of the leukocyte, the polarized distribution of Gβγ protein subunits is thought to lead through several intermediary effector proteins to actin polymerization at the leading end of the leukocyte, pushing it forward [80, 121].

Apart from polarization, this chemokine-mediated reorganization of the actin cytoskeleton is further involved in leukocyte adhesion to the endothelial layer followed by transendothelial migration, which enables leukocytes to travel and access the sites of injury or infection [71, 298, 305].

1.3.3 Establishing chemokine gradients for leukocyte recruitment

To fulfill their chemoattractant function by establishing gradients that guide circulating leukocytes to the injured tissue, chemokines concentrate on the endothelial surface by immobilization on glycosaminoglycans (GAGs) and oligomerization [100, 101, 122]. Both aspects seem to be critical for the chemotactic function, as experiments using engineered variants of chemokines with mutations rendering them incapable of GAG binding or oligomer formation, also showed an abrogated chemoattracting potential *in vivo* [12, 215, 221]. GAGs are negatively charged carbohydrate structures covalently attached to the core protein of proteoglycans, ubiquitously found on the surface of cells and in the extracellular matrix. Due to their vast structure variability with particular distribution patterns on the surface of various cell types as well as specific affinity for certain chemokines, GAGs affect chemokine localization and gradient formation [101]. Concentrated on the endothelial surface, chemokines can interact directly with their corresponding receptors on the leukocyte membrane, activating the internal signaling cascade. This causes the circulating leukocyte to first adhere to the endothelium by rapid increase in integrin binding, then to migrate across the endothelial wall into the tissue [100, 101, 221].

1.3.4 Chemokine-directed leukocyte migration on the example of monocytes

10% of the circulating leukocytes in human blood are monocytes, a pivotal component of the immune system in both steady-state maintenance and first line of defense [17]. Their recruitment to the site of injury is crucial for the control and clearance of pathogenic microorganisms and cellular debris, modulating the development and resolution of the inflammatory response [89, 309]. Their ability during inflammation to mobilize from the bone marrow into the bloodstream and then extravasate to distinct tissue sites, where they further differentiate into macrophages and DCs relies on chemotactic signals [257]. The distinct chemokine receptor expression on their surface allows a phenotypic and functional classification of monocytes.

1) Classical monocytes (cluster of differentiation (CD)14^{++} $CD16^{-}$): express high levels of CCR2 and of the LPS co-receptor CD14 ($CD14^{++}$) on their surface. In case of inflammatory challenge, these monocytes are recruited from the bone marrow in a CCL2/CCL7-dependent manner [277], while during homeostatic conditions

monocyte-derived macrophages patrol extravascular tissues, surveying for antigens that they transport to draining lymph nodes [115].

2) Non-classical monocytes (CD14^{+} CD16^{++}): express moderate levels of CD14 (CD14^{+}), lack CCR2, but express high levels of low-affinity IgG receptor CD16^{++} and of chemokine receptor CX3CR1 [317]. These are patrolling monocytes that constitutively migrate on the luminal surface of the endothelium, scavenging for particles and damaged cells and thus maintaining the endothelial integrity [45]. In homeostatic conditions, less than 1% of these monocytes cross the endothelial barrier into tissues [16].
3) Intermediate monocytes (CD14^{++} CD16^{+}) with high CD14 and moderate CD16 expression have also recently been reported, distinguishing themselves from the other subgroups by differences in inflammatory cytokine secretion [317, 318].

The classical CCR2-equipped monocytes are the ones to engage from the medullary or extramedullary haematopoetic sites into travelling towards distant affected tissues [257]. Their mobilization depends on the corresponding CCR2 ligands: monocyte chemotactic protein 1 (MCP-1) and 3 (MCP-3), alias CCL2 and CCL7 [118, 257, 269, 277]. CCR2-deficient mice show reduced trafficking of the monocyte subset corresponding to classical human monocytes, that in mice are also characterized by high CCR2 expression, as well as high expression of the surface protein LY6C (Ly6C^{++}) [144, 145]. The effect is similar when deleting either CCL2 or CCL7 which leads to a 40-50% reduction in monocyte recruitment [118, 257]. CCL2 is produced by many cell types including endothelial, epithelial, smooth muscle cells, fibroblasts etc., but the major source are monocytes/macrophages that are activated in the inflammatory milieu [9, 25, 55, 306]. It was expected that CCL2, driven through the blood stream from the site of inflammation triggers monocyte emigration from the bone marrow. Experimental proof provided by Shi et al. [256] indicates though, that bone marrow mesenchymal stem and progenitor cells are at least partially responsible for the CCL2 production that mobilizes monocytes and that this CCL2 production is induced by circulating low levels of TLR ligands [256].

As the primary scope of these mobilized monocytes is to exert their function at the site of injury, a correlation between the amount of mobilized, circulating and tissue infiltrating monocytes would be intuitive. However, the paradigmatic leukocytosis in inflammatory diseases is rather due to an increase in neutrophil numbers than due to monocytosis, and the extravasation of monocytes into tissues rather depends on the local barrier condition than on the sheer number of circulating monocytic cells [51, 150]. Certain chemokine axes

are crucial in coordinating monocyte movement across these physiological barriers, underlining their importance in temporal and spatial compartmentalization of the immune response [257].

The multi-step process that monocytes undergo to cross the endothelial barrier starts by selectin-mediated capturing of free-circulating monocytes to the vessel wall, followed by rolling, adhesion to endothelial cells, postadhesion strengthening, crawling, and finally transmigration [88, 108]. Chemokines and their corresponding receptors withhold explicit roles during this process of extravasation: CCR1 is responsible for monocyte arrest through integrin activation, CCR5 contributes to postadhesion strengthening, while both receptors support CCL5-mediated transmigration [257, 296]. The differential involvement of these chemokine receptors and their corresponding ligands in monocyte recruitment is of importance not only during the acute inflammatory process but also in the constantly rising number of chronic diseases, where sustained inflammation is proven to occupy center stage in pathogenesis. In murine models of chronic inflammatory diseases such as atherosclerosis or multiple sclerosis (MS), manipulating chemokine receptor expression or ligand levels can favorably influence outcome [39, 242, 296, 312].

Having crossed the endothelial barrier and arrived in an inflammatory setting, classical monocytes receive microenvironmental cues to differentiate into macrophages and DCs that further engage in the inflammatory response [115] by producing high levels of pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. In addition, they also exert phagocytic properties, clearing cellular debris and pathogens, while activating NK cells through IL-18 production and engaging adaptive immunity through antigen presentation [261].

1.3.5 Beyond the chemotactic role: CCL3, CCL4, CCL5 and their receptors

As mentioned previously, CCR1 and CCR5 and their respective ligands CCL3, alias macrophage inflammatory protein (MIP)-1α, CCL4 (MIP-1β) and CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES) exert key functions in monocyte chemotaxis and extravasation. Their implicit roles in development, sustenance and propagation of acute and chronic inflammation but also autoimmunity has pushed for sustained efforts to develop pharmaceutics to modulate their activity. Aside chemotaxis, these ligands have been discovered to be much more versatile in function, raising the importance of targeted function control.

CCR5 is an obligate co-receptor for the human immunodeficiency virus (HIV)-1 envelope fusion and entry into macrophages and activated T cells [28, 155]. Individuals carrying a naturally occurring homozygous CCR5 mutation, accounting for less than 1% of the population, are completely resistant to infection with HIV-1 [208]. CCL3, -4, or -5 binding to CCR5 block HIV-1 entry into the cells. Chemically modified CCL5 and small-molecule antagonists for CCR5 have proven to be efficient anti-HIV agents in pre-clinical trials and also lead to the first anti-chemokine therapy to be approved for clinical use [289].

Concerning tumor biology, chemokines have an implicit role in recruiting immune cells to the tumor microenvironment but can also directly target non-immune cells such as tumor stem-like cells, stromal and vascular endothelial cells inside the tumor [187]. In some types of cancer CCL2, CCL3 and CCL5 promote cancer cell extravasation by inducing matrix degradation through induction of increased metalloproteinase secretion [239], influence cancer invasiveness by directly targeting endothelial cells and sustain cancer cell motility and epithelial-mesenchymal transition once they accessed the vasculature [153, 278]. Murine experimental models of cancer showed increased survival due to inhibition of tumor angiogenesis and metastasis, when chemokine function was targeted, though they also showed that chemokine blockade alone does not suffice as an effective anti-tumor therapy [153, 187, 223]. A clinical trial combining a classical chemotherapy protocol with CCR2 blockade in patients with boarderline resectable and locally advanced pancreatic cancer showed promising results, though on a limited patient cohort [197].

While inflammation is intrinsic and necessary, it remains positive just as long as it is contained. Regulatory mechanisms such as the chemokine system are crucial for delivering either a self-resolving or self-maintaining and propagating inflammation. Chemokines have a major influence in compartmentalizing the immune response by controlling selective immune cell trafficking across different biologic compartments (bone marrow, blood, peripheral tissues) and by modulating the function of the immune cells inside these compartments. The most prominent differences in immune response characteristics are between the blood compartment and the injured tissue, setting the barrier between affected and non-affected. Monocytes, as one of the earliest responding leukocyte subset, secrete potent pro-inflammatory cytokines such as IL-1β with beneficial effect as a local, limited event. As soon as this pro-inflammatory process traverses into the blood compartment resulting in high systemic IL-1β levels, there is an increased risk

for SIRS and distant tissue damage to develop [149, 154, 261]. This accentuates the importance of regulatory mechanisms in preventing this spillage of the local pro-inflammatory process into the bloodstream.

1.4 IL-1β: a tightly controlled cytokine

Monocytes, macrophages and DCs, are among the cell types that produce and secrete IL-1β in response to infections and injuries. The role of IL-1β in microbe elimination was acknowledged early on, only for further discoveries to gradually set this cytokine at the core of the inflammatory process arising not only in infectious disease but also in trauma, chronic multifactorial disease and auto-inflammatory conditions [63, 91]. This potent pro-inflammatory cytokine, generated at the site of immunological challenge, affects cells and organs as distant from the site of injury as the hypothalamus, at remarkably low concentrations, causing fever, regulating pain threshold, sleep and appetite, coordinating cell recruitment and perpetuating the pro-inflammatory cytokine cascade, that can potentially lead to tissue damage [63, 82].

Considering its potent, pleiotropic and potentially damaging effect, IL-1β bioactivity is regulated at the level of its production and maturation, of receptor binding, and of post-receptor signaling [66]. Production and release into the extracellular environment is a multistep process underpinned by several control levels involving synthesis of an inactive precursor, pro-IL-1β, which undergoes proteolytic cleavage to mature IL-1β that is subsequently secreted [66, 91]. Pro-IL-1β synthesis does not occur in steady-state conditions. Instead, several factors signaling danger during an inflammatory challenge, such as endotoxins or endogenous cytokines, prime myeloid cells to produce pro-IL-1β [234]. This first danger signal is mediated by TLR or tumor necrosis factor receptors (TNFRs) leading to NF-κB activation and enhanced transcription then translation of pro-IL-1β [66, 94]. LPS binding to TLR4 is the classic example for myeloid cell priming, widely used in experimental settings to induce pro-IL-1β synthesis. TNF and IL-1β itself are equivalent endogenous pro-IL-1β inducers [97, 117].

A second signal is tipically required for IL-1β maturation through proteolytic cleaveage by caspase-1, whose activation is dependent on the assembly of inflammasomes, that are multiprotein complexes of the cytosolic compartment [97, 167].

1.4.1 Inflammasomes as intracellular inflammation sensors

The activation of several inflammasomes converge on the end point of caspase-dependent cytokine maturation but starts with a differentiated recognition of a wide array of second danger signals. This differentiated recognition is due to inflammasomes containing different sensor proteins that specifically identify endogenous or exogenous danger signals [167]. For example, the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) containing a caspase activating and recruitment domain (CARD) 4 (NLRC4) sensor protein directly recognizes bacterial flagellin, while absent in melanoma 2 (AIM2) identifies double-stranded deoxyribonucleic acid (DNA) when initiating inflammasome activation [142, 238]. Beside the sensor protein, a classical inflammasome contains an adaptor protein (apoptosis-associated speck-like protein containing a CARD [ASC]) and a caspase effector [226, 293].

The NLR family, pyrin domain (PYD) containing 3 (NLRP3) inflammasome is the best characterized and most efficient IL-1β producing mechanism in myeloid cells [91]. Its cytosolic sensor is a NLR that initiates inflammasome activation in the presence of a large array of stimuli, both of exogenous nature such as bacterial toxins, viral nucleic acids, fungal products, and of endogenous provenance such as ATP, cholesterol crystals, monosodium urate [138]. This links the NLRP3 inflammasome to the most common infectious disease such as staphylococcal infection, candidiasis and influenza, as well as to some of most challenging clinical entities in modern day medicine like SIRS, Alzheimer’s dementia or metabolic stress [96, 99, 126, 165, 316]. The ASC adaptor protein connects through its N-terminal PYD and its CARD the NLR sensor to the pro-caspase-1 effector, whose activation leads to the proteolytic maturation and secretion of IL-1β [97].

Activation of the NLRP3 inflammasome does not imply direct binding of the sensor protein to the enumerated PAMPs and DAMPs. Instead, these varied agents interact with receptors on the cell surface or lead to pore formation in the cell membrane inducing an array of signals including potassium efflux, elevated levels of reactive oxygen species (ROS) or lysosomal destabilization that mediate inflammasome activation [97, 216]. This model of inflammasome assembly was verified in the case of ATP, an endogenous molecule whose extracellular presence is an indicator of cell injury or stress and which induces potassium efflux in myeloid cells when interacting with the purinergic P_2X_7 receptor (P_2X_7R) on their surface [165].

1.4.2 Purinergic signaling in inflammation

Due to the high energy content of its pyrophosphate bonds, the role of ATP as the energy transfer unit of the cell has long been acknowledged. An amount of ATP equivalent to ones own body weight is being synthetized daily [275]. A fraction of this is consumed for intracellular signaling, as a substrate for kinases or for signal transduction of GPCRs, which are by far the most numerous in eukaryotes [111]. This function of ATP as an intercellular mediator however, has only recently shifted into focus, concordant with and supported by the danger model propagated by Matzinger [50, 172]. The first proposition of ATP as an extracellular messenger, responsible for non-adrenergic, non-cholinergic neurotransmission was made in the 1970s by Burnstock [43, 44]. The purinergic hypothesis remained unpopular until the specific nucleotide receptors were cloned in the 1990 leading to increasing acceptance of nucleotides as extracellular transmitters [38, 156, 283, 295].

Extracellular ATP is a sensitive indicator of cellular distress and has been shown to reach concentrations in the hundred micromolar range at inflammatory sites as opposed to healthy tissues, where it remains in a low nanomolar level [24, 211, 302]. These data further support the meanwhile well-established role of ATP as a DAMP at inflammatory sites, triggering IL-1β maturation. Apart from this, other critical immune events such as antigen-driven T-lymphocyte proliferation, neutrophil and macrophage chemotaxis have also been shown to rely on purinergic signaling [74, 123].

Despite the late discovery and protracted acceptance, equivalents to the human purinergic ligand-receptor system have even been found in primitive invertebrates, thus placing nucleotides among the primordial mediators of cell-to-cell communication [50, 288]. The receptor set responsible for extracellular nucleotide recognition comprises two subfamilies: the P_2Y receptors (P_2YRs), which are G protein-coupled metabotropic receptors, and the mainly ligand-gated ionotropic P_2XRs [4]. While P_2YRs interact with various nucleotide ligands such as adenosine diphosphate (ADP), uridine diphosphate (UDP), uridine-5'-triphosphate (UTP), UDP-glucose, or UDP-galactose, P_2XRs selectively engage with extracellular ATP [113].

Several members of the P_2XR subclass have been identified on the surface of immune cells, their function, in the wider context of metabolic regulation of T-lymphocytes or neutrophil extravasation still awaiting more precise definition [160, 304]. The function of P_2X_7R though, has been investigated at length and is well established. It was the discovery

of the NLRP3 inflammasome that shed light on the pivotal role of P_2X_7R, as part of the 'two signal model' of IL-1β production [75, 167]. In its functional state, the P_2X_7R is a trimer, that upon ATP binding undergoes a conformational rearrangement and forms a pore that allows the inward flux of Na^+ and Ca^{2+} and the outward flux of K^+ [103, 162]. The exact molecular mechanism connecting the decrease in intracellular K^+ concentration to inflammasome activation is still unknown, but it has been suggested that the change in the cytosolic microenvironment is what recruits the inflammasome components to the vicinity of P_2X_7R, where they assemble [182]. This suggestion has been strengthened by experimental data that co-localized P_2X_7R and NLRP3 using confocal microscopy and co-immunoprecipitation [77]. Several microbial toxins, such as nigericin, are also known to activate the inflammasome by causing depletion of intracellular K^+, which seems to be a necessary and sufficient condition for NLRP3 activation [182].

Besides triggering its production, the P_2X_7R also seems to play a significant role in IL-1β release into the extracellular environment. IL-1β lacks an N-terminal secretory sequence that would enable it to engage the canonical secretory pathway of the endoplasmatic reticulum and Golgi apparatus [243, 250]. Modified lysosomes, exosomes or plasma-derived microvesicles have been proposed as vehicles for transporting mature IL-1β across the cellular membrane, with P_2X_7 as a key component of these alternative secretory routes [62, 217]. The direct interaction of the P_2X_7R's intracellular C-terminal tail with membrane proteins such as pannexin-1 and connexin-43 hemichannels leads to the formation of large-conductance pores allowing the extracellular release of IL-1β and ATP [26, 210].

Further experimental proof involving P_2X_7R in key inflammatory events showed that it promotes the expression of several chemokines, such as the major monocyte attractants CCL2 and CCL3 [129, 204]. Furthermore, inflammasome activation can also trigger an inflammatory form of cell death known as pyroptosis [8, 218, 258]. In this process, mediated by caspase-1activity, pores of an estimated diameter of 1.1-2.4 nm are formed in the cell membrane, disrupting the cellular ionic gradients and increasing osmotic pressure. This leads to an influx of water, causing the cell to swell, then ultimatelly burst, spilling its pro-inflamatory content, including caspase-1-cleaved activated cytokines such as IL-1β [258].

1.4.3 Clinical significance of IL-1 blockade

The sheer importance of strict IL-1β control, from inflammasome assembly to receptor binding can easily be deduced from disorders with naturally occurring loss of these innate regulatory mechanisms. An example are infants born with a deficiency of the IL-1 receptor antagonist (IL-1RA) known as DIRA, a recessively inherited disease caused by loss-of-function mutations in the gene encoding IL-1RA. As a consequence, a severe systemic sterile inflammation develops within the first days of life, that proves fatal unless treated [229]. In his pioneering work set to characterize the function of IL-1β, Dinarello described in 1996 severe adverse effects in patients injected with low nanomolar doses of IL-1β such as fever, hypotension, anorexia, myalgias, arthralgias, fatigue and sleep disturbances [63].

The synthesis and clinical use of IL-1β blockers as monotherapeutics were effective in selected inflammatory disorders with IL-1β dysregulation at the core of their pathogenesis. These so termed 'autoinflammatory' syndromes are caused by mutations altering the intracellular apparatus involved in IL-1β synthesis and are uniquely responsive to IL-1β blockade [128, 169]. The available agents approved for IL-1β neutralization in a clinical setting are limited to three pharmaceuticals: Anakinra - the recombinant form of the naturally occurring IL-1RA [65], Rinolacept - a soluble IL-1 decoy receptor [195, 263] and Canakinumab - monoclonal anti-IL-1β antibodies [40, 69]. Many other IL-1β blockers are undergoing clinical trials and are pending approval. Their efficiency is incontestable in the treatment of autoinflammatory disease, whose distinct entities though varied in clinical manifestation all exhibit an elevated IL-1β secretion by blood monocytes when compared to healthy individuals [68, 83]. Amongst them the best known is familial mediterranean fever, caused by a mutation in the gene encoding for the intracellular protein pyrin, that regulates caspase-1 activation and therefore IL-1β maturation [48].

Selective IL-1β blockade has also proved beneficial in autoimmune disorders, ischemia/reperfusion injuries and several other chronic inflammatory disorders [5, 40, 67, 161]. This is due to intertwined inflammatory pathways and regulatory feedback loops binding innate and adaptive immunity, thus generating a continuum of inflammatory and immunological diseases.

To take example on the currently most prevalent and lethal health condition, acute ischemic heart disease also involves inflammatory events, that can be beneficially

influenced by IL-1β blockade. IL-1β negatively affects cardiac function by directly suppressing myocardium contractility and by amplifying local inflammation through recruitment of cytokine producing monocytes that contribute to myocardial necrosis. Working on experimental models of acute myocardial infarction in mice, Abbate et al. obtained beneficial outcomes when blocking IL-1β, during the acute insult as well as during the subsequent process of cardiac remodeling [1, 2]. Subsequent clinical trials showed a significant improvement of myocardial contractility and relaxation parameters as well as of coronary flow reserve and endothelial function after IL-1β blockade with Anakinra [3, 285].

Recently gathered data depicts inflammatory events as key contributors to the pathogenesis of a growing number of clinical entities such as stroke, diabetes, mental impairment, hearing loss, amyloidosis, MS; all of which are currently being evaluated for therapeutic response to IL-1β blockade [29, 65, 67, 161, 271].

Though trial results deliver a solid argument for the clinical use of IL-1β blockers in a growing number of inflammatory diseases, some major health issues quintessentially characterized by a cytokine storm astonishingly do not benefit from IL-1β blockade. In the treatment of sepsis, for example, where IL-1β attenuation was hoped to provide a major breakthrough, two phase III trials comparing Anakinra to placebo failed to meet the primary endpoint of improved survival [76, 201]. A healthy immune system incorporates many IL-1β regulatory mechanisms in order to maintain or reestablish immune homeostasis, providing therefore just as many niches for therapeutic IL-1β control. These might supply treatment options in disorders with clear IL-1β involvement, where currently available IL-1β blockers are inefficient. Therefore, the quest for IL-1β modulation is still underway. Recently, a new compound entitled CY-09 was identified to directly bind to the NACHT domain of NLRP3 inhibiting its ATPase activity, which is essential for NLRP3 oligomerization and inflammasome assembly [119]. The advantages over agents directly targeting IL-1β are argued to be an additional inhibition of IL-18 production or pyroptosis and lower immunosuppressive side-effects.

A further alternative for IL-1β control was delivered by the antiinflamatory effect of nicotinic agonists, mediated through nicotinic acetylcholine receptors (nAChRs).

1.4.4 Cholinergic regulation of IL-1β release

The first hints that nAChR agonists might exert an anti-inflammatory effect came from the simple clinical observation that patients with chronic inflammatory syndromes such as rheumatic arthritis or ulcerative colitis benefit from nicotine consumption [11, 27, 52, 102, 174, 194]. The presence of nAChRs on the surface of immune cells has been reported as early as the 1970s and since confirmed by radiolabeled ligand-binding studies [7, 168, 273], messenger ribonucleic acid (mRNA) expression [179, 247] and immonocytochemical analysis. Acetylcholine (ACh) has been established by Dale as the neurotransmitter of the cholinergic nervous system and was isolated by him and Dudley from the spleen of oxen in 1929 [58]. Earlier even, in 1910, the presence of lymphocytes and their circulation through the spleen, a secondary lymphoid tissue, was described [260]. When putting all of the above facts together, theories linking the ACh releasing efferents of the vagal nerve to the nAChRs of immune cells in the spleen seemed rational. These theories were further endorsed by the experimental results of Tracey's group showing that stimulation of the distal end of the vagal nerve dampens the release of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-18 in LPS-stimulated human macrophages [37, 276, 294]. But the theoretically presumed link between parasympathetic neurons and immune cells found no anatomic correlate as it currently accepted, that all primary and secondary immune organs, including the spleen, receive innervation only by sympathetic postganglionic neurons and no cholinergic vagal input [189]. More recent studies employing methods with increased sensitivity such as anterograde labelling also failed to detect a direct interaction between vagal nerve endings and macrophages in the gut or spleen [84, 166].

A non-neuronal origin of the splenic ACh isolated by Dale had not been taken into consideration for a long time. But later, ACh was identified in blood [127, 132], in the proximity of lymphocytes [133], that were soon after described to possess all essential components for a non-neural cholinergic system, including its synthetizing enzyme choline acetyltransferase (ChAT) [79, 235]. ChAT-producing T cells are currently thought responsible for Tracey's described anti-inflammatory cholinergic reflex. Rosas-Ballina et al. brought evidence that vagal stimulation triggers release of norepinephrine from sympathetic neurons of the splenic nerve, inducing ACh secretion through activation of β-adrenoceptors on T cells [241]. The released ACh acts on the nAChRs of splenic

macrophages, dampening cytokine production via the Janus kinase (Jak)2- signal transducer and activator of transcription (STAT3) signaling pathway [241].

From the various nAChR subunits identified on the surface of immune cells, α7 received the most attention, due to the immunemodulatory properties it was attributed with as part of the inflammatory reflex described by Tracey. Pro-inflammatory cytokine levels in endotoxemic α7 knockout mice significantly exceeded those of wild-type controls and were not influenced by cholinergic agonists [276, 294]. Further studies provided evidence of the dual inonotropic/metabotropic nature of α7 nAChRs, that elicit downstream effects by either transiently increasing intracellular Ca^{2+} or by engaging more prolonged signaling events [124, 139, 227].

Adding to the further characterization of immune cell nAChRs, our group suggested a cholinergic mechanism that potently inhibits ATP-mediated inflammasome activation and therefore IL-1β maturation in human and rat monocytes via the α7, α9, and α10 nAChR subunits [106, 233, 311]. Monocyte-lineage cells, as main IL-1β producers are also capable of producing ACh, depending on their tissue distribution and immunological status [141, 245, 299]. A marginal ChAT mRNA expression was detected in splenic, lung and alveolar macrophages and monocytes as well as in mature and immature DCs during resting conditions [107, 141, 301]. In contrast, peritoneal macrophages have no ChAT activity, neither at rest nor when activated [134]. Kawashima showed that LPS and other TLR agonists induce ACh synthesis in DCs and macrophages [134], whereas our group pointed out, that activated monocytes accumulating in blood vessels of rat renal grafts produce ACh during acute rejection [107, 301]. As monocytic cells express all five M_1–M_5 muscarinic AChR (mAChR) subtypes and various nAChR subunits, an autocrine modulation of pro-inflammatory cytokine release during immune challenge was suggested. Hecker et al. further provided evidence that certain pathogens, by producing phosphocholine (PC)-modified macromolecules can exploit this nicotinergic anti-inflammatory mechanism to evade the immune system of the host [106]. Backhaus et al. showed that the anti-inflammatory properties dipalmitoylphosphatidylcholine (DPPC), as the main lipid constituent of surfactant, are at least partially accounted for by a similar cholinergic mechanism [19].

All these findings extend the significance of the non-neural cholinergic system beyond the frame of a neuronal-immune reflex involving the vagus nerve and splenic immune cells. This prompted us to suggest that this cholinergic control mechanism might be involved in other key immunomodulatory events working to contain inflammation to a

local event and to properly compartmentalize the immune response. As leukocyte trafficking, inherently standing under the influence of chemokines, is central to many of these immune events, our present study is aimed at investigating the cholinergically mediated immunomodulatory properties of chemokines.

1.5 Aim of the study

Chemokines and ATP are among the first mediators released at inflammatory sites that can enter the circulation via damaged blood vessels. When encountering ATP, circulating primed monocytes receive their signal for inflammasome activation, but releasing IL-1β into the blood stream before having reached the site of injury would imply an inefficient immune response with enhanced systemic inflammation in the detriment of a reduced local immune cell infiltration.

Therefore, we predict a prioritization of the chemotactic signal that enables monocytic infiltration of the injured tissue but prevents premature release of IL-1β into the blood stream, thus avoiding SIRS. This prediction prompted us to investigate, whether chemokines can engage the already described non-neuronal cholinergic control mechanism to inhibit the ATP-dependent release of IL-1β by LPS-primed human monocytic cells.

In the current study we test whether the monocyte attracting chemokines CCL3, CCL4 and CCL5 inhibit the release of IL-1β by human monocytes, by conducting *in vitro* experiments using the monocytic U937 cell line as well as freshly isolated peripheral blood mononuclear cells (PBMCs). Furthermore, we aim to describe the molecular mechanism initiated down-stream of the chemokine/receptor interaction and ending with an inhibition of IL-1β release.

2 Material and Methods

2.1 Material

2.1.1 Cell line

U937 (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany)

2.1.2 Chemicals and reagents

[V11L; V16D]ArIB, kindly provided by J. Michael McIntosh from the Department of Biology, University of Utah, Salt Lake City, UT, USA

2′(3′)-O-(4-benzoyl-benzoyl)adenosin 5′-triphosphat-triethylammonium salt (BzATP) (Sigma-Aldrich, Taufkirchen, Germany or Jena Bioscience, Jena, Germany)

2-Mercaptoethanol (Roth, Karlsruhe, Deutschland)

α-bungarotoxin (Tocris Bioscience, Bristol, UK)

Acetic acid, 96% p.a. (pro analysi) (Riedel-deHaën, Hannover, Germany)

Acetylcholine (ACh) (Sigma-Aldrich)

Acrylamide 30% solution, Rotiphorese® Gel 30 (Roth)

Agarose (Invitrogen™, Life Technologies, Darmstadt, Germany)

Ammonium persulfate (APS) (Roth)

Apyrase (Sigma-Aldrich)

Aqua destillata (dH_2O) (B. Braun, Melsungen, Germany)

Arachidonyl trifluoromethyl ketone (ATK) (Enzo Life Sciences, Lausen, Switzerland)

Bovine serum albumin (BSA), ≥96% p.a. (Sigma-Aldrich)

Bromoenol lactone (BEL) (Enzo Life Sciences)

Bromophenol blue (Roth)

CCL3L1 (MIP-1α), human recombinant (R&D Systems, Wiesbaden, Germany)

CCL4 (MIP-1β), human recombinant (R&D Systems)

CCL5, human recombinant (R&D Systems)

CCR1 human siRNA (siRNA) ON-TARGETplus SMARTpool (GE Dharmacon, Lafayette, CO, USA)

CCR3 human siRNA ON-TARGETplus SMARTpool (GE Dharmacon)

CCR5 human siRNA ON-TARGETplus SMARTpool (GE Dharmacon)

CXCL12, human recombinant (R&D Systems)

CXCL16, human recombinant (R&D Systems)

Demineralized water (University Hospital Gießen, Germany)

DNA Gel Loading Dye 6X (Thermo Scientific™, Life Technologies)

DPBS, Dulbecco's phosphate buffered saline (PBS) without calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (Gibco®, Life Technologies)

Ethylenediaminetetraacetic acid (EDTA) (Serva, Heidelberg, Germany)

Fetal calf serum (FCS) (Biochrome, Berlin, Germany)

GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA)

GeneRuler 100 bp Plus DNA ladder (Thermo Scientific™, Life Technologies)

GlutaMAX™ (Gibco®, Life Technologies)

Glycerol, ≥99% p.a. (Sigma-Aldrich)

Glycine, Pufferan® ≥99% p.a. (Roth)

Heparin-Natrium, 25000 I.U./5 ml (Merckle, Blaubeuren, Germany)

Hydrochloric acid (HCl), 1 N Titripur® Reag (Merck, Darmstadt, Germany)

Lipopolysaccharide (LPS), *Escherichia coli,* ≥95% p.a., L2654 (Sigma-Aldrich)

M-MLV reverse transcriptase (RT) ribunoclease (RNase) H(-) Point Mutant (Promega, Mannheim, Germany)

M-MLV-RT reaction buffer 5x (Promega)

Magnesium chloride ($MgCl_2$), 25 mM (Promega)

Mecamylamine hydrochloride (Sigma-Aldrich)

Methanol, high pressure liquid chromatography (HPLC) grade, ≥99.9% (Sigma-Aldrich)

Mucocit® T (Schülke, Norderstedt, Germany)

Nigericin (Sigma-Aldrich)

ON-TARGETplus Nontargeting Control Pool (GE Dharmacon)

Polymerase chain reaction (PCR) Nucleotide Mix (dNTPs), cGMP-Grade (Promega)

PLA2G6 human siRNA ON-TARGETplus SMARTpool (GE Dharmacon)

Platinum® SYBR® Green qPCR SuperMixUDG (Invitrogen™, Life Technologies)

Potassium chloride (KCl), p.a. (Merck)

Potassium dihydrogen phosphate (KH_2PO_4), p.a. (Merck)

Powdered milk, blotting grade (Roth)

Precision Plus Protein™ Standards, Dual Color (Bio-Rad, München, Germany)

Protease inhibitor cocktail tablets, Complete® Mini (Roche Diagnostics, Mannheim, Germany)

Random primers (Promega)

RgIA4, kindly provided by J. Michael McIntosh from the Department of Biology, University of Utah, Salt Lake City, UT, USA

RNase-free water (Quiagen, Hilden, Germany)

RNasin® RNase inhibitor (Promega)

Sodium chloride (NaCl), ≥99%, p.a. (Sigma-Aldrich)

Sodium dodecylsulfate (SDS), ≥99.9% ultrapure (Roth)

Sodium hydroxide (NaOH) 1N Titripur® Reag (Merck)

Sodium dihydrogen phosphate (NaH_2PO_4), p.a (Merck)

Strychnine hydrochloride (Sigma-Aldrich)

Tetramethylethylenediamine (TEMED) (Roth, Karlsruhe, Germany)

Tris Pufferan®, ≥99.9% p.a. (Roth, Karlsruhe, Germany)

Trypan blue solution, 0.4% for microscopy (Sigma-Aldrich)

Türk's solution (Merck)

Tween® 20 (Merck)

X-ray developer concentrate, X-Ray (Adefo-Chemie, Dietzenbach, Germany)

X-ray fixer concentrate, X-Ray (Adefo-Chemie)

2.1.3 Antibodies

Antibody	**Type**	**Host species**	**Clonality**	**Supplier**	**Product number**
anti- iPLA2β	primary	rabbit	polyclonal	Sigma-Aldrich	SAB4200129
anti-β-actin	primary	mouse	monoclonal	Sigma-Aldrich	A1978
anti-rabbit Ig, HRP labeled	secondary	goat	polyclonal	Dako, Glostrup, Denmark	P0448
anti-mouse Ig, HRP labeled	secondary	rabbit	polyclonal	Dako	P0161

Table 1: *Primary and secondary antibodies used for western blotting: characteristics and suppliers.* iPLA2β, calcium-independent phospholipase A2β; Ig, immunoglobulin; HRP, horseradish peroxidase.

2.1.4 Consumables and expendables

Amicon® Ultra Centrifugal filters, 3 kDa cut-off (Merck)

Conical tubes BD Falcon™ 10/50 ml (Greiner bio-one, Frickenhausen, Germany)

Culture flask T75 160 ml (Sarstedt, Nümbrecht, Germany)

Electrophoresis chambers (Keutz Labortechnik, Reiskirchen, Germany)

Glass Pipettes 10/20 ml (Greiner bio-one)

High-performance chemiluminescence films (GE Healthcare Bio-Sciences, Uppsala, Sweden)

Leucosep™ 227 288 (Greiner bio-one)

Multiwell plates 12-/24-/96-wells (Greiner bio-one)

Neubauer cell-counting chamber 0.0025 mm² (LO-LaborOptik, Lancing, England)

Nitrile gloves Vasco® (B. Braun)

PCR plate, 96-well (Thermo Scientific™, Life Technologies)

Pipette filter tips np Nerbe-Plus 100/1250 µl (Novolab NV, Geraardsbergen, Belgium)

Pipette tips 10/200/1000 µl (Sarstadt, Nümbrecht, Germany)

Polyvinylidene difluoride (PVDF)-membrane Immobilon®- pore size: 0.45 µm (Merck)

Reaction tubes Safe Seal 0.5/1.5/2 ml (Sarstedt)

Sterile syringe 20 ml (B. Braun)

X-Ray cassette (Dr. Goos-Suprema GmbH, Heidelberg, Germany)

2.1.5 Instruments

Analogue tube roller SRT9 (Stuart, Staffordshire, UK)

Balance WL100826 (Kern & Sohn, Balingen, Germany)

Block heater S81B25034 (Peqlab Biotechnologie, Erlangen, Germany)

Centrifuges Rotina 420R, Mikro 220 and Mikro 200 R (Hettich, Tuttlingen, Germany)

Digital camera Olympus C4000-Zoom (Olympus, Hamburg, Germany)

Electrophoresis power supply Consort EV231/E835 (Von Keutz Labortechnik)

Epoch spektrophotometer (BioTek, Bad Friedrichshall, Deutschland)

FLUOstar OPTIMA spectophotometer (BMG Labtech, Offenburg, Germany)

Gel imaging system (Intas, Göttingen, Germany)

Incubator Heracell™ 240i (Thermo Scientific™, Life Technologies)

Laminar flow hood (Integra Biosciences GmbH, Konstanz, Germany)

Magnetic stirrer RH basic 2 (IKA®, Staufen, Germany)

Microscopes Labovert and Laborlux (Leitz, Wetzlar, Germany)

Mini Plate Spinner MPS 1000 (Labnet, Edison, USA)

Mini Spin centrifuge (Abimed, Langenfeld, Germany)

NanoDrop 1000 (Peqlab Biotechnologie)

Nucleofector® Device (Lonza Group Ltd., Basel, Switzerland)

PH-Meter UB-10 (Denver Instrument, Göttingen, Germany)

Pipettes Reference 100-1000; 10-100; 0.5-10 µl Pipetus® (Eppendorf, Hamburg, Germany)

Pipetus® (Hirschmann®, Eberstadt, Germany)

Step-One Real-time PCR-System (Applied Biosystems®, Life Technologies)

Rocking shaker ST5 (Kobe, Marburg, Germany)

Thermal Cycler G-Storm, GS482 (AlphaMetrix Biotech, Rödermark, Deutschland)

Tissue homogenizer MM301 (Retsch, Haan, Germany)

Transilluminator (Biozym Scientific, Oldendorf, Germany)

Ultrasonic bath Sonorex Super RK102H (Bandelin, Berlin, Germany)

Vortex Mixer Reax2000 (Heidolph, Schwabach, Germany)

Water bath (Köttermann Labortechnik, Uetze/Hänigsen, Germany)

2.1.6 Buffers and solutions

Buffer/ Solution	**Source/ Recipe**
APS solution	200 mg APS were dissolved in 400 ml demineralized water shortly before usage
PBS, 10x	1.37 M NaCl 27 mM KCl 80 mM NaH_2PO_4 15 mM KH_2PO_4 The pH of the resulting solution was adjusted to 7.2.
PBS, 1x	was obtained by a 1:10 dilution of PBS (10x) with dH_2O. The pH was maintained at 7.2.
PBS-T (Tween 0.1 %)	0.1% (v/v) was mixed into the PBS (1x) solution.

Resolving gel buffer	18.16 g Tris Pufferan® (1.5 M) were dissolved in 70 ml demineralized water and titrated with 1 N HCl until a pH of 8.8. 4 ml of the 10% SDS solution and dH_2O up to a volume of 100 ml were added.
RPMI 1640 cell culture medium	Gibco® by Life Technologies, Darmstadt, Germany
Sample buffer 1	62.5 mM Tris-HCl, pH 6.8 2.3% (w/v) SDS 1 protease inhibitor cocktail tablet per 20 ml
Sample buffer 2	62.5 mM Tris-HCl, pH 6.8 40% (v/v) glycerol 2.3% (w/v) SDS 16% (v/v) 2-mercaptoethanol 0.025% (w/v) bromphenol blue 1 protease inhibitor cocktail tablet per 10 ml
SDS stock solution (10 %)	10 g SDS were dissolved in 100 ml demineralized water.
Stacking gel buffer	0.5 M Tris-HCl, pH 6.8 0.4% (w/v) SDS
Transfer buffer	20 mM Tris 200 mM glycine (200 mM) 0.05 (w/v) SDS 20% (v/v) methanol
TAE, 50x	2 M Tris 0.06 M EDTA 5.71% (v/v) acetic acid if necessary, the the solution was adjusted by titration with either 1 N NaOH or 1 N HCl to a pH of 8.

TAE, 1x	was obtained by a 1:50 dilution of TAE (50x) with dH_2O.
Tris-HCl buffer, 1 M (pH 6.8)	obtained by dissolving 121.14 g Tris Pufferan® in 1 litre demineralized water and titrating with 1 N HCl until a pH of 6.8.

Table 2: *Buffers and solutions.* APS, ammonium persulfate; PBS, phosphate buffered saline; NaCl, sodium chloride; KCl, potassium chloride; NaH_2PO_4, sodium dihydrogen phosphate; KH_2PO_4, potassium dihydrogen phosphate; dH_2O, aqua destillata; PBS-T, phosphate buffered saline with Tween; HCl, hydrochloric acid; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; TAE, tris-acetate EDTA; NaOH, sodium hydroxide; w/v, weight per volume; v/v, volume per volume.

2.1.7 Kits

Amaxa® Cell Line Nucleofactor® Kit C (Lonza Group Ltd.)

CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega)

Lumi Light Western Blotting Substrate (Roche Diagnostics, Mannheim, Germany)

Micro BCA Protein Assay Kit (Thermo Scientific™, Life Technologies)

Min Elute® PCR Purification Kit (Qiagen)

Qiagen RNeasy Miniprep Kit (Qiagen)

Quantikine® ELISA Human IL-β/IL-1F2 (R&D Systems)

Silver Stain Plus (Bio-Rad Laboratories, Munich, Germany)

2.2 Methods

2.2.1 Cell culture experiments

2.2.1.1 Culture methods and cell counting

Cell culture experiments have been carried out on the U937 human histiocytic lymphoma cell line. The cells were cultured in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum (FCS), and 2 mM GlutaMAX™, at 37 °C and in humidified atmosphere with 5% CO_2. Change of the supplemented cell culture medium and passaging of the cells took place twice a week. All experiments have been carried out using sterile reagents and consumable materials under a laminar flow hood.

At the beginning of each experiment, haemocytometric counting was performed. For this, the cell suspension from a culture flask was centrifuged for 8 min at room temperature and 500 g. The supernatant was discarded and the cell pellet resuspended in 10 ml fresh

supplemented culture medium. 10 µl of the fresh cell suspension were stained with 90 µl 0.2% trypan blue solution and loaded onto a Neubauer cell-counting chamber. Viable cells were counted under the microscope, then, according to the calculated cell concentration, the cell suspension was set to a density of 10^6 cells/ml.

2.2.1.2 Experimental outline

The experimental workflow comprised the main steps depicted in Figure 1, namely cell priming with 1 µg/ml LPS from *Escherichia coli* for 5 h, followed by stimulation with 100 µM BzATP (2´(3´)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt), a specific ligand of ATP receptor P_2X_7. Chemokines, nicotinic antagonists or inhibitors of phospholipases A2 (PLA2s) were added to the cell suspension simultaneously with BzATP. After another 30 min of incubation, supernatants were separated by centrifugation for 8 min, at 500 g and room temperature and used for IL-1β quantification using an enzyme-linked immunosorbent assay (ELISA). Lactate dehydrogenase (LDH) concentrations as a measure of cell death were also determined from the supernatants using the CytoTox 96® colorimetric assay kit.

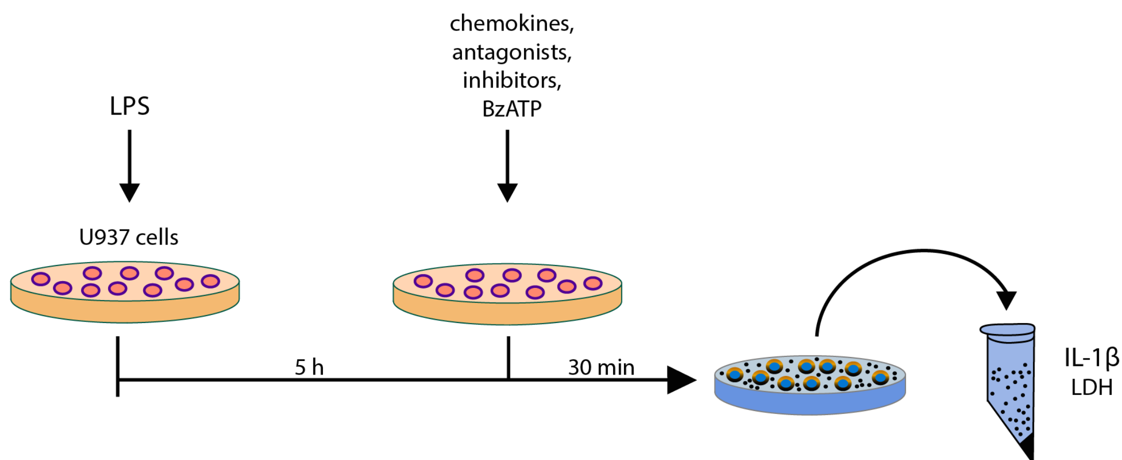

Figure 1: *Interleukin-1β (IL-1β) release experiments on U937 cells*. U937 cells were primed with lipopolysaccharide (LPS) for 5 h, after which chemokines in the presence or absence of nicotinic acetylcholine receptor (nAChR) antagonists or phospholipase A2 (PLA2) inhibitors were added, together with 2´(3´)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt (BzATP). Following another 30 min of incubation, supernatants were separated and used for interleukin-1β (IL-1β) and lactate dehydrogenase (LDH) measurement.

Each experiment included a set of control samples listed in Table 3.

To determine the total release of LDH, a sample consisting of 10^6 untreated cells was stored at -80 °C and thawed to ensure lysis of all contained cells with consequent maximum LDH release.

Control experiments	U937 cells	Ligands
1.	10^6 cells/ml	-
2.	10^6 cells/ml	LPS
3.	10^6 cells/ml	LPS, BzATP
4. total release of LDH	10^6 cells/ml	-
5. medium	-	-

Table 3: *List of control experiments.* Each experiment included a set of 3 controls as follows: 1. untreated cells, 2. lipopolysaccharide (LPS)-primed cells, 3. cells primed for 5 h with LPS and activated with 2´(3´)-O-(4-benzoyl-benzoyl)ATP trieethylammonium salt (BzATP, 100 µM, 30 min). For generating reference values in the subsequent assays for interleukin-1β (IL-1β) and lactate dehydrogenase (LDH) measurement, a total release of LDH sample consisting of untreated cells that were lysed at -80 °C (4) and a cell culture medium sample (5) were also included.

2.2.1.3 Dose-response experiments, nAChR antagonization and PLA2 inhibition

The experiments were carried out in 24-well plates. In a first step, 1 ml cell suspension containing 10^6 cells was loaded per well and 1 µl of previously sonicated LPS stock solution (1 µg/µl) was added per well. The cells were then incubated, as previously described, for 5 h at 37 °C and in humidified atmosphere with 5% CO_2. Chemokines CCL3, CCL4, CCL5, CXCL12, CXCL16 were delivered in lyophilized form and dissolved in 100 µl PBS with 0.1% bovine serum albumin (BSA) resulting in a stock solution with a concentration of 50 ng chemokine/µl, that was then divided in 10 µl aliquots. The chemokine solutions were stored at -20 °C, thawed and sonicated before usage. For the dose-response experiments LPS-primed U937 cells were treated with increasing concentrations (0.1, 0.5, 1, 5, 10 and 50 ng/ml) of each of the chemokines tested.

In a second set of experiments, the LPS-primed cells were sequentially treated with the following nAChR antagonists: 100 µM mecamylamine hydrochloride (Mec), 1 µM α-bungarotoxin (α-Bun), 10 µM strychnine (Stry) or the conotoxin-derived peptides [V11L; V16D]ArIB (500 nM) or RgIA4 (200 nM) [112, 233, 240, 300]. The effective concentrations of CCL3, CCL4 or CCL5 were subsequently added, followed by activation with 100 µM BzATP. To inhibit phospholipase A2 (PLA2), 50 µM arachidonyl trifluoromethyl ketone (ATK) or 50 µM bromoenol lactone (BEL) was applied prior to CCL3 and BzATP addition in a further experimental series. The cells were incubated with the applied reagents for another 30 min under standard conditions, then supernatants were harvested and stored at -20 °C until further processing. Each experiment was repeated

four to five times.

2.2.1.4 Experiments using nigericin

LPS-primed U937 cells were treated with apyrase (0.5 U/ml), an enzyme that cleaves ATP, in the presence or absence of the efficient inhibitory doses of chemokines CCL3, CCL4 and CCL5. Simultaneously, the pore-forming bacterial toxin nigericin (50 µM) was added and the cells were incubated for 30 min under standard conditions. The cell suspensions were centrifuged, supernatants collected and stored until further processing. Two additional samples of nigericin-treated cells and a medium sample with added nigericin were included to be used as control samples in the CytoTox 96® colorimetric assay for LDH measurement.

2.2.1.5 Gene silencing

In order to knock-down the expression of the chemokine receptor and iPLA2β genes, U937 cells were transfected with small interfering RNA (siRNA) ON-TARGETplus SMARTpool targeting human *CCR1* or *PlA2G6* using the Amaxa® Cell Line Nucleofector® Kit C. ON-TARGETplus Non-targeting Control Pool was used as a negative control. The lyophilized siRNA was resuspended in 250 µl RNAse-free water in order to obtain a 20 µM stock solution that was aliquoted into 5 µl portions. The siRNA aliquots were stored at -80 °C and thawed before usage.

An appropriate number of wells on a 12-well plate were filled with 1 ml supplemented culture medium (RPMI 1640 with 10% FCS and 2 mM GlutaMAX™ solution) and equilibrated in the humidified 37 °C/5% CO_2 incubator. The required number of cells (10^6 cells per sample) was centrifuged at 110 g for 10 min at room temperature. After discarding the supernatant, each cell pellet was resuspended in 100 µl Nucleofector® Solution per sample. This cell suspension was mixed with 2 µl siRNA stock solution targeting human *CCR1*, *PlA2G6* or with control siRNA, respectively. The cell/siRNA suspension was transferred into the certified cuvette and electroporation was performed using the appropriate programme of the Nucleofector® device. 500 µl of pre-equilibrated culture medium were added to each cuvette and each sample was transferred into the prepared 12-well plate. The cells were incubated under standard conditions for 48 h before further processing.

After incubation, the transfected cells were used in IL-1β release experiments according to the protocol outlined in section 2.2.1.2., which involves priming with LPS followed by addition of effective chemokine (CCL3, CCL4 and CCL5) concentrations and BzATP. Centrifugation (8 min, 500 g, room temperature) was performed after further 30 min of incubation and supernatants were collected for IL-1β and LDH measurement, whereas the cells were washed in DBPS and centrifuged in order to obtain cell pellets that were stored at -20 °C for future mRNA and protein extraction.

2.2.1.6 Conditioned medium

The required number of cells was centrifuged (8 min, room temperature, 500 g), the supernatant discarded and the cells resuspended in FCS-free medium. Cells were then primed with LPS as previously described, then 10 ng/ml CCL3 were added followed by 30 min of incubation. 2 ml fractions of the supernatant separated by centrifugation (8 min, 500 g, 4 °C) were loaded on to Amicon® Ultra Centrifugal filters with a cut-off of 3 kDa. A control ultrafiltrate was produced by adding the chemokine to the supernatant of LPS-primed U937 cells just shortly before ultrafiltration.
Ultrafiltration was performed at 4000 g for 20 min at 4 °C. The collected flow-through from the Amicon® filters was used in IL-1β release experiments, on LPS-primed cells with or without addition of RgIA4 or [V11L; V16D]ArIB. The efficiency of the ultrafiltration was controlled by SDS-gel electrophoresis followed by silver staining. For this purpose, 40 µl of each of the three fractions, namely the supernatant before ultracentrifugation, the ultrafiltrate (UF) and the high molecular mass fraction (HF) obtained after ultrafiltration were mixed with 10 µl sample buffer 2 and stored at -20 °C until further use.

2.2.2 Experiments on human PBMCs

Studies on human blood from male healthy non-smoking volunteers were approved by the local ethics committee of the University of Giessen (No. 81/13). For PBMC isolation, 10 ml of blood was drawn from a peripheral vein into a sterile syringe containing 175 I.U. Heparin. Following a dilution with PBS with 0.1% BSA up to a total volume of 25 ml, the blood was transferred into Leocosep™ gradient tubes. Through centrifugation (20 min, 800 g and room temperature), the enriched cell fraction containing the PBMCs covered by a plasma layer was separated above the porous barrier of the gradient tube.

The plasma was discarded and the PBMCs collected and suspended in 5 ml supplemented culture medium (RPMI 1640 with 10% FCS and 2 mM GlutaMAX™). The cells were counted following the same method described in section 2.2.1.1., except for using Türk's solution for cell staining. The cell suspension was accordingly diluted to obtain a cell density of 10^6 cells/ml cell suspension. Cell pellets for subsequent RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) experiments were obtained by centrifugation after washing with DPBS.

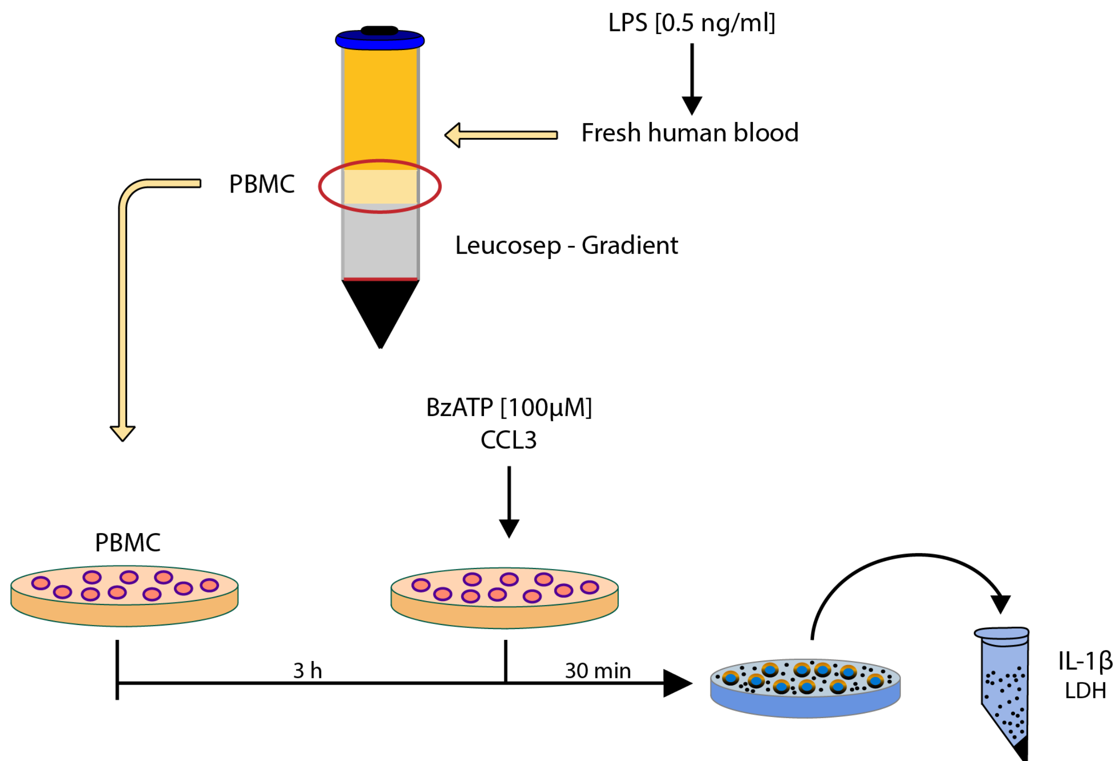

Figure 2: *Interleukin-1β (IL-1β) release experiments on primary human cells.* Human peripheral blood mononuclear cells (PBMCs) were freshly separated from lipopolysaccharide (LPS)-primed human blood by Leucosep™ gradients. After 3 h of incubation, 2´(3´)-O-(4-benzoyl-benzoyl)ATP triethylammonium salt (BzATP, 100 µM) was added to adherent cells in fresh culture medium for 30 min in the presence or absence of CCL3 (10 ng/ml). IL-1β and lactate dehydrogenase (LDH) were measured in cell culture supernatants.

In IL-1β release experiments, as depicted in Figure 2, 10 ml of heparinized blood were pulsed with 0.5 ng LPS/ml, then subjected to density gradient centrifugation as described above. The separated PBMCs were seeded on multi-well plates at a density of 10^6 cells per well and cultured for 3 h under standard conditions. Non-adherent cells were removed together with the culture medium, while fresh culture medium was added to the adherent cells that were then treated with BzATP (100 µM) in the presence or absence of CCL3 (10 ng/ml). After further 30 min of incubation, supernatants were collected for IL-1β and LDH measurement.

2.2.3 Measuring IL-1β concentration using ELISA

The IL-1β levels from the samples obtained in the cell culture experiments were determined using the Quantikine® ELISA Human IL-β immunoassay kit, which implies a solid phase quantitative sandwich enzyme technique. The assay was performed following the manufacturer's instructions. IL-1β present in the samples was bound by a monoclonal antibody specific to human IL-1β, immobilized on the surface of the provided 96-well microplate. A second, enzyme-linked polyclonal antibody specific to human IL-1β was added, whose reaction with the appropriate substrate resulted in color development proportional to the amount of IL-1β in the samples. The absorption was measured using the FLUOStar OPTIMA spectrophotometer at a wavelength of 450 nm. According to the data sheet, the analytical sensitivity of the assay is of 1 pg/ml with a detection range for cell culture supernatatns of 3.9 - 250 pg/ml.

2.2.4 Estimation of cell death by LDH measurement

Cell viability in the samples was determined by measuring the amount of the cytosolic LDH released to the sample supernatants by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay, according to the manufacturer's instructions. This colorimetric assay is based on the enzymatic formation of a red formazan product, in a manner proportional to the amount of active LDH. The amount of colour formed was determined by spectrophotometric absorbance at 490 nm. Cell viability was measured by calculating the ratio of the released LDH in each sample to the total LDH released by lysis of 10^6 cells.

2.2.5 mRNA quantification

Real-time RT-PCR was employed to analyze mRNA expression of the chemokine receptors in U937 cells. The target receptors for the studied chemokines are listed in Table 4.

Gene silencing efficiency in the transfection experiments was also evaluated through real-time RT-PCR.

Chemokine	Receptors
CCL3	CCR1, CCR5
CCL4	CCR1, CCR5
CCL5	CCR1, CCR3, CCR5
CXCL12	CXCR4, ACKR3 (CXCR7)
CXCL16	CXCR6

Table 4: *Target receptors for the studied chemokines*, adapted and modified from C. Schütt [251].

2.2.5.1 RNA isolation

RNA was isolated from untreated PBMCs and U937 cells as well as from siRNA-treated U937 cells using Qiagen RNeasy Miniprep Kit, a spin column based RNA purification method, following the instructions provided by the manufacturer. The concentration of the extracted RNA was measured spectophotometrically using Nano Drop 1000 at the wavelength of 260 nm. The samples were then stored at -80 °C until further usage.

2.2.5.2 Complementary DNA (cDNA) synthesis

Reverse transcription was performed to obtain cDNA transcripts from the extracted RNA using a two-step protocol. Firstly, 1 µg of purified RNA was mixed with 1 µg random primers then with dH_2O up to a volume of 10 µl. The samples were heated at a temperature of 70 °C for 5 min, then cooled down to 4 °C for 5 min using the Thermal Cycler G-Storm from AlphaMetrix Biotech. This step is intended to melt the secondary structure of the template. The samples were then immediately placed on ice and shortly vortexed. In a second step the components listed in Table 5 were mixed and then added to the pre-incubated 10 µl RNA samples, thus obtaining a final sample volume of 25 µl. The newly obtained samples were shortly vortexed then incubated in the Thermal Cycler G-Storm at 25 °C for 10 min, then at 42 °C for 1 h followed by 15 min at 70 °C for cDNA synthesis. The obtained cDNA was ready for direct use or could be stored at –20 °C.

Component	**Amount (µl)**
M-MLV-RT reaction buffer 5x	5
PCR nucleotide mix (dNTPs)	1.5
Recombinant RNasin® Ribonuclease Inhibitor	0.5
M-MLV RT RNase H(-) Point Mutant	0.5
dH_2O	7.5

Table 5: *Components of the master mix used for complementary deoxyribonucleic acid (cDNA) synthesis*. RT, reverse transcriptase; PCR, polymerase chain reaction; dNTP, deoxynucleotide triphosphate; RNase, ribonuclease, dH_2O, aqua destillata.

2.2.5.3 Real-time PCR

For each of the analyzed genes, the components for the real-time PCR were combined in a master mix using the formula and reagents listed in Table 6. The Platinum®SYBR® Green qPCR SuperMixUDG contains, according to the manufacturer, deoxyribonucleotide triphosphates (dNTPs), the recombinant *Taq* DNA polymerase and *SYBR® Green I Dye*.

Component	**Amount (µl)**
Platinum®SYBR® Green qPCR SuperMixUDG	13
dH_2O	8
50 mM $MgCl_2$	1
1 µM forward primer	0.5
1 µM reverse primer	0.5

Table 6: *Components of the master mix used for real-time polymerase chain reaction (PCR).* dH_2O, aqua destillata, $MgCl_2$, magnesium chloride.

All primers were synthetized by Eurofins Genomics (Ebersberg, Germany) and were delivered in lyophilized form. The employed primer sequences are indicated in Table 7. 2 µl cDNA were added to the master mix, resulting in a total sample volume of 25 µl that was loaded onto a 96-well plate. In negative controls the cDNA was replaced by dH_2O. cDNA from freshly isolated human PBMCs was included as a positive control. For the real-time PCR, evaluating the efficiency of gene silencing, cDNA from non-transfected cells was included. The plate was shortly centrifuged, then using Applied Biosystems Step-One the real-time PCR was initialized by heating the samples to 50 °C for 2 min then at 95 °C for 5 min followed by 45 amplification cycles, based on 3 steps:

- denaturation for 5 s at 95 °C;
- primer annealing for 5 s at 60 °C;
- elongation for 30 s at 72 °C.

Hydroxymethylbilane synthase (*HMBS*), alias porphobilinogen deaminase (*PBGD*) was used as housekeeping gene for data normalization. Analysis of the relative gene expression between two samples was facilitated by the attached software (StepOne v2.3). The $2^{\Delta CT}$ method was applied for calculation, using the threshold cycle (CT) values generated by the RT-PCR system, where ΔCT is the difference between the CT of the housekeeping gene and of the gene of interest. The obtained mean value for the cells treated with control siRNA was set to one arbitrary unit.

Gene Name (Accession number)	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size (bp)
CCR1 (NM_001295.2)	GGA CAA AGT CCC TTG GAA CC	GGA GTT GCA TCC CCA TAG TC	101
CCR3 (NM_178328.1)	ATC CGG GCA AGA ACT TAT CG	AGG ATG TGG TAC CAA AGG TCT C	114
CCR5 (NM_000579.3)	CTG GCC AGA AGA GCT GAG AC	GGG CTC CGA TGT ATA ATA ATT GA	116
CXCR4 (NM_003467.2)	GGA GAA CCA GCG GTT ACC AT	CAG GGT TCC TTC ATG GAG TC	100
CXCR6 (NM_006564.1)	GGA ACA AAC TGG CAA AGC AT	TGG CTG CTG TCA TTG AAA CT	107
*ACKR3 (*CXCR7) (NM_020311.2)	ACA GCA CAG CCA GGA AGG	AGT CGA AGA GAT GCA GAT CCA	107
PLA2G6 (NM_003560.2)	CAT CCG TAA CCA CCC CAG C	CGT TCT CCG CGC AAT TGG	109
HMBS (NM_001258209.1)	GGC GCA GCT ACA GAG AAA GT	AGC CAG GAT AAT GGC ACT GA	115

Table 7: *List of primers and their sequences used for real-time RT-PCR.* ***CCR1***, C-C motif chemokine receptor 1; ***CCR3***, C-C motif chemokine receptor 3; ***CCR5***, C-C motif chemokine receptor 5; ***CXCR4***, C-X-C motif chemokine receptor type 4; ***CXCR6***, C-X-C motif chemokine receptor type 6; ***ACKR3*** (***CXCR7)***, atypical chemokine receptor 3 or C-X-C motif chemokine receptor type 7; ***PLA2G6***, phospholipase A2 group VI; ***HMBS***, hydroxymethylbilane synthase; bp, base pairs.

2.2.5.4 Agarose-gel electrophoresis

To verify size and purity of the amplicons resulting from real-time PCR, these were separated by electrophoresis on a 1.5 % agarose gel. To prepare the gel, 1.2 g of agarose

were mixed with 80 ml Tris-acetate EDTA (TAE) 1x solution and heated for 3 min until the agarose was completely dissolved. 8 µl of GelRed™ Nucleic Acid Dye was added, the mixture was then cast into a tray and left to cool for 20 min. Afterwards, the gel was placed into the electrophoresis chamber filled with TAE 1x as a running buffer. 9 µl of each PCR product mixed with 2 µl of 6x DNA Gel Loading Dye were loaded into the gel pockets. 7 µl of a molecular mass marker (GeneRuler™ 100 bp DNA ladder) were loaded in the last gel pocket. The electrophoresis was performed for 30 to 40 min at 100 V. The DNA bands in the gel were viewed with an ultraviolet (UV) transilluminator, documented using the Olympus C4000-Zoom digital camera, processed and stored using AlphaDigiDoc 1201 software.

2.2.5.5 PCR product purification and sequencing

To further confirm the identity of the amplicons, DNA bands were excised from the gel and purified using MinElute® PCR Purification Kit from Qiagen, according the the manufacturer's instructions. The PCR products were then sequenced by SeqLab (Göttingen, Germany).

2.2.6 Protein biochemistry

2.2.6.1 Determining protein concentration

For protein extraction, each of the cell pellets collected from the transfection experiments were resuspended in 50 µl sample buffer 1, followed by a bead-beating homogenization process at 30 Hz for 5 min, heating at 95 ºC for 5 min and centrifugation (5 min, 5720 g, room temperature). The samples collected from the conditioned medium experiments (supernatant before ultracentrifugation, UF and HF) were thawed, heated and centrifuged under the same conditions. 10 µl from each of the resulting samples were taken to determine protein concentration by using the Micro BCA™ Protein Assay Kit. The assay involves a colorimetric reaction that is quantified by comparing absorbance at 562 nm to a set of standard BSA dilutions using spectrophotometry.

Following the manufacturer's instructions, a series of sample dilutions and BSA standard dilutions were incubated with the substrate, then the protein concentration was determined by absorbance measurement with the FluoStar OPTIMA spectrophotometer and data processing using the attached software. According to the determined protein concentrations, the samples were further diluted with a suitable amount of sample buffer

1 and sample buffer 2 (mixed in a 1:1 ratio) in order to obtain a final concentration of 0.8 g/µl.

2.2.6.2 SDS-gel electrophoresis

Samples were separated on reducing SDS-polyacrylamide gels. The acrylamide concentration was chosen according to the molecular size of the protein of interest: a 15% SDS-gel for CCL3 detection, and a 10% SDS-gel for iPLA2β detection.
The gels were prepared by mixing the substances in quantities listed in Table 8:

Component	15% SDS-gel	10% SDS-gel
dH_2O	1050 µl	1830 µl
resolving gel buffer	1076 µl	1080 µl
Acrylamide, 30% solution	1100 µl	1460 µl
APS solution	7.7 µl	7.7 µl
TEMED	3 µl	3 µl

Table 8*: Components and their appropriate quantities used for preparing 15% and 10% SDS resolving gels.*dH_2O, aqua destillata; APS, ammonium persulfate; TEMED, tetramethylethylenediamine.

After properly mixing the components, 3.75 ml of the resolving gel were poured into the gel assembly cassette, adding water on top of the resolving gel until the assembly cassette was filled. The gel was left to polymerize for about 40 min during which a stacking gel was prepared following the recipe depicted in Table 9. After removing the water, the stacking gel was poured onto the resolving gel so as to fill up the cassette, then a gel comb was introduced to form the gel pockets.

Component	Amount (µl)
dH_2O	1350
stacking gel buffer	790
acrylamide, 30% solution	312.5
APS solution	5.62
TEMED	4.5

Table 9: *Components and their appropriate quantities used for preparing the stacking gel.* dH_2O, aqua destillata; APS, ammonium persulfate; TEMED, tetramethylethylenediamine.

After another 40 min, the gel comb was removed and the gel pockets washed with running buffer. The gel cassettes were assembled onto the electrophoresis apparatus that was filled

with running buffer. The gel pockets were each filled with 10 µl of the protein samples that were previously heated at 70 °C for 5 min and then centrifuged (5 min, 5720 g, room temperature). The dual colour Precision Plus Protein™ Standards Marker was added to one of the gel pockets. Electrophoresis was run at 80 V for 10 min in order to concentrate the samples in the stacking gel, then at 120 V for 75 min for protein separation according to their molecular mass.

2.2.6.3 Silver staining

The 15% SDS-gel slabs containing the separated proteins from the samples obtained in the conditioned medium experiments were stained with the Silver Stain Plus kit according to the manufacturer's instructions and documented using a gel imaging system (Intas, Göttingen, Germany).

2.2.6.4 Western blotting

The proteins from the cells resulting from the transfection experiments, separated on 10% SDS-gels for iPLA2β detection were transferred onto Immobilon® polyvinylidene difluoride (PVDF) membranes. For this, each of the gel slabs was stacked onto a membrane, previously activated in methanol, between filter paper and sponges soaked with transfer buffer. The assembled stacks were then mounted onto the electrophoresis apparatus filled with transfer buffer. Protein transfer was performed over 90 min at 90 mA. The membranes were then left to dry for 1 h, then activated for 1 min in methanol, rinsed with PBS, then blocked with 5% BSA diluted in PBS. The primary polyclonal rabbit antibodies directed to human iPLA2β were diluted 1:5000 in PBS and 2.5% powdered milk then incubated with the membranes overnight, at a temperature of 4 °C, under constant rotation. The next day, the membranes were washed 4 times for 7 min in PBS-T followed by 90 min of incubation with the secondary horseradish peroxidase-labeled goat anti-rabbit Ig antibodies, diluted 1:5000 in PBS-T and 2.5% powdered milk. After three rinsing cycles with PBS-T and one with PBS, the membranes were incubated for 3 min with SuperSignal West Dura Extended Duration Substrate. Chemoluminescent detetection was used to visualize protein bands on film.

To determine whether samples have been loaded equally across all wells, the membranes were rinsed three times with PBS, then incubated for 75 min with monoclonal mouse anti-human β-actin antibodies (1:50000), as a loading control. After four further rinsing

cycles, bound primary antibodies were detected with secondary rabbit anti-mouse Ig horseradish peroxidase-labeled antibodies (1:5000) and the Lumi-Light substrate on High Performance Chemiluminescence Films.

2.2.6.5 Densitometry

To quantify Western blot signals, films were imaged using the AlphaEase software that measured the optical density (OD) of the immunepositive bands. iPLA2β expression was normalized by dividing the OD of iPLA2β protein bands to the OD of the β-actin loading control. The average of the determined values for cells treated with control siRNA was set to one arbitrary unit and the values for cells transfected with siRNA targeting *PLA2G6* were statistically compared to this unit for determining fold difference in protein expression.

2.2.7 Statistical analyses

Results are presented as individual data points, median and percentiles 25 and 75. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test using the SPSS software (IBM SPSS statistics, version 23, Munich, Germany). Data obtained from primary leukocytes were analyzed by the Wilcoxon signed-rank test (SPSS software). p-values below 0.05 were considered as statistically significant. IC_{50} values were determined using GraphPad Prism® (Version 6, GaphPad Software) by fitting log-transformed concentration values and the original effect data.

3 Results

3.1 CCL-induced inhibition of IL-1β release

3.1.1 CCL chemokines dose-dependently inhibit IL-1β release

First experiments were performed to determine whether CCL chemokines with an established role in monocyte chemotaxis influence IL-1β maturation and release by LPS-primed and ATP-stimulated human monocytic U937 cells. U937 cells at a density of 10^6 cells/ml were primed with LPS (1 µg/ml) for 5 h followed by activation with the P_2X_7R agonist BzATP (100 µM) for 30 min. Chemokines CCL3, CCL4 and CCL5 were added at increasing concentrations ranging from 0.1 to 50 ng/ml concomitantly with BzATP. IL-1β and LDH levels in the sample supernatants were determined using ELISA and a colorimetric assay, respectively. Basal IL-1β secretion, from the supernatants of untreated cells, was barely detectable. LPS priming alone did not increase IL-1β release into the cell culture medium when compared to basal levels. The consecutive application of LPS and BzATP, induced IL-1β secretion (median IL-1β concentration of 41 pg/ml, range 34 – 57 pg/ml; n = 8) (Figure 3 a, c, e). In line with our hypothesis, the BzATP-induced release of IL-1β by LPS-primed human monocytic U937 cells was dose-dependently and efficiently inhibited by chemokines CCL3 (IC_{50} = 9.2 ng/ml), CCL4 (IC_{50} = 11.5 ng/ml) and CCL5 (IC_{50} = 51.3 ng/ml) ($p = 0.029$; n = 4, each). Notably, among the three tested CCL-chemokines, CCL3 was most potent in inhibiting IL-1β secretion, showing a significant inhibition from a concentration of 1 ng/ml ($p = 0.029$; n = 4) and reaching a full inhibitory effect at a concentration of 10 ng/ml (Figure 3 a). CCL4 and CCL5 were needed in higher concentrations of 5 ng/ml and 10 ng/ml respectively, to induce a significant inhibitory effect on IL-1β secretion, both chemokines reaching a fully inhibitory effect at a concentration of 50 ng/ml (Figure 3 c, e). When fully inhibitory concentrations of chemokines CCL3 (10 ng/ml), CCL4 (50 ng/ml) and CCL5 (50 ng/ml) were applied to LPS- primed U937 cells in the absence of BzATP, no IL-1β was released into the cell culture medium (Figure 3 a, c, e).

LDH levels in cell culture supernatants, as a measure of cell death, remained below 4% of the total LDH content of lysed cells. Furthermore, there was no significant variation of LDH levels among the analyzed samples (Figure 3 b, d, f).

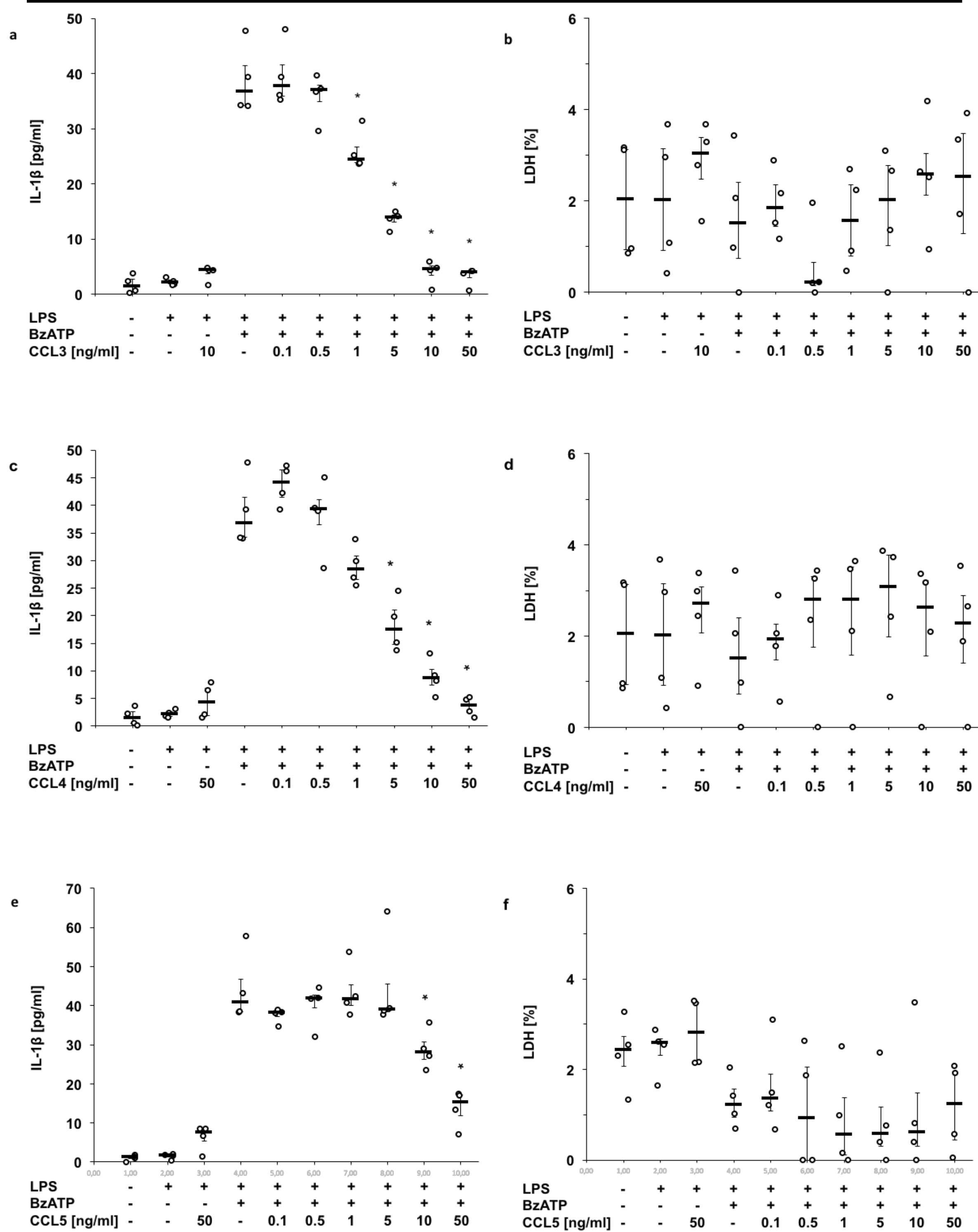

Figure 3. *CCL chemokines dose-dependently inhibit the ATP-induced IL-1β release in U937 cells.* Human monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated with 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'- triphosphate (BzATP; 100 µM, 30 min) in the presence of increasing CCL chemokine concentrations. The levels of interleukin-1β (IL-1β) and lactate dehydrogenase (LDH) in the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) and CytoTox 96® Non-Radioactive Cytotoxicity Assay, respectively. CCL3 (a), CCL4 (c), and CCL5 (e) dose-dependently and efficiently inhibited the BzATP-induced release of IL-1β. LDH levels throughout all performed experiments (b, d, f) did not exceed 4% of the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; n = 4; *p = 0.029, in comparison to cells treated with LPS and BzATP. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

3.1.2 CXCL chemokines do not inhibit ATP-dependent IL-1β release

Tested in the same experimental setting on LPS-primed and ATP stimulated U937 cells, chemokines CXCL12 and CXCL16 did not inhibit IL-1β secretion, regardless of the concentration used (0.1 to 50 ng/ml) (Figure 4 a, c). Cell death throughout the samples was minimal, as reflected by the LDH levels of below 4% of the total LDH content of lysed cells (Figure 4 b, d).

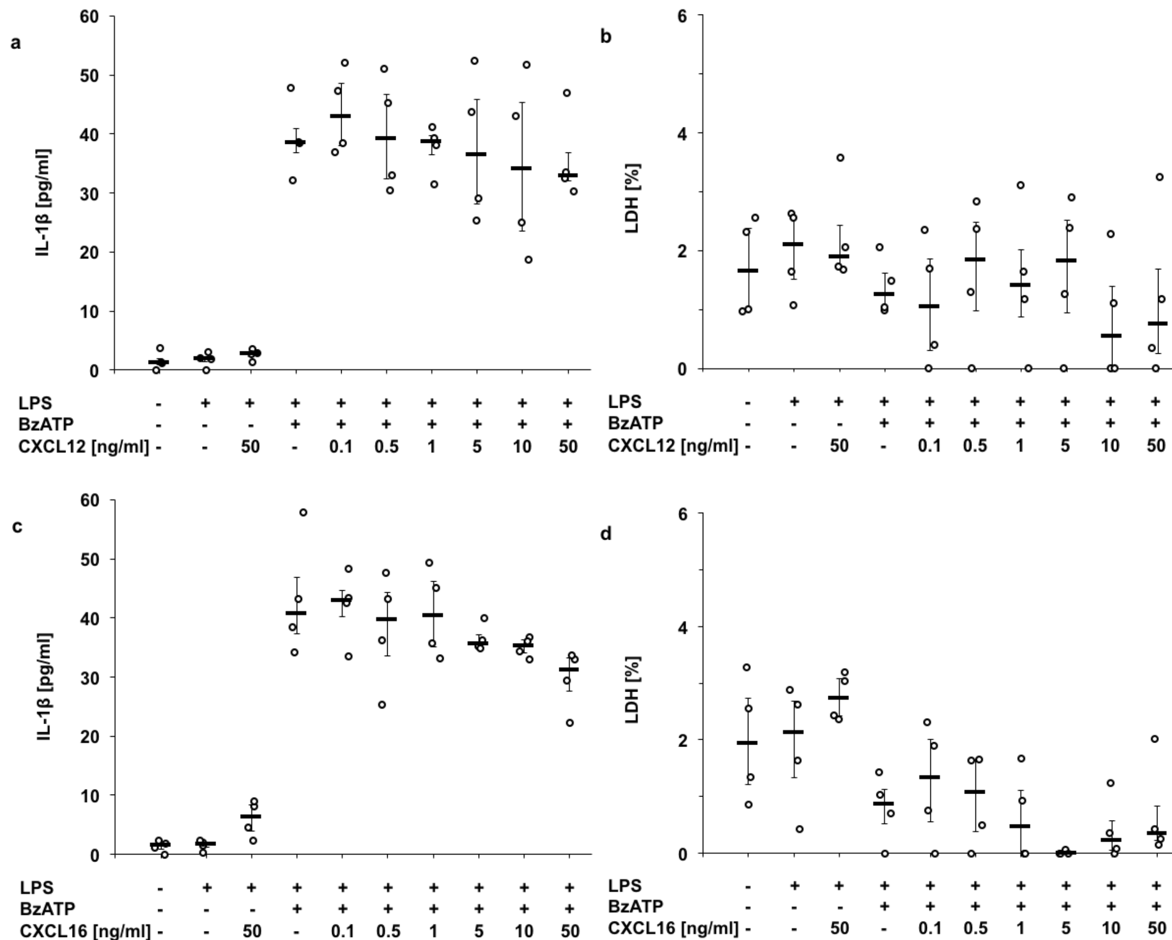

Figure 4. *CXCL chemokines do not inhibit the ATP-induced IL-1β release in U937 cells.* Lipopolysaccharide-primed (LPS; 1 µg/ml, 5 h) human monocytic U937 cells were stimulated with 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'- triphosphate (BzATP; 100 µM, 30 min) and CXCL12 or CXCL16 were simultaneously added in concentrations ranging from 0.1 - 50 ng/ml. The levels of interleukin-1β (IL-1β) and lactate dehydrogenase (LDH) in the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) and CytoTox 96® Non-Radioactive Cytotoxicity Assay, respectively. CXCL12 (a) and CXCL16 (c) did not influence IL-1β secretion to the cell culture medium regardless of the applied concentration. LDH levels remained below 4% of the total LDH of lysed cells (b, d). Data are presented as individual data points, bars indicate median, whiskers percentiles 25 and 75; n = 4. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

3.2 CCL3-induced inhibition of IL-1β release in human PBMCs

To test if the inhibitory effect of CCL3 on BzATP-mediated release of IL-1β also applies to primary mononuclear cells, experiments were conducted on human PBMCs (Figure 5). PBMCs were separated by gradient centrifugation from heparinized blood collected from healthy male donors. Adherent LPS-pulsed PBMCs secreted a small amount of IL-1β (median 344 pg/ml, range 247 – 468 pg/ml; n = 4) within 30 min, that did not differ from the amount of IL-1β secreted by untreated cells. Application of BzATP (100 µM) induced an increase in the IL-1β secretion when compared to untreated cells or cells treated just with LPS (median 3.6 ng/ml, range 0.6 – 9.2 ng/ml; n = 4). CCL3, applied in the previously determined effective inhibitory concentration of 10 ng/ml significantly reduced the ATP-dependent IL-1β release by LPS-primed PBMCs (median 2.2 ng/ml, range 0.4 – 4.2 ng/ml; p = 0.029; n = 4) (Figure 5 a). A median 44% reduction in measured IL-1β levels was induced when adding CCL3 to LPS-primed and ATP stimulated PBMCs. Cell death was slightly higher than in U937 cells with maximum LDH levels not surpassing 8% of the total LDH content of lysed cells (Figure 5 b).

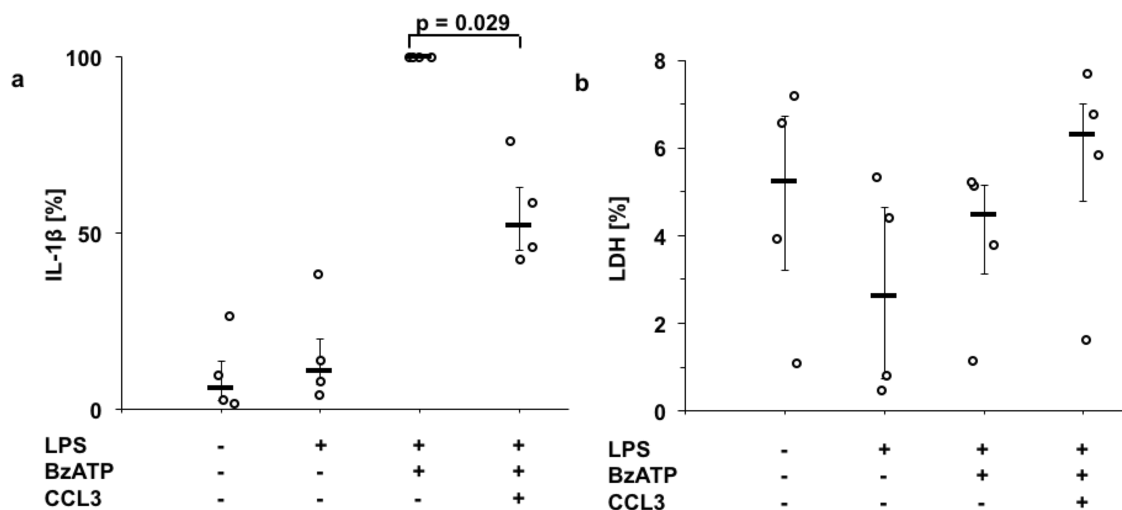

Figure 5: *CCL3 inhibits the ATP-induced IL-1β release by human peripheral blood mononuclear cells (PBMCs).* Blood from healthy volunteers was pulsed with 0.5 ng lipopolysaccharide (LPS)/ml before purification of PBMCs. PBMCs were cultured for 3 h, and 2′(3′)-O-(4- benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM) was added for 30 min in the presence or absence of CCL3 (10 ng/ml). Interleukin-1β (IL-1β) was measured in cell culture supernatants, and the values obtained in the supernatants of cells treated with LPS and BzATP were set to 100%. CCL3 efficiently inhibited BzATP-induced release of IL-1β by LPS-primed human PBMCs (a). Lactate dehydrogenase (LDH) was measured in cell culture supernatant and calculated as a percentage of the total LDH content of lysed cells (b). Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75; n = 4, each. Data were analyzed by the Wilcoxon signed-rank test. p-values below 0.05 were considered as statistically significant.

3.3 Signaling via CCR

3.3.1 Chemokine receptor expression in U937 and PBMCs

To elucidate the mechanism underlying the CCL chemokine-induced inhibition on ATP-dependent IL-1β release by human monocytes, we first studied the role of their cognate receptors. The mRNA expression of receptors corresponding to all previously tested chemokines, irrelevant of their IL-1β inhibitory potential, was analyzed by real-time RT-PCR. This panel of receptors included *CCR1, CCR3, CCR5, CXCR4, CXCR6* and *CXCR7*. In U937 cells, we were able to detect the mRNA for *CCR1*, *CCR3*, *CCR5*, *CXCR4* and *CXCR7* whereas no mRNA for *CXCR6* was detected after 45 cycles of amplification. PBMCs, that include both monocytes and lymphocytes, served as a positive control and expressed the mRNA of all chemokine receptors, including *CXCR6* (Figure 6).

No DNA was amplified in negative controls in which cDNA was replaced by dH_2O. The amplicons resulting from the real-time RT-PCR were separated on 1.5% agarose gels by electrophoresis and DNA bands of the expected size (between 100 and 120 bp, as listed in Table 7) were detected. DNA sequencing confirmed product identity

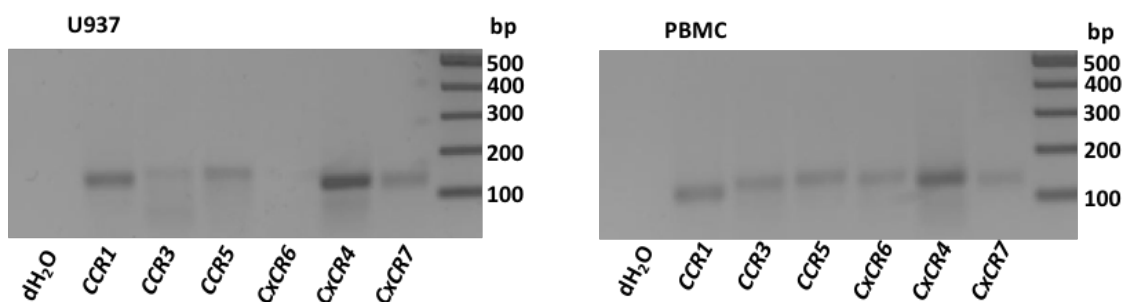

Figure 6: *Expression of chemokine receptor mRNA in U937 cells and peripheral blood mononuclear cells (PBMCs)*. The mRNA expression of chemokine receptors *CCR1*, *CCR3*, *CCR5*, *CXCR4*, *CXCR6*, and *CXCR7* was investigated in U937 cells by real-time RT-PCR, and the amplicons were separated by gel electrophoreses together with a base pair (bp) ladder and detected by GelRedTM Nucleic Acid Dye. PBMCs obtained from healthy volunteers served as positive control.

3.3.2 CCR1 mediates the inhibitory effect of CCL3 on ATP-dependent IL-1β release

To determine whether CCR1 participates in the CCL-induced inhibition of IL-1β release, we transfected U937 cells with siRNA targeting *CCR1* gene expression. We selected *CCR1* for gene silencing, because it is a common receptor for all three CCL-chemokines included in the study and because the mRNA of this receptor seemed to be more abundant

in U937 cells than *CCR3* and *CCR5* mRNA (Figure 7). Control cells were transfected with irrelevant control siRNA. To verify transfection efficiency, cells were harvested after 48 h of incubation and the mRNA expression of *CCR1* was analyzed using real-rime RT-PCR. In contrast to control siRNA, siRNA targeting *CCR1* significantly reduced the mRNA expression, as shown in Figure 6 (p = 0.029; n = 4). The transfection process itself did not alter *CCR1* gene expression, as *CCR1* mRNA levels were similar in cells that have not been transfected and in control siRNA transfected cells.

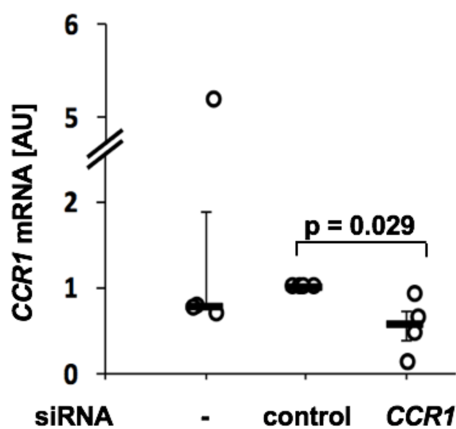

Figure 7: *Efficient reduction of CCR1 expression in U937 cells*. U937 cells were transfected with control siRNA or with siRNA targeting *CCR1*. The efficiency of the siRNA transfection was verified 48 hours post-transfection by real-time RT-PCR. A significant down-regulation of the *CCR1* gene expression was obtained for cells treated with siRNA targeting *CCR1* when compared to those transfected with control siRNA. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75, n = 4, each. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

Having proven transfection efficiency, U937 cells transfected with control siRNA or with siRNA targeting *CCR1* were used in IL-1β release experiments, 48 h post-transfection. Cells that have not been transfected and that were stimulated with either LPS alone or LPS and BzATP were included as controls. BzATP-dependent release of IL-1β by LPS-primed cells was not impaired and was maintained at equivalent levels in cells transfected with either control siRNA or with *CCR1* targeting siRNA when compared to cells that were not manipulated. ACh, as a classical nicotinic receptor agonist, has been previously proven to efficiently inhibit ATP-dependent IL-1β release in human monocytic cells [106]. Therefore, ACh was included as a positive control. The ACh-mediated inhibition of IL-1β release was just as efficient in cells where the *CCR1* gene was silenced as in control-transfected cells (Figure 8 a). In contrast to this, the inhibitory effect of CCL3 was significantly blunted in cells with down-regulated *CCR1* expression when compared to control-transfected cells, as depicted in Figure 8 a (p = 0.008; n = 5).

LDH concentrations in the cell culture supernatants did not surpass 10% of the total LDH content of lysed cells (Figure 8 b). There was also no significant difference in cell death between cells transfected with *CCR1* targeting siRNA or control-transfected cells.

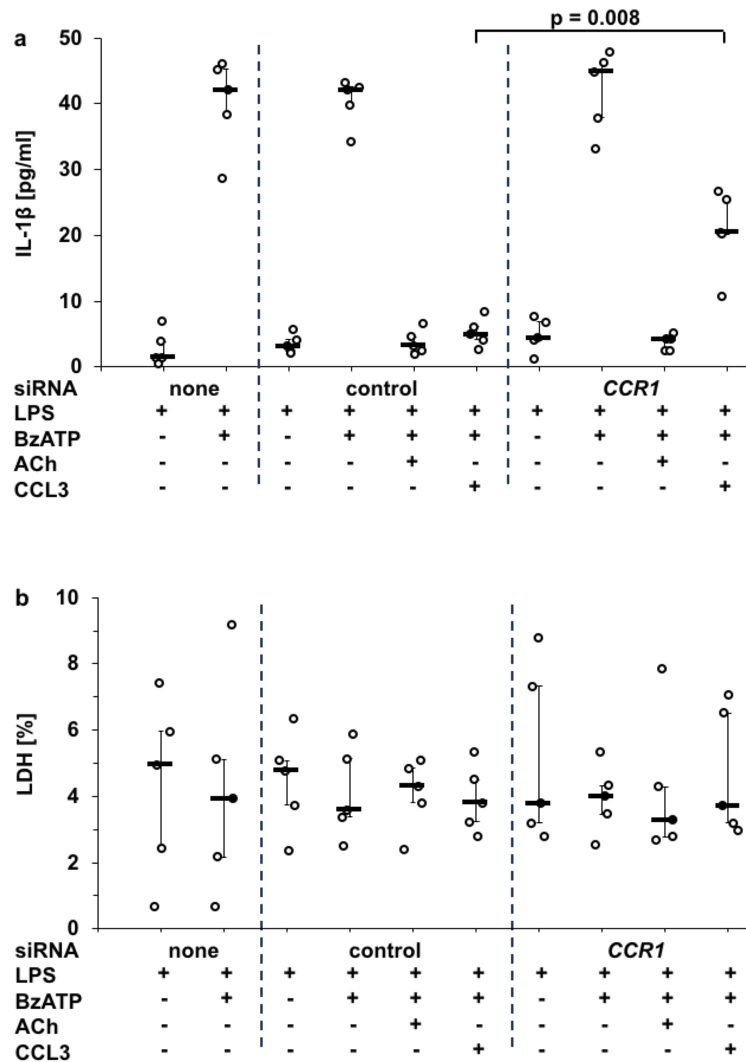

Figure 8: *The inhibitory effect of CCL3 on ATP-induced IL-1β release by U937 cells is dependent on CCR1.* U937 cells were transfected with control siRNA or with siRNA targeting *CCR1*. After 48 hours of incubation, these cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) followed by activation with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). a. Interleukin-1β (IL-1β) in the cell culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). In control-transfected cells, CCL3 (10 ng/ml) fully inhibited the BzATP-induced release of IL-1β, whereas silencing of *CCR1* significantly blunted the inhibitory effect of CCL3. In all experiments, acetylcholine (ACh; 10 µM) was included as a positive control. b. Cell death was quantified by measuring lactate dehydrogenase (LDH) concentration in cell culture supernatants using the CytoTox 96® Non- Radioactive Cytotoxicity Assay. LDH levels were expressed as a percentage from the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75; n = 5. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

3.3.3 CCR1 is not mandatory for the inhibitory effect of CCL4 and CCL5 on ATP-dependent IL-1β release

In the same experimental setting, the previously determined efficient inhibitory concentration of CCL4 (50 ng/ml) were applied to LPS-primed and BzATP stimulated U937 cells, transfected with either control siRNA or siRNA targeting *CCR1*. In contrast

to CCL3, the inhibitory effect of CCL4 was not influenced by *CCR1* gene silencing, implying that this receptor in not an essential part of the mechanism leading to CCL4-induced inhibition of IL-1β release (Figure 9 a). LDH levels remained below 10% of the total LDH content of lysed cells (Figure 9 b).

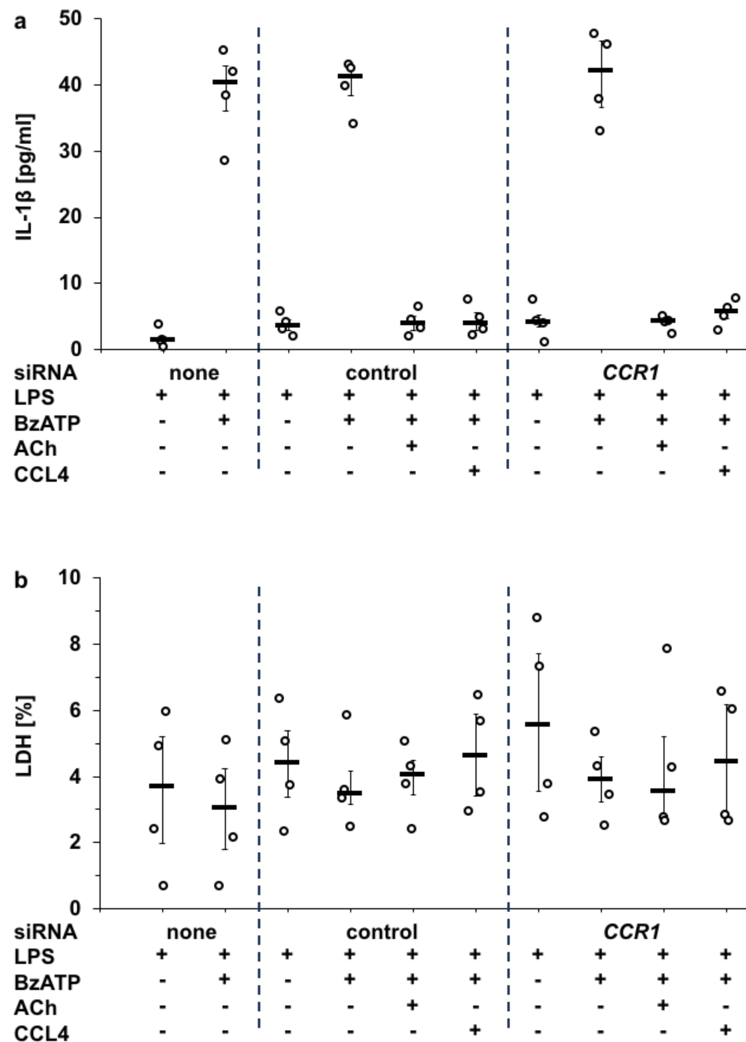

Figure 9: *CCR1 silencing does not influence the inhibitory effect of CCL4 on ATP-induced IL-1β release by U937 cells.* U937 cells were transfected with control siRNA or with siRNA targeting *CCR1*. After 48 hours of incubation, these cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) followed by activation with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). a. Interleukin-1β (IL-1β) in the cell culture supernatants was measured by ELISA. In control-transfected cells as well as in cells with down-regulated *CCR1* gene expression, CCL4 (50 ng/ml) fully inhibited the BzATP-induced release of IL-1β. In all experiments, acetylcholine (ACh; 10 µM) was included as a positive control. b. Cell death was quantified by measuring lactate dehydrogenase concentration in cell culture supernatants using the CytoTox 96® Non- Radioactive Cytotoxicity Assay. LDH levels were expressed as a percentage from the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75; n = 4. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

The involvement of CCR1 in the CCL5-induced inhibition of ATP-dependent IL-1β release was also tested by down-regulating *CCR1* gene expression. CCL5 fully inhibited

BzATP-dependent IL-1β secretion in cells transfected with control siRNA as well as in cells transfected with *CCR1*-targeting siRNA, showing just as in the case of CCL4, no essential contribution of the CCR1 receptor signaling to its inhibitory effect (Figure 10 a). The viability of the cells was not impaired (Figure 10 b).

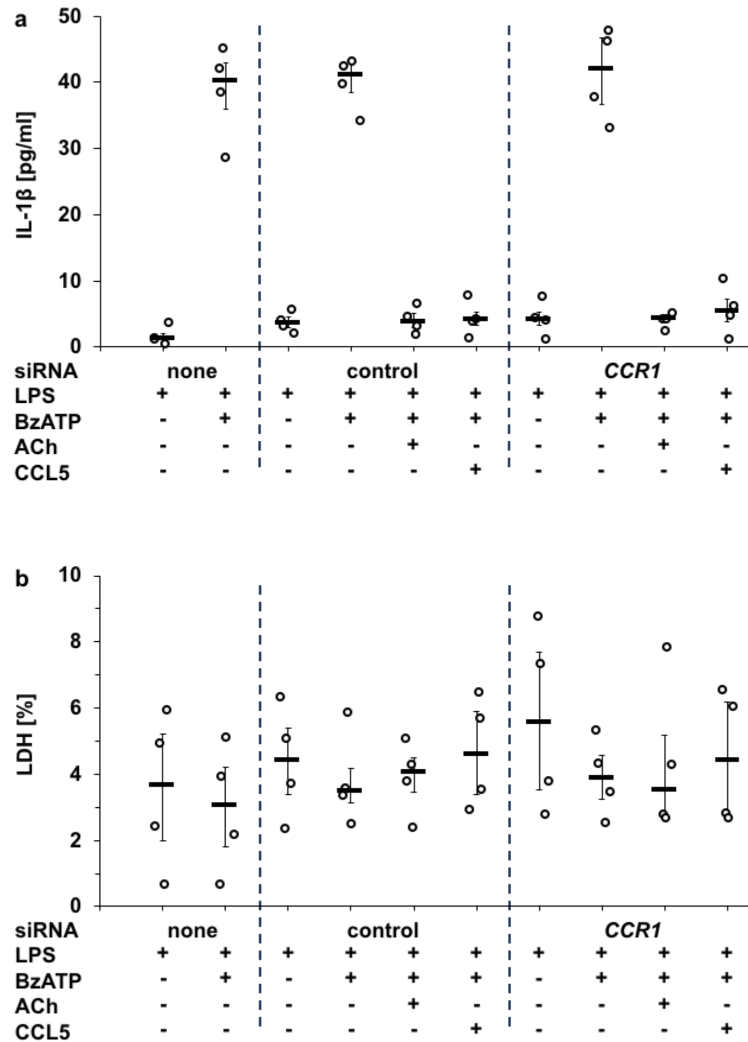

Figure 10: *CCR1 silencing does not influence the inhibitory effect of CCL5 on ATP-induced IL-1β release by U937 cells.* U937 cells were transfected with control siRNA or with siRNA targeting *CCR1*. After 48 hours of incubation, these cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) followed by activation with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). a. Interleukin-1β (IL-1β) in the cell culture supernatants was measured by ELISA. In control-transfected cells as well as in cells with down-regulated *CCR1* gene expression, CCL5 (50 ng/ml) fully inhibited the BzATP-induced release of IL-1β. In all experiments, acetylcholine (ACh; 10 µM) was included as a positive control. b. Cell death was quantified by measuring lactate dehydrogenase concentration in cell culture supernatants using the CytoTox 96® Non- Radioactive Cytotoxicity Assay. LDH levels were expressed as a percentage from the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75; n = 4. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

CCL3 was selected for the following elucidation of the signaling pathway.

3.4 Involvement of nAChRs

3.4.1 CCL3 signaling involves nAChRs

We hypothesized that chemokine-induced inhibition of IL-1β secretion is mediated by a cholinergic mechanism involving nAChRs, similar to the one described by Hecker et al. and Richter et al. [106, 233]. These two studies conducted in our laboratory established that stimulation of the α7 and α9/α10 nAChR subunits triggers a cholinergic pathway that prevents LPS-primed monocytes from secreting IL-1β upon receiving an ATP stimulus. We therefore set out to verify whether CCL3 signaling also involves these nAChR subunits by using a panel of nAChR antagonists that either preferentially or specifically act upon nAChR containing subunits α7 and α9/α10.
The usual set of controls showed an adequate and significant release of IL-1β by U937 cells incubated for 5 h with LPS and stimulated for 30 min with BzATP compared to untreated or LPS-primed cells. The inhibitory effect of CCL3 on the BzATP-induced release of IL-1β by U937 cells was indeed sensitive to mecamylamine (100 µM; $p = 0.001$, $n = 5$ versus $n = 9$), a general nAChR antagonist [90, 259]. Similarly, α-bungarotoxin (1 µM; $p = 0.001$, $n = 5$ versus $n = 9$) and strychnine (10 µM; $p = 0.001$, $n = 5$ versus $n = 9$), reagents that preferentially antagonize nAChR containing subunits α7 and α9 [171, 281] were efficient (Figure 11 a). To differentiate between those subunits, we made use of the antagonistic peptides [V11L; V16D]ArIB (500 nM), specific for nAChR containing an α7 subunit, and RgIA4 (200 nM), an antagonist of α9/α10 nAChR [112, 240, 300]. Both peptides antagonized the effect of CCL3 ($p = 0.003$, $n = 5$ versus $n = 9$), suggesting that signal transduction involves nAChR subunits α7 and α9/α10 (Figure 11 a). These nAChR subunits have been previously shown to be expressed by U937 cells and by human monocytes [106, 131]. The viability of the cells in this set of experiments remained unimpaired, regardless of the applied reagents with LDH values remaining below 4% of the total LDH content of lysed cells (Figure 11 b).

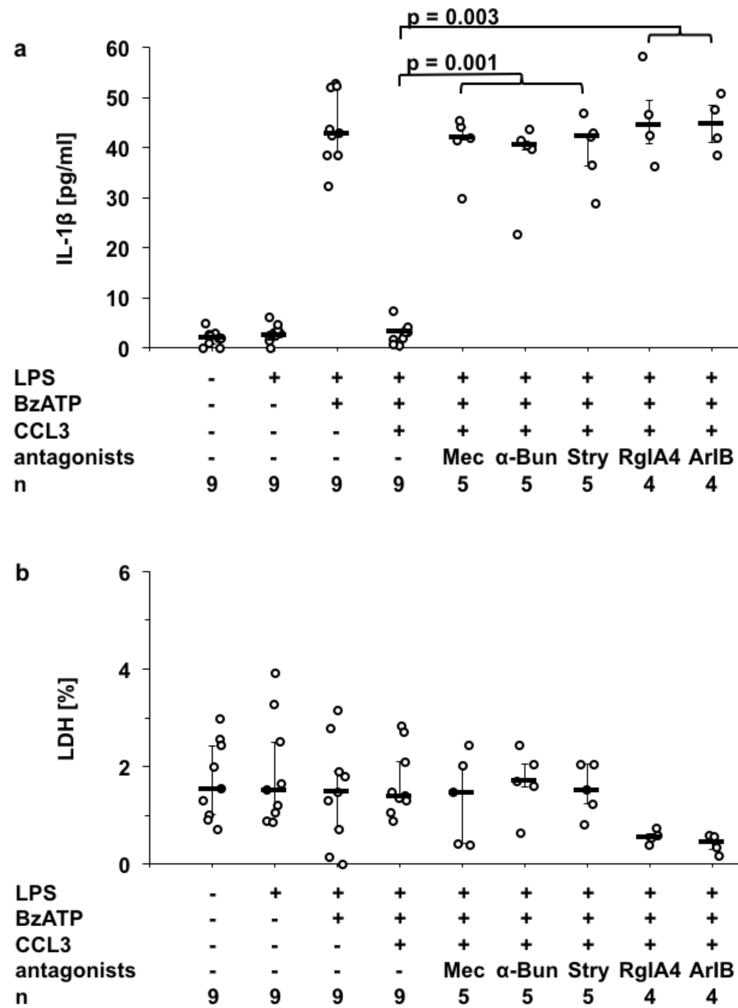

Figure 11: *CCL3 signaling involves nicotinic acetylcholine receptors.* Human monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). The release of IL-1β to the cell culture supernatant was measured by ELISA. a. Nicotinic antagonists mecamylamine (Mec; 100 µM), α-bungarotoxin (α-Bun; 1 µM), and strychnine (Stry; 10 µM), as well as conotoxin-derived peptides [V11L; V16D]ArIB (200 nM) and RgIA4 (200 nM), significantly reversed the inhibitory effect of CCL3. b. Lactate dehydrogenase (LDH) levels remained under 4% of the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

3.4.2 Nigericin-mediated release of IL-1β is not inhibited by CCL3

As a pore-forming bacterial toxin, nigericin induces K^+ efflux that activates the inflammasome independent of BzATP [182]. As expected, stimulation of LPS-primed U937 cells with nigericin, in the presence of the ATP-cleaving enzyme apyrase resulted in a significant increase of IL-1β levels in the cell culture supernatant, when compared to untreated or LPS-treated cells ($p = 0.02$, $n = 6$). The addition of CCL3 did not affect the

nigericin-induced secretion of IL-1β (Figure 12 a). LDH levels were even and did not exceed 4% of the total LDH content of lysed cells (Figure 12 b).

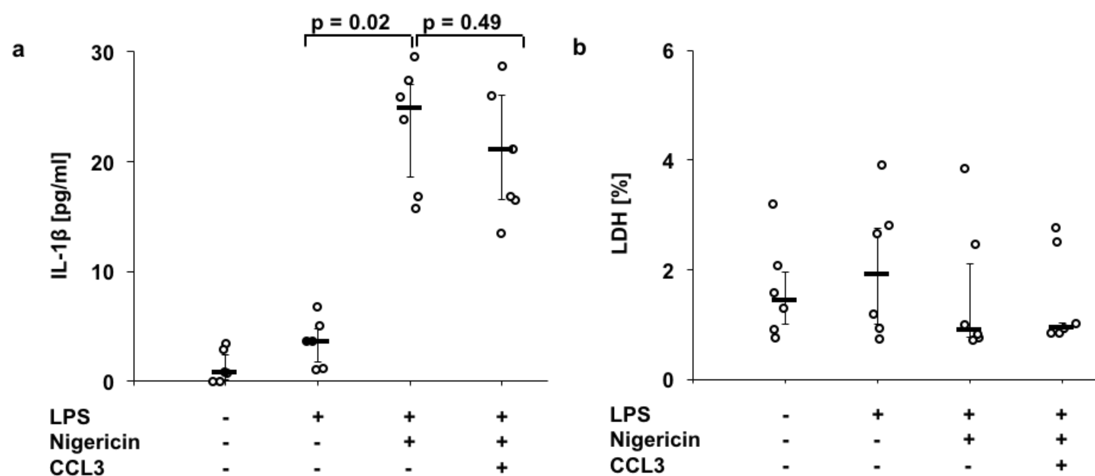

Figure 12: *CCL3 does not inhibit the ATP-independent IL-1β release by U937 cells.* Human monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated with the pore-forming bacterial toxin nigericin (50 µM) in the presence of apyrase (0.5 U/ml). a. The release of interleukin-1β (IL-1β) into the cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Nigericin induced the release of IL-1β by LPS-primed U937 cells, but the chemokine CCL3 (10 ng/ml) did not impair the nigericin-triggered release of IL-1β (n = 6). b. Lactate dehydrogenase (LDH) levels remained below 4% of the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

3.5 Release of soluble factors

We wanted to solve the question of how CCR activation links to cholinergic signaling. As at least some of the effective nicotinic antagonists such as α-bungarotoxin and RgIA4 probably do not enter the cytoplasm of the target cell due to their size and hydrophobicity, we assume that relevant nAChRs are localized in the plasma membrane. Hence, we postulated the involvement of soluble small nicotinic agonists that are released to the cell culture medium in response to CCL3.

To test this hypothesis, LPS-primed U937 cells were stimulated with CCL3 (10 ng/ml), and conditioned cell culture medium was harvested 30 min later. A low molecular weight fraction of the conditioned cell culture medium devoid of CCL3 was produced by ultrafiltration with a cut-off of 3.5 kDa (Figure 13 a).

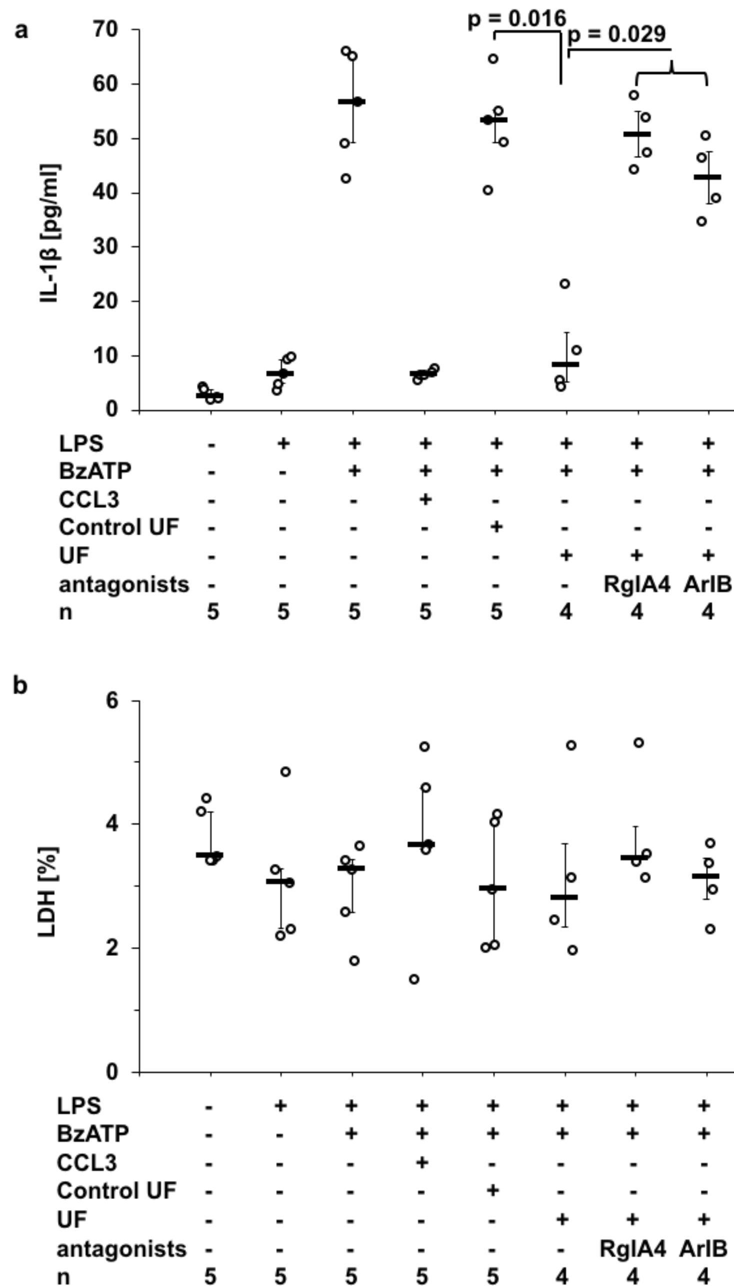

Figure 13: *Release of small mediators in response to CCL3*. U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated with 2′(3′)-O-(4- benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). An ultrafiltrate (UF) was produced containing the low molecular mass fraction (<3 kDa) of the cell culture supernatant (S) of LPS-primed U937 cells treated with CCL3 (10 ng/ml) for 30 min. For the production of control UF, CCL3 was added to the cell-free supernatant of LPS-primed U937 cells shortly before ultrafiltration. a. The release of interleukin-1β (IL-1β) to the cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). CCL3, which was included as a positive control, inhibited the BzATP-induced release of IL-1β. Control UF had no effect on the BzATP-induced release of IL-1β by LPS-primed U937 cells, whereas the UF significantly reduced the IL-1β release. The inhibitory effect of the UF was reversed by antagonistic peptides [V11L; V16D]ArIB (200 nM) and RgIA4 (200 nM). b. Cell death was quantified through lactate dehydrogenase (LDH) measurement and remained at levels below 6% of the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

As a control, conditioned medium was produced in the absence of CCL3 but CCL3 was added to the cell-free supernatant shortly before ultrafiltration. In line with our hypothesis, the ultrafiltrate of conditioned medium significantly inhibited the BzATP-stimulated release of IL-1β by LPS-primed U937 cells, whereas the ultrafiltrate of the control conditioned medium was ineffective ($p = 0.016$; $n = 4$ versus $n = 5$) (Figure 13 a). We wondered if this small mediator acts as a nicotinic agonist at nAChR subunits and were indeed able to demonstrate that the effect of conditioned medium is antagonized by peptides [V11L; V16D]ArIB and RgIA4 ($p = 0.029$; $n = 4$ each) that are specific for nAChR subunits α7 and α9, respectively (Figure 13 a). These results suggest that CCL3 induces the secretion of nicotinic agonists by U937 cells. Cell viability was not affected in these experiments as LDH levels, determined using the CytoTox 96® Non- Radioactive Cytotoxicity Assay remained below 6% of the total LDH content of lysed cells (Figure 13 b).

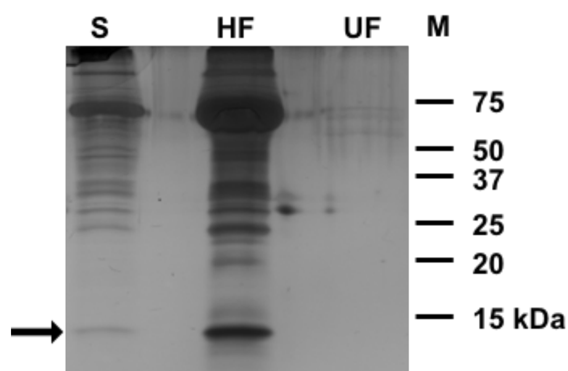

Figure 14. *Ultrafiltration efficiently depleted CCL3 from the conditioned cell culture supernatant.* U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). An ultrafiltrate (UF) was produced containing the low molecular mass fraction (<3 kDa) of the cell culture supernatant (S) of LPS-primed U937 cells treated with CCL3 (10 ng/ml) for 30 min. The UF and the high molecular mass fraction (HF) obtained by ultrafiltration were separated in a 15% SDS- polyacrylamide gel along with a molecular mass marker (M) followed by silver staining. The arrow is pointing to the band corresponding to CCL3. Proteins with higher molecular mass are bovine serum albumin (66.5 kDa) and its contaminations that were added to the CCL3 preparation for stabilization.

To verify whether the ultrafiltration process efficiently depleted CCL3 from the conditioned cell culture supernatant, the ultrafiltrate and the high molecular mass fraction obtained by ultrafiltration were separated in a 15% SDS- polyacrylamide gel along with molecular mass standards. The conditioned cell culture medium, harvested 30 min after CCL3 application was used as a control. Because of the low amount of CCL3 (approximately 20 ng) used for the conditioning of the cell culture supernatant, the protein bands were detected by silver staining. A protein band corresponding to CCL3 was detected in the conditioned supernatant and was enriched as expected in the high

molecular mass fraction, but absent from the ultrafiltrate, proving the efficiency of ultrafiltration. (Figure 14). The protein bands in the higher molecular mass range are at least in part due to bovine serum albumin that was used for stabilization of CCL3. The silver staining was kindly performed by Dariusz Zakrzewicz, Department of Biochemistry, Faculty of Medicine, Giessen, Germany.

3.6 Involvement of iPLA2β

Knowing from previous studies conducted in our laboratory that phospholipase-dependent metabolites of phosphatidylcholines can engage nAChRs [106, 233, 311] and that chemokine receptors signal through downstream phospholipases, we tested whether iPLA2 links CCL3 signaling to the cholinergic inhibition of IL-1β release. For this purpose, we used ATK (50 µM), an inhibitor of cytosolic, calcium-dependent (cPLA2) and calcium-independent (iPLA2) classes of phospholipase A2 [157, 266]. ATK enabled BzATP-induced IL-1β release in despite of the presence of CCL3 ($p = 0.029$, $n = 4$; Figure 15 a). BEL (50 µM), a more specific inhibitor of iPLA2 [6], was also effective, suggesting that iPLA2 plays an essential role ($p = 0.029$, $n = 4$; Figure 15 a) in the CCL3-mediated inhibition of IL-1β secretion by LPS-primed and ATP-stimulated U937 cells. The included controls showed a significant release of IL-1β by LPS-primed U937 cells upon activation with BzATP that was reversed by the inhibitory effect of CCL3. LDH levels were minimal, proving that cell viability was not affected throughout the experiment (Figure 15 b).

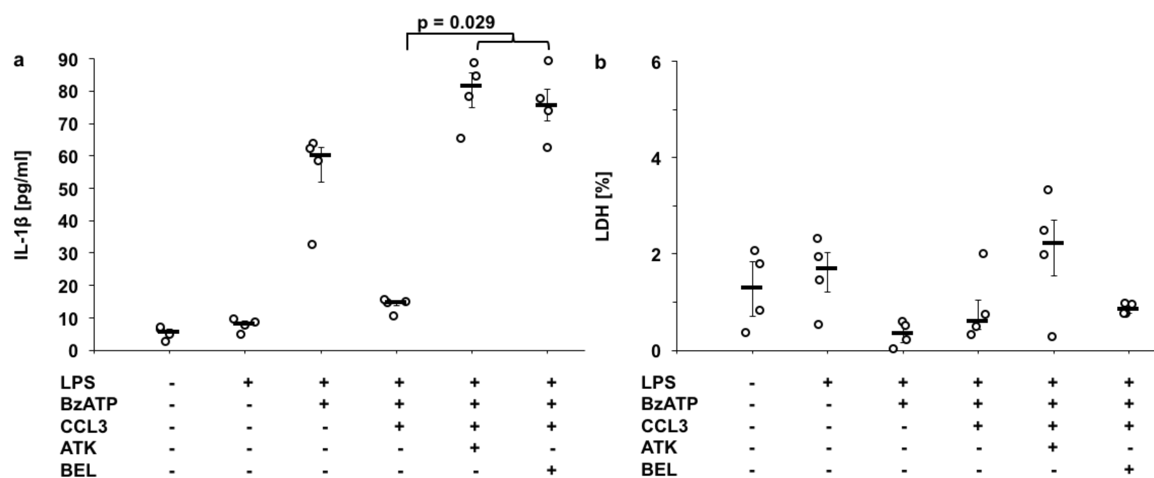

Figure 15. *CCL3 signaling involves calcium-independent phospholipase A2 (iPLA2).* Human monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated 2′(3′)-O-(4-Benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). a. The release of IL-1β to the cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). CCL3 (10 ng/ml) inhibited the release of IL-1β in response to BzATP. The general PLA2 inhibitor arachidonyl

trifluoromethyl ketone (ATK) and the specific iPLA2 inhibitor bromoenol lactone (BEL) reversed CCL3-dependent inhibition. b. Cell death was quantified through lactate dehydrogenase (LDH) measurement and remained at levels below 4% of the total LDH content of lysed cells. Data are presented as individual data points, bars indicate median, and whiskers percentiles are 25 and 75, n = 4, each. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

To further assess the importance of iPLA2 for the inhibitory effect of CCL3 on IL-1β secretion by LPS-primed and BzATP-activated U937 cells, we silenced iPLA2β expression by transfecting U937 cells with *PLA2G6* targeting siRNA via electroporation. Cells transfected with irrelevant siRNA and untreated U937 cells were included in the experiments as controls and had similar *PLA2G6* mRNA expression levels. Transfection efficiency was first verified by comparing *PLA2G6* gene expression levels in cells transfected with siRNA targeting the gene of interest (*PLA2G6*) with control-transfected cells by using real-time RT-PCR. A significant down-regulation of the *PLA2G6* gene was detected, proving transfection efficiency (Figure 16, p = 0.029, n = 4). The expression of the housekeeping gene *HMBS*, used as a positive control in the real time RT-PCR experiments was not affected by siRNA transfection.

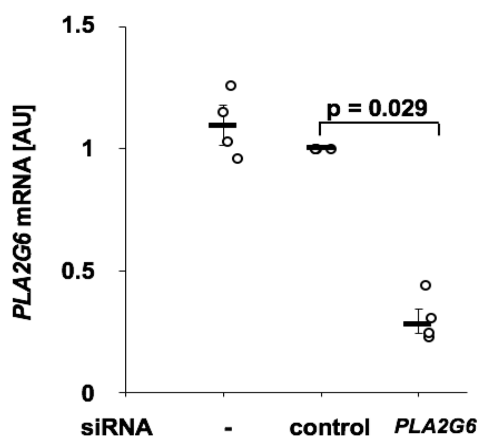

Figure 16: *Efficient PLA2G6 silencing by siRNA transfection.* Expression of iPLA2 by U937 cells was silenced by siRNA (*PLA2G6*). Silencing of *PLA2G6* expression was efficient as revealed by real-time RT-PCR. Values obtained for cells treated with siRNA targeting *PLA2G6* were statistically compared to those transfected with control siRNA. Data are given as arbitrary units, bars indicate median, and whiskers percentiles 25 and 75, n = 4. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

The effects of *PLA2G6* silencing were also verified at the protein level by Western blotting. U937 cells were lysed, and proteins were separated on 10% SDS-polyacrylamide gels, then transferred onto polyvinylidene membranes together with dual color protein standards. iPLA2β immobilized on the membranes was detected by polyclonal rabbit antibodies to iPLA2β (1 : 5000), then detected with secondary HRP-labeled antibodies (1 : 5000), lumi-light substrate, and high-performance chemiluminescence films. β-actin served as a loading control and was detected by mouse monoclonal antibodies to β-actin (1 : 50000) and the same secondary antibody and substrate. The optical density (OD) of the immunopositive bands was measured, and the ratio of the OD of iPLA2β and β-actin

was determined, showing a significant decrease in the iPLA2β levels in cells with silenced *PLA2G6* expression compared to control-transfected cells (Figure 17 b, p = 0.029, n = 4). A typical result of 4 experiments is shown in Figure 17 a with the protein band corresponding to iPLA2β detected at about 80 kDa, that is visibly attenuated in cells transfected with siRNA targeting *PLA2G6* when compared to untreated or control-transfected cells.

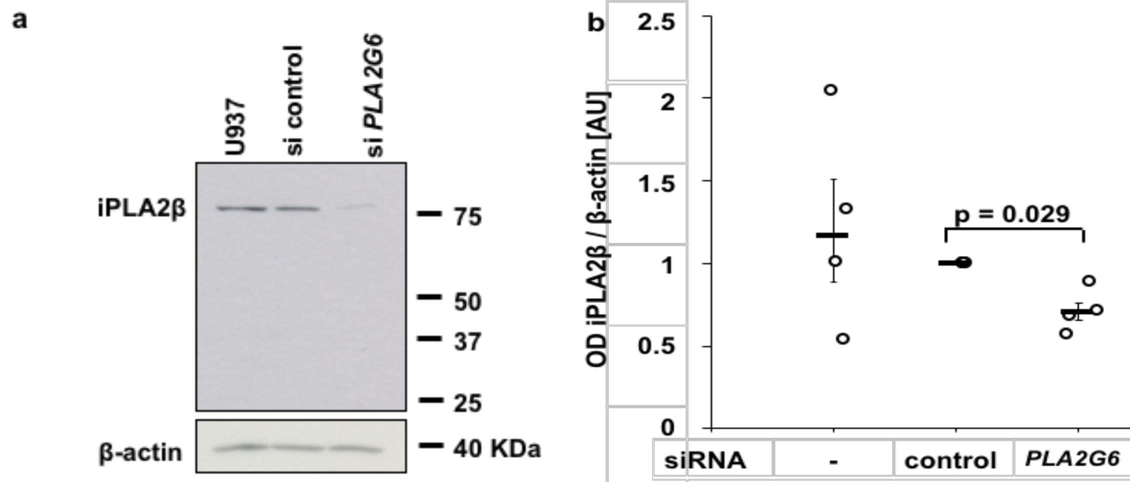

Figure 17: *Efficient silencing of iPLA2β expression in U397 cells by RNA interference.* U937 cells were transfected with either control siRNA or siRNA targeting *PLA2G6*. a. Silencing of *PLA2G6* expression resulted in a significant decrease in iPLA2β levels as shown by Western blotting. β-actin served as a loading control. b. The optical density (OD) of the immunopositive bands was measured, and the ratio of the OD of iPLA2β and β-actin was formed. Values obtained for cells treated with siRNA targeting *PLA2G6* were statistically compared to those transfected with control siRNA. Data are given as arbitrary units, bars indicate median, and whiskers percentiles 25 and 75, n = 4. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

The U937 cells with down-regulated iPLA2β expression were used in IL-1β release experiments. The obtained IL-1β levels were compared to those resulting from cells transfected with control siRNA. BzATP-dependent IL-1β secretion was unimpaired regardless if the LPS-primed cells were transfected or not. CCL3 showed a fully inhibitory effect in untreated cells and control-transfected cells but this inhibitory effect was significantly blunted in the case of cells that were transfected with siRNA targeting *PLA2G6* (Figure 18 a; p = 0.029, n = 4). LDH levels remained below 7% of the total LDH content of lysed cells (Figure 18 b). There was no significant difference between the LDH levels detected in the supernatants of untreated cells and those of transfected cells.

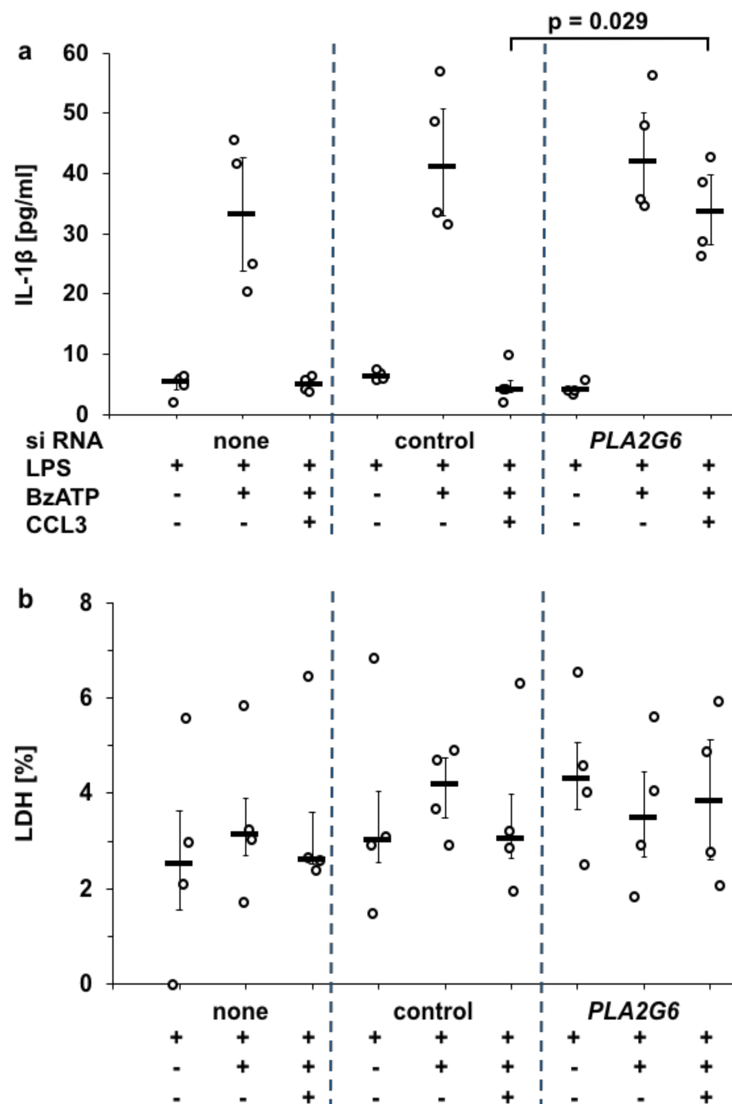

Figure 18: *CCL3 signaling involves calcium-independent phospholipase A2β (iPLA2β).* Human monocytic U937 cells were primed with lipopolysaccharide (LPS; 1 µg/ml, 5 h) and activated 2′(3′)-O-(4-Benzoylbenzoyl)adenosine-5′-triphosphate (BzATP; 100 µM, 30 min). a. The release of interleukin-1β (IL-1β) to the cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). After treatment with siRNA targeting *PLA2G6* expression, the inhibitory effect of CCL3 on IL-1β release was blunted. In untreated control cells and upon transfection of control siRNA, CCL3 was effective. b. LDH levels remained below 7% of the total LDH content of lysed cells and did not significantly vary between individual samples. Data are presented as individual data points, bars indicate median, and whiskers percentiles 25 and 75, n = 4, each. Data were analyzed by non-parametric Kruskal-Wallis test followed by the Mann-Whitney rank sum test. p-values below 0.05 were considered as statistically significant.

Taken together, these results are in line with the hypothesis that iPLA2β is involved in CCL3 signaling and might be a key enzyme for the production of the bioactive soluble factor(s) that presumably stimulate nAChR.

4 Discussion

4.1 The rationale behind a cholinergic anti-inflammatory effect of chemokines

In this study, we provide evidence for a novel immunomodulatory property of CCL-chemokines, that makes use of nAChR in order to control the ATP-induced release of IL-1β into the appropriate biological compartment.

A lot of data has been gathered recently on the cholinergic system of immune cells, supporting its critical contribution to the regulation of immune function and suggesting novel niches for therapeutic manipulation. The emphasis was put on the anti-inflammatory cholinergic reflex advanced by Tracey's group, encompassing vagal-immune interactions that result in a down-regulation of pro-inflammatory cytokine synthesis by splenic macrophages [37, 276, 294]. However, cholinergic macrophages are not the only ones equipped to respond to cholinergic stimuli, as gene expression for nAChR subunits was detected in all human mononuclear cells and in leukemic cell lines [131, 247]. As neuroanatomically no immune organ, spleen included, disposes of a direct parasympathetic innervation [189], most of the nAChR-equipped mononuclear cells would be out of reach for vagal ACh. An alternative explanation, that complies with the lack of parasympathetic innervation of the spleen, was provided by Rosas-Balina in 2011, supporting Tracey's concept of an anti-inflammatory cholinergic reflex [241]. As stated by the authors, efferent signal transmission occurs via the splenic nerves that release norepinephrine, in response to which a specific subset of T-cells releases ACh that acts on the α7 nAChRs of splenic macrophages, suppressing TNF-α expression [200, 241]. Introducing the $CD4^+$ T cells as the source of ACh uncovered further controversial issues [30], such as lacking evidence for a synapse between sympathetic nerves and $CD4^+$ T cells, so that a final verdict in the matter of the vagal anti-inflammatory reflex is still pending.

Meanwhile, a ready and able ACh producing machinery was described in immune cells as distant from the spleen as alveolar macrophages [299], prompting the idea of local, non-neural cholinergic mechanisms that control immune cell function. Furthermore, ACh production by certain immune cells, including machrophages and DCs, was shown to be induced by immune challenging events such as exposure to the classic TLR4 agonist, LPS

[78, 228]. These innate immune cells might therefore be able to directly engage their cholinergic equipment at the site of injury, as a negative feed-back mechanism that contains local inflammation. Further supporting this hypothesis, Hecker et al. [107] demonstrated on allogenic renal grafts that endogenous ACh supresses the release of proinflammatory cytokine IL-1β by allograft monocytes. Immune challenge, in this case in the form of acute transplant rejection, also lead to increased ACh production and release. Bringing forward the arguments of a short ACh half-life due to its rapid degradation by ACh esterases [159, 255] and of surgically denervated grafts that exclude neuronal ACh spillage, the detected ACh in mononuclear leukocytes isolated from the vascular bed of renal allografts was argued to be of non-neuronal origin [107].

Building upon these results, further compounds have been identified that act as nAChR agonists, causing an inhibition of inflammasome-mediated IL-1β maturation and secretion by human monocytes. Among these is lysophosphatidylcholine (LPC), a common PLA2-dependent metabolite of phosphatidylcholines [311]. It has long been acknowledged that PLA2 plays an essential role in inflammation by generating lipid mediators, that thus far are recognized to have both pro- and anti-inflammatory effects [61, 252, 253]. Several isoforms of the PLA2 family are known to act as downstream mediators of chemokine receptors [49, 284]. A further compound, β-nicotinamide adenine dinucleotide (β-NAD), released during inflammation by damaged cells, by interacting with P_2Y receptors on human monocytes also inhibits IL-1β synthesis, by engaging a very similar cholinergic signaling pathway [109]. The P_2Y receptors are part of the family of GPCRs, as are chemokine receptors [4, 289].

Chemokines play their central role in coordinating the spaciotemporal dynamic of inflammatory cells during tissue injury. Considering that their signaling pathways have at least some common elements with the above mentioned compounds, we set out to investigate whether chemokines themselves might engage the same cholinergic mechanism to reduce IL-1β release by human monocytes. Such immunomodulatory action might be of utmost importance and pathophysiologically reasonable at sites of endothelial disruption, where spillage of inflammasome activating substances, such as ATP, might lead to premature IL-1β release from monocytes.

4.2 Anti-inflammatory effects of CCL-chemokines

The experimental rat renal allografts delivered the fundament for the postulated non-neuronal cholinergic IL-1β inhibiting mechanism, with all the advantages of an *in vivo* model, yet it depicts a rather particular immune reaction and it is difficult to reproduce *ex vivo*. Subsequent experiments elucidating further aspects of the nAChR-mediated IL-1β inhibition were successfully translated to and carried out on U937 cells. The current study therefore also employs this human monocytic cell line as well as freshly isolated primary mononuclear cells.

Our hypothesis presumes that chemokines prevent monocytes receiving both chemotactic and inflammasome-activating signals from prematurely releasing their IL-1β load before having crossed the endothelial barrier into the injured tissue. It is known that monocyte emigration from the bone marrow is CCR2-dependent, driven by a CCL2 chemotactic gradient [118, 258]. CCR1 and CCR5, as shown by *in vitro* transmigration assays as well as *in vivo* experiments, have non-redundant roles in monocyte locomotion on the endothelial surface, where they mediate the arrest and spreading of the monocytes in the presence of shear flow, before transmigration [70, 296, 313]. The highest concentration of ATP leaked from inflamed and injured tissues into the circulation would likely be reached at the endothelial interface. Therefore, we chose CCL3, CCL4 and CCL5 as shared ligands of CCR1 and CCR5 to test their inhibitory potential on ATP-induced monocyte IL-1β secretion.

All three of the tested ligands (CCL3, CCL4 and CCL5) effectively and dose-dependently inhibited IL-1β secretion from LPS-primed, BzATP-stimulated U937 cells, with CCL3 standing out as the most potent inhibitor. The IC_{50} values were in the range of 1 – 5 ng/ml for CCL3 and CCL4 and about 10 ng/ml for CCL5. These IC_{50} values are in the range of the EC_{50} values typical for the activation of their cognate receptors [280].

Several studies describe CCR1, CCR5 and their corresponding ligands to be involved in immune events that prevent or at least attenuate a further propagation of the inflammatory response. None of these imply direct inhibition of pro-inflammatory cytokine production, as our results do. D'Amico et al. reported that CCR1, CCR2 and CCR5 on the surface of monocytes can be uncoupled from further down-stream signaling by IL-10, with the consequence of an impaired monocyte recruitment. The deactivated receptors then act as functional decoys for their corresponding ligands, preventing them from exerting their pro-inflammatory chemotactic functions [56].

A further example for CCR1 mediating anti-inflammatory effects comes from a mouse model of nephrotoxic nephritis where CCR1 deficiency unexpectedly exacerbated the course of disease causing greater renal impairment. The results were attributed to an enhanced mononuclear TNF-α production and increased Th1 immune response of CCR1-deficient mice [274]. Discrepant findings were delivered by a model of pulmonary inflammation secondary to acute pancreatitis that CCR1-deficient mice were protected from, the authors associating this effect with decreased TNF-α levels [86].

Examples supporting either pro- or anti-inflammatory effects resulting from the interaction of CCR1 and CCR5 with their corresponding ligands are abundant. Seemingly, this plasticity of their effect has much to do with the context or with the inflammatory microenvironment that surrounds the immune cells that express these chemokine receptors. Another point to consider is that most inflammation models that generate results are murine models and that mouse and human orthologs may differ in biological function. For example, human CCL3 and CCL5 unlike their murine counterparts are poor agonists for neutrophils, meaning that the parallels drawn between mouse disease models and human pathology will at least partially be blurred by neutrophil activity [53, 310].

Knowing that cell lines can also considerably differ from primary cells, we sought to confirm our result on freshly isolated PBMCs from healthy donors. IL-1β released by LPS-pulsed and ATP-stimulated PBMCs was significantly reduced when adding CCL3, leading us to conclude that primary mononuclear cells are equipped with and can engage the inhibitory pathway triggered by CCL chemokines. CCL3 applied in a concentration of 10 ng/ml reduced the IL-1β secretion of U937 cells to baseline levels compared to a 48% reduction achieved for primary cells. CCL3 induced IL-1β inhibition in LPS-pulsed and ATP-stimulated PBMCs was therefore less effective but significant nonetheless ($p = 0.029$).

4.3 No effect of CXCL-chemokines on ATP-induced monocytic IL-1β secretion

Monocytes also possess a panel of receptors for CXCL chemokines that includes CXCR1, CXCR2, CXCR4 and CXCR7 [18]. CXCR1 and CXCR2 are prototypic neutrophil chemotactic receptors and the physiologic relevance of their expression on the surface of monocytes has not thoroughly been investigated. CXCL12 (stromal cell-derived factor-1, SDF-1), the ligand for CXCR4 and CXCR7, is believed to be the primordial

chemokine, having evolved as a regulating signal for stem cell homing [185]. Primarily a homeostatic chemokine, it can be induced as a chemoattractant for monocytes, B cell precursors and T-cells. Most studies define CXCL12 therefore as being a pro-inflammatory cytokine with implications in various autoimmune diseases as well as in cardiovascular pathologies involving a pro-inflammatory milieu such as ischemia/reperfusion injuries, thrombosis or the pro-angiogenetic tumor environment [20, 33, 315]. One of the few experiments providing evidence of CXCL12 exerting an anti-inflammatory effect involves a murine model of autoimmune encephalitis, in which CXCL12 administration lead to rapid disease remission. The observed effect was attributed to the polarization of $CD4^{+}$ T cells and macrophages to become $IL10^{high}$-producing regulatory T cells (Tregs) [176].

Due to the fact that its encoding gene is unique in that it shows an equidistant sequence homology to both CCL and CXCL chemokines, we considered CXCL12 to be a good candidate to test whether the cholinergic inhibitory mechanism is restricted to the domain of CCL chemokines. In our experiments, CXCL12 regardless of the applied concentration (0.1 to 50 ng/ml) did neither inhibit nor increase IL-1β secretion from LPS- and BzATP-stimulated U937 cells.

CXCL16 is not involved in monocyte migration and its corresponding receptor, CXCR6, is not expressed on the surface of myeloid cells but on T cells [18]. This fact was confirmed by the real-time RT-PCR analysis that showed no mRNA expression for CXCR6 in U937 cells as opposed to the PBMC fraction, that also includes lymphocytes (Figure 6). This made CXCL16 a valid negative control for the dose-response experiments conducted with CCL chemokines and CXCL12 on U937 cells. As expected, CXCL16 had no inhibitory effect on the IL-1β release from LPS-primed, ATP-stimulated U937 cells.

4.4 CCL-mediated IL-1β inhibition commences with CCL/CCR-interaction

We further investigated whether the described pathway inhibiting the ATP-induced secretion of IL-1β is triggered by the canonical interaction of CCL3, CCL4 or CCL5 with their cognate GPCRs. *CCR1*, *CCR3* and *CCR5* mRNA expression was detected, as expected, in both U937 and primary PBMCs, with *CCR1* being expressed most abundantly and chosen therefore for further pathway elucidation. Down-regulation of *CCR1* expression diminished the inhibitory potential of CCL3 on IL-1β release but did not influence that of CCL4 and CCL5. These results suggest that the anti-inflammatory

effect of CCL3 is at least in part mediated by its interaction with CCR1. The fact that *CCR1* silencing did not fully counteract the inhibitory effect of CCL3 may be explained by the fact that siRNA-treatment did not fully abolish the expression of *CCR1* and/or that apart from CCR1 other chemokine receptors such as CCR3 and CCR5 are involved.

Though all three of the studied chemokines bind to CCR1, they do so at different affinities. In fact, only a N-terminally truncated form of CCL4, secreted by activated human lymphocytes acts as a low-affinity CCR1 ligand, whereas the full-length variant does not bind to the receptor at all [98]. This does explain why CCR1 silencing in our experiments did not attenuate the inhibitory effect of CCL4 on ATP-mediated IL-1β release. Since there is a level of redundancy in the chemokine/receptor system of CCL3, CCL4, CCL5/CCR1, CCR3, CCR5, silencing of each or of any combination of the three receptors would probably be necessary to determine how CCL4 or CCL5 trigger the inhibitory mechanism.

4.5 Linking CCL/CCR interaction to the inhibition of ATP-induced IL-1β release through iPLA2β enzymatic activity

We focused on the CCL3/CCR1 interaction to further investigate the pathway leading to the inhibition of ATP-induced IL-1β release. Ligand-binding to the CCR1 receptor triggers the classic chemokine signaling pathway through the $G_{i/o}$ class of G proteins, context-dependently selecting one of various potential downstream phospholipid-modifying enzymes including phospholipase A2, C or D, MAPKs and tyrosine kinases [190].

The role of signal-activated PLA2 enzymes as down-stream mediators of chemokine receptor signaling has recently shifted into focus, when they emerged as regulators of monocyte chemotaxis [46, 152, 180]. Monocytes dispose of secretory (sPLA2), cPLA2 and iPLA2 classes of phospholipase A2, that generate free fatty acids and lysophospholipids by hydrolysing phospholipids [61]. Unlike other PLA2 members, that exert their enzymatic activity at a specific position in the phospholipid structure, iPLA2 has no substrate preference and can act as a lysophospholipase, transacylase or thioesterase [183]. Therefore, the products of its enzymatic activity are highly varied and their biologic activity and significance not fully understood.

Most studies observing iPLA2, more specifically iPLA2β involvement in monocyte chemotaxis are modeled on the chemotactic activity of CCL2 during its interaction with its cognate CCR2 receptor, leaving this aspect of CCR1/CCR3 post-receptor signaling

uncharacterized. Monocytes rendered deficient in iPLA2β no longer migrated along the CCL2 gradient, though chemotaxis could be restored by lysophosphatidic acid (LPA) [180]. This lipid metabolite of iPLA2β was therefore considered essential for monocyte chemotaxis, especially in terms of speed and directionality, that involved rearranging of the cytoskeleton for controlling the direction of forward membrane protrusion and cell orientation. The mechanism through which iPLA2β coordinates these changes in monocyte morphology includes its translocation from the cytosol to the plasma membrane, where it colocalizes with proteins regulating actin organization and is provided with substrates for its enzymatic activity. The resulting LPA was speculated to leave the cell in order to further deliver the chemotactic signal by interacting with LPA receptors on the surface of monocytes thus creating a membrane traversing signaling path [47, 180].

We also confirmed the importance of iPLA2β mediation in the signal transduction starting from CCL3 engaging its receptor and leading to the inhibition of ATP-mediated IL-1β release. This was achieved both by silencing iPLA2β expression using small interfering RNA and by blocking its activity using ATK or BEL. The inhibitory effect of CCL3 on IL-1β secretion was blunted in both instances, proving that iPLA2β is a necessary component of our chemokine-induced anti-inflammatory mechanism.

Similar to the chemotactic signaling pathway of CCL2, we assumed that metabolites resulting from the enzymatic activity of iPLA2β on lipid substrates from the cell membrane leave the cell and interact with receptors on the surface of monocytes. As mentioned before, our work group has already identified a broad panel of such metabolites, including LPC, glycerophosphocholine (G-PC) and PC, that act as nicotinic agonists of monocytic nAChRs that control the ion channel function of the P_2X_7R [106, 233, 311]. Hence, it is conceivable that a similar iPLA2β -derived lipid mediator can cholinergically inhibit ATP-induced release of IL-1β downstream of the CCL3/CCR1 interaction. The supernatant of CCL3-stimulated U937 cells was subjected to ultrafiltration in order to deplete it of residual CCL3. The ultrafiltrate significantly inhibited the BzATP-stimulated release of IL-1β, confirming that a mediator with a molecular molecular mass below 3.5 kDa was releasead extracellularly as a result of CCL3/CCR1 interaction and that this mediator was responsible for the inhibitory effect of CCL3 on ATP-induced IL-1β release. The efficacy of the ultrafiltration process in removing residual CCL3, was confirmed by silver staining that proved the ultrafiltrate to be devoid of CCL3 and by the fact that the control ultrafiltrate remained ineffective in

inhibiting BzATP-induced IL-1β release. The nature of the product, resulting from iPLA2β enzymatic activity, involved in our anti-inflammatory mechanism is still to be determined.

There are several other reports placing iPLA2-dependent fatty acids and lysophospholipids in an immunomodulatory context. Mostly based on experiments on U937 cells, iPLA2 was found to play a role in both monocytic proliferation and apoptosis [21, 22, 213] LPC, as a product of iPLA2 activity in apoptotic U937 cells was shown to act as a clearance signal promoting macrophage phagocytosis [212]. An anti-inflammatory effect of LPC was also reported by Yan [307] experimenting on a murine sepsis model. LPC administration enhanced bactericidal activity in neutrophils and reduced TNF-α an IL-1β release upon LPS administration. The reduction in pro-inflammatory cytokine synthesis was mediated by a non-cholinergic mechanism involving the G_2A LPC receptor [307]. The other major metabolite of iPLA2 enzymatic activity, archidonic acid (AA) is the precursor of prostaglandins, leukotrienes, and related compounds, which have important roles in inflammation and in the regulation of immunity [23, 61]. In an experimental model of sterile inflammation using mouse macrophage-like $P388D_1$ cells, Akiba showed that prostaglandin D2 (PGD2), a metabolite of AA is significantly attenuated by iPLA2 antisense oligonucleotide [10]. PGD2 is reported to have anti-inflammatory effects by inhibiting the recruitment of dendritic cells and neutrophils [184]. Under the influence of the anti-inflammatory cytokines IL-4 and IL-10 AA is also involved in the biosynthesis of anti-inflammatory lipid mediators such as lipoxins, resolvins and protectins, actively involved in the resolution of inflammation [254].

4.6 Cholinergic mediation of CCL3 activity

To test for cholinergic mediation in our chemokine induced anti-inflammatory mechanism, we evaluated the inhibitory effect of CCL3 on IL-1β release against a panel of nicotinic antagonists. Indeed, mecamylamine, a general nicotinic blocker, as well as α-bungarotoxin and strychnine, antagonists of α7 and α9 nAChR subunits [171, 281], antagonized the CCL3-induced inhibition of IL-1β secretion from LPS-primed, BzATP-stimulated U937 cells. To further discriminate between the nAChR subunits involved, we made use of conotoxin-derived peptides [V11L; V16D]ArIB and RgIA4 that specifically anatagonize nAChRs containing the α7 and α9/α10 subunits respectively. As both peptides blunted the effect of CCL3, we concluded that signal transduction in our proposed mechanism involves nAChR subunits α7 and α9/α10. Furthermore, the

inhibitory properties of the iPLA2β -derived mediator separated in the ultrafiltrate of conditioned medium were also antagonized by [V11L; V16D]ArIB and RgIA4.

In a previous study, Zakrzewicz et al. [311] established the nicotinic receptor requirements for the anti-inflammatory effect induced by several iPLA2-metabolites, including G-PC and 1-palmitoyl-*sn*-glycero-3-phosphocholine, a member of the LPC class of phospholipids. By using the same panel of nAChR antagonists as applied in our study and corroborating the results with those of gene-silencing experiments using siRNA directed at specific nAChR subunits, it was shown that α9 and α10 are mandatory for the inhibitory mechanism mediated by this specific LPC and by G-PC. In contrast, α7 seemed to play no significant role in the cholinergically mediated inhibition of ATP-induced IL-1β release by the two phospholipids. Therefore it is safe to conclude that the product of iPLA2β enzymatic activity released extracellularly downstream of the CCL3/CCR1 interaction differs from 1-palmitoyl-*sn*-glycero-3-phosphocholine and G-PC, as the α7 subunit is mandatory for its inhibitory effect. In a similar manner, ACh, nicotine and PC require all three receptor subunits (α7, α9 and α10) in order to inhibit ATP-induced IL-1β by monocytes [106, 233, 311]. It is to be noted that α9 is present in each of these examples, but regardless of the combination of nAChR subunits the effect remains a clear inhibition of ATP-induced IL-1β release in LPS-primed BzATP stimulated human monocytes.

The question of how nAChR activation leads to inhibition of ATP-induced IL-1β release is not clearly answered. It has been shown that in myenteric neurons, nicotinic ACh receptors can functionally interact with ATP-sensitive P_2XRs by forming a heteromeric complex, in which the function of the partner receptor is altered [60]. nAChRs are canonically described as ionotropic receptors that upon activation by an agonist induce rapid increase in membrane permeability to Na^+, K^+ and Ca^{2+}. By using patch-clamp experiments to monitor ion currents across the celluar membrane, it was shown that PC does not provoke any ion changes at the nAChRs in U937 cells and therefore metabotropic signaling is responsible for the inhibition of the P_2X_7R function [233]. In support of the metabotropic signaling of nAChRs it has been shown that the cytoplasmatic loop of certain nAChR subunits can interact with G proteins that might account for further signal transduction [140].

The matter of whether metabotropic or ionotropc signaling downstream of nAChRs was responsible for CCL3-induced inhibition ATP-dependent IL-1β release was not further pursued. We did verify whether the inhibitory effect of CCL3 on IL-1β synthesis was preserved in case of ATP-independent inflammasome activation. For this purpose we

made use of nigericin, a toxin produced by *Streptomyces hygroscopicus*, that forms pores in the cell membrane, leading to a depletion of intracellular K^+ that induces NLRP3 inflammasome assembly in the absence of extracellular ATP [93]. Nigericin-induced secretion of IL-1β was not reduced by CCL, supporting our assumption that CCL3 triggers a mechanism that is specific for ATP-dependent inflammasome activation.

Together, these results are in line with the idea, that chemokine signaling triggers a recently described mechanism involving activation of metabotropic functions of noncanonical nAChR containing subunits α7 and α9/ α10 that inhibit P_2X_7R signaling and, consequently, BzATP-induced release of IL-1β.

4.7 Limitations of the study and perspectives for future research

This study has several limitations, and certainly, more research is needed to substantiate the intriguing concept that chemokines attracting monocytes inhibit ATP-mediated inflammasome activation. Here, we only investigated chemokines CCL3, CCL4, and CCL5 with a focus on CCL3. The concept should be confirmed for other relevant chemokines involved in monocyte chemotaxis, especially for CCL2 that is regarded as the main attractant of human monocytes.

Further, we used the monocytic cell line U937 as a model for human blood monocytes in most experiments. These cells were chosen as they possess the cholinergic control mechanism of IL-1β release typical for human blood monocytes [106, 267]. U937 cells, however, secrete very low amounts of IL-1β in response to ATP in comparison to primary human and mouse cells. Therefore, we corroborated the inhibitory potential of CCL3 on IL-1β release by primary human PBMCs *in vitro*. The levels of IL-1β released upon stimulation of the same amount of cells with the same BzATP concentration (100 µM) reached a median of 3.6 ng/ml in experiments using primary cells and of 38 pg/ml when using U937 cells. The LPS concentration used for priming primary cells was considerably lower than that used for U937 cells, as previous experience showed that PBMCs are primed through the process of isolation itself, especially during gradient centrifugation and adherence to tissue culture dishes [106, 114, 136]. The molecular mechanism undelying the inhibitory effect of chemokines on IL-1β secretion is therefore intact in the U937 cell line, just as in primary cells, with notable quantitative differences in the LPS concentration required for priming and the secreted IL-1β levels. In support of this hypothesis, the experiments accounting for the involvement of iPLA2β and nAChR in the proposed mechanism should be reproduced using primary cells.

Going one step further, the inhibitory mechanism should be tested *in vivo*, where difficulties are bound to arise due to the intricacies of the chemokine system. As mice are the *in vivo* experimental model of choice, several differences in the murine chemokine systems are to be noted when compared to the human counterpart. Refering strictly to chemokine and chemokine receptors acting on monocytes and monocyte-derived cells, CCL13 also known as MCP-4, binding to chemokine receptors CCR1, CCR2 and CCR3 has been identified in humans but not in mice [178]. CCL14 that is 46% identical in amino acid composition to CCL3 and CCL4 and is involved in monocyte activation is also human specific and lacks a murine counterpart [18]. Conversely CCL12 or MCP-5 is a chemokine binding to CCR2 identified in mice but not in humans [178].
In addition, there are numerous open questions regarding the details of the proposed novel signal transduction mechanism, including the identification of the bioactive soluble factor(s) activating nAChR and the signaling mechanisms down-stream of nAChR.

4.8 Biological and clinical relevance

Inflammasome activation, resulting in the production of inflammasome-dependent pro-inflammatory mediators is a double-edged sword that is required for host defense against infections but is also associated with the risk of inducing life-threatening SIRS. A strict quantitative, temporal and spacial control of IL-1β release, translating into the right amount of secreted IL-1β, at the right time, into the appropriate biological compartment might be at least partially responsible for whether the balance tips towards the resolution of inflammation or SIRS. Release of monocytic IL-1β to the blood stream is expected to be of limited use as the cytokine is swept away from the site of inflammation, but in contrast, the risk of inducing harmful systemic inflammation should be higher. In this study, we demonstrate that chemokines block the ATP-dependent IL-1β release by blood monocytes, a mechanism that would contribute to the prevention of systemic inflammation. In contrast, release of IL-1β by inflammatory macrophages within the tissue would fight against local infections causing less systemic effects. We expect that IL-1β can be released to inflamed tissue by macrophages despite high local chemokine levels, as pathogens induce inflammasome activation by several ATP-independent mechanisms that are probably not sensitive to chemokines [147, 268, 290]. Furthermore it is well known that upon maturation into tissue macrophages, human monocytes undergo a reconfiguration of the chemokine receptors expressed on their surface, with a loss of CCR2 expression [73]. It is also known that polarized tissue macrophages differ in patterns

of cytokine production and regulation of pro- and anti-inflammatory components of the IL-1 system [64]. The low oxygen concentrations characterizing the inflammatory tissue milieu also affects how macrophages respond to chemokines [279]. There are therefore several reasons to hypothesize that chemokines do not inhibit the ATP-dependent IL-1β release of tissue macrophages, a topic that certainly deserves more investigation.

A number of components involved in our proposed mechanism of chemokine induced IL-1β inhibition have already been targeted for the development of anti-inflammatory drugs, resulting in a series of studies ranging from *in vitro* to approved pharmaceutics, as illustrated in Table 10. In the case of selective CCR1-antagonists, the encouraging results obtained in preclinical models of MS, rheumatoid arthritis (RA) or heart transplantation failed to be reproduced by clinical trials. The argument most frequently brought forward to explain the discrepancy in results was incomplete receptor coverage [57, 148].

Only a few iPLA2 inhibitors have been developed up to date, since the importance of the enzyme in a number of medical pathologies has only recently come to focus. An anti-inflammatory effect was suggested by in vivo studies on murine models of MS [125]. Clinical trials have not yet been initiated. Relying on cholinergically mediated anti-inflammatory effects selective α7nAChR agonists have been developed with promising results in experimental clinical trials. No doubt, the most successful of the below listed componds are the ones targeting IL-1β, that partially have already been approved for clinical use in a spectrum of autoinflammatory and systemic inflammatory conditions. There are therefore reasons to believe that the immunemodulatory effect of CCL3 might also be used to develop novel therapeutic agents.

Target	Compound	Mechanism of action	Addressed pathology	Type of study	Status	Ref.
CCR1	BX 471	CCR1-antagonists	MS, psoriasis	phase II clinical trials	no efficacy	151, 209
	MLN 3897		RA		no efficacy	287
	CP-481, 715		RA		no efficacy	42
	AZD4818		COPD		no efficacy	137
	CCX354		RA		ongoing	57
	C-4462		RA		no efficacy	191
	C-6448		MS		no efficacy	191
iPLA2	FKGK11	iPLA2-inhibitors	EAE	*in vivo*, murine model	slows disease progression	125
	FKGK18		diabetes	*in vivo*, murine model	prevents beta-cell apoptosis	272
	GK 187		none	*in vitro,* micelle-based assays		158
nAChR	nicotine	nAChR agonist	experimental endotoxemia	phase I clinical trial	attenuated febrile response	303
	GTS-21	selective-α7nAChR agonists	experimental endotoxemia	phase I clinical trial	no efficacy	143
	PNU-282987		acute lung injury	*in vivo*, murine model	anti-inflammatory	85
IL-1β	Anakinra	receptor antagonist for IL-1RI	RA CAPS Still's disease	approved	anti-inflammatory	66
	Rilonacept	soluble IL-1 receptor	CAPS	approved	anti-inflammatory	195, 263
	Canakinumab	neutralizing anti-IL-1β IgG1 mAb	CAPS	approved	anti-inflammatory	40, 69
	Gevokizumab	neutralizing anti-IL-1β IgG2 mAb	RA	phase II clinical trial	anti-inflammatory	54
NLRP3 inflammasome	CY-09	inhibitor of NLRP3 ATPase activity	CAPS Type 2 diabetes	*in vivo*, murine models	anti-inflammatory	119
	OLT1177	specific NLRP3 inihibition	gouty arhritis	*in vivo*, murine models	anti-inflammatory	164

Table 10: *Compounds targeting components related our proposed chemokine induced anti-inflammatory mechanism, their mechanism of action and achieved results in preclinical and clinical trials.* CCR1, C-C motif chemokine receptor 1; MS, multiple sclerosis; RA, rheumatoid arthritis; COPD, chronic obstructive pulmonary disease; iPLA2, calcium-independent phospholipase A2; EAE, experimental autoimmune encephalomyelitis; nAChR, nicotinic acetylcholine receptor; IL-1β, interleukin-1β; IL-1RI, interleukin-1

receptor, type I; CAPS, cryopyrin-associated autoinflammatory syndrome; NLRP3, NOD-like receptor family, pyrin domain containing 3.

4.9 Conclusion

Our results are in line with the hypothesis that CCL3, CCL4, and CCL5 inhibit BzATP-induced maturation and release of IL-1β by LPS-primed monocytic cells. CCL signaling seems to depend on binding to cognate CCR, activation of iPLA2β, and release of soluble agonists of nAChR containing subunits α7 and α9/α10 that inhibit IL-1β release (Figure 19). It has been shown before that activation of monocytic nAChR is a potent way to inhibit ATP- induced activation of P_2X_7R, inflammasome activation, and release of IL-1β. The control of inflammasome activation in despite of the presence of ATP is of outstanding clinical interest. A better understanding of the underlying mechanisms might lead to the development of therapeutic strategies for the prevention and treatment of inflammatory diseases. With all due caution, we suggest a novel CCL-induced anti-inflammatory triple-membrane-passing signaling pathway inhibiting premature inflammasome activation in monocytes in response to extracellular ATP.

This mechanism might reduce trauma-induced release of IL-1β into the circulation and thereby prevent sterile SIRS. As trauma is often associated with infection, infiltrating monocytes/macrophages and local IL-1β release at the site of inflammation are desirable. As PAMP-induced inflammasome activation is typically ATP-independent, local secretion of inflammasome-dependent cytokines by infiltrating monocytes/macrophages should be enabled, in despite of the presence of chemokines.

5 Graphical summary

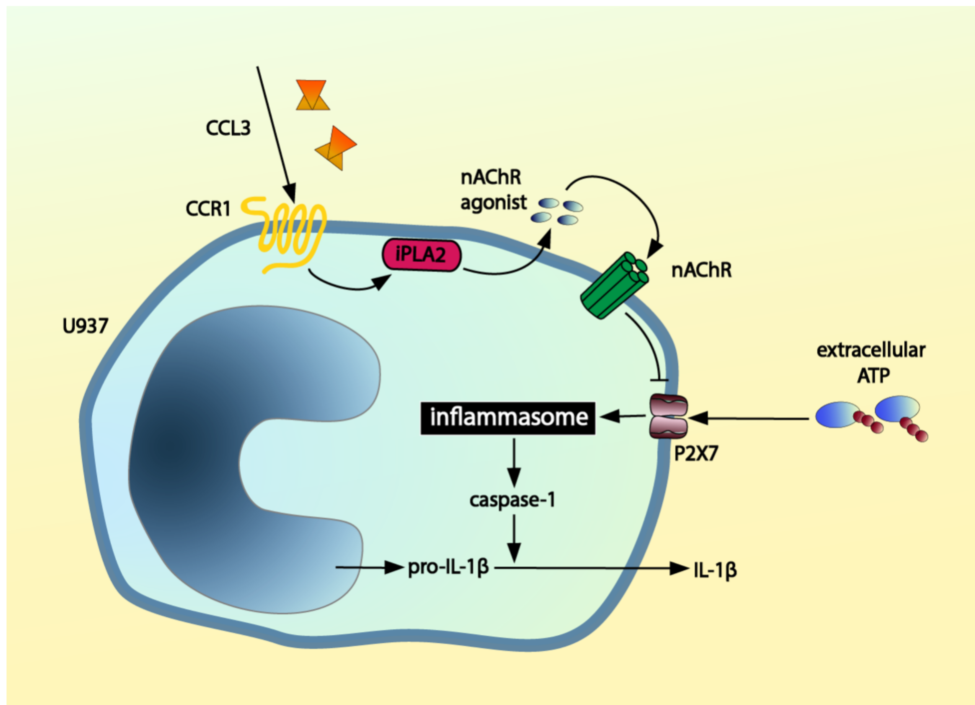

Figure 19: *Schematic presentation of the proposed mechanism.* The binding of extracellular ATP to P_2X_7R on LPS-primed human monocytic U937 cells results in formation of a multiprotein complex called inflammasome that, in turn, activates caspase-1. Caspase-1 catalyzes the proteolytic maturation of pro-IL-1β and enables the release of mature, bioactive IL-1β. We propose that, chemokine CCL3 binding to its cognate CCR1 chemokine receptor, on the surface of LPS-primed U937 cells leads to downstream activation of calcium-independent phospholipase A2β (iPLA2β) and the secretion of a small agonist of nicotinic acetylcholine receptors (nAChR). Stimulation of nAChR containing subunits α7 and α9/α10 inhibits P_2X_7R function and, hence, maturation and secretion of ATP-dependent IL-1β. It is still unclear if nAChR subunits of monocytic cells actually form conventional pentamers as shown in the schematic drawing

6 Summary

ATP and chemokines are among the first inflammatory mediators that can enter the circulation via damaged blood vessels at the site of injury, leading to an activation of the host's immune response. The main function of chemokines is leukocyte mobilization, guiding immune cells towards the injured tissue along a chemotactic concentration gradient. In monocytes, ATP typically triggers inflammasome assembly, a multiprotein complex necessary for the maturation and secretion of IL-1β. IL-1β is a potent inflammatory cytokine of innate immunity, essential for pathogen defense. However, excessive IL-1β may cause life-threatening systemic inflammation. Here, we hypothesize that chemokines control ATP-dependent secretion of monocytic IL-1β, by engaging a cholinergic signaling pathway.

LPS-primed human monocytic U937 cells were treated with chemokines in the presence or absence of nAChR antagonists or iPLA2β inhibitors and concomitantly stimulated with the P_2X_7 agonist BzATP. IL-1β concentration was determined in the cell culture supernatants. Silencing of the chemokine receptor and iPLA2β gene expression was achieved by transfecting cells with the appropriate siRNA.

CCL3, CCL4, and CCL5 dose-dependently inhibited BzATP-stimulated release of IL-1β, whereas CXCL16 was ineffective. The effect of CCL3 was confirmed for primary mononuclear leukocytes. The inhibitory effect of CCL3 was blunted after silencing CCR1 or iPLA2β gene expression by siRNA and was sensitive to antagonists of nAChRs containing subunits α7 and α9/α10. U937 cells secreted small factors in response to CCL3 that mediated the inhibition of IL-1β release.

We suggest that CCL chemokines inhibit ATP-induced release of IL-1β from U937 cells by a triple-membrane-passing mechanism involving CCR, iPLA2, release of small mediators, and nAChR subunits α7 and α9/α10. We speculate that whenever chemokines and ATP enter the circulation concomitantly, systemic release of IL-1β is minimized.

Zusammenfassung

ATP und Chemokine gehören zu den ersten Entzündungsmediatoren, die bei Verletzungen in den Blutstrom gelangen und zu einer Aktivierung des Immunsystems führen. Chemokine sind chemotaktisch wirksame Botenstoffe, die Leukozyten entlang von Konzentrationsgradienten zum entzündeten Gewebe führen. ATP löst in Monozyten typischerweise die Bildung des Inflammasoms aus, ein Multiproteinkomplex, der zur Reifung und Freisetzung von IL-1β benötigt wird. IL-1β ist ein potentes pro-inflammatorisches Zytokin, das bei der Infektabwehr eine zentrale Rolle spielt. Hohe systemische IL-1β-Spiegel lösen jedoch das lebensbedrohliche systemische SIRS aus. Wir überprüfen hier die Hypothese, dass Chemokine die ATP-induzierte IL-1β-Freisetzung aus Monozyten kontrollieren, indem sie einen kürzlich von uns beschriebenen cholinergen Signalweg aktivieren.

Humane U937-Zellen wurden mit LPS vorstimuliert und mit ATP aktiviert. Gleichzeitig mit ATP wurden Chemokine, nAChR-Antagonisten oder Inhibitoren der iPLA2 eingesetzt. Die IL-1β-Konzentration wurde im Zellkulturüberstand mittels ELISA bestimmt. Die Herunterregulation der Expression von Chemokinrezeptoren und der iPLA2 erfolgte durch Transfektion spezifischer siRNA.

CCL3, CCL4, und CCL5 inhibieren dosisabhängig die ATP-induzierte IL-1β-Freisetzung, während CXCL16 unwirksam ist. Der Effekt von CCL3 konnte an frisch isolierten primären peripheren mononukleären Blutzellen bestätigt werden. Die inhibitorische Wirkung von CCL3 hängt vom Chemokinrezeptor CCR1, von der iPLA2 und von den nAChR-Untereinheiten α7, α9 und α10 ab. U937-Zellen sezernierten nach Stimulation mit CCL3 niedermolekulare bioaktive Faktoren, die die Inhibition ATP-abhängigen IL-1β-Freisetzung vermitteln.

In menschlichen Monozyten hemmen CCL-Chemokine die ATP-induzierte IL-1β-Freisetzung über einen Mechanismus, der dreimal die Cytoplasmamembran überspannt und über Chemokinrezeptoren, iPLA2 und nAChR vermittelt wird. Wir vermuten, dass immer wenn CCL-Chemokine und ATP zusammen in den Blutstrom gelangen, die systemische Freisetzung von IL-1β und damit der Gefahr eines SIRS reduziert wir

7 Literature

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8 Declaration

Erklärung zur Dissertation

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Ort, Datum Unterschrift

9 Acknowledgements

After long hours spent with mixed feelings of frustration and enthusiasm that finally amounted to the completion of the current work, I would like to thank the many people who guided and supported me during this time.

Prof. Veronika Grau, my supervisor, exemplary in her scientific knowledge and thoroughness, I thank for offering me the first insight into the world of experimental research that she constantly infuses with exciting, intrugiung ideas and with her work, obviously done with passion. She invariably provided solid guidance, accompanied by friendly, encouraging words whilst being supportive of my demanding work schedule as a trainee surgeon, which at times caused interruptions and delays. To the rest of the team of the Laboratory of Experimental Surgery, I would like to express my sincere gratitude for helping me in these first steps of scientific endeavour. I especially thank Anka Zakrzewicz and Sigrid Wilker for providing excellent material, together with logistical and human conditions for completing the experimental part of my work.

I thank Prof. Padberg for encouraging and providing the work frame for those expressing scientific interest in his department, despite the difficulties and added pressure that it involves. Andreas Hecker I thank as a collegue and friend for inspiring with the ease of balancing a successful scientific and surgical career and for the optimism and positivity with which he supports others in achieving the same goals.

Most of all I am greatfull to my partner, Sebastian, for providing sense and perperspective throughout all of the ups and downs of my research. I thank my sister for her artistic contribution to this work and her intellectual input, and both her and my mother for their unconditional support.

I dedicate this work to the memory of my father, whom by confessing to his unfulfilled dream of becoming a surgeon pushed me on to my current path and kept me travelling on it.