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Role of Sphingosine-1-Phosphate Receptor 1 and Downstream Heme Oxygenase-1 Induction in Alternative Macrophage Activation Induced by Apoptotic Cells

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ABBREVIATIONS

ABC	ATP binding cassette
AC	Apoptotic cells
ACAMP	Apoptotic cell-associated molecular patterns
AC-CM	Conditioned medium form apoptotic cells
ACy	Adenylyl cyclase
APAF-1	Apoptotic protease activating factor-1
APS	Ammonium persulfate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated protein x
Bcl-2	B cell leukemia/lymphoma-2
Bcl-X_L	B cell leukaemia/lymphoma-x long
BH	Bcl-2 homology domain
Bid	BH3-interacting domain death agonist
BM	Bridging molecules
CDase	Ceramidase
CDSyn	Ceramide synthase
CM	Conditioned medium
CO	Carbon monoxide
CORM-2	Tricarbonyldichlororuthenium(II) dimer
COX	Cyclooxygenase
Cre	Cyclization recombinase
DAPI	4´,6-diamidino-2-phenylindol
Deta-NO	Diethylenetriamine-NO
DISC	Death-inducing signaling complex
DMS	Dimethylsphingosine
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
GC	Glucocorticoids
GPC	G protein-coupled

HO	Heme oxygenase
IC	Immune complexes
iCORM-2	Inactivated product of tricarbonyldichlororuthenium(II) dimer
IL-1RL	Interleukin-1 receptor ligands
iNOS	Inducible NO-Synthase
KLF	Krüppel-like factor
loxP	Locus of X-over of P1
MΦ	Macrophage
MΦ-CM	Conditioned medium form macrophages
NC	Necrotic cells
NOS	NO synthase
Nrf2	NF E2-related factor-2
p38	p38 MAPK
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PK	Protein kinase
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
Ptm	Point mutation
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RR	Recognition receptor
RT	Room temperature
S1P	Sphingosine-1-phosphate
si	small interferring
SMase	Sphingomyelinase
SMSyn	Sphingomyelin synthase
SphK	Sphingosine kinase
SPP	S1P phosphohydrolase
SUMO	Small ubiquitin-like modifier

T_A	Annealing temperature
TEMED	Tetraethylendiamine
TLRL	Toll-like receptor ligand
VC	Viable cells
VEGF	Vascular endothelial growth factor

Standard abbreviations were defined according to the instructions for authors of the Journal of Immunology and are used without definition.

1 SUMMARY

Macrophages show a remarkable functional plasticity, which enables them to change their phenotype in response to environmental signals. They are key players during infection by initiating inflammation through the release of pro-inflammatory mediators. Furthermore, macrophages contribute to the resolution of inflammation by phagocytosis of apoptotic granulocytes. Phagocytosis of apoptotic cells (AC) induces an anti-inflammatory phenotype in macrophages and protects them against apoptosis. However, mechanistic details provoking these phenotype alterations are incompletely understood. Therefore, the aim of my Ph.D. thesis was to investigate the molecular basis of anti-inflammatory macrophage polarization.

In the first part of my studies, I investigated the expression of heme oxygenase (HO)-1 in macrophages following treatment with supernatants from AC. HO-1 catalyzes the first and rate-limiting step of heme degradation and potentially bears anti-inflammatory as well as anti-apoptotic potential. I was able to show biphasic upregulation of HO-1 by AC supernatants. The first phase of HO-1 induction at 6 h required activation of p38 MAPK and was accomplished by the bioactive lipid sphingosine-1-phosphate (S1P) engaging S1P receptor 1 ($S1P_1$). However, the second wave of HO-1 induction at 24 h was attributed to autocrine signaling of vascular endothelial growth factor (VEGF) A, whose expression was facilitated by S1P. The release of VEGFA from macrophages was STAT1-dependent, whereas VEGFA itself acted on the macrophage HO-1 promoter via STAT1/STAT3 heterodimer binding. Knockdown of HO-1 revealed its relevance in promoting enhanced expression of the anti-apoptotic proteins B cell leukemia/lymphoma-2 (Bcl-2) and B cell leukaemia/lymphoma-x long ($Bcl\text{-}X_L$), as well as the anti-inflammatory adenosine receptor A_{2A}. MHC II and indoleamine 2,3-dioxygenase expression were also affected by AC-supernanatants, but were not HO-1 dependent.

Unexpectedly, $S1P_1$ was also upregulated following treatment with AC supernatants. Thus, I considered whether $S1P_1$ induction could specifically be mediated by alternative macrophage activating factors. The expression of $S1P_1$ was enhanced in the presence of the alternative activation stimuli IL-4 as well

as IL-10, whereas it was unchanged following incubations with LPS, interferon-γ or S1P. My next aim was to investigate the expression of the different S1P receptor isoforms in macrophages following treatment with supernatants form AC. While the expressions of $S1P_1$ as well as $S1P_3$ were induced by exposure to supernatants from AC, $S1P_2$ expression was unaffected. As $S1P_{1/3}$ and $S1P_2$ are conflictively involved in the regulation of cell migration, I asked for a correlation between increased S1P receptor expression and enhanced migration rate. Indeed, macrophages showed enhanced motility following treatment with supernatants form AC, which was inhibited in $S1P_1$ knockout macrophages.

In summary, my findings indicate that HO-1, which is induced by AC-derived S1P, is critically involved in macrophage polarization towards an alternatively activated macrophage phenotype. $S1P_1$ seems to represent a central checkpoint during macrophage activation. On the one hand, $S1P_1$ is induced by supernatants form AC and promotes migration of macrophages. On the other hand, it mediates the induction of HO-1, which is accompanied by anti-inflammatory as well as anti-apoptotic signaling. Furthermore, my studies provide evidence that upregulation of HO-1 and $S1P_1$ in macrophages may contribute to the resolution of inflammation by establishing an anti-inflammatory macrophage phenotype and provoking macrophage migration along the vascular S1P gradient out of an inflammatory environment into the lymph.

2 ZUSAMMENFASSUNG

Makrophagen weisen eine bemerkenswerte funktionelle Plastizität auf, welche es ihnen ermöglicht, ihren Phänotyp als Antwort auf Umweltreize zu ändern. Sie spielen eine zentrale Rolle während der Infektion, indem sie die Entzündungsreaktion durch die Freisetzung von pro-inflammatorischen Mediatoren initiieren. Darüber hinaus tragen Makrophagen durch die Phagozytose apoptotischer Zellen zur Auflösung der Entzündung bei. Phagozytose von apoptotischen Zellen induziert einen anti-inflammatorischen Phänotyp in Makrophagen und schützt sie vor Apoptose. Jedoch sind die mechanistischen Details, welche diese Phänotyp-Veränderungen bewirken, nur unvollständig bekannt. Daher war es das Ziel meiner Doktorarbeit, die molekulare Basis der anti-inflammatorischen Makrophagen-Polarisierung zu untersuchen.

Im ersten Teil meiner Studien untersuchte ich die Expression der Häm Oxygenase (HO)-1 in Makrophagen nach Behandlung mit Überständen von apoptotischen Zellen. Die HO-1 katalysiert den ersten und limitierenden Schritt des Häm-Abbaus und weist anti-inflammatorisches sowie anti-apoptotisches Potential auf. Ich war in der Lage eine biphasische Induktion der HO-1 durch Überstände von apoptotischen Zellen zu zeigen. Die erste Phase der HO-1-Induktion nach 6 h erforderte die Aktivierung der p38 MAPK und wurde durch Interaktion des bioaktiven Lipids Sphingosin-1-Phosphat (S1P) mit dem S1P-Rezeptor 1 ($S1P_1$) vermittelt. Die zweite Welle der HO-1-Induktion nach 24 h war dem „vascular endothelial growth factor“ (VEGF) A zuzuschreiben, dessen Expression durch S1P ausgelöst wurde. Während die Freisetzung von VEGFA aus Makrophagen STAT1-abhängig war, wirkte VEGFA autokrin über STAT1/STAT3-Heterodimer-Bindung auf den HO-1-Promotor. Ein Knockdown der HO-1 enthüllte ihre Bedeutsamkeit für der Förderung der Expression der anti-apoptotischen Proteine „B cell leukemia/lymphoma-2“ (Bcl-2) und „B cell leukaemia/lymphoma-x long“ (Bcl-X_L) sowie des anti-inflammatorischen Adenosin-Rezeptors A_{2A}. Die Expression des MHC II und der Indolamin 2,3-dioxygenase wurden auch von Überständen von apoptotischen Zellen beeinflusst, waren aber nicht HO-1-abhängig.

Unerwartet war auch der $S1P_1$ nach Behandlung mit Überstanden von apoptotischen Zellen hochreguliert. Folglich erwägte ich, ob die Induktion des $S1P_1$ spezifisch durch Faktoren vermittelt werden konnte, welche Makrophagen alternativ aktivieren. Die Expression des $S1P_1$ war in Gegenwart der alternativ-aktivierenden Stimuli IL-4 und IL-10 gesteigert, hingegen war sie nach Inkubation mit LPS, Interferon-γ oder S1P unverändert. Mein nächstes Ziel war es, die Expressionsrate der verschiedenen S1P-Rezeptor-Isoformen in Makrophagen nach Behandlung mit Überständen von apoptotischen Zellen zu untersuchen. Während die Expression des $S1P_1$ sowie des $S1P_3$ durch Stimulation mit Überständen von apoptotischen Zellen induziert waren, war die Expression des $S1P_2$ unberührt. Da der $S1P_{1/3}$ und der $S1P_2$ gegensätzlich in die Regulation der Zell-Migration involviert sind, fragte ich nach einer Korrelation zwischen der erhöhten S1P-Rezeptor-Expression und einer gesteigerter Migrationsrate. In der Tat, wiesen Makrophagen nach Behandlung mit Überständen von apoptotischen Zellen eine gesteigerte Motilität auf, welche in $S1P_1$-Knockout-Makrophagen gehemmt war.

Zusammenfassend zeigen meine Entdeckungen, dass die HO-1, welche durch von apoptotischen Zellen freigesetztes S1P induziert wird, kritisch in die Makrophagen-Polarisierung in Richtung eines alternativ-aktivierten Makrophagen-Phänotyps involviert ist. S1P scheint einen zentralen Kontrollpunkt während der Makrophagen-Aktivierung darzustellen. Einerseits wird der $S1P_1$ durch Überstände von apoptotischen Zellen induziert und fördert die Migration der Makrophagen. Andererseits vermittelt er die Induktion der HO-1, welche anti-inflammatorische sowie anti-apoptotische Antworten auslöst. Darüber hinaus liefern meine Studien Hinweise dafür, dass die Induktion der HO-1 und des $S1P_1$ in Makrophagen die Etablierung eines anti-inflammatorischen Makrophagen-Phänotyps und das Auslösen von Makrophagen-Migration entlang des vaskulären S1P-Gradienten aus der entzündlichen Umgebung in die Lymphe bewirkt, und somit zur Auflösung einer Entzündung beitragen kann.

3 INTRODUCTION

3.1 Cell death

In metazoans, tissue homeostasis is ensured by two central mechanisms. While ‘new’ cells are generated during the cell cycle of progenitor cells, followed by their differentiation into specialists, redundant, damaged or infected cells are eliminated by cell death (1, 2).

A vast number of distinct modes of cell death associated with varying morphological characteristics has been reported. These modes include necrosis, autophagy and mitotic catastrophe (3). However, in multicellular organisms apoptosis, a physiological and programmed form of cell death, is the predominant mode of cell death (4).

In 1842, naturally occurring cell death was first described by Carl Vogt (5). However, after a long period of neglect, more than one decade later this process was termed apoptosis, the Greek word for the fall of leaves in autumn, by Kerr, Wyllie and Currie (6).

The characteristic and stereotypical morphology of apoptosis involves nuclear chromatin condensation and fragmentation, cell shrinkage and controlled cell disintegration through the formation of membrane vesicles, so-called ‘apoptotic bodies’, whose membrane integrity is maintained (6, 7). Apoptosis is a tightly regulated means, by which cells actively orchestrate their own demise (8). Apoptotic cells (AC) provide signals for the rapid clearance of apoptotic debris by professional phagocytes *in vivo*. Thus, ‘quiet clearance’ of this process is ensured (9).

In contrast, necrosis classically has been defined as accidental or murderous cell death owing, for example, to noxious insults such as heat, irradiation or toxins provoking uncontrolled cell swelling and membrane rupture. The consequence is leakage of cytosolic, organelle and nuclear components into the interstitial space, which may cause severe inflammation. Membrane disintegration is also observed during secondary necrosis, which is a result of defective phagocytosis of AC. Secondary necrosis occurs *in vivo* when the

number of apoptotic cells exceeds the local capacity for phagocyte-mediated clearance (10, 11).

3.1.1 Induction of apoptosis

Caspases, a family of cystein proteases, which cleave their substrates after aspartic acid residues, play a central role in the induction of apoptotic cell death (12). They are highly conserved throughout evolution and can be found even in hydra, a member of the ancient metazoan phylum Cnidaria (13). Caspases are synthesized as enzymatically inert zymogens, so-called pro-caspases. They are usually activated upon proteolytic processing at cleavage sites containing aspartic acid residues by other active caspases (14). This activation strategy is termed ‘caspase cascade’ and involves the activation of effector caspases by initiator caspases to amplify and integrate pro-apoptotic signals (15). Activation of the main initiator caspases caspase-8 and caspase-9 defines the sensitive step in apoptosis induction. Starting from this point, two principally different, eventually converging pathways can be pursued (16) (Figure 1).

3.1.1.1 Extrinsic pathway of apoptosis induction

The extrinsic cell death pathway starts with coupling of death ligands, which belong to the TNF family, to their specific death receptors on the cell surface (17). Receptor ligation is followed by the recruitment of adapter proteins such as fas-associated death domain and pro-caspase-8 to form a complex termed death-inducing signaling complex (4, 18). Consequently, pro-caspase-8 is cleaved to yield enzymatically active caspase-8, which then processes effector caspases including caspase-3, -6 and -7. Activation of these caspases finally results in the execution of apoptosis, as they initiate the cleavage of different substrates such as DNA repair enzymes, structural proteins and endonuclease inhibitors (4).

3.1.1.2 Intrinsic pathway of apoptosis induction

Reactive oxygen species (ROS) and/or reactive nitrogen species, DNA damage or growth factor withdrawal are initiators of the intrinsic cell death pathway (19-21). Cytochrome c is released from mitochondria in response to these stimuli, which then is accompanied by the activation of caspase-9 (19). Members of the B cell leukemia/lymphoma-2 (Bcl-2) protein family serve to tightly regulate the leakage of pro-apoptotic cytochrome c from mitochondria. The Bcl-2 protein family includes proteins with pro- as well as anti-apoptotic properties and thus, proteins of this family are considered as essential regulators of apoptosis (22). Based on their domain architecture, the members of the Bcl-2 protein family were classified into three distinct groups (23). While the anti-apoptotic members such as Bcl-2 and B cell leukaemia/lymphoma-x long (Bcl-X_L) share all four Bcl-2 homology domains (BH1-BH4), the pro-apoptotic ones like Bcl-2-associated protein x or Bcl-2 homologous antagonist/killer share three domains (BH1-BH3). The members of the so-called BH-3-only proteins like Bcl-2 antagonist of cell death, Bcl-2-interacting mediator of cell death or BH3-interacting domain death agonist (Bid) possess only the BH3 domain (24). As Bcl-2 proteins form heterodimers between pro- and anti-apoptotic family members to neutralize each other, the balance between pro- and anti-apoptotic Bcl-2 proteins determines cell fate (15). Oversimplyfied, BH3-only proteins are activated by intrinsic death stimuli, which enables them to inhibit anti-apoptotic or to activate pro-apoptotic Bcl-2 family members with high specifity. As a consequence, the mitochondrial outer membrane is permeabilized and cytochrome c is released into the cytosol (25). Subsequently, cytochrome c induces ATP-dependent heptamerization of apoptotic protease activating factor to form a ‘wheel-shaped’ signaling platform, which is termed the apoptosome (26). After recruitment of pro-caspase-9 to the apoptosome and its activation, caspase-9 activates effector caspases (27). Notably, dependent on cell type and apoptosis-inducing stimulus, a connection between the extrinsic and the intrinsic cell death pathway has been observed. Caspase-8, which is activated via the extrinsic pathway can promote cleavage of the BH3-only protein Bid. The resulting truncated Bid can insert into the mitochondrial outer membrane to induce its permeabilization and thus activate the intrinsic pathway of apoptosis induction (28).

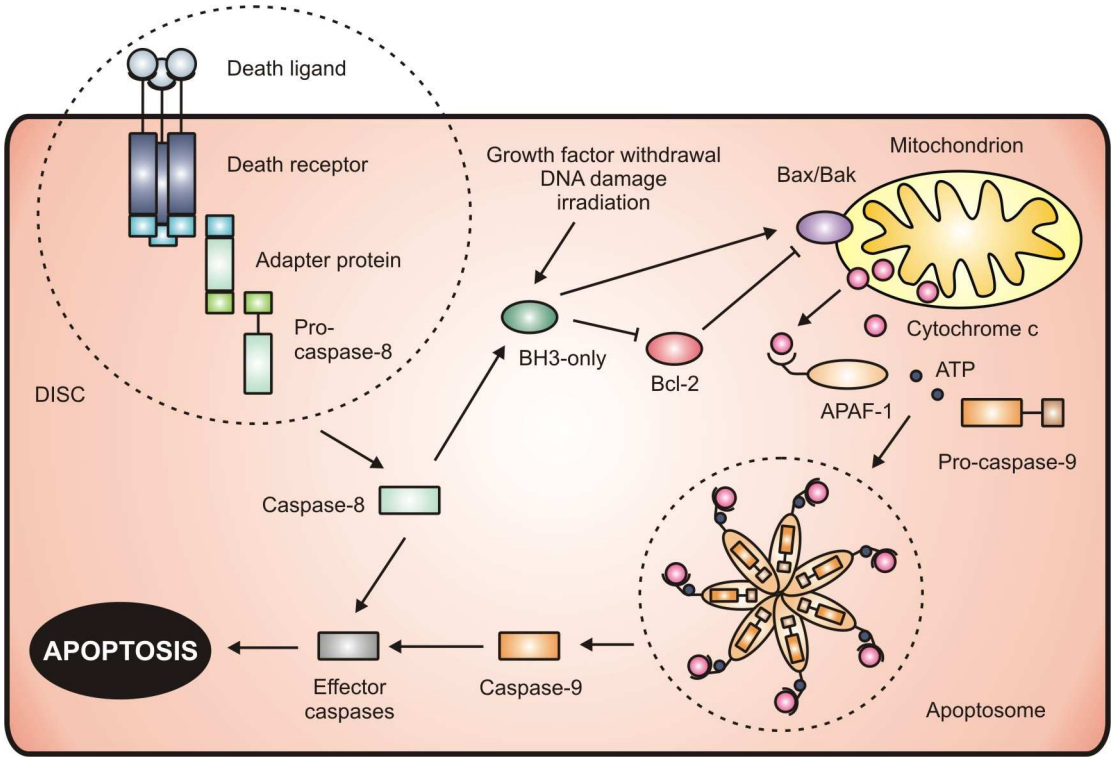

Figure 1. Induction of apoptosis. Apoptosis can be initiated by two distinct pathways. The extrinsic pathway is initiated upon ligation of death receptors by specific death ligands. After recruitment of adapter proteins and pro-caspase 8, the death-inducing signaling complex (DISC) is formed, which provokes activation of caspase-8 via proteolytic cleavage of pro-caspase 8. Caspase-8 then activates effector caspases resulting in the execution of apoptosis. Caspase-8 also may cross-activate the intrinsic death pathway, which is induced in response to growth factor withdrawal, DNA damage as well as irradiation and involves activation of Bcl-2 homology domain (BH)3-only members of the B cell leukemia/lymphoma-2 (Bcl-2) family. Thereupon, cytochrome c is released from mitochondria mainly through the action of Bcl-2-associated protein x (Bax) and Bcl-2 homologous antagonist/killer (Bak), which constitute pro-apoptotic members of the Bcl-2 family. Anti-apoptotic members such as Bcl-2 can be inhibited by BH3-only proteins and can themselves inhibit pro-apoptotic members of the Bcl-2 family like Bax or Bak. Cytochrome c together with apoptotic protease activating factor-1 (APAF-1), ATP and pro-caspase 9 forms a heptameric complex termed the apoptosome. After the activation of caspase 9, effector caspases are activated, which results in the execution of apoptosis.

3.1.2 Diseases associated with apoptosis

Recognition and clearance of AC by phagocytes is vital to the morphological shaping of tissues during development and plays a pivotal role in the maintenance of tissue homeostasis, the regulation of the immune system and the resolution of inflammation (1, 29). Autoimmune diseases such as systemic lupus erythematosus may arise due to defects in AC clearance (30). Furthermore, disruption of the balance between cell proliferation and cell death can result in the development of neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease, different forms of cancer, autoimmune disorders such as rheumathoid arthritis and increased susceptibility to bacterial and viral infections including aquired immune deficiency syndrome or ebola (31).

3.2 Phagocytosis of apoptotic cells

Clearance of AC represents the last step in the apoptotic program. Like the initiation and execution of apoptosis, also clearance of AC is a tightly regulated process. Three critical steps are traversed to ensure proper clearance of AC. First, phagocytes are attracted to the site of tissue damage. Second, AC are specifically recognized by phagocytes. Finally, AC or their fragments are engulfed by phagocytes. The following paragraphs will elaborate on the underling mechanisms of these steps.

3.2.1 Attraction

As professional phagocytes such as macrophages may not be in close proximity of dying cells, the secretion of chemoattractants by AC is of enormous importance to ensure the removal of AC and therefore to avoid secondary necrosis (Figure 2).

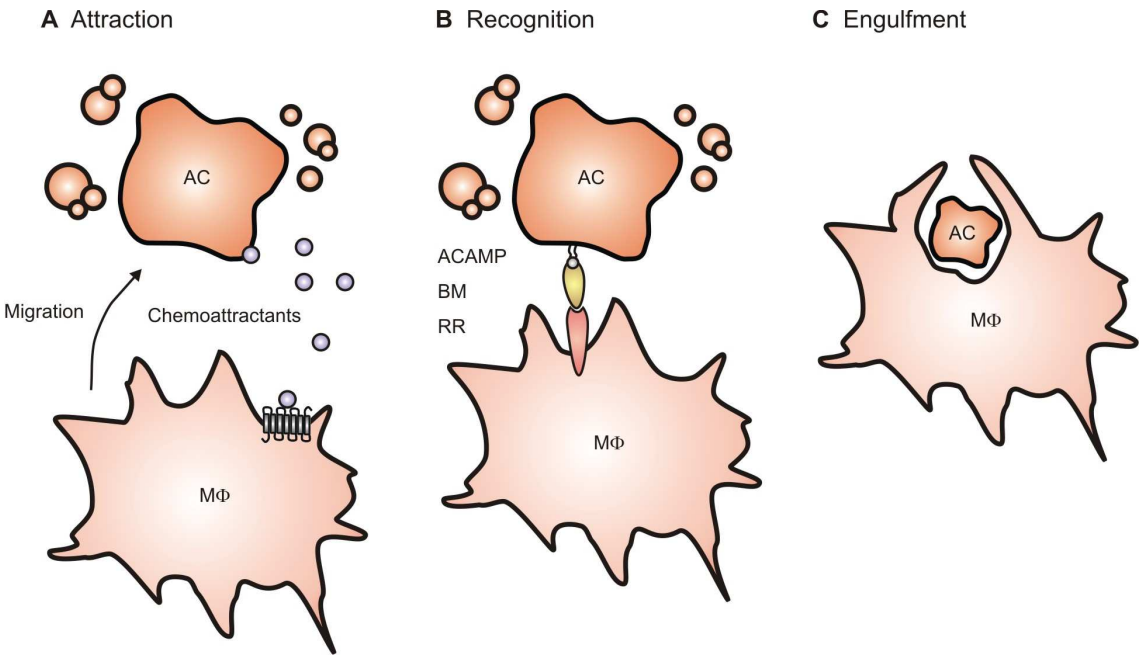

Figure 2. Phagocytosis of apoptotic cells. (A) Apoptotic cells (AC) secrete a multitude of chemoattractants to direct macrophages (MΦ) to sites of tissue damage. (B) Different apoptotic cell-associated molecular patterns (ACAMP) allow phagocytes to distinguish viable or nerotic cells from AC. Bridging molecules (BM) often serve to strengthen the interaction of ACAMP and recognition receptors (RR) on the phagocyte surface. (C) Rearrangement of the phagocyte's cytoskeleton finally results in a 'zipper-like' ingestion process.

A plethora of chemoattractants, which are secreted by AC have been described. These factors include thrombospondin 1, lysophosphatidylcholine and endothelial monocyte-activating polypeptide II (32-34). Thrombospondin 1 as a heterodimer with CD36 was also shown to serve as a signal for the recognition of AC (32). Furthermore, sphingosine-1-phosphate (S1P) is secreted by AC (35). Besides its role in promoting monocyte/macrophage migration *in vitro* (36), S1P was reported to exert potent anti-inflammatory activities, as will be discussed in section 3.3.2.3. AC not only provide attraction signals for mononuclear cells, but they also contribute to the resolution of inflammation by secreting lactoferrin, which was reported to potently inhibit granulocyte migration *in vitro* and *in vivo* (37). Thus, AC on the one hand actively attract phagocytes to secure their clearance and on the other hand prevent the attraction of additional inflammatory cells.

3.2.2 Recognition

Discrimination between pathogens, necrotic cells (NC) or AC defines an important mechanism enabling phagocytes to elicit a proper immunological response. AC-associated molecular patterns, which are also known as ‘eat me’ signals, allow phagocytes to distinguish viable cells (VC) or NC from AC (8, 9). Bridging molecules such as thrombospondin, growth arrest-specific 6 and milk-fat globule epidermal growth factor 8 often serve to strengthen the interaction of AC-associated molecular patterns and phagocyte receptors (38).

Externalization of the membrane phospholipid phosphatidylserine (PS) was considered a hallmark of apoptosis, as in VC, PS is mostly confined to the inner leaflet of the plasma membrane (39). However, in some cell types necrosis was also associated with PS externalization resulting in engulfment by macrophages and prevention of an inflammatory response (40). In general, externalization of PS regardless of the type of cell death might be a trigger for the clearance by macrophages and associated with anti-inflammatory signaling. Besides PS receptors, numerous other receptors expressed by macrophages play an important role during the recognition of AC. Most of these receptors are involved in the recognition and engulfment of pathogens or the recognition of oxidized lipoproteins. Among these receptors are integrin receptors (38), scavenger receptor CD36 (41), scavenger receptor A (42) and oxidized low-density lipoprotein receptor 1 (43).

In contrast to ‘eat me’ flags, which are expressed by apoptotic cells, the expression of CD31 on the surface of VC serves as detachment or ‘don’t eat me’ signal via homophilic interaction with macrophage CD31 (44). Apoptosis provokes modification of CD31, thereby disrupting this interaction and allowing the phagocytosis of the respective cell.

3.2.3 Removal

After the interaction of ‘eat me’ signals with phagocyte receptors, the cytoskeleton of the phagocyte is rearranged, resulting in a ‘zipper-like’ ingestion process. However, mechanistic studies are obscure. Nevertheless, a

mechanistic link between phagocytic receptors and actin filaments was suggested. Soon after internalization, F-actin is depolymerized from the phagosome. Then, by a series of fusion and fission events with components of the endocytic pathway, the phagosome matures, resulting in the formation of the phagolysosome (45).

3.3 Macrophage polarization

Macrophages originate from myeloid progenitor cells in the bone marrow, which differentiate into monocytes dependent on lineage-determining cytokines such as GM-CSF. After the entry of monocytes into the blood stream, they can be allured by an appropriate stimulus to migrate into different tissues. There, monocytes differentiate into tissue macrophages under the influence of growth factors like GM-CSF or M-CSF. Then, the microenvironment of the respective tissue serves to shape the functionality of these macrophages (46, 47).
Macrophages, as part of the body's innate immune system, fulfill a variety of different tasks. They represent prodigious phagocytic cells, which efficiently clear cells, that have undergone cell death. Their ability to migrate along chemokine gradients allows macrophages to intervene in inflammatory events. Moreover, by the presentation of endogenous and exogenous antigens and the secretion of different cytokines, macrophages are capable of linking and shaping innate and adaptive immune responses (48).

3.3.1 Macrophage phenotypes

Macrophages show remarkable plasticity, which offers them the possibility to change their phenotype in response to environmental signals (49). Thus, different stimuli polarize macrophages towards various phenotypes. In the 1960s, Mackaness first described classically activated macrophages, which arose in the presence of microbes (50, 51). However, innate and adaptive immune responses can give rise to a second type of macrophage, which is

more susceptible to pathogen infections and less equipped to produce cytokines, that enhance the immune response. These two macrophage phenotypes were initially defined by Mantovani and co-workers as extremes of a continuum of functional states. Mirroring the nomenclature of Th lymphocytes, classically activated macrophages were denominated as M1 macrophages, while alternatively activated macrophages were termed M2 macrophages (52, 53). Along a linear scale, M1 macrophages were classified to represent one extreme of macrophage activation, whereas M2 macrophages represented the other extreme (54). While M1 macrophages are associated with pro-inflammatory reactions, pathogen killing and tumor resistance, M2 macrophages promote tissue remodeling and tumor formation (54).

Classically activated macrophages arise in response to injury or infection. Th1 lymphocyte-derived IFN-γ alone or in concert with TNF-α or microbial products such as LPS can induce classically activated macrophages, which results, among other parameters, in the production of NO, ROS and the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 (54) (Figure 3).

In contrast, alternatively activated macrophages comprise a broad range of phenotypes with different biochemistry and function. Different immunomodulators such as the Th2 lymphocyte-derived cytokines IL-4, IL-13 or IL-10 can provoke polarization towards an alternatively activated phenotype. Treatment of macrophages with IL-4 and IL-13 induced a macrophage phenotype, which was characterized by the enhanced expression of IL-10 and arginase and was assigned as the M2a phenotype (54). Arginase contributes to the production of the extracellular matrix and to wound-healing by the conversion of arginine to ornithine, which is a precursor of polyamines and collagen (55). M2b macrophages can be generated by immune complexes and TLR ligands or IL-1 receptor ligands. These macrophages exhibit enhanced secretion of TNF-α, IL-1, IL-6 and IL-10. The M2c macrophage phenotype is induced by IL-10, which is followed by the secretion of high levels of IL-10 and TGF-β by these macrophages. While MHC II, which is needed for antigen presentation, is a feature of M1 as well as M2a and M2b macrophages, it is not expressed by M2c macrophages (54).

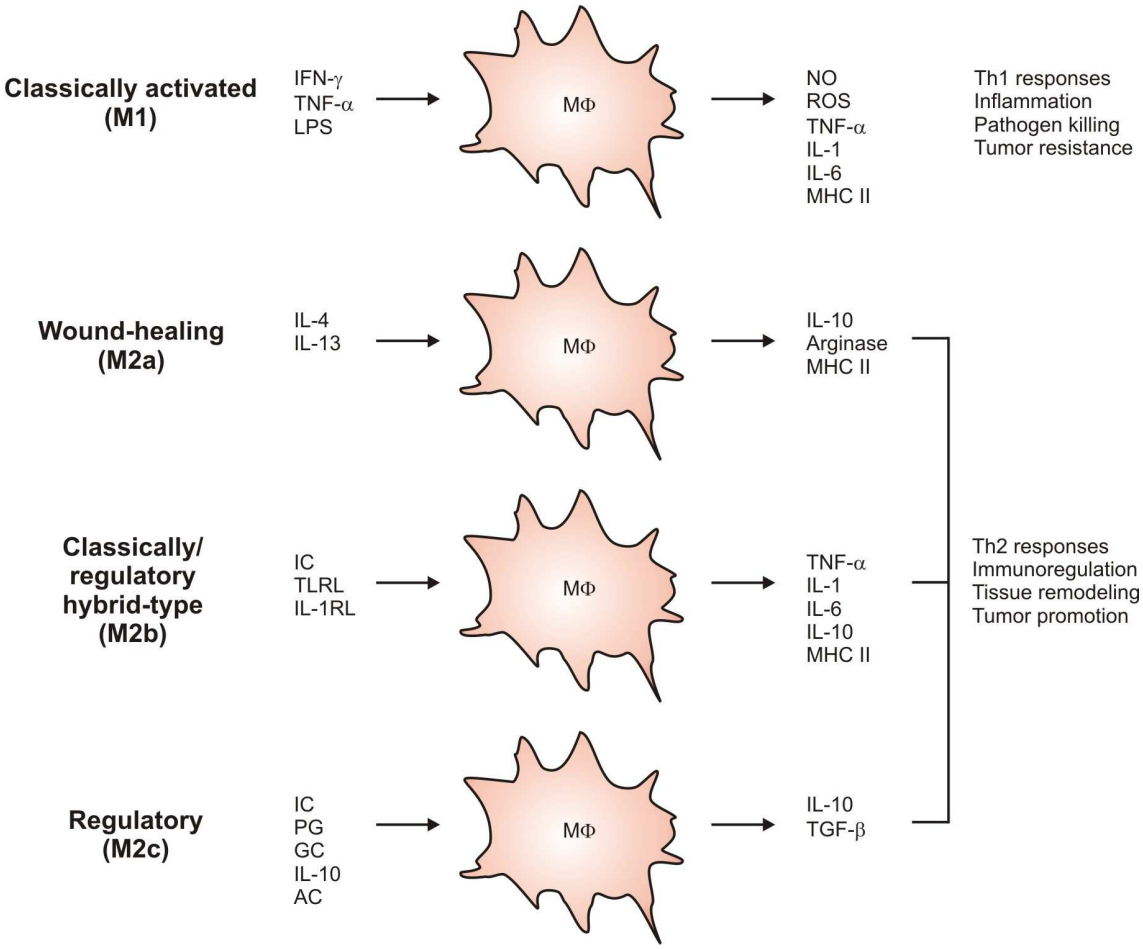

Figure 3. Macrophage phenotypes. Classically activated macrophages (M1 macrophages) can be induced by IFN-γ alone or together with TNF-α or LPS. This results, among other parameters, in the production of NO, reactive oxygen speciies (ROS) and the pro-inflammatory cytokines TNF-α, IL-1 and IL-6. Treatment of macrophages with IL-4 and IL-13 provokes polarization towards the wound-healing or M2a macrophage phenotype. This phenotype is characterized by the enhanced expression of IL-10 and arginase. Immune complexes (IC) and TLR ligands (TLRL) or IL-1 receptor ligands (IL-1RL) can induce the formation of a hybrid-type macrophage phenotype. This phenotype shows M1 as well as M2c characteristics and was termed M2b phenotype. Among these features are the enhanced secretion of TNF-α, IL-1, IL-6 as well as IL-10. Regulatory macrophages or M2c macrophages can be generated by immune complexes (IC), PG, glucocorticoids (GC), IL-10 or apoptotic cells (AC), which is followed by the secretion of IL-10 and TGF-β. MHC II is expressed by all phenotypes except the M2c phenotype.

Recently, three functional states of macrophage activation were proposed for classification as an alternative for the M1/M2 pattern, which are host defense, wound-healing and immune regulation (56). In this model, three basic

macrophage phenotypes, denoted classically activated macrophages, wound-healing macrophages and regulatory macrophages, were illustrated as the three primary colors in a color wheel. This model is useful for the classification of macrophages, which exhibit characteristics that are shared by more than one macrophage population, as these macrophages are illustrated analogous to secondary colors in the color spectrum, finally resulting in 'hybrid-type' macrophages (56). Thus, this classification reflects the high plasticity in macrophage responses, which is why I prefer to use this classification. Therefore, in the following I will distinguish classically activated macrophages from wound-healing macrophages, regulatory macrophages and hybrid-type macrophages.

3.3.2 Macrophage polarization by apoptotic cells

AC modulate the functional response of macrophages apart from simply being removed and thus shape their phenotype.

3.3.2.1 Attenuation of pro-inflammatory responses

Notably, phagocytosis of AC, in contrast to pathogen phagocytosis, is associated with an active anti-inflammatory response. This response is characterized by an attenuated release of pro-inflammatory mediators and an increase in the secretion of anti-inflammatory mediators.

Following co-culture with apoptotic lymphocytes, LPS-induced TNF-α and IL-1β expression in monocytes was attenuated, whereas IL-10 expression was enhanced (57). This finding was supported by many others, showing that AC provide signals to directly influence macrophages, i.e. to repress their pro-inflammatory response (Figure 4). The autocrine signaling of TGF-β, PGE_2 or platelet activating factor, all of which are secreted from macrophages in response to AC, plays a central role in the inhibition of pro-inflammatory cytokine expression (58). These factors were reported to be responsible for the reduction of IL-1β, IL-8 as well as TNF-α after co-incubation periods of more than 18 h.

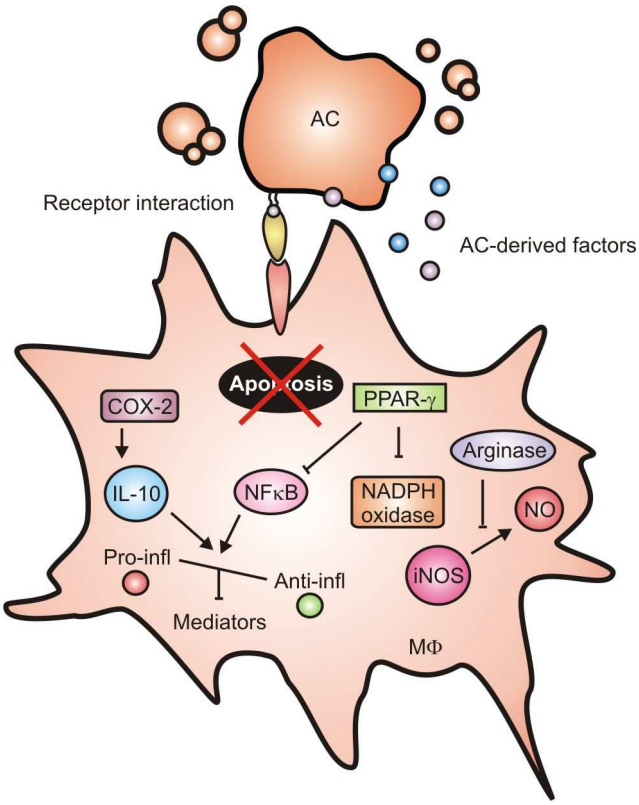

Figure 4. Macrophage polarization by apoptotic cells. Apoptotic cells (AC) polarize macrophages (MΦ) by the secretion of soluble factors and/or via cell-cell contact-dependent mechanisms. The most prominent pathways involve induction of cyclooxygenase (COX)-2 expression, which was suggested to enhance IL-10 levels. The activation of peroxisome proliferator-activated receptor (PPAR)-γ inhibits LPS-induced NF-κB signaling, further contributing to shift the balance between pro-inflammatory (Pro-infl) and anti-inflammatory (Anti-infl) mediators towards the anti-inflammatory ones. Furthermore, PPAR-γ also inhibits NADPH oxidase and thus the production of reactive oxygen species. Besides providing protection of macrophages against apoptosis, AC also induce arginase expression, which results in the inhibition of inducible NO synthase (iNOS)-associated NO production.

NF-κB forms a major transcriptional regulator of pro-inflammatory cytokine expression (59). LPS-induced NF-κB activation was demonstrated to be impaired by AC, although NF-κB binding to DNA as well as inhibitor of κB degradation were not affected by AC implying the involvement of co-activators and/or co-repressors (60). Recently, mechanistic details were provided by the observation that AC induce small ubiquitin-like modifier (SUMO)ylation of peroxisome proliferator-activated receptor (PPAR)-γ, which prevents LPS-induced nuclear co-repressor removal from κB binding sites within the

promoters of pro-inflammatory cytokines (61). However, at present the receptors and molecular pathways initiating PPAR-γ SUMOylation remain to be identified.

Even though alternative macrophage activation after interaction with apoptotic cells was observed by a multitude of reports, the type of macrophage/AC interaction as well as the underlying molecular signaling circuits are ill defined. Several reports showed an involvement of PS, Mer tyrosin kinase receptor and vitronectin receptor in immunosuppression, although phagocytosis *per se* was not essential (62). However, generalized effects and details are still controversial.

3.3.2.2 Attenuation of ROS and NO formation

The bactericidal capacity of macrophages is partly defined by the generation of NO (63) and ROS (64). Treatment of macrophages with LPS and/or IFN-γ initiates the upregulation of inducible NO synthase (iNOS), which catalyzes the oxidation of L-arginine to L-citrulline and NO (65). Several reports showed reduced NO levels in response to AC (66, 67). Arginase seems to play a crucial role in this process, as it metabolizes L-arginine to urea and ornithine, thus competing with iNOS for the same substrate (68). On the one hand, it was reported that AC induce arginase 1 expression in a PS-dependent manner, while simultaneously attenuating iNOS expression (69). On the other hand, it was shown that arginase 2 was upregulated by AC (70). However, arginase 2 expression was cell-cell-contact-independent. Instead soluble factors, which were secreted by AC, were suggested to mediate arginase 2 induction. Interestingly, in this report iNOS expression was unaffected.

ROS are generated during the oxidative burst, which is initiated by protein kinase-dependent assembly of the NADPH oxidase complex (71). Attenuated ROS formation, which was observed after interaction of macrophages with AC, was proposed to be attributed to a cell-cell-contact-dependent mechanism (72). This mechanism was suggested to involve PPAR-γ, although mechanistic details are still unknown (73). Taking into consideration that PPAR-γ also plays a central role in the inhibition of NF-κB and the generation of pro-inflammatory

cytokines, PPAR-γ can be considered as an eminent factor shaping the phenotype of regulatory macrophages.

3.3.2.3 S1P and IL-10 in macrophage polarization

As mentioned before, not only recognition-dependent signals, but also soluble factors released by AC were proven to induce macrophage polarization. Among these factors S1P and IL-10 play an important role.

Co-culture of tumor cells with primary human macrophages resulted in apoptosis induction in tumor cells and alternative activation of macrophages (74). Apoptosis induction was essential for the induction of the alternative macrophage phenotype, whereas conditioned medium from AC also polarized macrophages, which argued for a soluble factor. S1P was identified as the crucial factor, which is secreted from AC and provides protection of macrophages against apoptosis (35). Moreover, AC-derived S1P was suggested to reduce TNF-α and IL-12p70 secretion and to enhance the expression of IL-8 and IL-10 (74).

IL-10 is also produced by AC (75) and has emerged as a key regulator for the suppression of Th1 responses (76), since the IL-10-induced DNA binding of STAT3 homodimers is followed by the attenuation of transcription of pro-inflammatory mediators (77). Cyclooxygenase (COX)-2 seems to be involved in increasing IL-10 levels. COX-2 constitutes the rate-limiting enzyme in the conversion of arachidonate to PGH_2, which can be further metabolized towards anti-inflammatory mediators such as the PPAR-γ agonist 15d-PGJ_2 (78, 79). COX-2 expression in macrophages due to interaction with AC was shown to be either facilitated via a cell-cell-contact-dependent mechanism (66) or via AC-derived S1P (80). Increased PGE_2 production has been linked to impaired pathogen clearance by lung alveolar macrophages and to elevated levels of IL-10, which were dependent on E prostanoid receptor 2-induced cAMP generation (81). cAMP signaling is considered a hallmark of regulatory macrophages in the resolution phase of inflammation and is generally accompanied by COX-2 expression and the secretion of high levels of IL-10 (82).

3.4 Sphingosine-1-phosphate

The bioactive lipid mediator S1P is derived from plasma membrane sphingolipids (83). S1P was originally considered to be just an inert structural component of the cell membrane (84). However, today the important role of S1P as a signaling molecule in a plethora of biological processes, including Ca^{2+} mobilization, cell growth, survival and cell motility, has become clear (85).

3.4.1 S1P metabolism

Cellular S1P levels are low and tightly regulated by the balance between synthesis and degradation (86). Ceramide constitutes an important progenitor in the synthesis of S1P. The crucial step in the *de novo* synthesis of ceramide occurs by condensation of serine and palmitoyl-CoA to form 3-ketosphinganine (Figure 5). Followed by multiple catalytical steps, 3-ketosphinganine is converted into ceramide. The second way of ceramide production is the hydrolysis of membrane sphingomyelin by sphingomyelinase, a reaction, which can be reversed by sphingomyelin synthase. Ceramide is subsequently deacylated by ceramidase to sphingosine. Sphingosine can be reconverted to ceramide by ceramide synthase. Finally, phosphorylation of sphingosine by sphingosine kinase (SphK) results in S1P generation. The turnover of S1P is mediated via two different pathways. S1P can either be reversibly dephosphorylated to sphingosine by specific S1P phosphohydrolase or it can be irreversibly cleaved by S1P lyase to form phosphoethanolamine and hexadecenal (87, 88).

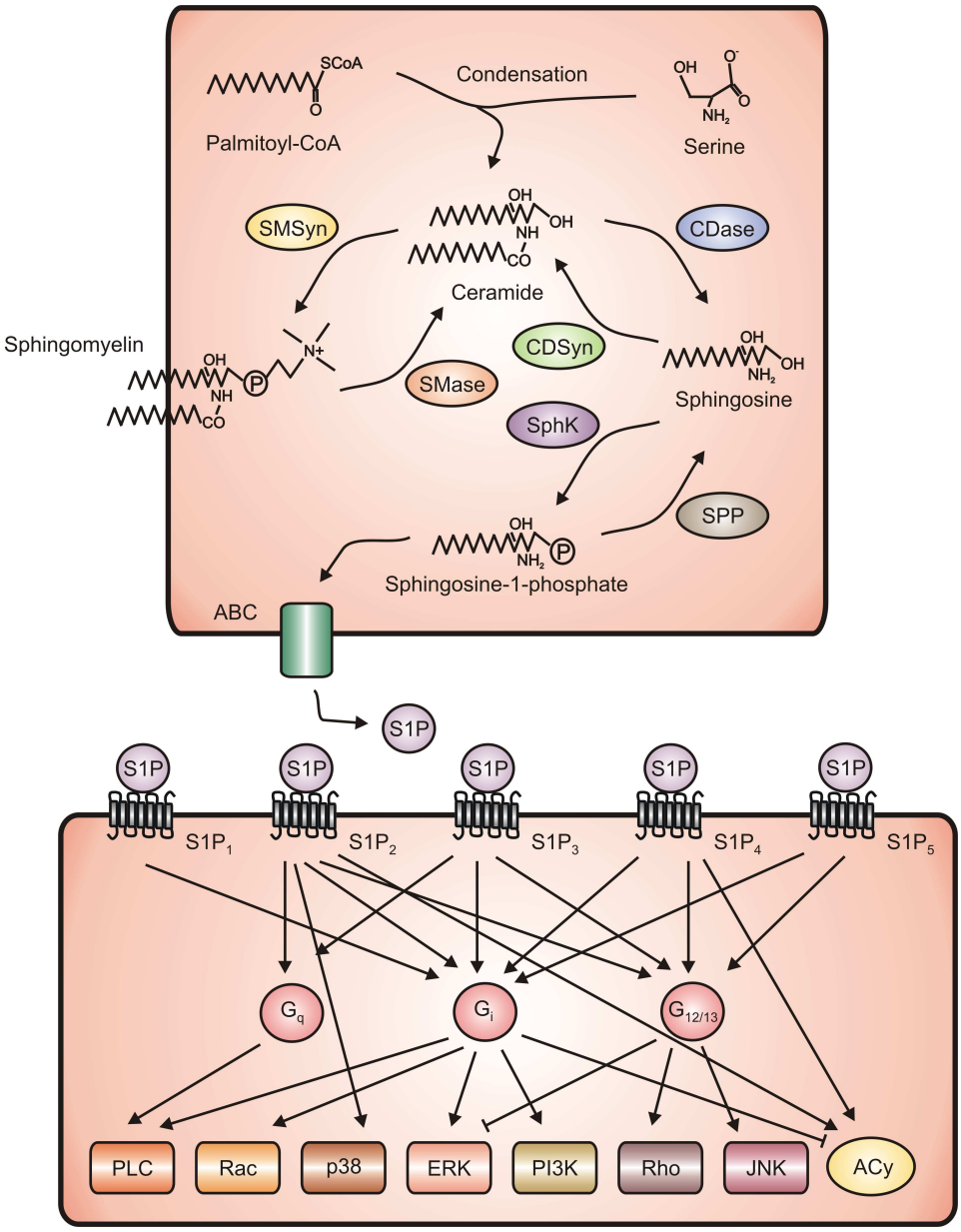

Figure 5. Synthesis and action of S1P. Sphingosine-1-phosphate (S1P) can be synthesized *de novo* by condensation of serine and palmitoyl-CoA or by sphingomyelinase (SMase)-mediated hydrolysis of sphingomyelin to ceramide. Ceramide can either be reconverted to sphingomyelin by sphingomyelin synthase (SMsyn) or it can be converted into sphingosine by ceramidase (CDase). The latter reaction can be reversed by ceramide synthase (CDSyn). Sphingosine kinase (SphK) catalyzes the phosphorylation of sphingosine to S1P. S1P degradation can either be mediated by dephosphorylation by S1P phosphohydrolase (SPP) or by irreversible cleavage catalyzed by S1P lyase. After the export of S1P via ATP binding cassette (ABC) transporter S1P can occupy five specific S1P receptors ($S1P_{1\text{-}5}$). Ligation of S1P receptors can result in the activation of a various cellular signaling pathways, which are activated directly or via G-proteins (G_q, G_i, $G_{12/13}$). These pathways include phospholipase C (PLC), Rac, p38 MAPK (p38), ERK, PI3K, Rho, JNK and adenylyl cyclase (ACy).

3.4.2 Sphingosine kinases

S1P is generated by phosphorylation of sphingosine by sphingosine kinases, an enzyme family consisting of two isoenzymes, namely SphK1 and SphK2. SphKs exhibit different kinetic properties and substrate specificities and they differ in their temporal expression patterns during development as well as in their tissue distribution (86, 89). Moreover, at the cellular level SphK isoforms may fulfill distinct or even antithetic biological virtues, although facilitating the same reaction (90).

SphKs constitute evolutionary conserved enzymes. They were proven in humans as well as in mice, yeast and plants, having also homologs in worms and flies (86). In humans, alternative splice variants for both isoenzymes have been described (87).

SphKs are regulated on transcriptional level and also via posttranslational modifications. After treatment with platelet-derived growth factor (PDGF) or cytokines such as TGF-β (91) and IL-1 (92) or under hypoxia (93), enhanced mRNA levels of SphK1 were detected. The induction of SphK activity was shown to be predominantly regulated by growth factors like epidermal growth factor, nerve growth factor and PDGF. Moreover, SphK activity, among others, is also stimulated by G protein-coupled (GPC) receptor agonists, cross-linking of immunoglobulin receptors, TNF-α and even S1P itself (88).

The activity of SphK1 has been associated with enhanced growth, proliferation and protection against apoptosis in a multitude of cellular systems (86). In the following, I will focus on the regulation of apoptosis, as in this aspect the opposing roles of the two SphK isoforms appear most prominent. As SphK1 has a substantial basal activity and stimulation with agonists often provokes only a slight increase in catalytic activity, it was suggested that translocation to subcellular compartments might be the important step in SphK1 regulation (89). Phosphorylation of SphK1 by ERK was shown to result in increased activity of SphK1, which was accompanied by its translocation from cytosol towards the plasma membrane, where it phosphorylated sphingosine to S1P (94). After externalization of S1P, it can occupy its receptors, a process which is called ‘inside-out’ signaling (86). However, during apoptosis SphK1 can be cleaved, resulting in the downregulation of its pro-survival properties (95).

On the contrary, SphK2 promotes apoptosis when being overexpressed. This pro-apoptotic property can be attributed to its N-terminal BH3 domain, which allows the physically interaction of SphK2 with Bcl-$X_{L,}$ an anti-apoptotic Bcl-2 family protein, causing its inactivation and thus induction of apoptosis (96). Nevertheless, disruption of this interaction only partly reduced the apoptosis-inducing capability of SphK2. This raised the proposal that SphK2 and S1P phosphohydrolase act in concert with ceramide synthase at the endoplasmic reticulum (ER) to convert S1P to sphingosine, and subsequently to pro-apoptotic ceramide. Thus, the subcellular localization of S1P formation may define its impact on apoptosis. Furthermore, SphK2 overexpression was reported to suppress cell growth via its nuclear localization which was accompanied by inhibition of DNA synthesis (97). Interestingly, S1P, which was generated by AC and protected macrophages against apoptosis, was mainly derived from SphK2 (35).

Genetic deletion of both, SphK1 and SphK2 together, in mice resulted in prenatal death (98) as a consequence of severely disturbed neurogenesis and angiogenesis, which reinforces the regulatory impact of S1P on several complex biological circuits such as survival, proliferation or migration (86). Interestingly, deletion of either SphK1 or SphK2 alone was not associated with an obvious phenotype under unstressed conditions, indicating that SphKs may compensate for each other (98).

3.4.3 S1P as a signaling molecule

In mammals, S1P is enriched only in the blood and lymph (99). In the plasma, S1P is mainly bound to high-density lipoprotein or albumin and can reach micromolar concentrations (99, 100). Tissue levels of S1P are not easy to determine without causing tissue cell stress or tissue disruption (100). However, they are believed to be below the amount necessary for receptor activation, although all cells synthesize S1P in association with the turnover of membrane sphingolipids through the endocytic recycling pathway (86). This indicates that S1P secretion could be performed by specialized cells such as erythrocytes, which may secrete S1P into the plasma (101), or endothelial cells, which deliver

S1P to the lymph and/or plasma (102). However, the low tissue levels of S1P could also be a consequence of enhanced S1P degradation.

Most of the S1P-dependent modulations of immune responses are attributed to S1P receptor ligation. Upon its generation by SphK from plasma membrane sphingolipids, S1P can either engage S1P receptors in an auto- and/or paracrine fashion or can act as a second messenger with intracellular activity, which was originally suggested to be the task of S1P (103, 104). A direct intracellular target for S1P had for a long time not been identified. Nevertheless, several studies described intracellular S1P actions that were not imitated by extracellular S1P and could not be attributed to coupling to S1P receptors (89). Intracellular S1P could simply shift the balance between pro-apoptotic ceramid/sphingosine and anti-apoptotic S1P, the so-called 'sphingolipid rheostat', towards S1P and survival (86). Furthermore, activation of SphK was reported to mediate intracellular signaling via MAPK (105) or calcium currents (106). According to this observation, TNF-α activated SphK1 resulting in NF-κB activation and protection against apoptosis (107). However, recently the molecular mechanism of the action of intracellular S1P was revealed. S1P generated by SphK2 was shown to inhibit histone deacetylases resulting in enhanced expression of the cyclin-dependent kinase inhibitor p21 (108).

Coupling of S1P to its specific receptors is important for directed cell movement, angiogenesis, vascular maturation and immunity (86). However, mechanistic details revealing how S1P is released from cells to occupy its specific receptors are limited. In mast cells and platelets, which store S1P, its release is facilitated by ATP binding cassette family transporters (109, 110). Alternatively, in human endothelial cells a splice variant of SphK1 was reported to be exported from the cells, provoking extracellular production of S1P (102). Furthermore, it was hypothesized that S1P could also be flipped from the inner leaflet to the outer leaflet of the plasma membrane (99).

Over the last years it became apparent that the SphK, S1P, S1P receptor signaling axis in macrophages might play a central role in the pathogenesis of inflammatory diseases such as atherosclerosis, rheumatoid arthritis, asthma as well as cancer (111).

3.4.4 Sphingosine-1-phosphate receptors

S1P receptors belong to the family of hepta-helical GPC transmembrane receptors. In vertebrates, five isoforms of S1P receptors termed $S1P_{1-5}$ have been identified. S1P receptors differ in their association with different G-proteins and also in their binding affinity for S1P, which is in the nanomolar range. In general, activation of S1P receptors is followed by different G-protein cascades in which, among others, the activation of phospholipase C, PI3K, adenylyl cyclase, ERK 1/2, p38 MAPK and JNK are involved. Although S1P receptors activate common GPC pathways, they are only partly redundant in their cellular signaling capacity (99, 112). Thus, the S1P receptor expression profile on the respective cells represents a critical parameter determining the influence of S1P on distinct cell populations.

While $S1P_{1-3}$ have a wide tissue distribution, $S1P_4$ is predominantly expressed in lymphoid tissue and platelets and $S1P_5$ is restricted to the nervous system, NK cells and some dendritic cells (84, 113, 114). In mononuclear cells, species-dependent alterations in the S1P receptor expression profile were detected. While human monocytes express $S1P_1$, $S1P_2$ and $S1P_4$, human macrophages express $S1P_{1-4}$ (115), whereas murine macrophages are restricted to $S1P_1$ and $S1P_2$ (116).

S1P receptor gene deletion studies underlined the importance of S1P receptors. $S1P_1$ single and $S1P_{2/3}$ double knockout mice died *in utero* as a consequence of severe developmental vascular defects, which were similar to the picture seen in SphK1/2 knockout mice (117).

The activation of S1P receptor signaling is a transient process. In $S1P_1$ overexpressing human embryonic kidney cells exogenous S1P treatment, in a dose-dependent manner, induced receptor translocation to perinuclear vesicles (118). $S1P_1$ was suggested to be phosphorylated at its C-terminal tail, which is followed by the recruitment of the adapter protein arrestin and the induction of receptor internalization via clathrin coated pits. However, $S1P_1$ recycled back to the plasma membrane. Thus, S1P specifically induces the reversible trafficking of $S1P_1$ via the endosomal pathway.

3.4.5 The important role of $S1P_1$ during trafficking of immune cells

$S1P_1$ receptor mRNA was originally isolated from human endothelial cells as an immediate-early gene product which was induced by phorbol 12-myristate 13-acetate (119). In vascular cells, $S1P_1$ was shown to be upregulated following treatment with growth factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (120, 121). However, the pathways mediating $S1P_1$ induction remain to be determined. The only transcription factor, that has been described to provide $S1P_1$ induction is krüppel-like factor (KLF), a member of the zinc finger family of transcription factors, which plays diverse roles during differentiation and development (122, 123).

The vascular S1P gradient, i.e. S1P is enriched in the circulatory system, whereas it is estimated to be much lower in interstitial fluids of tissues, is a spatial cue for lymphocyte and hematopoietic cell trafficking (99). In the lymph, S1P was suggested to serve as a chemoattractant to allow lymphocyte egress from the thymus, secondary lymphoid organs and Peyer's patches (99). Furthermore, B cell recruitment from the bone marrow to the periphery, dendritic cell trafficking from the periphery to the lymph nodes and hematopoietic progenitor cell recirculation are regulated by S1P (99). Lymphocyte $S1P_1$ levels are downregulated during transit through the blood, upregulated in lymphoid organs, and downregulated again in the lymph (124).

The expression of $S1P_1$ is critical for T cell egress from the thymus and homing to the lymph nodes (Figure 6). T cells must complete negative selection before they can leave the thymus and they aquire the ability to migrate towards S1P only at the mature single-positive stage (100). Following the entry of naïve lymphocytes into a secondary lymphoid organ from the blood, they travel to separate subcompartments where they survey for antigen. In the absence of antigen encounter, lymphocytes leave the respective organ via the efferent lymphatics or in the case of the spleen, via the red pulp (124). However, in an inflamed lympoid organ $S1P_1$ is internalized. After activation by TCR stimulation, T cells downregulate $S1P_1$ mRNA, thus loosing their ability to migrate towards S1P. Three days after activation, $S1P_1$ mRNA starts to recover, T cells regain their S1P responsiveness and reappear in the circulation (100).

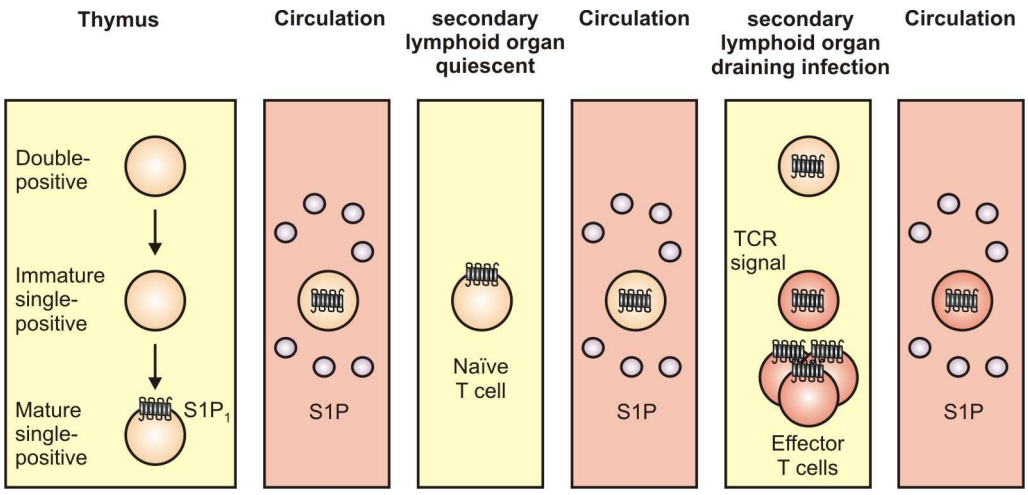

Figure 6. Role of S1P receptor 1 during T lymphocyte trafficking. Mature single-positive T lymphocytes express S1P receptor 1 ($S1P_1$) on their surface, which enables them to migrate into the blood along S1P gradients. There, due to high S1P concentartions $S1P_1$ is internalized. Following entry of naïve T cells into secondary lymphoid organs they survey for antigens, while $S1P_1$ reappears on the cell surface. In the absence of antigens T cells leave the secondary lymphoid organ and enter the blood again. However, when T cells arrive in an inflamed lymphoid organ $S1P_1$ is internalized and $S1P_1$ transcription decreases in response to an activating TCR signal. After three days $S1P_1$ is expressed on T cell surface again, allowing the newly generated effector T cells to enter the blood.

3.5 Heme oxygenase

Heme (iron-protoporphyrin IX) constitutes an essential molecule for all nucleated cells, as it senses or uses oxygen (125). It functions as a prosthetic moiety of various hemoproteins including hemoglobin, myoglobin, cytochromes, COX, NOS and soluble guanylyl cyclase (126). As heme cannot be recycled among different cells, it must be synthesized and degraded within an individual cell. An exception to this rule is formed by senescent erythrocytes, which are phagocytosed by macrophages in the reticuloendothelial system, in which hemoglobin is separated to the heme and globin moieties. While the globin moiety is hydrolyzed to amino acids, heme breakdown is catalyzed by heme oxygenase. (125). Hemoglobin derived from senescent erythrocytes forms the major source of heme for macrophages (125), although it was postulated that AC in general could release heme (127).

Heme oxygenase (HO) was discovered in 1968 as a microsomal enzyme, which catalyzes the initial and rate-limiting step in the oxidative degradation of heme to equimolar quantities of biliverdin IXα, Fe^{2+} and carbon monoxide (CO) (Figure 7) (128, 129). In mammals, HO forms complexes with NADPH: cytochrome P-450 reductase, which provides reducing equivalents required for the catalytic turnover as well as with NAD(P)H: biliverdin reductase, which catalyzes the further metabolization of biliverdin to bilirubin (130, 131). Bilirubin is transported to the liver, where it is conjugated with glucuronic acid and excreted into bile. Fe^{2+} can be used for heme synthesis in the cell or it can be transported via transferrin to other tissues, mainly the bone marrow. CO is transported to the lung, where it is exhaled (125, 132).

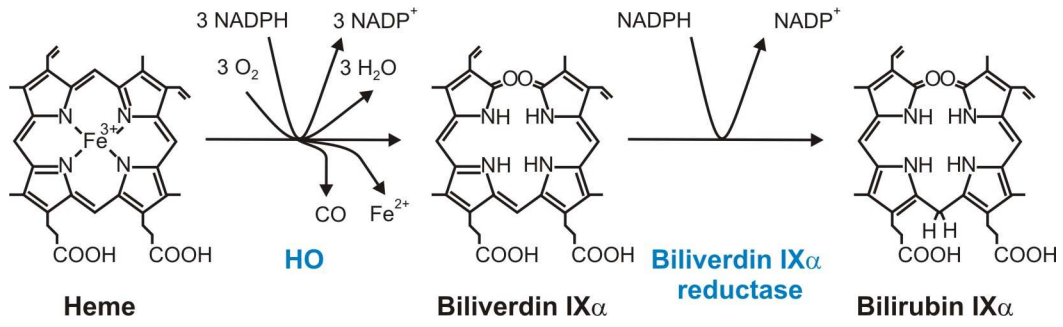

Figure 7. The pathway of heme metabolism. Using the reducing capacity of NADPH, which is delivered by NADPH:cytochrome P-450 reductase, heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme to equimolar quantities of biliverdin IXα, Fe^{2+} and carbon monoxide (CO). Biliverdin-IXα is further metabolized to bilirubin IXα by biliverdin IXα reductase under the use of NADPH.

3.5.1 Tissue distribution of HO

In recent years, three isoforms of HO have been described. HO-1 and HO-2 are the products of distinct genes (133). However, as stop codons are present within the coding region of the HO-3 gene and a lack of detectable mRNA or protein product was observed in rat tissues, HO-3 was considered to be a pseudogene originating from HO-2 transcripts (134).

HO-1 constitutes an inducible enzyme, which is expressed ubiquitously at low levels, whereas HO-2 is constitutively expressed (135). HO-1 is found in high amounts in liver, spleen and bone-marrow, where relatively high heme concentrations due to continuous erythrocyte turnover are present (135). Large amounts of HO-2 were found in the brain, testes and endothelial cells (136). Interestingly, in the rat brain HO-2 was shown to co-localize with soluble guanylyl cyclase, suggesting a role for CO in neurotransmission through the cGMP pathway (137). Furthermore, the high expression of HO-2 in endothelial cells points to a role of CO in vasorelaxation, as exogenously applied CO lowered blood pressure in spontaneously hypertensive rats (138). Moreover, a role for HO-2 as an oxygen sensor for calcium-sensitive potassium channels in carotid cells was also reported (139).

The focus of my work was the inducible isoform HO-1, which plays a cytoprotective role by modulating tissue response to injuries in various pathological states (140).

3.5.2 Subcellular localization of HO-1

HO-1 was originally reported to localize to the smooth ER (141). It is embedded in the ER membrane with an insertion sequence near its C-terminal portion, while a large domain containing the N-terminus is exposed cytoplasmically (142).

However, HO-1 was also demonstrated to localize to the mitochondria (143). Furthermore, among other stimuli, hemin was shown to provoke translocation of HO-1 into the nucleus (144). Nuclear entry was facilitated by protease-mediated C-terminal cleavage and was also associated with reduction of HO activity. Nevertheless, nuclear and cytoplasmic HO-1 similarly provided protection of cells against hydrogen peroxide-mediated injury. Thus, nuclear localization of HO-1 was suggested to serve for upregulation of genes that promote cytoprotection against oxidative stress. HO-1 was reported to activate oxidant responsive transcription factors including AP-1, although mechanistic details were not provided. As the structure of HO-1 contains no traditional DNA binding motifs, a potential mechanism involving binding of HO-1 to a transcription factor

or a protein complex was suggested. Another study showed that the interaction of HO-1 with caveolin-1 in the plasma membrane of rat endothelial cells also decreased the enzymatic activity of HO-1 (145). Recently, it was demonstrated that HO-1 forms dimers/oligomers in the ER (146). Truncation of the C-terminal transmembrane segment provoked translocation of HO-1 into the cytosol and nucleus indicating that the transmembrane segment is crucial for the stabilization of HO-1 in the ER.

3.5.3 Regulation of HO-1

The original characterization of HO-1 as an inducible enzyme by non-heme agents involved the use of heavy metals (147). Besides their potential to increase HO-1 activity, heavy metal ions can enhance the *de novo* synthesis of HO-1 (148). Today, a broad spectrum of inducers of HO-1 has been identified, including various noxious stressors, such as ROS (149), NO (150), LPS (151), ultraviolet (UV) radiation (149) as well as its physiological substrate heme (152). HO-1 is regulated primarily at the level of gene transcription (153). However, hypoxia was reported to regulate HO-1 gene expression by stabilization of mRNA, although hypoxia also induces HO-1 protein expression through p38 MAPK and PKC (154, 155). Furthermore, the involvement of translational control of HO-1 expression by cobalt ions has been suggested (148). Besides its role in regulating HO-1 expression at the transcriptional level, Akt/PKB mediates posttranslational phosphorylation of HO-1 at its serine-188 residue and thus enhances its activity (156).

The diversity of HO-1 inducers is reflected by the variety of pathways and transcription factors, which are involved in HO-1 gene regulation. HO-1 is a representative phase II detoxification enzyme, whose stress-responsive element is structurally and functionally similar to the antioxidant responsive element (135, 157). As the redox-sensitive transcription factor NF E2-related factor-2 (Nrf2) plays a major role in the transcriptional activation of ARE-driven genes (158, 159), current research indicates that Nrf2, besides various other transcription factors with minor roles, is a critical regulator of HO-1 gene expression (135).

The fact that HO-1 can also be induced by quaint stimuli such as cigarette smoke (160), diesel exhaust particles (161) and broccoli extracts (162) confronts us with HO-1 ever during our whole life. Importantly, the medicinal effects of pharmaceutical compounds such as aspirin (163) and statins (164) may be imparted indirectly through HO-1 induction, which then mediates their therapeutic effects.

3.5.4 Cytoprotective effects of HO-1

As HO-1 is induced by various oxidative stressors, the assumption was raised that the induction of HO-1 is associated with protection of cells against these insults and thus with the limitation of tissue damage (165). In fact, in several disease models, including cardiac ischemia and reperfusion injury as well as pulmonary inflammation, hypertension and sepsis, the expression of HO-1 was shown to be potently cytoprotective (166, 167). Thus, the induction of HO-1 can be regarded as an adaptive response, i.e. inducible defense, to several injurious stimuli. Besides its anti-oxidative role, anti-inflammatory as well as anti-proliferative and anti-apoptotic properties have also been attributed to HO-1 (166).

An interesting research field has opened to the scientific community, as it was discovered that the by-products of heme degradation are not just obscure waste products with potential toxicological implications, but can also be associated with numerous biological functions (135). Thus, the interest in HO enzymes has shifted from their well-defined metabolic function of heme catabolism to their potential cytoprotective fuction in different models of cellular stress or organ pathology.

The products of heme degradation were shown to mimic the cytoprotective effects, which were ascribed to HO-1 involving a plethora of different disease models and pathways (166). In the following, I will focus on the cytoprotective effects, which are directly associated with heme turnover.

One key effect is the degradation of the potentially dangerous intracellular pro-oxidant heme by HO-1, which was the initial explanation for the numerous protective effects provided by HO-1 (168). Bilirubin may act as a potent peroxyl

radical scavenger and was considered as an important antioxidant molecule (169). Furthermore, bilirubin can also counteract nitrosative reactions (170). As an indirect effect, the generation of Fe^{2+} is balanced by a strong induction of the synthesis of ferritin, an iron-sequestering protein (171). Thus, participation of free iron in the so-called 'Fenton reaction', which generates highly reactive hydroxyl radicals from hydrogen peroxide, might be limited (172). Furthermore, an ATPase is co-localized in the ER with HO-1 and transports Fe^{2+} to the luminal side of the ER. It was suggested that within the lumen of the ER Fe^{2+} may be oxidized to Fe^{3+}, bind transferrin and return to the extracellular fluid via the exocytotic pathway (173).

Although functional roles for the other heme degradation products have been reported, CO has received increasing attention (174). CO was first considerd as an inert gaseous molecule with little physiological relevance (175). It was rather associated with lethality, as it bears a high affinity for hemoglobin, which is approximately 245 times that of oxygen in humans (176), thus preventing oxygen delivery to tissues and organs and creating tissue hypoxia (166). Furthermore, CO can act as a neurotoxin poisoning the respiratory chain in the mitochondria, thereby suppressing aerobic glycolysis (177). However, according to the statement of Paracelsus that 'the right dose differentiates a poison from a remedy' (176) it was shown that in macrophages CO suppresses the expression of the LPS-induced pro-inflammatory cytokines TNF-α, IL-1β and macrophage inflammatory protein-1β, while increasing the LPS-induced expression of the anti-inflammatory cytokine IL-10 (167). Furthermore, CO inhibits platelet aggregation, which might be accompanied by the suppression of thrombosis and the pro-inflammatory damage induced by activated platelets (178). Other effects elicited by CO include prevention of apoptosis in several types of cells, such as endothelial cells, fibroblasts and macrophages (179-181). Furthermore, CO exerts potent anti-proliferative effects on cells, including T lymphocytes and airway smooth muscle cells (182, 183). It was shown that predominantly cGMP and p38 MAPK play a role in mediating the cytoprotective effects of CO (158). However, mechanistic details are still elusive.

3.5.5 Involvement of HO in diseases

A single case report described a patient diagnosed with genetic deficiency of the HO-1 gene, who exhibited serious impairment of iron metabolism. The phenotype was accompanied by severe persistent leukocytosis and increased vulnerability to oxidant injury besides growth retardation and anemia. The patient eventually died of intracranial hemorrhage at the age of six years (184, 185).

HO-1-deficient mice exhibit a similar phenotype compared to this patient, which underscores the importance of HO-1 in cellular homeostasis (186). As VEGF synthesis was shown to be partially dependent on HO-1, it was suggested that the embryonic lethality of HO-1 knockout mice may involve defects in angiogenesis or vasculogenesis (187). Furthermore, it was also hypothesized that impairment of angiogenesis, required for proper ovulation and male fertility, could play a role in reducing the fertility of HO-1 knockout mice (187).

HO-1 is upregulated in a multitude of tumors including human prostate cancer and squamous carcinoma (136). Interestingly, in different forms of cancer such as human melanoma, HO-1 was specifically expressed in macrophages, but only slightly in tumor cells (188). Unfortunately, HO-1 expression in cancer cells can be further increased in response to chemotherapy, radiation or photodynamic therapy (189, 190) and then may offer cancer cells a growth advantage.

To date, three polymorphisms in the 5'-flanking region of the HO-1 gene have been discovered in humans, a $(GT)_n$ dinucleotide repeat polymorphism and two single nucleotide polymorphisms, G(-1135)A and T(-413)A (191). The $(GT)_n$ polymorphism was suggested to influence the basal promoter activity as well as the induction level of the HO-1 gene expression in response to stress. This was supposed to be attributed to Z-DNA, left-handed helices, which long $(GT)_n$ repeats are likely to form, and which may reduce the transcription of the HO-1 promoter (125). Indeed, i*n vitro*, short HO-1 alleles (<25 GT repeats) show stronger promoter activity following treatment with H_2O_2, compared to longer alleles (192). Interestingly, in most cases long $(GT)_n$ repeats are associated with susceptibility to pathological conditions such as the presence of emphysema in smokers (193). However, a human study on the risk for malignant melanoma

revealed that the homozygous short allele was found more frequently in the melanoma group (192). However, the short HO-1 allele also conveyed protection against various disease entities such as restenosis as well as local vascular inflammation following percutaneous transluminal angioplasty (194, 195). Thus, the increased HO-1 expression associated with short HO-1 alleles on the one hand might provide protection of tumor cells against apoptosis but on the other hand exhibits potent beneficial roles in a vast number of other pathologies. While the G(-1135)A polymorphism has not been reported to cause any pathologies, the T(-413)A polymorphism was involved in coronary artery disease as well as hypertension in women (191).

Targeting HO-1 has been proven to be potently cytoprotective in ameliorating the pathology associated with a plethora of disease processes. Tin-protoporphyrin, a potent competitive inhibitor of HO-1, has been successfully used for the treatment of hyperbilirubinemia in newborn rats (196). Furthermore, the administration of biliverdin and/or bilirubin in rodent models was shown to elicit protection in several disease states including ischemia/reperfusion injury, transplant rejection and inflammatory bowel disease (176). CO also coveyed protection in several rodent disease models including sepsis, different lung injury models, ischemia/reperfusion injury and organ transplantation (135). The exogenous administration of CO and/or biliverdin and bilirubin appears promising in treating human diseases. However, standard clinical phase trials are required to determine efficacy, toxicological profiling, tissue distribution and half-life of these agents (176).

3.6 Aims of this study

The objective of my work was to examine whether the expression of HO-1 and $S1P_1$ contribute to alternative macrophage activation. In this regard, the underlying molecular pathways of HO-1 and $S1P_1$ induction and their relevance for macrophage polarization should be identified. The following main points were experimentally addressed.

- In the first part of my studies, I analyzed whether HO-1 was induced in primary human macrophages following treatment with supernatants from AC.

- Next, I investigated the signaling pathways, which are involved in the expression of HO-1 following incubation with supernatants from AC.

- Then, I identified the target genes of HO-1 being affected by treatment with supernatants from AC.

- As unexpectedly $S1P_1$ expression was also fostered by AC supernatants, I worked out whether this induction was specific for alternative macrophage activating factors and whether the S1P receptor expression profile was altered in general.

- Finally, I analyzed the impact of $S1P_1$ upregulation and trans-localization, which was affected by AC supernatants, on the migratory potential of macrophages.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

All chemicals were of the highest grade of purity commercial available.

40% acrylamide/bis-acrylamide (29%:1.0% w/v)	Carl Roth GmbH & Co. KG, Karlsruhe
40% acrylamide/bis-acrylamide (37.5% : 1.0% w/v)	Carl Roth GmbH & Co. KG, Karlsruhe
4′,6-Diamidino-2-phenylindol (DAPI)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
ABsolute™ QPCR SYBR® Green	ABgene®, Hamburg
Adenosine triphosphate (ATP)	Roche Diagnostics GmbH, Mannheim
Agarose	PeqLab Biotechnologie GmbH, Erlangen
Ammonium chloride (NH_4Cl)	Merck KGaA, Darmstadt
Ammonium persulfate (APS)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Ampicillin	Biomol® GmbH, Hamburg
BSA	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Brome phenol blue	AppliChem GmbH, Darmstadt
Calcium chloride ($CaCl_2$)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Chloroform	Merck KGaA, Darmstadt
Coelenterazine	BIOTREND® Chemikalien GmbH, Köln
Coenzyme A (lithium salt)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Diethyl pyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe
DMSO	Carl Roth GmbH & Co. KG, Karlsruhe
Disodium hydrogen phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt
Dithiothreitol (DTT)	Carl Roth GmbH & Co. KG, Karlsruhe
D-luciferin	AppliChem GmbH, Darmstadt
DNA loading dye (6x)	Fermentas GmbH, St. Leon-Rot
Ethanol	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Ethidium bromide	Sigma-Aldrich® Chemie GmbH, Deisenhofen
EDTA	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Ficoll™ PM 400	Amersham Biosciences Europe GmbH, Freiburg

Forene® (Isoflurane)	Abbot GmbH & Co. KG, Wiesbaden
GeneRuler™ 100bp DNA Ladder	Fermentas GmbH, St. Leon-Rot
Glucose	Carl Roth GmbH & Co. KG, Karlsruhe
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
Glycine	Serva Electrophoresis GmbH, Heidelberg
HEPES	Carl Roth GmbH & Co. KG, Karlsruhe
Hydrochloric acid (HCl)	Merck KGaA, Darmstadt
Isopropanol	Merck KGaA, Darmstadt
JetPEI™	Polyplus-Transfection™, Illkirch (France)
LB agar (Luria/Miller)	Carl Roth GmbH & Co. KG, Karlsruhe
LB medium (Luria/Miller)	Carl Roth GmbH & Co. KG, Karlsruhe
Magnesium carbonate hydroxide [$(MgCO_3)_4$ x $Mg(OH)_2$]	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Magnesium chloride ($MgCl_2$)	AppliChem GmbH, Darmstadt
Magnesium sulfate ($MgSO_4$)	Carl Roth GmbH & Co. KG, Karlsruhe
Methanol (MeOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Nonidet P-40	ICN Biomedicals GmbH, Eschwege
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
Paraformaldehyde (PFA)	Merck KGaA, Darmstadt
peqGOLD RNAPure™	Peqlab Biotechnologie GmbH, Erlangen
PMSF	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Phosphatase inhibitor mix PhosSTOP	Roche Diagnostics GmbH, Mannheim
Phosphoric acid (H_3PO_4)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Poly(dI-dC)	Pharmacia, Uppsala (Sweden)
Potassium chloride (KCl)	Merck KGaA, Darmstadt
Potassium hydrogen carbonate ($KHCO_3$)	Merck KGaA, Darmstadt
Potassium hydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt
Potassium hydroxide (KOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Protease inhibitor mix complete	Roche Diagnostics GmbH, Mannheim
Proteinase K	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Sodium borate ($Na_2B_4O_7$)	Merck KGaA, Darmstadt
Sodium chloride (NaCl)	Merck KGaA, Darmstadt
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium fluoride (NaF)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt
Sucrose	Carl Roth GmbH & Co. KG, Karlsruhe
Tetraethylendiamine (TEMED)	Carl Roth GmbH & Co. KG, Karlsruhe
Tricine	Carl Roth GmbH & Co. KG, Karlsruhe
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Triton X-100	Carl Roth GmbH & Co. KG, Karlsruhe

Trypan blue	Biochrom AG, Berlin
Tryptone	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Tween® 20	Carl Roth GmbH & Co. KG, Karlsruhe
Urea	Sigma-Aldrich® Chemie GmbH, Deisenhofen
VECTASHIELD® Hard Set™ Mounting Medium	Vector Laboratories, Inc., Burlingam (USA)
Yeast extract	Life Technologies, Paisley (Scotland)

4.1.2 Buffers and solutions

Unless indicated otherwise, all buffers and solutions were manufactured and diluted in bidestilled H_2O and adjustment of pH was performed with HCl or NaOH.

Antibody solution:

TTBS	50 % (v/v)
Blocking buffer	50 % (v/v)

Blocking buffer	Rockland Immunochemicals Inc., Gilbertsville (USA)

Blotting buffer (10 x):

Tris	250 mM
Glycine	1.9 M
Methanol	20% (v/v)
pH	8.3

Buffer D:

HEPES	20 mM
Glycerol	20 % (v/v)
KCl	100 mM
EDTA	0.5 mM
Nonidet P-40	0.25 % (v/v)
DTT	2 mM
PMSF	0.5 mM
NaF	10 mM
Protease inhibitor mix	1 x
Phosphatase inhibitor mix	1 x
pH (adjusted with KOH)	7.9

Buffer F:

Ficoll^{TM} PM 400	20 % (w/v)
HEPES	100 mM
KCl	300 mM
DTT	10 mM
PMSF	0.5 mM
NaF	10 mM
Protease inhibitor mix	1 x
Phosphatase inhibitor mix	1 x
pH (adjusted with KOH)	7.9

DEPC-treated H_2O

DEPC	1 ml
H_2O	999 ml

Firefly assay reagent:

ATP	530 µM
Coenzyme A	213 mg/l
D-luciferin	470 mM
DTT	33.3 µM
EDTA-K^+	100 µM
$(MgCO_3)_4$ x $Mg(OH)_2$	1.07 mM
$MgSO_4$	2.67 mM
Tricine	20 mM
pH	7.8

Firefly lysis buffer (5 x):

DTT	10 mM
Glycerol	50% (v/v)
Tris	125 mM
Triton X-100	5% (v/v)
pH (adjusted with H_3PO_4)	7.8

Genotyping lysis buffer

NaOH	25 mM
EDTA	0.2 mM
pH	12.0

Genotyping neutralization buffer

Tris	40 mM
pH	5.0

Hypotonic cell lysis buffer:

HEPES	10 mM
$MgCl_2$	2 mM
EDTA	100 µM
KCl	10 mM
DTT	1 mM
PMSF	0.5 mM
NaF	10 mM

Protease inhibitor mix	1 x
Phosphatase inhibitor mix	1 x
pH (adjusted with KOH)	7.9

Leukocyte washing buffer:

EDTA	2 mM
in PBS	

Lower Tris buffer:

Tris	1.5 M
pH	8.8

Nuclear lysis buffer:

HEPES	50 mM
KCl	50 mM
NaCl	300 mM
EDTA	100 µM
Glycerol	10% (v/v)
DTT	1 mM
PMSF	0.5 mM
NaF	10 mM
Protease inhibitor mix	1 x
Phosphatase inhibitor mix	1 x
pH	7.9

Passive lysis buffer Promega GmbH, Mannheim

PBS:

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	8.1 mM
KH_2PO_4	1.5 mM
pH	7.4

PFA (4%):

PFA	4% (v/v)
Sucrose	10% (v/v)
in PBS	
pH	7.4

Protein lysis buffer:

Tris	50 mM
EDTA	5 mM
NaCl	150 mM
Nonidet P-40	150 mM
PMSF	0.5 mM
DTT	1 mM
Protease inhibitor mix	1 x
pH	8.0

***Renilla* assay reagent:**

Tris	25 mM
NaCl	100 mM
$CaCl_2$	1 mM
Coelenterazine	90 µM
pH	7.5

Running buffer (glycerol tolerant) Amersham Biosciences Europe GmbH, Freiburg

SDS-PAGE sample buffer (4 x):

Tris	125 mM
10% SDS	2% (v/v)
Glycerol	20% (v/v)
Brome phenol blue	0.002% (w/v)
DTT	5 mM
pH	6.9

SDS-PAGE running buffer (10 x):

Tris	250 mM
Glycine	1.9 M
SDS	7 mM
pH	8.3

STE (2 x):

Tris (pH 8.0)	10 mM
NaCl	50 mM
EDTA	1 mM

TBS (10 x):

Tris	500 mM
NaCl	1.5 M
pH	7.4

TBE (5 x):

Tris	445 mM
$Na_2B_4O_7$	445 mM
EDTA	10 mM

TE:

Trypsin	0.5 g/l
EDTA	0.2 g/l
in PBS	

TTBS:

TBS	1 x
Tween 20	0.06% (v/v)

Upper Tris buffer:

Tris	0.5 M
pH	6.8

Whole cell protein lysis buffer:

Urea	6.65 M
Glycerol	10% (v/v)
SDS	1% (v/v)
Tris (pH 6.8)	10 mM
pH	7.4

4.1.3 Stimulants and inhibitors

Bilirubin	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Tricarbonyldichlororuthenium(II) dimer (CORM-2)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Diethylenetriamine-NO (Deta-NO)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Dimethylsphingosine (DMS)	Biomol® GmbH, Hamburg
Fludarabine	Sigma-Aldrich® Chemie GmbH, Deisenhofen
IFN-γ (human)	Roche Diagnostics GmbH, Mannheim
IL-4 (human)	PeproTech GmbH, Hamburg
IL-10 (human)	PeproTech GmbH, Hamburg
JAK Inhibitor I (InSolution™)	Merck KGaA, Darmstadt
LPS *(Escherichia coli*, serotype 0127:B8)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
SB203580	Alexis® Biochemicals, Grünberg
Sphingosine-1-phosphate (S1P)	Avanti® Polar Lipids, Inc., Alabaster (USA)
Staurosporine	Sigma-Aldrich® Chemie GmbH, Deisenhofen
STA-21	Biomol® GmbH, Hamburg
VPC 23019	Avanti® Polar Lipids, Inc., Alabaster (USA)

CORM-2 was freshly dissolved in DMSO for each experiment. As a negative control, CORM-2 was inactivated (iCORM) according to a previously described method (197).

4.1.4 Cell lines and primary cells

Primary human monocytes:

Primary human monocytes were isolated from buffy coats, which were obtained from DRK-Blutspendedienst Baden Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main.

Primary murine peritoneal macrophages:

Murine peritoneal macrophages were isolated from 6-10 week-old C57Bl/6 EDG-$1^{fl/fl}$ x F4/$80^{Cre/+}$ or C57Bl/6 EDG-$1^{fl/fl}$ x F4/$80^{+/+}$ mice.

Jurkat T cells (DSMZ GmbH, Braunschweig):

Jurkat T cells are derived from a 14 year-old boy with acute lymphatic leukemia. This T cell line was established in 1977 (198).

4.1.5 Mice

Mice with a $S1P_1$ knockout in F4/80 positive cells (C57Bl/6 EDG-$1^{fl/fl}$ x F4/$80^{Cre/+}$) and control littermates (C57Bl/6 EDG-$1^{fl/fl}$ x F4/$80^{+/+}$) were kindly provided by Dr. Bodo Levkau (Institute of Pathophysiology, University of Essen Medical School). These mice were generated by breeding F4/80 cyclization recombinase (Cre) knockin mice (199) with $S1P_1^{loxP}$ mice, in which exon 2 of the $S1P_1$ gene was flanked by locus of X-over of P1 (*loxP*) sites (200). Mice were kept in the Zentrale Forschungseinrichtung of the Johann Wolfgang Goethe-University Frankfurt, Frankfurt am Main in polysulphon cages with filter cover containing 5-10 mice in each cage. Cages were equipped with litter and pellet food as well as water *ad libitum*. Experiments followed the guidelines of the Hessian animal care and use committee.

4.1.6 Bacteria

XL1-Blue supercompetent bacteria [Stratagene GmbH, Amsterdam (Niederlande)]. Genotype: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB $lacI^qZ\Delta M15$ $Tn10(Tet^R)$].

XL-10-Gold® ultracompetent bacteria [Stratagene GmbH, Amsterdam (Niederlande)]. Genotype: Tet^R Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB $lacI^qZ\Delta M15$ $Tn10$ (Tet^R) Amy Cam^R].

4.1.7 Reagents for cell culture

FCS Gold	PAA Laboratories GmbH, Cölbe
Human plasma (AB positive donor)	DRK-Blutspendedienst Baden Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main
L-glutamine	PAA Laboratories GmbH, Cölbe
Lymphocyte separation medium LSM 1077	PAA Laboratories GmbH, Cölbe
PBS (Instamed 9.55 g/ml)	Biochrom AG, Berlin
Penicillin/streptomycin	PAA Laboratories GmbH, Cölbe
Trypsin/EDTA (3.5 U/mg, porcine)	PAA Laboratories GmbH, Cölbe

4.1.8 Media for cell culture

Different culture media were used on the basis of Roswell Park Memorial Institute (RPMI) 1640, which was obtained from PAA Laboratories GmbH (Cölbe). FCS and human plasma were heat-inactivated by incubation at 56℃ for 30 min.

RPMI with FCS

RPMI 1640	
FCS	10%
L-Glutamin	2 mM
Penicillin	100 U/ml
Streptomycin	0,1 mg/ml

RPMI with human plasma

RPMI 1640	
Human plasma	10%
L-Glutamin	2 mM
Penicillin	100 U/ml
Streptomycin	0,1 mg/ml

RPMI without plasma

RPMI 1640	
L-Glutamin	2 mM
Penicillin	100 U/ml
Streptomycin	0,1 mg/ml

4.1.9 Media for bacteria culture

SOC medium

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
$MgCl_2$	10 mM
KCl	2.5 mM
Glucose	2 mM

4.1.10 Antibodies

Primary antibodies:

Anti-actin (rabbit, polyclonal)	Sigma-Aldrich® Chemie GmbH, Deisenhofen
Anti-Adora A_{2A} (rabbit, polyclonal)	Merck KGaA, Darmstadt
Anti-Bcl-X_L (mouse, monoclonal)	BD Transduction Laboratories™, Heidelberg
Anti-HO-1 (rabbit, polyclonal)	Biomol® GmbH, Hamburg
Anti-$S1P_1$ (rabbit, polyclonal)	Orbigen Inc., San Diego (USA)
Anti-$S1P_1$ (rabbit, polyclonal)	ProSci Inc., Poway (USA)
Anti-$S1P_1$ (mouse, monoclonal)	Abcam® plc, Cambridge (UK)

Secondary antibodies:

IRDye® 800-labeled donkey anti-rabbit	LI-COR® Biosciences GmbH, Bad Homburg
IRDye® 800-labeled donkey anti-mouse	LI-COR® Biosciences GmbH, Bad Homburg
Alexa Fluor® 546-labeled goat anti-mouse	Invitrogen™ GmbH, Karlsruhe

4.1.11 Oligonucleotides

Labeled oligonucleotides were obtained from metabion GmbH (Martinsried), whereas unlabeled oligonucleotides were purchased from biomers.net GmbH (Ulm).

IRDye®700-labeled oligonucleotides for EMSA analysis:

The sequence of the human HO-1 promoter was obtained using the online transcriptional regulatory element database CSH. The online tool TFSearch provided the putative STAT binding sites located within this sequence. According to this sequence the following oligonucleotides for the STAT binding site at -2361 bp to -2369 bp of the human HO-1 promoter were purchased.

5'- IRDye®700-AGG CAC TAT TCC AGG AAC TGG GAA T-3'

5'- IRDye®700-ATT CCC AGT TCC TGG AAT AGT GCC T-3'

Unlabeled oligonucleotides for competitive EMSA analysis:

STAT1 oligonucleotides were designed according to the sequence sc-2573 provided by Santa Cruz Biotechnology®, Inc., Heidelberg (consensus site in boldface letters):

5'-CAT GTT ATG CAT ATT **CCT** GTA AGT-3'

5'-ACT TAC **AGG** AAT ATG CAT AAC ATG-3'

STAT3 oligonucleotides were designed according to the sequence sc-2571 provided by Santa Cruz Biotechnology®, Inc., Heidelberg (consensus site in boldface letters):

5'-GAT CCT TCT GGG **AAT** TCC TAG ATC-3'

5'-GAT CTA GGA **ATT** CCC AGA AGG ATC-3'

Oligonucleotides for the STAT binding site at -2361 bp to -2369 bp of the human HO-1 promoter with a point mutation to impair STAT3 binding (point mutation in boldface letters):

5’-AGG CAC TAT TCC AGG **CC**C TGG GAA T-3’

5’-ATT CCC AG**G G**CC TGG AAT AGT GCC T-3’

Primers for quantitative PCR:

For human ribosomal 18S RNA, actin, HO-1, $S1P_2$, $S1P_3$ and VEGF the following primers were obtained from biomers.net GmbH (Ulm).

Human ribosomal 18S RNA:

5’-GTA ACC CGT TGA ACC CCA TT-3’

5’-CCA TCC AAT CGG TAG TAG CG-3’

Human actin:

5’-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3’

5’-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3’

Human HO-1:

5’-GCC ACC AAG TTC AAG CAG CT-3’

5’-CAG TGC CCA CGG TAA GGA AG-3’

Human $S1P_2$:

5’-AGT TGC ACT ATT TGG GGC AC-3’

5’-GGT GGC TGT TTT TGA AAG GA-3’

Human $S1P_3$:

5’-GCT TCA GGA AAT GGA AGC TG-3’

5’-TCA GGA TGC TGT GAA ACT GC-3’

Human VEGF:

5’-TAC CTC CAC CAT GCC AAG TG-3’

5’-AAG ATG TCC ACC AGG GTC TC-3’

For human adenosine receptor A_{2A} (Adora A_{2A}), Bcl-2, Bcl-X_L, HLA-DMB, indoleamine-2,3-dioxygenase (IDO) and $S1P_1$, validated QuantiTect Primer Assays were purchased from QIAGEN GmbH (Hilden). Sequence information is restricted to the manufacturer.

Primers for the detection of the Cre allele (199):

5’-CTG ATG GTG GCA ACT CAG C-3’ (F4/80 3.4)

5’-GCA TGC ACC GGT AAT GCA GGC-3’ (Cre rev 1)

Primers for the detection of the F4/80 wild-type allele (199):

5’-CTG ATG GTG GCA ACT CAG C-3’ (F4/80 3.4)

5’-AGA GGA GCA GCC AAA AGC CCC-3’ (F4/80 exon 1)

Primers for the detection of the loxP site flanked exon 2 of the $S1P_1$ allele:

5’-GAG CGG AGG AAG TTA AAA GTG-3’ (P1)

5’-CCT CCT AAG AGA TTG CAG CAA-3’ (P2)

Primers for site-directed mutagenesis:

5’-CCA GGC ACT ATT CCA GGC CCT GGG AAT TTA CAA AGC-3’

5’-GCT TTG TAA ATT CCC AGG GCC TGG AAT AGT GCC TGG-3’

Using these primers, a point mutation was introduced into the putative STAT binding site at position -2361 bp to -2369 bp within the human HO-1 promoter, to impair STAT3 binding. Thus, sequence was mutated from 5’-TTC CAG GAA-3’ to 5’-TTC CAG GCC-3’.

Specific small interferring (si)RNA:

Commercially available validated siRNA against HO-1 (QIAGEN GmbH, Hilden), VEGFA (QIAGEN GmbH, Hilden) or $S1P_1$ (Ambion®/Applied Biosystems, Darmstadt) was used. Sequence information is restricted to the manufacturer. Specificity was assured using siCONTROL non-targeting Duplex #1 [Dharmacon Inc., Lafayette (USA)].

4.1.12 Reporter plasmids

The plasmids p1.0-Luc and p1.3-Luc were a gift from Dr. Jianzhu Chen (123). The plasmids phHOLUC40, phHOLUC(-2782) and phHOLUC(-1976) were kindly provided by Dr. Shigeru Takahashi (201).

p1.0-Luc:

The plasmid is based on the vector pGL3-basic and carries a 1 kb fragment of the 5'-flanking region of the murine $S1P_1$ gene.

p1.3-Luc:

The vector contains a 1.3 kb fragment of the 5'-flanking region of the murine $S1P_1$ gene and is based on the vector pGL3-basic.

phHOLUC40:

The plasmid carries a 4 kb fragment of the 5'-flanking region of the human HO-1 gene and is based on the vector pGL3-basic.

phHOLUC(-2782):

On the basis of the vector pGL3-basic the plasmid contains 2782 bp of the 5'-flanking region of the human HO-1 gene.

phHOLUC(-1976):

The plasmid carries 1976 bp of the 5'-flanking region of the human HO-1 gene and is based on the vector pGL3-basic.

phHOLUC(-2782ptm):

This plasmid was created based on the vector phHOLUC(-2782) by site-directed mutagenesis (4.2.9). A point mutation of the putative STAT binding site at position -2361 bp to -2369 bp within the human HO-1 promoter was introduced to impair STAT3 binding.

pRL-CMV (Promega GmbH, Mannheim):

The vector was used in combination with the HO-1 or $S1P_1$ reporter vector to co-transfect mammalian cells. It contains a cDNA encoding *Renilla* luciferase, whose expression is very high due to a CMV enhancer.

4.1.13 Kits

BD™ Cytometric Bead Array, Human VEGF Flex Set	BD Biosciences, Heidelberg
Human Macrophage Nucleofector® Kit	Lonza AG, Köln
HiSpeed® Plasmid Maxi Kit	QIAGEN GmbH, Hilden
iScript™ cDNA Synthesis Kit	Bio-Rad Laboratories GmbH, München
Taq DNA Polymerase Kit	Invitrogen™ GmbH, Karlsruhe
QuikChange® II XL Site-Directed Mutagenesis Kit	Stratagene GmbH, Amsterdam (Netherlands)
Standard DC Protein Assay Kit	Bio-Rad Laboratories GmbH, München

4.1.14 Instruments

ApoTome system	Carl Zeiss Microimaging GmbH, Offenbach/Main
Analysis scale AE 163	Mettler-Toledo GmbH, Giessen
Autoclave ELC 5075	Tuttnauer Co. Ltd., New York (USA)
Bacteria clean bench HERA guard	Heraeus Holding GmbH, Hanau
Bacteria incubator innova® 44	New Brunswick Scientific GmbH, Nürtingen
Cell counter CASY®, model TT	Innovartis AG, Reutlingen
Cell culture clean bench HERA safe	Heraeus Holding GmbH, Hanau
Cell culture incubator HERA cell 240	Heraeus Holding GmbH, Hanau
Cell homogenizer Sonifier® 250	Branson Ultraschall, Dietzenbach
Centrifuge 5415 R	Eppendorf AG, Hamburg
Centrifuge 5810 R	Eppendorf AG, Hamburg
Elektroblotter Trans-Blot® SD Semi-Dry Transfer Cell	Bio-Rad Laboratories GmbH, München
Falcon shaker RM5	Karl Hecht KG, Sondheim/Röhn
Flow cytometer FACSCanto™	BD Biosciences GmbH, Heidelberg
Fluorescence microscope Axiovert 200M	Carl Zeiss Microimaging GmbH, Offenbach/Main
Fluorescence microscope camera AxioCam MRm	Carl Zeiss Microimaging GmbH, Offenbach/Main
Freezer Premium (-20°C)	Liebherr Hausgeräte GmbH, Ochsenhausen
Freezer VIP™ Series (-80°C)	Sanyo Fisher Sales GmbH , München
Gel documentation system Image Documentation & Analysis	Raytest GmbH, Straubenhardt
Ice machine MF 30	Scotsman®, Vernon Hills (USA)

Infrared imaging system Odyssey	LI-COR® Biosciences GmbH, Bad Homburg
Magnetic stirrer MR 3000	Heidolph Instruments GmbH & Co. KG, Schwabach
Microscope CKX31	Olympus Deutschland GmbH, Hamburg
Multilabel reader Mithras LB 940	Berthold Technologies GmbH, Bad Wildbad
Nucleofector® II	Lonza Cologne AG, Köln
Orbit Shaker Titramax 100	Heidolph Instruments GmbH & Co. KG, Schwabach
PCR machine Mastercycler	Eppendorf AG, Hamburg
pH meter CG 842	Schott AG, Mainz
Pipettes (10 µl, 100 µl, 1000 µl)	Eppendorf AG, Hamburg
Pipetus®-akku	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
Power Supply PowerPac™ HC	Bio-Rad Laboratories GmbH, München
Real-Time PCR Detection System MyiQ™ Single Color	Bio-Rad Laboratories GmbH, München
Refrigerator Profi Line	Liebherr Hausgeräte GmbH, Ochsenhausen
Rocker platform Titramax 100	Heidolph Instruments GmbH & Co. KG, Schwabach
SDS gelelectrophoresis system Mini-PROTEAN 3	Bio-Rad Laboratories GmbH, München
Spectrophotometer NanoDrop® ND-1000	NanoDrop Technologies, Inc., Wilmington
Sub-Cell® GT Agarose Gel Electrophoresis System	Bio-Rad Laboratories GmbH, München
Table centrifuge Micromax	IEC, Needham Heights (USA)
Thermomixer compact	Eppendorf AG, Hamburg
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell	Bio-Rad Laboratories GmbH, München
Water system PURELAB Classic UVF	Elga Labwater, Celle
Vortexer REAX top	Heidolph Instruments GmbH & Co. KG, Schwabach
Water bath SW-20C	Julabo Labortechnik GmbH, Seelbach

4.1.15 Software

AxioVision Rel. 4.4	Carl Zeiss Microimaging GmbH, Offenbach/Main
BD FACSDivaTM Software	BD Biosciences GmbH, Heidelberg
FCAP Array 1.0.1	Soft Flow, Burnsville (USA)
Gene Expression Macro 1.1	Bio-Rad Laboratories GmbH, München
ND-1000 3.2.1	NanoDrop Technologies, Inc., Wilmington (USA)

Odyssey 2.1	LI-COR® Biosciences GmbH, Bad Homburg
MyiQ™ 1.0	Bio-Rad Laboratories GmbH, München
TESS	http://www.cbil.upenn.edu/cgi-bin/tess/tess)
TFSearch	http://www.cbrc.jp/research/db/TFSEARCH.html
Transcriptional regulatory element database CSH	http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home
MikroWin 2000	Mikrotek Laborsysteme GmbH, Overath
AxioVision Rel. 4.4	Carl Zeiss Microimaging GmbH, Offenbach/Main
BD FACSDivaTM Software	BD Biosciences GmbH, Heidelberg
FCAP Array 1.0.1	Soft Flow, Burnsville (USA)

4.1.16 Other material

ABgene® PCR Plates (96-well)	ABgene®, Hamburg
ABsolute™ QPCR Seal	ABgene®, Hamburg
Cover slips	Menzel-Gläser GmbH & Co. KG, Braunschweig
MILLEX®-HV PVDF Filters (0.22 µm pore)	Millipore GmbH, Schwalbach
Glassware	Schott AG, Mainz
Membrane PROTRAN®	Whatman® Schleicher & Schuell, Dassel
Needles (20-gauge)	Becton Dickinson GmbH, Heidelberg
Object slides	Süsse GmbH, Gudensberg
Pellet food (10 mm pellets, rat/mouse)	Ssniff Spezialdiäten GmbH, Soest
Plastic material	Becton Dickinson GmbH, Heidelberg Eppendorf AG, Hamburg Greiner bio-one GmbH, Frickenhausen Sarstedt AG & Co., Nümbrecht
Polysulphon cage with filter cover	Tecniplast GmbH, Hohenpreißenberg
Ring-shaped stickers	Avery® Dennison Zweckform, Holzkirchen
Syringes (10 ml)	B.Braun Melsungen AG, Melsungen
Whatman® paper GB 003	Whatman® Schleicher & Schuell, Dassel

4.2 Methods

4.2.1 Cell culture

4.2.1.1 Culture of Jurkat T cells

Jurkat T cells were cultured in RPMI with FCS. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37℃ and were transferred twice a week. Cell numbers were determined using the CASY® cell counter system.

4.2.1.2 Human monocyte isolation and culture

Human monocytes were isolated as described previously (202). In brief, using Ficoll-Hypaque gradients, monocytes were isolated from buffy coats. 50 ml-Leukosep® tubes were layered with 15 ml lymphocyte separation medium and blood cells were added, followed by density gradient centrifugation (440 x *g*, 35 min, RT, without brake). Peripheral blood mononuclear cells were collected, washed twice with leukocyte washing buffer, seeded in 10-cm dishes in RPMI without plasma and subsequently incubated for 1 h at 37℃ to allow their adherence to culture dishes. After removing non-adherent cells, monocytes were differentiated into macrophages with RPMI with human plasma for 7 days.

4.2.1.3 Murine peritoneal macrophage isolation and culture

Following anesthetization of mice with Forene®, the spinal cord was severed by cervical dislocation. Mice were injected with 10 ml ice cold PBS intraperitoneal. Then, PBS was spaced in the peritoneum and removed with a syringe. After centrifugation (500 x *g*, 5 min), cells of one mouse were seeded in two wells of a 12-well plate in RPMI with FCS. After 4 h of incubation, cells were washed twice with PBS to remove non-adherent cells. Cells were incubated for 3-5 days, washing them each two days with PBS before starting the experiments.

4.2.1.4 Generation of conditioned media

Apoptotic Jurkat cells were generated by incubating cells for 3 h with 0.5 µg/ml staurosporine in RPMI without serum (35). Necrosis in Jurkat cells, cultured in RPMI with FCS, was induced by incubating cells for 30 min at 56°C (35). AC or NC were washed twice with PBS and incubated for another 3-h period in RPMI with human plasma (for further treatment of primary human macrophages) or RPMI with FCS (for further treatment of primary murine peritoneal macrophages). Conditioned medium (CM) was harvested by centrifugation (1000 x *g*, 10 min) and filtration through 0.22 µm pore filters to remove apoptotic bodies. The procedure to generate CM from VC was equivalent. Jurkat cells were incubated in RPMI with FCS, omitting a death stimulus. DMS, an inhibitor of sphingosine kinases, was used to generate AC-CM without S1P. 20 µM DMS was added simultaneously with staurosporine to Jurkat cells during the initiation of apoptosis (35). To obtain macrophage CM (MΦ-CM), macrophages were incubated with AC-CM for 2 h, washed twice with PBS and incubated for another 4-h period in RPMI with human plasma. MΦ-CM was harvested by centrifugation (1000 x *g*, 10 min). For protein degradation, MΦ-CM was incubated with 50 µg/ml proteinase K at 37°C for 1 h, followed by inactivation of proteinase K at 100°C for 1 h. Cells were exposed to CM for the times indicated. Unless indicated otherwise, inhibitors were pre-incubated for 1 h each. CM was generated using 1 x 10^7 Jurkat cells and later on added to 2 x 10^6 macrophages (ratio 5:1). MΦ-CM was directly transferred from generator to recipient cells.

4.2.2 Transient transfection of eukaryotic cells

4.2.2.1 Transfection of plasmid DNA

For reporter assay 10% of one buffy coat monocyte preparation were seeded in one 12-well plate. After 7 days, macrophages were co-transfected with 900 µg of HO-1 or $S1P_1$ promoter constructs and 100 µg of *Renilla* luciferase control vector pRL-CMV using the jetPEI™ transfection reagent. Briefly, for each well, DNA and 2 µl jetPEI™ transfection reagent were filled-up each to 50 µl with

150 nM NaCl. Mixtures were vortexed and spinned-down briefly. Then, jetPEI™ solution was added to DNA mixture, vortexed and spinned down. After incubation for 30 min at RT 100 µl/well of the solution were added to macrophages. Macrophages were incubated for 24 h, medium was changed and macrophages were incubated for another 24 h followed by individual stimulation. Following individual incubations firefly luciferase as well as *Renilla* luciferase activity were determined.

4.2.2.2 Transfection of siRNA

For Western blot analysis (4.2.3) and quantitative PCR (4.2.5), primary human macrophages were transfected with the respective siRNA using Human Macrophage Nucleofector® Kits and the Nucleofector® II. Primary human macrophages were removed from dishes by washing them with PBS prior to their incubation with TE for 15 min at 4°C. Then, macrophages were incubated for 1 h at 37°C. Thereafter, macrophages were rinsed-off and 1.5 x 10^6 cells were harvested by centrifugation (200 x *g*, 10 min). Pellet was resuspended Human Macrophage Nucleofector® Solution and mixed with 3 µg siRNA. After transfer of the mixture into an appropriate cuvette, nucleofection was performed. Cells were seeded in 10-cm dishes in 5 ml pre-warmed RPMI with human plasma and incubated for 48 h prior to the use in the individual experiments.

4.2.3 Western immunoblotting

To investigate changes in protein expression, proteins were isolated, separated by SDS-PAGE and detected by Western blotting.

4.2.3.1 Isolation of proteins

For Western immunoblotting, cells were incubated as indicated in the individual experiments, scraped-off, lysed in various volumes (75-200 µl, depending on the mass of the cell pellet) ice cold protein lysis buffer (or in the case of analyzing $S1P_1$ in RT whole cell protein lysis buffer) and sonicated for 3 sec.

Lysates were incubated on ice for 30 min and vortexed each 10 min for 10 sec. After centrifugation (16000 x *g*, 30 min, 4℃) supernatants were collected and protein content was determined according to the Lowry method.

4.2.3.2 Protein determination (Lowry method)

The protein content of cell lysates was determined using the Standard DC Protein Assay Kit, which is based on the Lowry method (203). Briefly, a standard dilution series of BSA in H_2O was prepared (0.625-10 mg/ml). 2 µl of samples as well as standards were pipetted in duplicates into a 96-well plate and 20 µl solution A were added. The colorimetric reaction was started by the addition of 160 µl solution B. After incubation for 15 min (RT, shaking), extinction was measured at 750 nm using the Mithras LB 940 multilabel reader.

4.2.3.3 SDS-PAGE

40-80 µg protein was filled-up to 30 µl with H_2O and 10 µl 4 x SDS-PAGE sample buffer was added. Samples were denatured at 100℃ for 10 min and immediately put on ice for 2 min. Proteins were resolved on SDS polyacrylamide gels consisting of an upper stacking gel and a lower separating gel with 1 x SDS-PAGE running buffer using the Mini-PROTEAN 3 system. The gel composition is listed in table 1.

Table 1. Composition of SDS polyacrylamide gels.

	Separating gel	**Stacking gel**
	12%	4%
40% acrylamide/bis-acrylamide (37.5%:1.0% w/v)	3 ml	300 µl
Lower Tris buffer	2.5 ml	-
Upper Tris buffer	-	750 µl
10% (w/v) SDS	100 µl	30 µl
H_2O	4.4 ml	1.95 ml
10% (w/v) APS	50 µl	50 µl
TEMED	5 µl	5 µl

4.2.3.4 Western blotting

Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes by semi-dry blotting. Unspecific binding was blocked with blocking buffer for 2 h. Primary antibody was added in antibody solution according to the manufacturer's instructions and incubated either for 2 h at RT or overnight at 4°C. Membranes were washed three times for 5 min each with TTBS. Thereafter, membranes were incubated with a secondary near infrared dye (IRDye®) conjugated antibody in antibody solution for 1 h and again washed three times for 5 min each with TTBS. Specific proteins were detected and their expression was quantified compared to actin expression using the Odyssey infrared imaging system.

4.2.4 Electrophoretic mobility shift assay (EMSA)

4.2.4.1 Oligonucleotide annealing

For oligonucleotide annealing 50 µl of both oligonucleotides of the respective pair (100 pmol/µl) were mixed with 100 µl 2 x STE and incubated at 90°C for 10 min. Afterwards, oligonucleotides were cooled-down to 15°C with 1°C/min.

4.2.4.2 EMSA analysis

EMSA analysis was performed according to a previously described method (204). Briefly, cells were incubated as indicated in the individual experiments, scraped-off, resuspended in 200 µl ice cold hypotonic cell lysis buffer and incubated on ice for 10 min. After centrifugation (500 x *g*, 30 min, 4°C), the nucleic pellet was resolved in 100 µl nuclear lysis buffer, incubated on ice for 20 min, vortexed each 2 min for 10 sec, and pelleted (16000 x *g*, 5 min, 4°C). Supernatants were collected and protein concentration was determined by the Lowry method (4.2.3.2). Nuclear protein (10 µg) was incubated for 30 min at room temperature with 2 µg poly(dI-dC), 2 µl buffer D, 4 µl buffer F, and 250 fmol 5'-IRDye®700-labeled oligonucleotide in a final volume of 20 µl. In the case of competitive EMSA, additionally 2500 or 25000 fmol of unlabeled oligonucleotide were added prior to 250 fmol 5'-IRDye®700-labeled

oligonucleotide. Thereafter, samples were incubated on ice for 5 min. DNA-protein complexes were resolved on native 6% polyacrylamide gels at 80 V for 1 h and analyzed with the Odyssey infrared imaging system. The gel composition is listed in table 2.

Table 2. Composition of EMSA polyacrylamide gels.

	6%
20 x Running buffer	500 µl
40% acrylamide/bis-acrylamide (29%:1.0% w/v)	1.5 ml
H_2O	8 ml
10% (w/v) APS	100 µl
TEMED	20 µl

4.2.5 Determination of mRNA contents of cells

For the evaluation of changes in mRNA expression, RNA was isolated, followed by reverse transcription and quantitative PCR.

4.2.5.1 RNA isolation

Cells were incubated as indicated in the individual experiments, scraped-off, resuspended in 1 ml peqGOLD RNAPure™ and incubated for 5 min at RT. Then, 200 µl chloroform were added, samples were vortexed thoroughly for 15 sec and incubated for another 10 min at RT. To separate the RNA-containing water-phase from the phenol-phase and the intermediate lipid-layer samples were centrifuged (12000 x *g*, 5 min, 4℃). After collecting the water-phase, RNA was precipitated by adding 500 µl isopropanol followed by incubation for 30 min at RT and centrifugation (16000 x *g*, 15 min, 4℃). Pellet was washed twice in 1 ml 75% ethanol in DEPC-treated H_2O by vortexing and subsequent centrifugation (16000 x *g*, 10 min, 4℃). Then, pellet was dried at 70℃ and resuspended in 30 µl DEPC-treated H_2O by incubation at 60℃ for

30 min. RNA content was determined using the NanoDrop® spectrophotometer system, which calculated RNA content according to the fact that at 260 nM an OD of 1 is equivalent to 40 µg/ml RNA. Furthermore, quality was checked by determining the ratios of OD_{260}/OD_{280} and OD_{260}/OD_{230}.

4.2.5.2 Reverse Transcription

Reverse Transcription was performed using iScript™ cDNA Synthesis Kits. 1 µg total RNA in 15 µl nuclease-free H_2O was mixed with 4 µl 5 x reaction buffer and 1 µl iScript™ reverse transcriptase. Mixture was incubated for 5 min at 25℃, 30 min at 42℃ and finally 5 min at 85℃ to i nactivate the enzyme. The resulting cDNA was diluted 1:5 before being used for quantitative PCR.

4.2.5.3 Quantitative PCR

Quantitative PCR was performed using ABsolute™ QPCR SYBR® Green fluorescein mix. Depending on the primers different reaction mixtures were pipetted in 96-well PCR plates. The reaction mixtures are listed in table 3.

Table 3. Reaction mixtures of quantitative PCR.

	Primers from biomers	**QuantiTect Primer Assay**
cDNA	2 µl	2 µl
Forward primer (10 pmol/µl)	0.6 µl	-
Reverse primer (10 pmol/µl)	0.6 µl	-
Primer assay	-	2 µl
ABsolute™ QPCR SYBR® Green	10 µl	10 µl
H_2O	6.8 µl	6 µl

In the case of the reference gene (actin or ribosomal 18S RNA) only 1 µl of cDNA was used. After briefly spinning down the plate, it was sealed with optical tape. PCR was performed using the MyiQ™ Single Color Real-Time PCR Detection System according to the following program.

Activation of polymerase	2 min	50℃	
Initial denaturation	15 min	95℃	
Denaturation	15 sec	95℃	45 x
Primer annealing	30 sec	T_A℃	
Elongation	30 sec	72℃	
Final denaturation	1 min	95℃	
Final renaturation	55℃	1 min	
Melting curve	+0.5℃	10 sec	

Annealing temperature (T_A) was dependent on the used primer pairs. For all primer pairs except the ones for human $S1P_2$ and $S1P_3$ T_A was 55℃. In the case of $S1P_2$ and $S1P_3$ primers T_A was 54℃.

As SYBR® Green intercalates into double-stranded DNA molecules, fluorescence intensity, which was measured at the end of each elongation step, reflected the quantum of PCR products.

A melt curve was created to show the melting points of PCR products and therefore to confirm the specificity of the reaction. Samples were analyzed using the MyiQ™ 1.0 and Gene Expression Macro 1.1 software with actin or ribosomal 18S RNA expression as internal control.

4.2.6 Genotyping of mice

Tail tips were heated for 20 min at 100℃ in 70 µl genotyping lysis buffer. After incubation for 1 min on ice, 70 µl of genotyping neutralization buffer were added. Non-lysed tissue was sedimented by centrifugation (12000 x *g*, 15 sec, 4℃). Supernatant containing genomic DNA was used for PCR analysis. According to the following schemes two different PCRs were performed.

Supernatant	2.5 µl
$MgCl_2$ (25 mM)	5 µl
dNTPs (10 mM)	1 µl
Primer P1 (10 pmol/µl)	1 µl
Primer P2 (10 pmol/µl)	1 µl
Taq DNA Polymerase (5 U/µl)	2 µl
PCR Buffer (5 x)	10 µl
H_2O	27.5 µl

Supernatant	2.5 µl
$MgCl_2$ (25 mM)	5 µl
dNTPs (10 mM)	1 µl
Primer F4/80 exon 1 (10 pmol/µl)	1 µl
Primer F4/80 3.4 (10 pmol/µl)	1 µl
Primer Cre rev 1	1 µl
Taq DNA Polymerase (5 U/µl)	2 µl
PCR Buffer (5 x)	10 µl
H_2O	26.5 µl

PCR was performed according to the following parameters.

Denaturation	1 min	94℃	30 x
Primer annealing	30 sec	T_A℃	
Elongation	1 min	72℃	
Final elongation	5 min	72℃	

T_A was 50℃ for primers P1 and P2 and 63℃ for primer s F4/80 exon 1, F4/80 3.4 and Cre rev 1. After the addition of 10 µl DNA loading dye, PCR products were resolved on agarose gels (2% in 0.5 x TBE) for 2 h at 120 V. Gels were stained for 15 min in an ethidium bromide bath (1 µg/ml) and DNA fragments were detected using an UV light gel documentation system.

The PCR, in which primer pairs P1 and P2 were used, amplified a 200 bp fragment for the wild-type $S1P_1$ allele and an approximately 250 bp fragment for

the $S1P_1^{loxP}$ allele. The second PCR amplified a 100 bp fragment for the F4/80 wild-type allele and a 145 bp fragment for the Cre allele.

4.2.7 Quantification of VEGF release from macrophages

5 x 10^6 macrophages were incubated in 3-cm dishes with AC-CM for 2 h, washed twice with PBS, and incubated for another 16-h period. Supernatants were harvested by centrifugation (16000 x *g*, 10 min, 4℃). Measurement of VEGF release from macrophages was performed using human VEGF Cytometric Bead Array Flex Sets. Briefly, 50 µl of the supernatant were incubated with beads coated with antibodies against VEGF and a phycoerythrin-labeled detection reagent for 3 h. Samples were analyzed with the FACSCanto™ flow cytometer and quantitated using the FCAP Array 1.0.1 software.

4.2.8 Amplification of plasmids in bacteria

Before being used for transfection of macrophages, plasmids had to be amplified in bacteria.

4.2.8.1 Transformation of bacteria by heat shock

Plasmids were transformed into supercompetent bacteria using heat shock method. Therefore, 50 µl of bacteria stocks were thawed on ice, 100 ng of plasmid DNA were added and mixed gently. After incubation on ice for 30 min, heat shock (45 sec, 42℃) was performed and bacteri a were incubated on ice for another 2 min. Then, 400 µl of SOC medium were added, followed by incubation for 60 min at 37℃ with shaking (250 rpm). For the selection of plasmid-carrying clones, 200 µl of the cultures were inoculated on ampicillin-containing (100 µg/ml) LB agar plates and incubated overnight at 37℃.

4.2.8.2 Bacteria culture

For the preparation of plasmids, a single colony from the LB agar plate was picked, transferred into 3 ml LB medium supplemented with 100 µg/ml ampicillin and cultured overnight at 37℃ with shaking (250 rp m). Then, this pre-culture was transferred into 200 ml ampicillin-containing (100 µg/ml) LB medium and again grown overnight under the same conditions.

4.2.8.3 Preparation of plasmid DNA

The HiSpeed® Plasmid Maxi Kit was used to isolate plasmids from bacteria. The procedure followed the protocol of the manufacturer. DNA content was measured using the spectrophotometer NanoDrop® ND-1000.

4.2.9 Site-directed mutagenesis

Using the online transcriptional regulatory element database CSH and the online tool TFSearch potential STAT binding sites in the human HO-1 promoter (STAT response elements [STATx]) were identified.

A point mutation of the putative STAT binding site at position -2361 bp to -2369 bp within the human HO-1 promoter, to impair STAT3 binding, was generated using the QuikChange® II XL Site-Directed Mutagenesis Kit. The phHOLUC(-2782) construct served as a template. Primers were designed according to the manufacturer's instructions and are listed in 4.1.11. 10 ng of the template DNA, 125 ng of each primer, 5 µl 10 x reaction buffer, 1 µl dNTP mix, 3 µl QuikSolution™ reagent and 1 µl *PfuUltra* HF DNA polymerase were mixed and filled-up with H_2O to 50 µl. PCR was performed according to the following parameters.

Initial denaturation	1 min	95℃	
Denaturation	50 sec	95℃	18 x
Primer annealing	50 sec	60℃	
Elongation	15 min	68℃	
Final elongation	7 min	68℃	

Thereafter, the nonmutated parental DNA template was digested by incubation with 10 U Dpn I for 1 h at 37℃. Then, mutated plas mids were transfected into XL-10-Gold® ultracompetent bacteria using the heat shock method (4.2.8.1). After the selection of positive clones the plasmid DNA was extracted using the HiSpeed® Plasmid Maxi Kit. Correct sequence of the generated vectors was confirmed by sequencing, which was performed by AGOWA GmbH (Berlin).

4.2.10 Reporter assay

After individual incubations, medium was removed from 12-well plates, 100 µl H_2O/well was added followed by subsequent storage at -80℃. After at least 30 min of freezing, plates were thawed by shaking for 15 min at RT and subsequent centrifugation (3220 x *g*, 10 min, 4℃). Then, 50 µl of each well were transferred into two different 1.5-ml reaction tubes. The addition of 50 µl 2 x firefly lysis buffer to the first tube and 50 µl 2 x passive lysis buffer to the second tube was followed by vortexing. 20 µl of each sample was pipetted in duplicates into white 96-well plates. In the Mithras LB 940 multilabel reader 50 µl of firefly assay reagent or *Renilla* assay reagent were automatically added, the plate was shaken for 2 s and luminescence was measured. To control transfection efficiency as well as cell number of each well, firefly luciferase activity was normalized to *Renilla* luciferase activity.

4.2.11 Microscopy

For immunofluorescence analysis, primary human macrophages were seeded in 10-cm dishes and differentiated for 7 days. Then, macrophages were removed from dishes as described in section 4.2.2.2. Slides were sterilized with 75% ethanol and placed in fresh 10-cm dishes, in which macrophages were seeded once again. After incubation for 2 days macrophages were used for experiments. Following individual stimulations, macrophages were fixed in 4% PFA for 1 h. Macrophages were permeabilized by incubation in 1% Triton X-100 (in PBS) for 15 min, washed three times with PBS and unspecific antibody

staining was blocked by incubation with 30% FCS (in 1% Triton X-100) for 1 h. $S1P_1$ antibody was incubated in PBS overnight at 4°C. Following three washes with PBS, Alexa Fluor® 546-labeled goat anti-mouse antibody was incubated in PBS for 2 h in the dark. Nuclei were labeled by DAPI staining (20 ng/ml in PBS) for 20 min. For isotype staining, cells were only stained with the secondary antibody and DAPI. After three additional washed with PBS, slides were dabbed with VECTASHIELD® and covered with cover slips.

For scratch assays, 10% of one buffy coat monocyte preparation were seeded in 4 wells of one 12-well plate. Primary murine macrophages were seeded as described in section 4.2.1.3. Scratch assay was performed after differentiation of primary human monocytes for 7 days or after culture of primary murine macrophages for 3-5 days. Cells were starved by cultivation in RPMI without serum for 8 h (primary human macrophages) or 16 h (primary murine macrophages). Then, scratch was drawn in the middle of each well using a small pipette tip. This was followed by twice washing with PBS to remove detached cells. Then AC-CM was added and scratch width was microscopically documented. In the case of primary human macrophages, macrophages were washed three times with PBS after 1 h of incubation with AC-CM and 1 µM VPC 23019, an $S1P_{1/3}$ antagonist (205), was added. Ring-shaped stickers served to recover scratches after incubation periods, which were 9 h for primary murine macrophages or 16 h for primary human macrophages, respectively.

The immunofluorescence of fixed cells or the phase contrast of living cells was visualized with the fluorescence microscope Axiovert 200M together with the ApoTome unit.

For scratch asssays the area between the two sides of the scratch was calculated using AxioVision Rel. 4.4 software.

4.2.12 Statistical analysis

Each experiment was performed at least three times. For Western, EMSA and microscopy analysis one representative experiment is displayed. p-values were calculated using the paired Student's *t* test and considered significant at $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

5 RESULTS

5.1 Apoptotic cell supernatants provoke a biphasic upregulation of HO-1

My studies originated from a report showing that FTY720, a sphingosine analog, which can be phosphorylated by SphK2 to act similar to S1P (206), enhanced HO-1 expression (207). Furthermore, it was proven that S1P is released from AC (35) and provides anti-apoptotic as well as anti-inflammatory potential similar to the effects seen with CO (discussed in 3.5.4). Therefore, I first addressed whether HO-1 is induced in primary human macrophages after treatment with AC supernatants (AC-CM).

5.1.1 HO-1 is upregulated in a time-dependent manner

In a first set of experiments, HO-1 protein expression in primary human macrophages was analyzed following their exposure to AC-CM. HO-1 expression showed a biphasic response. While the first peak was noticed after 6 h, a second peak was observed after 24 h of incubation with AC-CM (Figure 8).

Figure 8. Induction of HO-1 by AC-CM in macrophages. Western analysis of HO-1 expression in primary human macrophages following treatment with AC-CM for times as indicated. The Western blot is representative for three individual experiments.

5.1.2 HO-1 upregulation is mediated by autocrine and paracrine signaling

The biphasic expression scheme of HO-1 led to the assumption that the second peak of HO-1 expression might be mediated by an autocrine factor, which was released by macrophages following treatment with AC-CM. To verify this hypothesis, supernatants from macrophages (MΦ-CM), which were previously stimulated with AC-CM, were harvested and MΦ-CM was transferred to fresh, resting macrophages. Indeed, not only AC-CM but also MΦ-CM enhanced HO-1 protein expression in primary human macrophages (Figures 8 and 9). Pronounced HO-1 expression in response to MΦ-CM was detected after 12-18 h, which corresponded to the second peak of HO-1 expression in response to AC-CM, seen after 24 h. Thus, paracrine signaling by AC as well as autocrine signaling of macrophages contributed to the induction of HO-1.

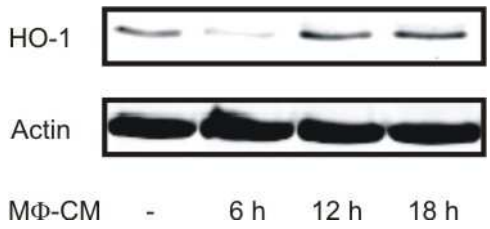

Figure 9. Induction of HO-1 by MΦ-CM in macrophages. Western analysis of HO-1 expression in primary human macrophages after incubation with MΦ-CM for times as indicated. One representative Western blot out of three individual experiments is displayed.

5.1.3 HO-1 induction is specifically mediated by AC but not by VC or NC

Importantly, HO-1 expression was only seen in response to AC-CM, but was not elicited by conditioned medium from necrotic cells (NC-CM) or viable cells (VC-CM) (Figure 10). Consequently, HO-1 expression can be attributed to a factor, which is uniquely produced by AC. The production of this factor may need a complex release mechanism, as it is not released as a result of membrane rupture, which is observed in NC.

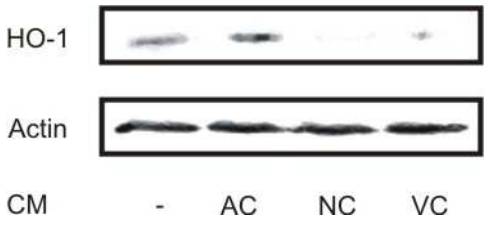

Figure 10. Induction of HO-1 by different forms of CM in macrophages. HO-1 expression in primary human macrophages treated with CM of AC, NC or VC for 24 h. The Western blot is representative for three individual experiments.

5.1.4 AC-CM enhances transcription of the HO-1 promoter

The induction of HO-1 by MΦ-CM after 18 h was further corroborated by reporter assays. Using the luciferase-coupled promoter constructs phHOLUC40 as well as phHOLUC(-2782), a significant induction of luciferase activity was noticed after treatment with MΦ-CM for 18 h (Figure 11). However, no activity was observed with the shorter luciferase construct phHOLUC(-1976). Results so far indicate that AC supernatants not only caused HO-1 protein expression, but also enhanced the transcription of the HO-1 promoter.

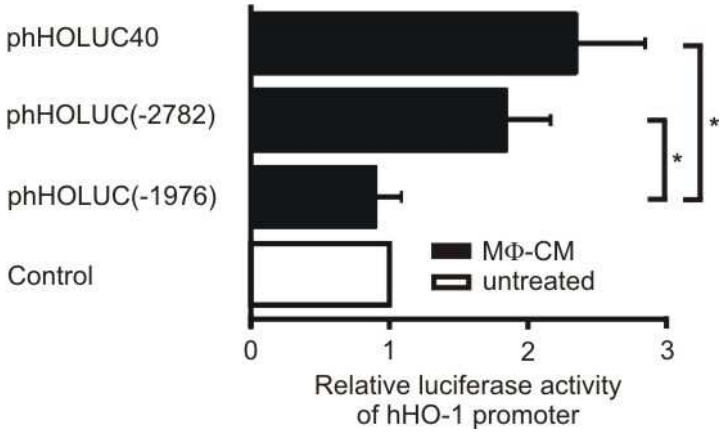

Figure 11. Induction of HO-1 promoter by MΦ-CM in macrophages. HO-1 promoter activity in primary human macrophages after transfection of individual phHOLUC reporter constructs and stimulation with MΦ-CM for 18 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are means ± SEM of at least four independent experiments. Asterisks mark statistically significant differences ($p \leq 0.05$).

5.1.5 p38 MAPK and Jak pathways are involved in HO-1 expression

Next, I investigated signal transduction pathways contributing to HO-1 expression. Therefore, macrophages were pre-incubated with inhibitors of pathways known to be associated with HO-1 induction, before stimulating them with AC-CM. The early expression of HO-1, after 6-h treatment with AC-CM, was reversed by SB203580, an inhibitor of p38 MAPK (Figure 12 A). The late-phase HO-1 expression at 24 h was partially attenuated by inhibiting Janus kinase signaling (Figure 12 B). Thus, the early HO-1 induction in response to an AC-CM-derived soluble factor was p38-mediated, whereas the late and second phase of HO-1 expression was facilitated by an autocrine factor, signaling via the Jak pathway.

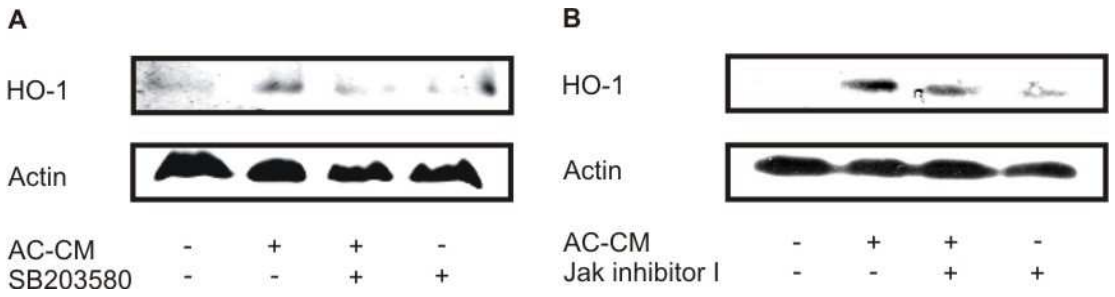

Figure 12. Inhibition of AC-CM-induced HO-1 expression in macrophages. (A) HO-1 expression in primary human macrophages after incubation with AC-CM for 6 h in the presence/absence of 5 µM SB203580. (B) Treatment of macrophages with AC-CM for 24 h with 1 µM Jak inhibitor I being present. Western blots are representative for at least three individual experiments.

5.2 Apoptotic cell-derived S1P is crucial in provoking HO-1 induction

As mentioned in section 5.1, my hypothesis was that S1P derived from AC-CM was responsible for the enhanced HO-1 expression in macrophages.

5.2.1 Apoptotic cell-derived S1P induces HO-1 protein expression

In a first approach primary human macrophages were stimulated with authentic S1P for 24 h (Figure 13). S1P at 100 nM and 1 µM as well as AC-CM facilitated HO-1 protein expression. DMS, an inhibitor of sphingosine kinases, was used to block their activity in Jurkat cells during the generation of AC-CM and thus to validate the contribution of S1P in AC-CM. AC-CM generated in the presence of DMS failed in inducing HO-1 expression in macrophages.

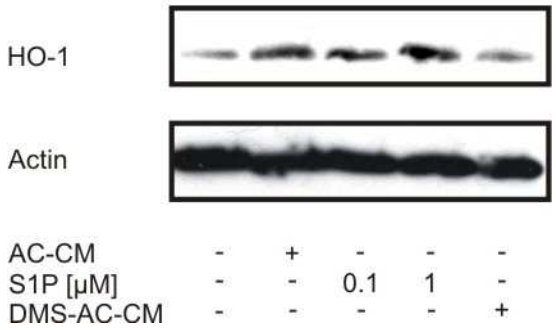

Figure 13. Apoptotic cell-derived S1P mediates HO-1 protein induction in macrophages. Western analysis of HO-1 expression in primary human macrophages treated with AC-CM, S1P or DMS-AC-CM for 24 h. 20 µM DMS were used to block the release of S1P into the supernatant of apoptotic cells (DMS-AC-CM). One representative Western blot of three individual experiments is outlined.

5.2.2 Authentic S1P enhances transcription of the HO-1 promoter

S1P, supplied for 24 h, also induced luciferase reporter activity (Figure 14), which further supported the data obtained at the protein level. Like AC-CM, S1P induced phHOLUC40 and phHOLUC(-2782) HO-1 reporter activity, while phHOLUC(-1976) HO-1 reporter activity was not affected.

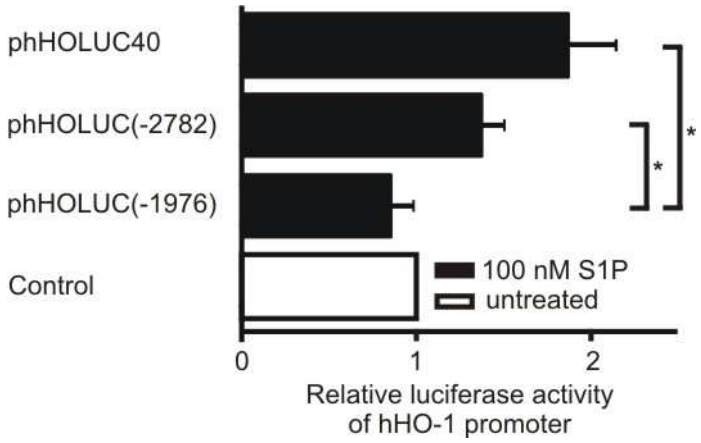

Figure 14. S1P induces HO-1 promoter activation in macrophages. HO-1 promoter activity of corresponding phHOLUC reporter constructs in primary human macrophages treated with 100 nM S1P for 24 h. Firefly luciferase activity normalized to *Renilla* luciferase activity is displayed. Data are means ± SEM of at least three independent experiments. Asterisks mark statistically significant differences ($p \leq 0.05$).

5.2.3 The induction of HO-1 is mediated via $S1P_1$

Activation of $S1P_1$ was reported to limit the expression of pro-inflammatory cytokines (116) and to protect macrophages from apoptosis induced by the combination of TNFα and cycloheximide (35). As it is widely accepted that the enhanced expression of HO-1 is accompanied by anti-inflammatory and anti-apoptotic effects, I suggested that $S1P_1$ could play an important role in the induction of HO-1 in my system. Therefore, knockdown of $S1P_1$ was performed to asses its role in HO-1 induction by AC-CM. Transfection of macrophages with non-targeting siRNA allowed the induction of HO-1 expression by AC-CM after 6 h, whereas knockdown of $S1P_1$ significantly reduced the HO-1 amount (Figure 15). Interestingly, I noticed that AC-CM enhanced $S1P_1$ expression in primary human macrophages, an effect suppressed by siRNA directed against $S1P_1$. I will elaborate on this effect in section 5.6.

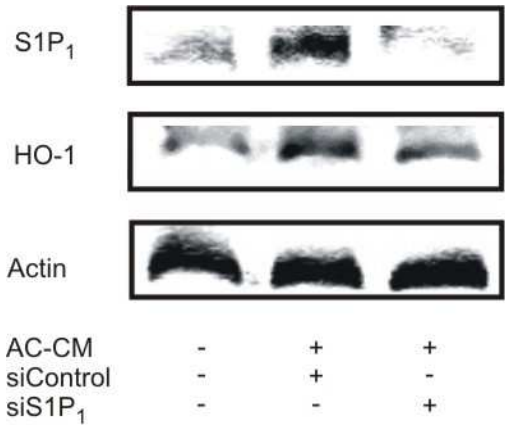

Figure 15. $S1P_1$ mediates HO-1 protein induction in macropages. Primary human macrophages were transfected with non-targeting siRNA or siRNA against $S1P_1$. Western analysis of $S1P_1$ and HO-1 was performed after 6-h treatments with AC-CM. The Western blot is representative for three individual experiments.

5.3 STAT1 and STAT3 provoke autocrine transcription of the HO-1 gene

As shown in figure 12 B, the induction of HO-1 after 24 h was dependent on Jak signaling. Considering that HO-1 induction at 24 h was facilitated by an autocrine factor (Figure 9), I wanted to discern whether the release or the action of the putative autocrine factor would be Jak-dependent. Therefore, using STAT1 and STAT3 inhibitors, signaling downstream of Janus kinases was analyzed.

5.3.1 The autocrine factor is released in a STAT1-dependent way and acts via STAT1 and STAT3

To attenuate the release of autocrine mediators, macrophages were pre-incubated for 1 h with either fludarabine, a specific inhibitor of STAT1 (208), or STA-21, a specific STAT3 inhibitor (209), prior to the addition of AC-CM. After incubation with AC-CM for 2 h, macrophages were washed twice with PBS, followed by continuing incubations for 4 h in full medium, without the further

addition of inhibitors. Thereupon, MΦ-CM was harvested from these cells and added to fresh, unstimulated macrophages for 18 h. Inhibition of STAT1 during the production of MΦ-CM with fludarabine eliminated its potential to upregulate HO-1, indicating that the production of the autocrine mediator was STAT1-dependent (Figure 16 A, lane 3). In contrast, blocking STAT3 by STA-21 during the production of MΦ-CM did not diminish HO-1 expression (Figure 16 B, lane 3). Interestingly, inhibition of STAT1 or STAT3 attenuated the expression of HO-1 in response to MΦ-CM (Figure 16 A and B, lane 4), suggesting that the autocrine factor demands active STAT1 and STAT3 for signaling.

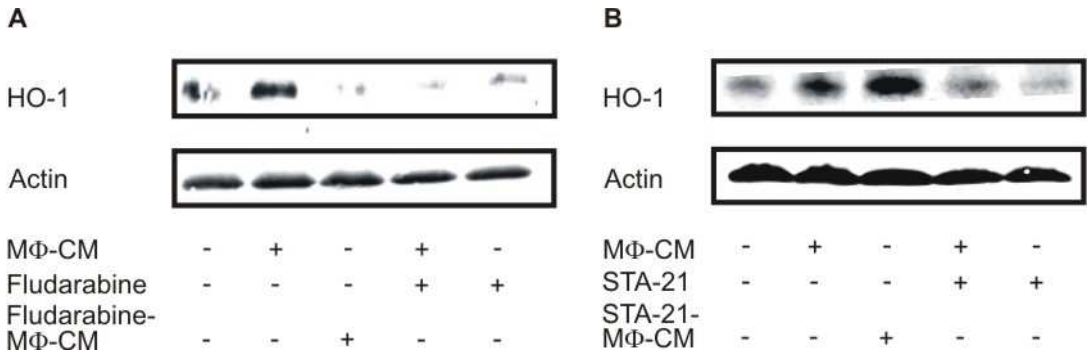

Figure 16. STAT1 and STAT3 signaling are involved in HO-1 promoter activation in macrophages. Primary human macrophages were incubated for 6 h with AC-CM with or without the addition of (A) 20 µM fludarabine or (B) 10 µM STA-21. MΦ-CM was harvested and added to fresh macrophages for 18 h with or without the addition of fludarabine (A) or STA-21 (B). Western blots are representative for at least three individual experiments.

5.3.2 STAT binding sites located within the human HO-1 promoter

These initial data suggested a role of STAT1 and STAT3 in late-phase (24 h) HO-1 expression by AC-CM. Therefore, I screened the human HO-1 promoter for potential STAT binding sites (Figure 17). Luciferase activity after treatment with MΦ-CM for 18 h was not induced following transfection of phHOLUC(-1976) into macrophages (Figure 11), thus excluding the three putative STAT binding sites located within this promoter construct as candidates involved in enhanced HO-1 transcription.

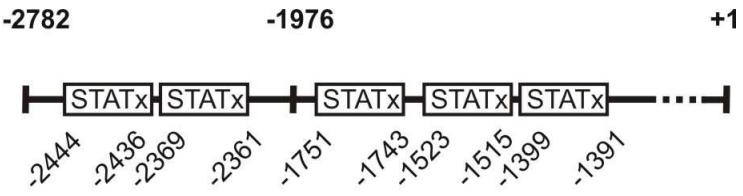

Figure 17. Putative STAT binding sites in the human HO-1 promoter. The human HO-1 promoter contains five STAT binding sites located within the sequence 2782 bp upstream from the transcription start including three STAT binding sites withing the sequence -1976 bp to +1 bp.

5.3.3 Identification of the STAT binding site responsible for HO-1 induction

Concerning the two remaining potentially critical STAT binding sites in the HO-1 promoter, only oligonucleotides resembling the STAT binding site at -2361 bp to -2369 bp, but not the ones containing the STAT binding site at -2436 bp to -2444 bp recruited transcription factors in EMSA analysis (Figure 18 A). Supporting the notion that HO-1 induction by the autocrine factor present in MΦ-CM was inhibited by fludarabine and/or STA-21 (Figure 16 A and B), EMSA analysis revealed that transcription factor binding to the oligonucleotides spanning the STAT binding site at -2361 bp to -2369 bp was slightly reduced when macrophages where incubated with fludarabine (Figure 18 A). Stronger inhibition was observed when macrophages were treated with STA-21 prior to MΦ-CM stimulation, whereas the combined application of both STAT inhibitors reduced transcription factor binding most efficiently. These observations imply that STAT1/STAT3 heterodimer binding to the putative STAT binding site located at -2361 bp to -2369 bp at the human HO-1 promoter affects HO-1 induction following treatment with MΦ-CM.

To reinforce these results, I performed competitive EMSA analysis with unlabeled oligonucleotides specific for STAT1 or STAT3 or with oligonucleotides for the putative STAT binding site at -2361 bp to -2369 bp containing a point mutation for STAT3 (Figure 18 B). STAT binding to the oligonucleotides containing the STAT binding site at -2361 bp to -2369 bp was strongly reduced with increasing concentrations of specific STAT1 and STAT3

oligonucleotides. In contrast, when using oligonucleotides with the STAT3 point mutation, STAT binding was not impaired.

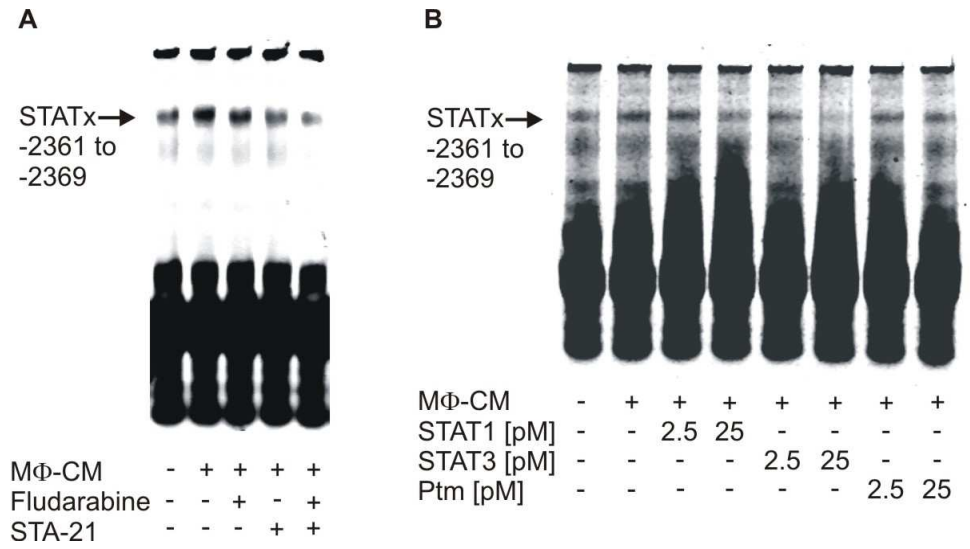

Figure 18. STAT1/STAT3 heterodimers mediate HO-1 promoter activation in macrophages. (A) EMSA analysis in primary human macrophages using 250 fmol of the oligonucleotides resembling the putative STAT binding site at -2361 bp to -2369 bp of the human HO-1 promoter in the presence/absence of 20 µM fludarabine and/or 10 µM STA-21. (B) Competitive EMSA analysis using 2.5 pM or 25 pM of unlabeled oligonucleotides specific for STAT1, STAT3 or oligonucleotides for the putative STAT binding site at -2361 bp to -2369 bp, which contained a STAT3 point mutation (Ptm), in addition. One representative EMSA out of three is displayed.

In addition, reporter assays were performed in primary human macrophages with the construct phHOLUC(-2782ptm). This construct contained a point mutation within the STAT binding site at -2361 bp to -2369 bp to eliminate STAT3 binding (Figure 19). Transfection of this construct into macrophages confirmed the results obtained by EMSA analysis, as luciferase activity elicited by MΦ-CM was significantly lower compared with transfection of the non-mutated phHOLUC(-2782). Thus, the STAT binding site located at position -2361 bp to -2369 bp of the human HO-1 promoter can be considered the critical binding site responsible for the induction of HO-1 after treatment with AC-CM.

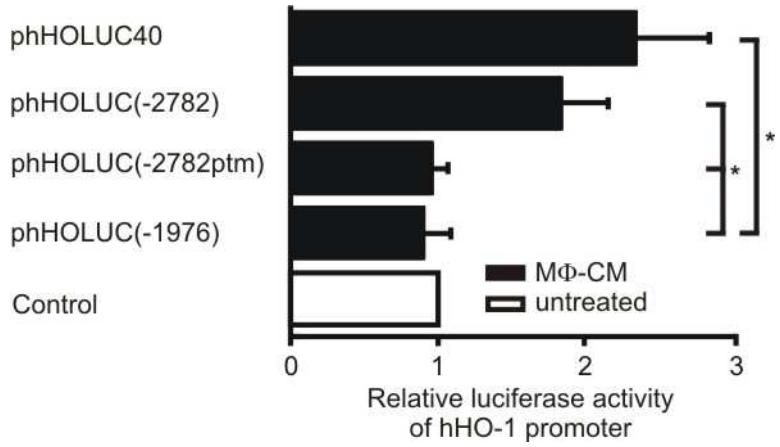

Figure 19. The STAT binding site at -2361 bp to -2369 bp is responsible for HO-1 promoter activation in macrophages. HO-1 promoter activity in primary human macrophages following transfection of the corresponding phHOLUC promoter constructs and stimulation with MΦ-CM for 18 h. Histograms show firefly luciferase activity normalized to *Renilla* luciferase activity. Data are means ± SEM of at least four independent experiments. Asterisks mark statistically significant differences ($p \leq 0.05$).

5.4 Late-phase HO-1 induction in macrophages requires autocrine VEGFA signaling

5.4.1 The autocrine induction of HO-1 by MΦ-CM is mediated by a protein factor

To elucidate which factor is released by macrophages following treatment with AC-CM to induce HO-1 in an autocrine way, I first degraded proteins in MΦ-CM with proteinase K digestion. MΦ-CM, deprived by functional proteins, revealed a significantly lower ability to express HO-1 compared with untreated MΦ-CM (Figure 20), implying that the autocrine factor might be a protein.

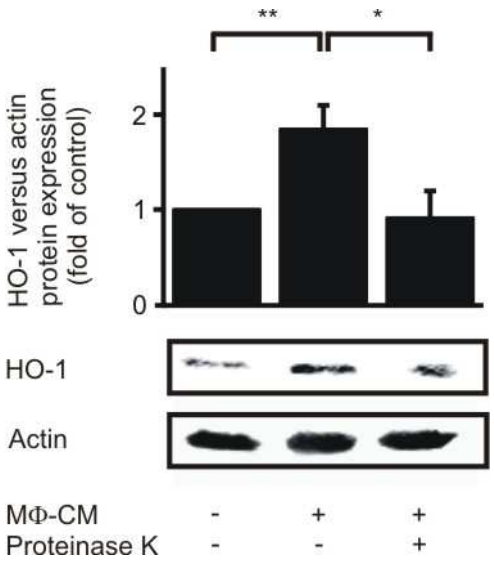

Figure 20. A protein factor is important for the late-phase induction of HO-1 protein in macrophages. Primary human macrophages were controls or treated with either MΦ-CM or de-proteinated MΦ-CM (50 µg/ml proteinase K) for 18 h. One representative Western blot out of seven is displayed. The graph shows the densitometric analysis. Asterisks indicate statistically significant differences (*$p \leq 0.05$ and **$p \leq 0.01$).

5.4.2 VEGF secretion by macrophages is S1P- and STAT1-dependent

My previous results (Figure 16 A) suggested that the release of the autocrine factor mediating HO-1 induction was STAT1-dependent. This corresponds to the observations of Funamoto and colleagues, who identified VEGF as a target gene of STAT1 signaling in cardiac myocytes (210). Furthermore, VEGFA secretion from mouse mammary epithelial cells after phagocytosis of AC was previously demonstrated (211) and signaling of VEGF in chronic lymphocytic leukaemia B cells enhanced STAT3 actions (212). These observations match my data, indicating that the autocrine protein factor in MΦ-CM activated STAT1/STAT3 (Figure 16 A and B). Finally, VEGF induced HO-1 in a model of hyperoxic acute lung injury (213). Therefore, I proposed VEGF as a candidate being responsible for the late phase of HO-1 expression observed in my system.

Indeed, VEGF mRNA was significantly elevated in macrophages treated with AC-CM for 1 h, compared with controls. Augmentation of VEGF mRNA was

blocked when macrophages were pretreated with fludarabine (Figure 21 A). Additionally, I quantified the release of VEGF into supernatants of AC-CM-treated macrophages by FACS analysis using human VEGF Cytometric Bead Array Flex Sets (Figure 21 B). AC-CM-stimulated macrophages secreted significant amounts of VEGF protein. Accompanying experiments revealed that 100 nM authentic S1P, added to macrophages for 1 h, enhanced VEGF mRNA expression, consequently supporting the notion that S1P in AC-CM may induce VEGF (Figure 21 C). Since HO-1 as well as VEGF expression were induced by S1P (Figures 13 and 21 C), an induction of HO-1 by autocrine VEGF signaling in primary human macrophages seemed likely.

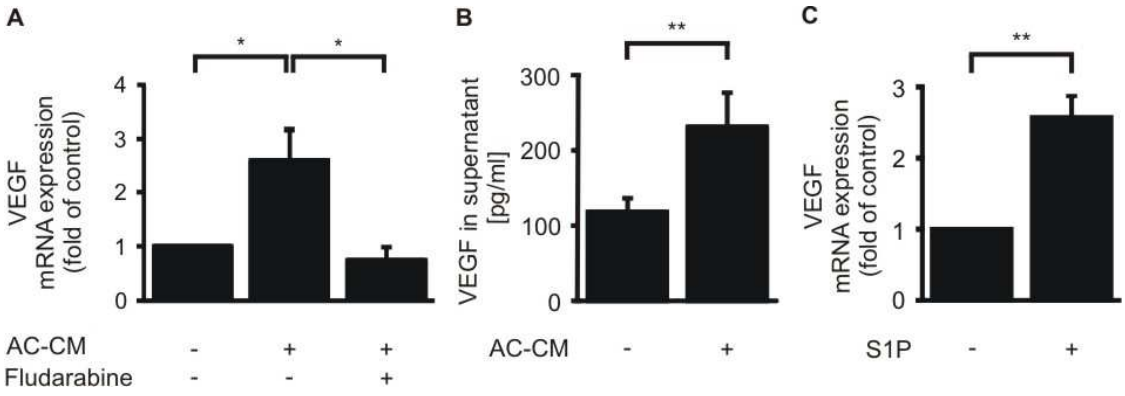

Figure 21. VEGF induction in macrophages is dependent on S1P and STAT signaling. (A) VEGF mRNA expression after stimulation of primary human macrophages with AC-CM or AC-CM together with 20 µM fludarabine for 1 h. (B) Quantitation of VEGF secretion by control or AC-CM-stimulated (18 h) macrophages. (C) VEGF mRNA expression in control or S1P-stimulated (100 nM, 1 h) macrophages. Graphs display mean values ± SEM of at least four independent experiments and asterisks indicate statistically significant differences *$p \leq 0.05$ and **$p \leq 0.01$.

5.4.3 VEGFA is the crucial factor of MΦ-CM provoking HO-1 induction

To further scrutinize this hypothesis, VEGF was knocked down in primary human macrophages prior to their incubation with AC-CM (Figure 22 A). VEGF mRNA expression was induced in macrophages following incubation with AC-CM. However, this response was abrogated by siRNA directed towards VEGFA.

Finally, to prove that VEGF was the autocrine factor secreted by macrophages, I analyzed the expression of HO-1 protein in macrophages, which were incubated with MΦ-CM generated from VEGF knockdown or siControl transfected macrophages. When macrophages were transfected with non-targeting siRNA and incubated with AC-CM, MΦ-CM derived from these cells induced HO-1 in fresh, unstimulated cells. However, HO-1 was not induced with MΦ-CM from VEGFA knockdown macrophages (Figure 22 B). Thus, these experiments suggest that VEGF mediated the autocrine induction of HO-1 after stimulation with AC-CM.

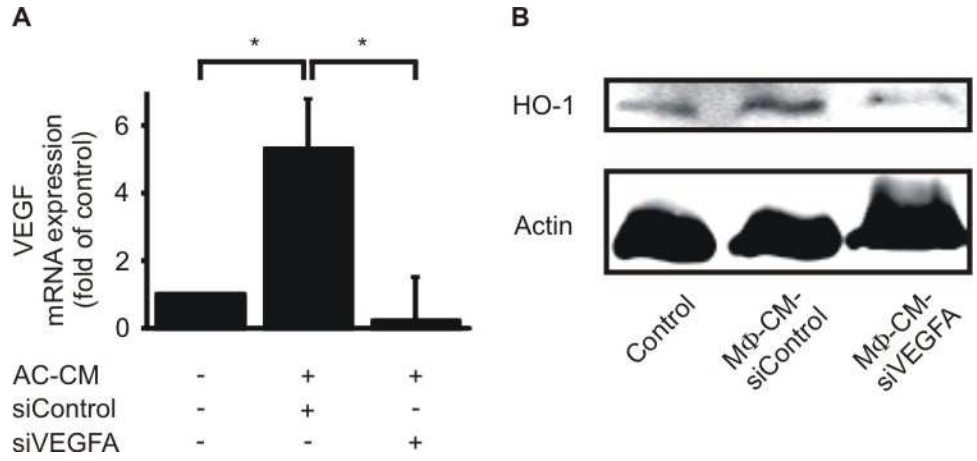

Figure 22. HO-1 expression in macrophages by autocrine VEGFA signaling. Primary human macrophages were transfected with non-targeting siRNA or siRNA against VEGFA. (A) VEGF mRNA expression in control or AC-CM-treated macrophages after 6 h. Graph displays mean values ± SEM of six independent experiments. Asterisks indicate statistically significant differences ($p \leq 0.05$). (B) Western analysis of HO-1 expression (18 h) following treatment with MΦ-CM. One representative blot out of three is displayed.

5.5 HO-1 affects anti-inflammatory and anti-apoptotic pathways in macrophages

HO-1 is well-known for its anti-apoptotic and anti-inflammatory actions (140, 167, 214). As shown recently, S1P from AC-CM induced Bcl-2 and Bcl-X_L in macrophages (35), I addressed the question whether the induction of Bcl-2 and/or Bcl-X_L required HO-1.

5.5.1 HO-1 affects the expression of Bcl-2, Bcl-X_L and Adora A_{2A}

HO-1 in primary human macrophages was knocked down by using siRNA technology. Transfection with siRNA directed against HO-1 efficiently blocked the mRNA increase of HO-1 after treatment with AC-CM for 9 h (Figure 23).

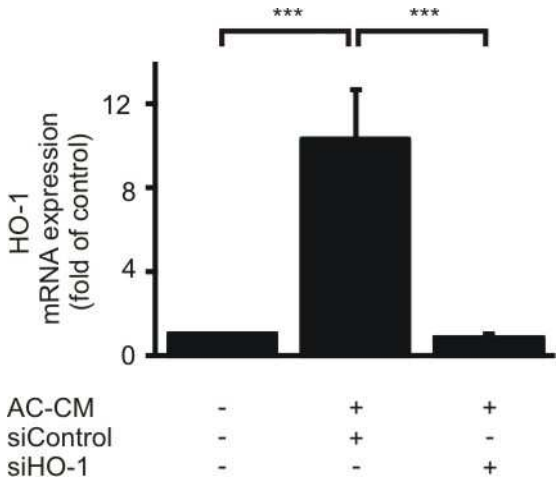

Figure 23. Downregulation of HO-1 in macrophages by siRNA transfection. Primary human macrophages were controls or transfected with siRNA against HO-1 or non-targeting siRNA followed by treatment with AC-CM for 9 h. Graph displays mean values ± SEM of 16 independent experiments. Asterisks indicate statistically significant differences ($p \leq 0.001$).

AC-CM induced Bcl-2 and Bcl-X_L mRNA in macrophages transfected with non-targeting siRNA, whereas their upregulation was significantly diminished when macrophages were transfected with siRNA against HO-1 (Figure 24 A). This suggests a role of HO-1 in contributing to the anti-apoptotic phenotype of macrophages elicited by AC-CM (35).

To examine whether the increased HO-1 expression also conveyed anti-inflammatory properties in my system, I analyzed the expression of the three markers Adora A_{2A}, IDO and HLA-DMB, which are generally linked to anti-inflammatory responses in macrophages. While the expression of Adora A_{2A} was enhanced in control macrophages incubated with AC-CM, knockdown of HO-1 prevented this increase (Figure 24 B). However, there was no correlation between the amount of HO-1 and the expression of either IDO or HLA-DMB (Figure 24 B and C). Expression of IDO mRNA was enhanced in response to

AC-CM in macrophages either transfected with non-targeting siRNA or with siRNA directed against HO-1 (Figure 24 B). In contrast, HLA-DMB expression was decreased following stimulation with AC-CM, which was unaffected by knockdown of HO-1 (Figure 24 C).

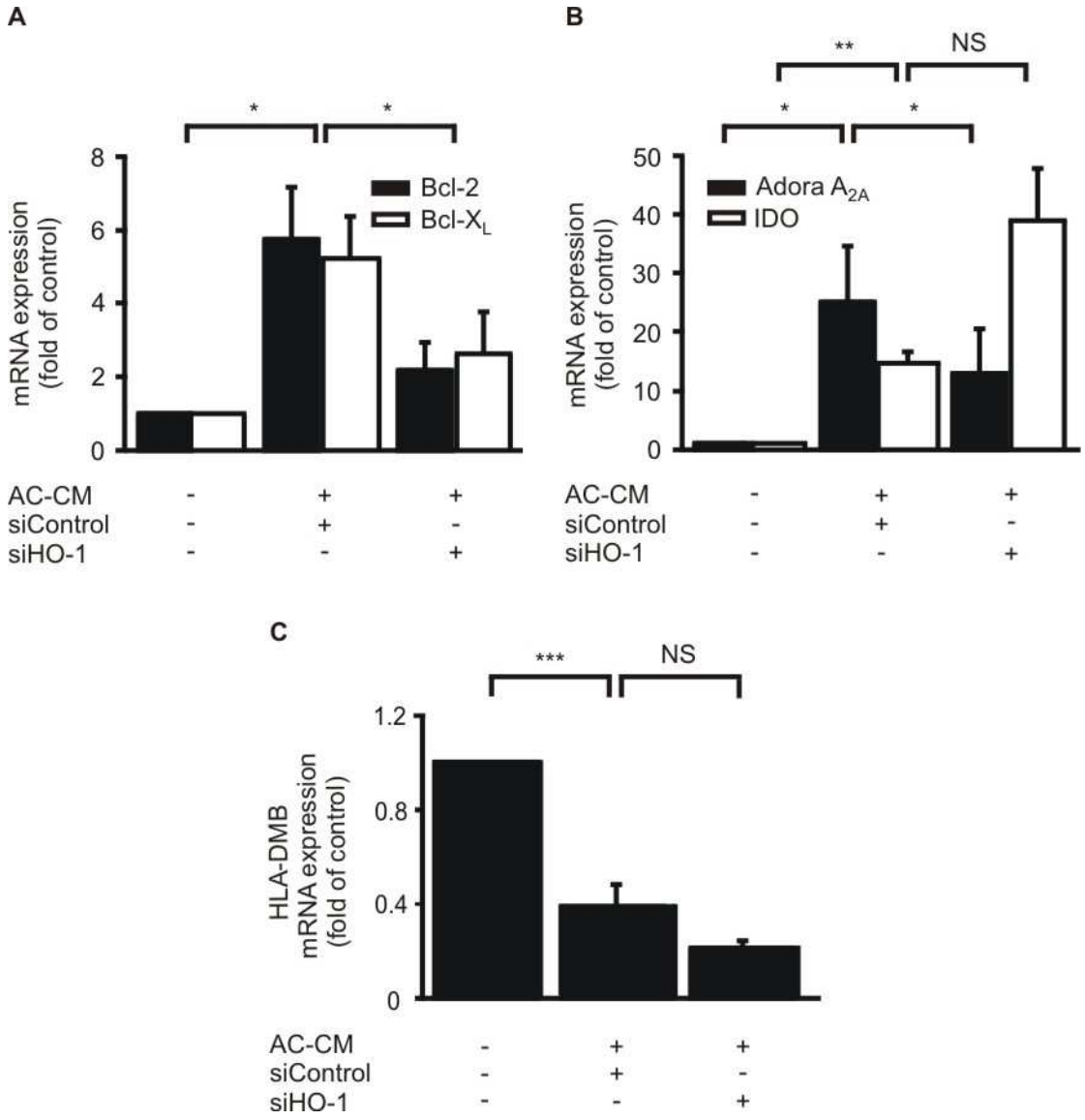

Figure 24. Regulation of Bcl-2, Bcl-X_L, Adora A_{2A}, IDO and HLA-DMB mRNA levels by HO-1 in macrophages. Primary human macrophages remained as controls or were transfected with siRNA against HO-1 or non-targeting siRNA. Graphs indicate mRNA expression of Bcl-2, Bcl-X_L (A), Adora A_{2A}, IDO (B) and HLA-DMB (C) in macrophages following incubations with AC-CM for 9 h. Data are mean values ± SEM of at least five independent experiments. Significant differences in mRNA expression are marked by *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ or were not significant (NS).

In addition, I examined whether HO-1 also affected protein expression of Bcl-X_L and Adora A_{2A}. Therefore, macrophages were transfected with non-targeting siRNA or HO-1-specific siRNA and incubated with AC-CM for 16 h. Both, Bcl-X_L and Adora A_{2A}, were significantly induced by AC-CM in macrophages transfected with non-targeting siRNA, whereas their expression remained equivalent to controls in macrophages with siRNA-mediated knockdown of HO-1 (Figure 25).

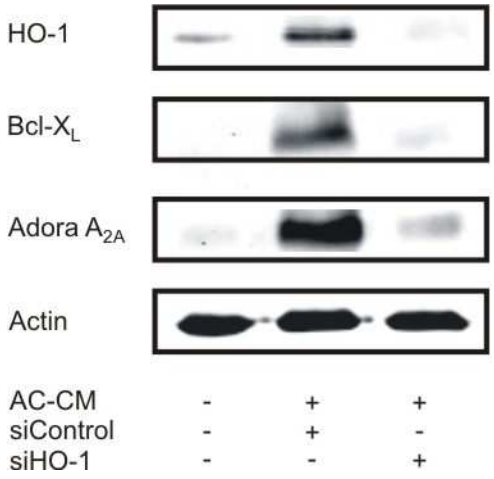

Figure 25. Regulation of Bcl-X_L and Adora A_{2A} protein levels by HO-1 in macrophages. Primary human macrophages were controls or transfected with siRNA against HO-1 or non-targeting siRNA. Western analysis of HO-1, Bcl-X_L and Adora A_{2A} expression in macrophages after stimulation with AC-CM for 16 h. One representative Western blot out of three is shown.

5.5.2 Impact of NO, bilirubin and CO on Adora A_{2A} expression

In inflammatory macrophages, HO-1 is induced as a response to LPS-induced NO production to initiate an anti-inflammatory negative feedback loop (215). Thus, I investigated whether NO reproduced HO-1-depedent effects on gene expression in my system. Indeed, Deta-NO induced Adora A_{2A}. However, this induction was less pronounced compared with AC-CM (Figures 24 B and 26). To determine which product of the HO-1-catalyzed reaction mediated Adora A_{2A} induction, bilirubin and the CO-releasing molecule CORM-2 were used. Both agents significantly elevated Adora A_{2A} mRNA expression (Figure 26), although the expression was low compared to the impact of AC-CM. Notably, the

inactivated, i.e. decomposed product of CORM-2 (iCORM-2) exerted no effect (Figure 26).

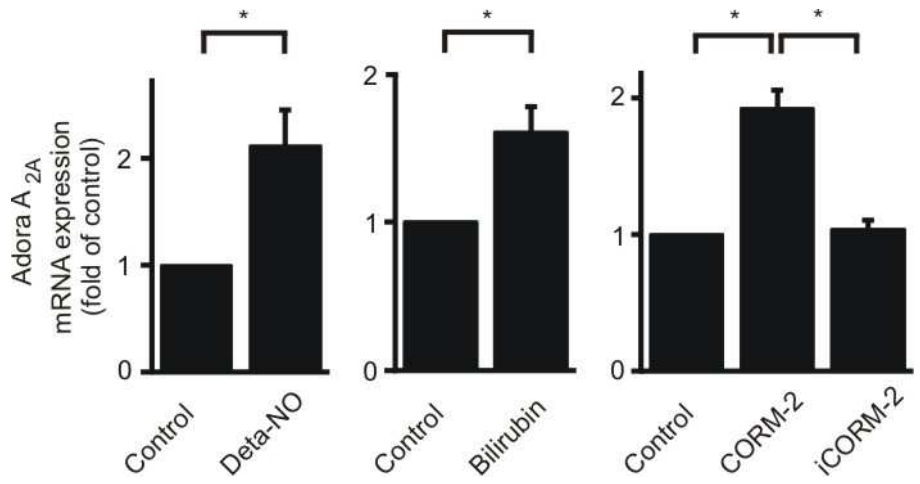

Figure 26. Adora A_{2A} mRNA expression in macrophages following treatment with Deta-NO, bilirubin or CORM-2. Primary human macrophages were incubated with 500 µM Deta-NO for 9 h, 10 µM bilirubin for 1 h or 100 µM CORM-2 for 24 h. Graphs display mean values ± SEM of at least three independent experiments. Significant differences in mRNA expression are marked by asterisks ($p \leq 0.05$).

5.6 Impact of AC supernatants on S1P receptor expression

Investigating the dependence of HO-1 induction on $S1P_1$ signaling, I unexpectedly observed that also $S1P_1$ was upregulated in primary human macrophage following treatment with AC-CM (Figure 15).

5.6.1 AC supernatants provoke upregulation of $S1P_1$ and $S1P_3$ mRNA

As $S1P_{1-3}$ are the most important S1P receptors in myeloid cells (115), I investigated the expression schemes of these receptor isoforms in primary human macrophages in response to AC-CM. $S1P_1$ and $S1P_3$ showed a time-dependent mRNA induction pattern being most prominent after incubation for 3 h. However, $S1P_2$ mRNA expression was unaffected (Figure 27). As I observed that $S1P_1$ transcript levels were generally much higher than those of

$S1P_3$ in primary human macrophages, I focussed on the investigation of $S1P_1$ in the following.

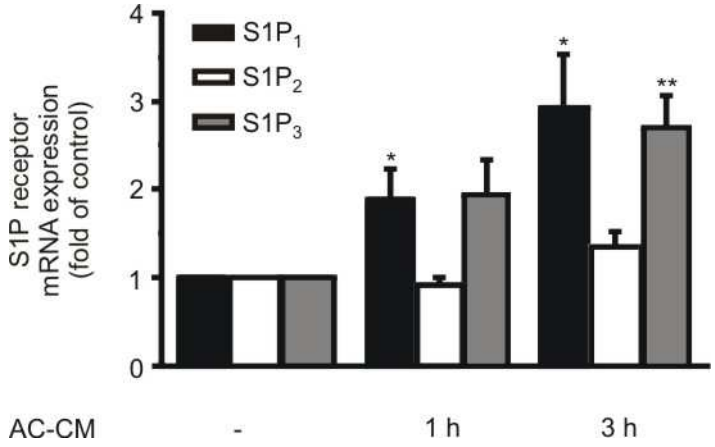

Figure 27. $S1P_1$, $S1P_2$ and $S1P_3$ mRNA expression in macrophages after treatment with AC-CM. Primary human macrophages were incubated with AC-CM for 1 h or 3 h, respectively. Graphs display mean values ± SEM of at least five independent experiments. Significant differences in mRNA expression are marked by *$p \leq 0.05$ and ** $p \leq 0.01$.

5.6.2 Induction of $S1P_1$ protein expression by AC supernatants

Treatment of primary human macrophages with AC-CM resulted in a time-dependent induction of $S1P_1$ protein expression reaching a maximum after 6 h (Figure 28).

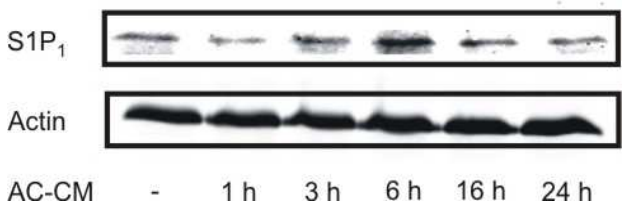

Figure 28. Induction of $S1P_1$ protein expression in macrophages following treatment with AC-CM. Western analysis of $S1P_1$ expression after incubations of primary human macrophages with AC-CM for times as indicated. The Western blot is representative for three individual experiments.

5.7 Alternative macrophage activating factors upregulate $S1P_1$ mRNA

As I observed that macrophages were alternatively activated following incubation with supernatants from AC, I asked whether the induction of $S1P_1$ can specifically be attributed to alternative macrophage inducing factors. Therefore, primary human macrophages were treated with LPS or INF-γ, which induce classical activation, or with IL-4 or IL-10 to provoke alternative activation, for 3 h. While incubation with IL-4 or IL-10 significantly induced $S1P_1$ mRNA expression, LPS or IFN-γ treatment exhibited no influence on $S1P_1$ mRNA levels (Figure 29). However, the induction of $S1P_1$ was more pronounced after AC-CM compared to IL-4 or IL-10 (Figures 27 and 29). Interestingly, also authentic S1P showed no effect on $S1P_1$ mRNA expression.

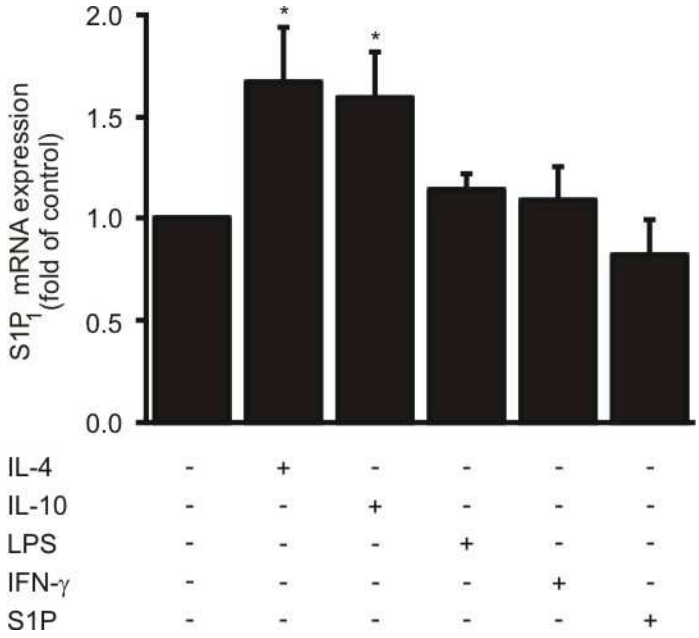

Figure 29. Induction of $S1P_1$ mRNA expression in macrophages by alternative macrophage activating stimuli. Primary human macrophages were treated for 3 h with 10 ng/ml IL-4, 20 ng/ml IL-10, 1 µg/ml LPS, 100 U/ml IFN-γ or 100 nM S1P. Graphs show mean values ± SEM of five independent experiments. Significant differences in mRNA expression are marked by asterisks ($p \leq 0.05$).

5.8 Krüppel-like factor is a potential transcription factor mediating $S1P_1$ transcription in response to AC supernatants

To corroborate the induction of $S1P_1$ by AC-CM and to identify the transcription factors mediating $S1P_1$ induction, reporter assays were performed in primary human macrophages. $S1P_1$ promoter contructs were a kind gift from the group of Jianzhu Chen (123). However, these contructs contained the murine promoter instead of the human promoter.

5.8.1 Transrciption factor binding sites located on the sequence -1000 bp to -1300 bp on the murine promoter provoke induction of $S1P_1$ luciferase activity

After transfection of the murine promoter contructs into primary human macrophages, $S1P_1$ promoter activation following treatment with AC-CM was noticed only when transfecting the fragment containing -1300 bp of the 5'-flanking region, but not with the -1000 bp fragment (Figure 30).

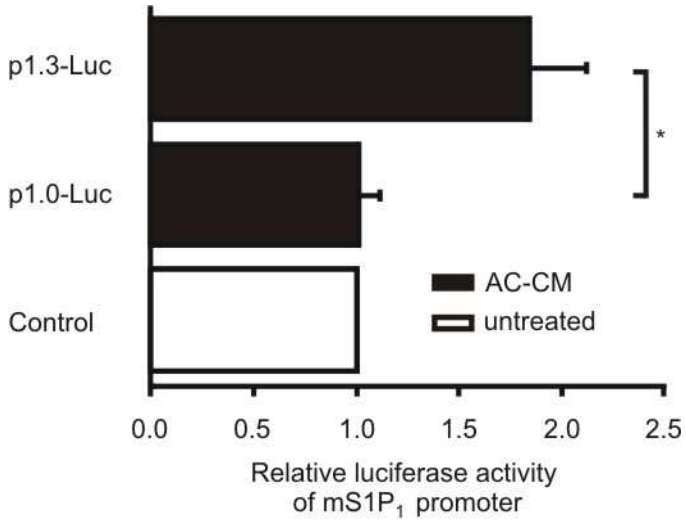

Figure 30. $S1P_1$ promoter activity in macrophages after stimulation with AC-CM. Primary human macrophages were transfected with the respective reporter constructs and incubated with AC-CM for 8 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are means ± SEM of five independent experiments. Asterisks indicate statistically significant differences ($p \leq 0.05$).

5.8.2 Human and murine $S1P_1$ promoter contain a highly conserved KLF binding site

To clarify whether the data obtained using murine promoter contructs can also be transferred to the human system, the online-tool TESS was used to compare the human and the murine $S1P_1$ promoter. The sequence located between -1050 bp and -1300 bp upstream from the transcription start is highly conserved between mice and humans and contains three binding sites for KLF, a transcription factor, which is known to induce $S1P_1$ expression (123). Interstingly, a sequence spanning 28 bp is completely identical in the human and the murine promoter (Figure 31). This sequence, among others, contains a binding site for KLF. Thus, this sequence appears likely to be responsible for the induction of $S1P_1$ in primary human macrophages.

Figure 31. Conserved sequence contained in the human and the murine $S1P_1$ promoter. A sequence of 28 bp is completely identical in the human and the murine $S1P_1$ promoter (blue) and, among others, contains a KLF binding site (red).

5.9 $S1P_1$ is located in pseudopodia-like structures of macrophages following treatment with AC supernatants

To correlate receptor expression to function its subcellular localization has to be considered, as signaling will only be possible if the respective ligand is available. In the case of S1P receptors, this is of special interest, as they are internalized upon treatment with S1P (118). Furthermore, S1P receptors can also be cross-activated by growth factors such as PDGF (216) or insulin-like growth factor-1 (217).

$S1P_1$ in primary human macrophages was located predominantly in the plasma membrane and the nucleus (Figure 32). I used the fact that $S1P_1$ is internalized upon treatment with S1P to confirm the specificity of the antibody staining of $S1P_1$. $S1P_1$ was internalized following incubation with 200 nM S1P for 45 min. Interestingly, $S1P_1$ was localized to the plasma membrane, in particular to pseudopodia-like structures, after treatment with AC-CM for 6 h.

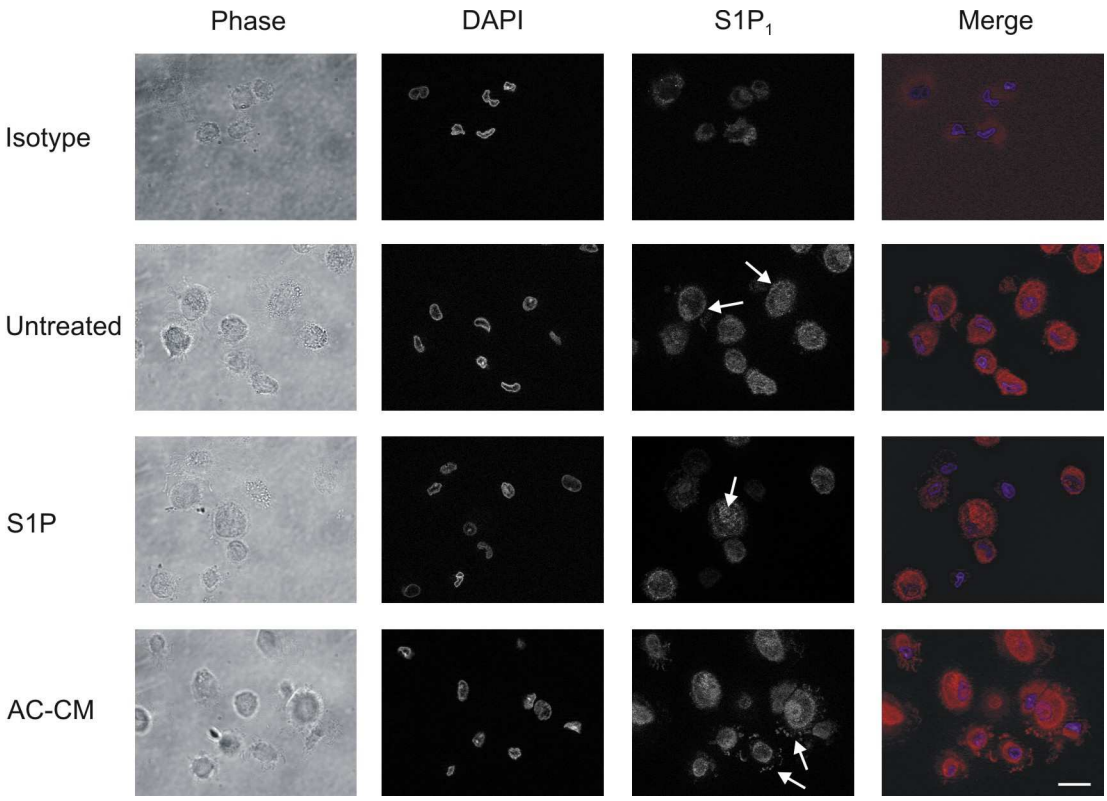

Figure 32. $S1P_1$ translocates to pseudopodia-like structures of macrophages in response to treatment with AC-CM. Primary human macrophages were stimulated with 200 nM S1P for 45 min or with AC-CM for 6 h. Immunofluorescence revealed the localization of $S1P_1$. Nuclei were labeled by DAPI-staining. Displayed cells are representative of three independent experiments. Bar indicates 20 µm.

5.10 $S1P_1$ is crucially involved in macrophage migration in response to AC supernatants

$S1P_1$ and $S1P_2$ have opposing roles in migration. While $S1P_1$ was associated with enhanced chemotaxis, $S1P_2$ was shown to counteract this process (83,

217). Following treatment with AC-CM, the expression of $S1P_1$ was much higher than the expression of $S1P_2$ and $S1P_1$ was localized to pseudopodia-like structures. Thus, it was tempting to speculate that $S1P_1$ could enhance the migratory potential of macrophages following their exposure to AC-CM. Therefore, a scratch assay was performed to analyze migration of primary human macrophages in response to treatment with AC-CM. AC-CM fostered scratch closure, whereas this was inhibited by the $S1P_{1/3}$ receptor antagonist VPC 23019 (Figure 33 A and B).

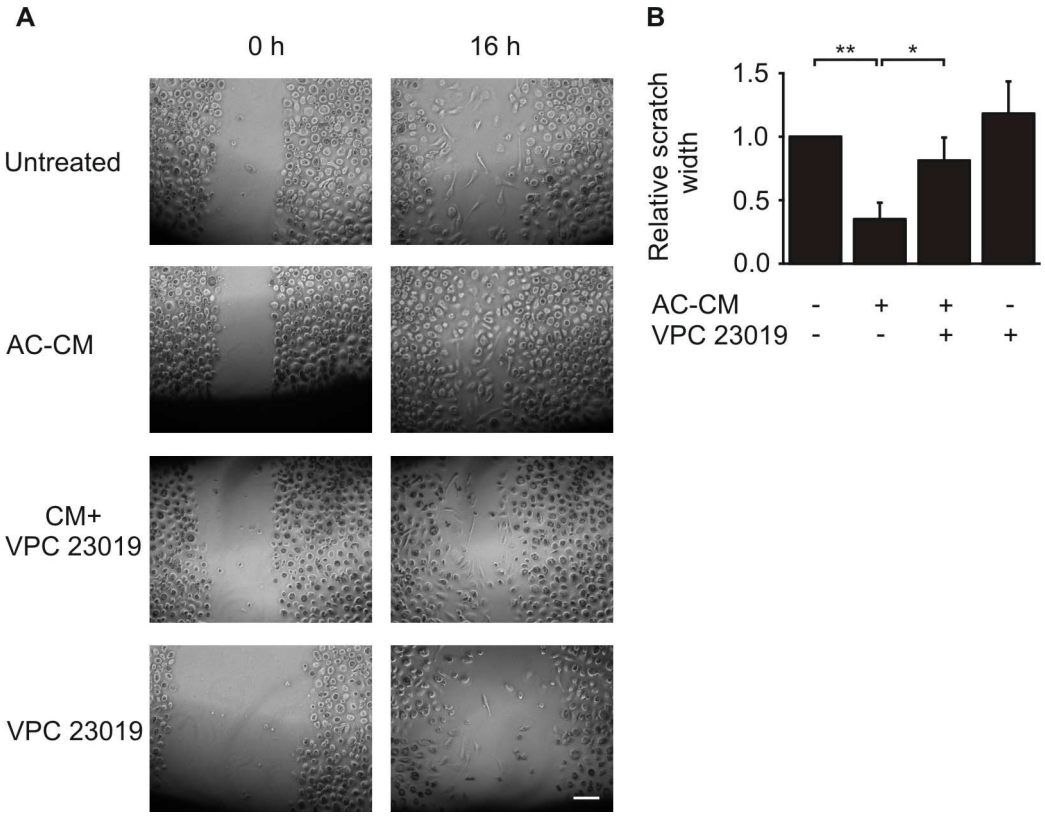

Figure 33. Enhanced migration of primary human macrophages in response to AC-CM is dependent on S1P receptor. Scratch assay in primary human macrophages incubated with AC-CM for 16 h in the presence/absence of 1 µM VPC 23019 (A) and statistical analysis (B). Photographs are representaitive of four individual experiments. Bar indicates 100 µM. Significant differences in scratch width are marked by *$p \leq 0.05$ and ** $p \leq 0.01$.

To confirm that $S1P_1$ was the S1P receptor responsible for migration, I used peritoneal macrophages from $S1P_1$ deficient mice (Figure 34 A). While macrophages from wild-type mice showed migration in response to AC-CM, migration was absent in $S1P_1$ knockout macrophages (Figure 34 B and C).

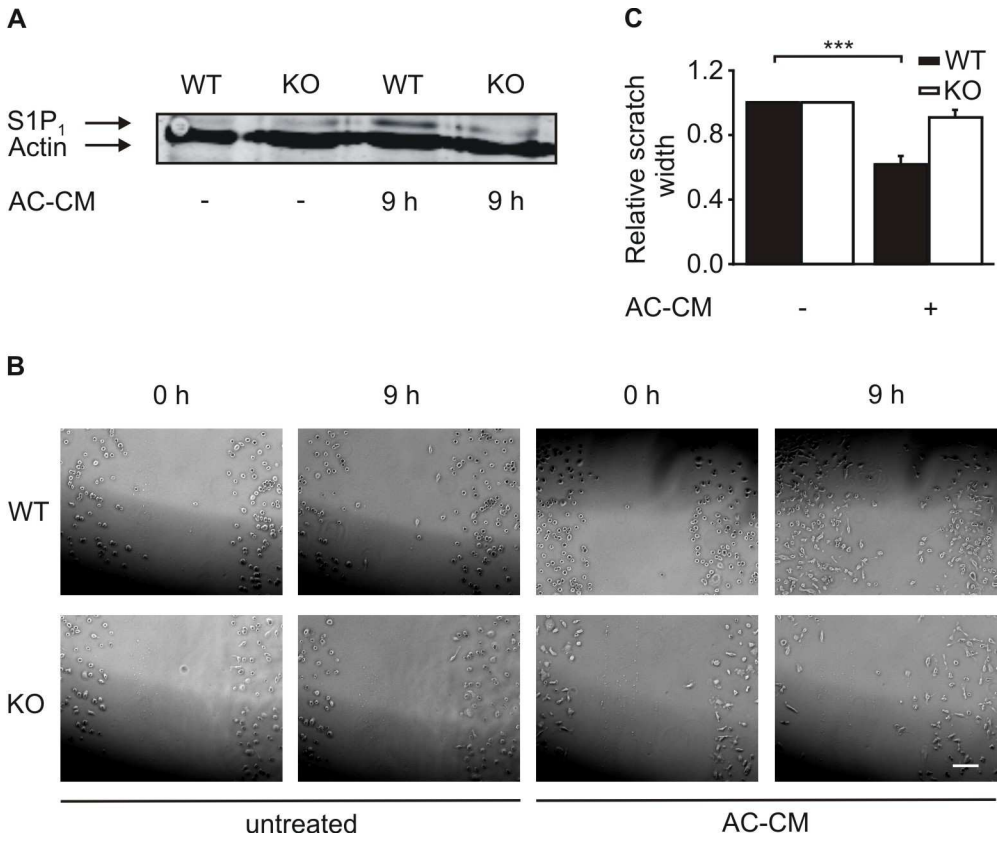

Figure 34. Enhanced migration of primary murine peritoneal macrophages in response to AC-CM is dependent on $S1P_1$. (A) Knockout of $S1P_1$ in peritoneal macrophages of $S1P_1$ knockout mice was confirmed by Western blot. One representative blot out of three individual experiments is displayed. Scratch assay was performed in primary murine macrophages derived from wild-type (WT) or $S1P_1$ knockout (KO) mice incubated with AC-CM for 9 h (B) and statistically analyzed (C). Photographs are representaitive of seven individual experiments. Bar indicates 100 µM. Asterisks indicate statistically significant differences ($p \leq 0.001$).

6 DISCUSSION

My data provide evidence for $S1P_1$ to be a key player during alternative activation of macrophages. Following treatment of primary human macrophages with AC-CM, $S1P_1$ was the crucial receptor being responsible for the induction of HO-1, which in turn increased the amount of anti-apoptotic and anti-inflammatory proteins such as Bcl-2 or Bcl-X_L and Adora A_{2A}. Furthermore, $S1P_1$ itself was induced in macrophages after treatment with AC-CM and provoked migration of macrophages.

6.1 HO-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants

The notion that authentic or AC-derived S1P induced HO-1 is in line with a report by Man and coworkers, showing that the sphingosine analog FTY720 enhanced HO-1 expression (207). Human macrophages express the S1P receptor subtypes 1-4 (218). Considering that activation of $S1P_1$ attenuated the expression of pro-inflammatory cytokines in murine macrophages (116) in conjunction with the observation that $S1P_1$ signaling in macrophages protected from apoptosis (35) fits well to my results, demonstrating that $S1P_1$ provoked an increase in HO-1 expression, which in turn triggered anti-apoptotic as well as anti-inflammatory signals (Figure 35).

Inhibitor studies revealed that in primary human macrophages p38 MAPK, a pathway well known for HO-1 induction (190, 219), facilitated S1P-evoked HO-1 expression. However, other studies have shown p38 MAPK activation downstream of $S1P_2$ (112), which might explain that siRNA-mediated knockdown of $S1P_1$ could not completely reduce HO-1 expression after stimulation with AC-CM. This preserves the option that residual $S1P_2$ signaling occurred.

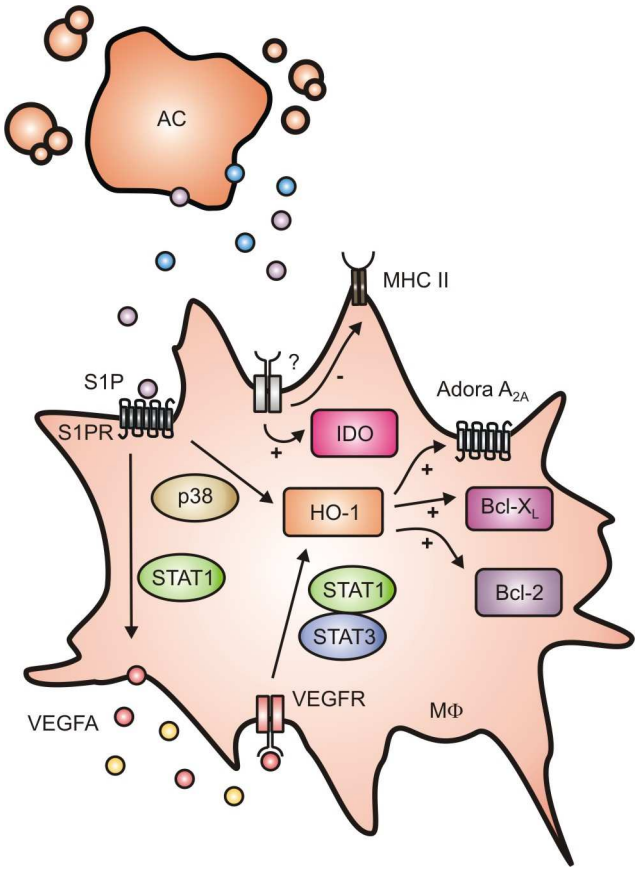

Figure 35. Heme oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. Following treatment with supernatants from apoptotic cells (AC) macrophages (MΦ) demonstrate a biphasic expression pattern of heme oxygenase (HO)-1. Whlie the first-phase induction at 6 h is mediated by p38 MAPK (p38) and is accomplished by AC-derived S1P engaging S1P receptor (S1PR) 1, the second wave of HO-1 induction at 24 h is attributed to autocrine signaling of vascular endothelial growth factor A (VEGFA). Whereas the expression of VEGFA is facilitated by S1P and its release is dependent on STAT1, VEGFA itself acts on the macrophage HO-1 promoter via STAT1/STAT3 heterodimer binding. HO-1 contributes to alternative macrophage activation by regulating the expression of adenosine receptor A_{2A} (Adora A_{2A}), Bcl-X_L and Bcl-2. MHC II and indoleamine 2,3-dioxygenase (IDO) expression are also fostered by AC supernatants, but are not dependent on HO-1.

The use of DMS was discussed recently. Besides inhibiting SphK1, DMS also has some undesirably side-effects, such as inhibition of PKC (205), enhancement of EGF receptor autophosphorylation (220) and even activation of SphK2 (221). As S1P mediating the anti-apoptotic effect of AC was mainly derived form SphK2 (35), a better approach would be to generate CM form

SphK2-deficient Jurkat cells. However, my data with authentic S1P also strongly point to S1P being the decisive factor.

A candidate that would fulfil the characteristics of a transcription factor being involved in the early-phase induction of HO-1 might be PPAR-γ. Two independent studies in RAW264.7 macrophages revealed that PPAR-γ was activated following co-culture with apoptotic Jurkat cells for 15 minutes (73) and that the selective PPAR-γ agonist $\Delta^{12}PGJ_2$-mediated HO-1 induction (222). Thus, as I observed HO-1 protein expression after 6 h of incubation with AC-CM, PPAR-γ could be the responsible transcription factor. EMSA analysis would help to clarify the involvement of PPAR-γ in the early-phase HO-1 induction.

My studies suggest a cross-talk between S1P-signaling and VEGFA secretion from human macrophages. A connection between S1P and VEGF signaling was recently also put forward for ML-1 thyroid follicular cancer cells as well as human FRO anaplastic thyroid cancer cells, in which S1P stimulated VEGFA secretion (223). Furthermore, VEGF secretion from epithelial cells after phagocytosis of AC was demonstrated previously (211). However, neither signal cross-talk between AC and epithelial cells nor signaling consequences were fully understood. In my system, there is evidence for S1P generated by AC, rather than cell-cell contacts or phagocytosis, being required for VEGF secretion. A connection between S1P and VEGF signaling is highlighted, especially for models of tumor angiogenesis, proposing that tumor-derived S1P stimulates VEGF formation in endothelial cells (224, 225). Macrophages, as cells in the tumor microenvironment, are critical players stimulating angiogenesis in a variety of human tumors, where they exhibit a pronounced alternatively activated anti-inflammatory and anti-apoptotic phenotype (226). My finding that VEGFA was not only secreted from macrophages in response to S1P, but also caused autocrine signaling to further induce HO-1, implies an important role of S1P in macrophage polarization, with particular relevance in the tumor setting. Besides mechanisms such as tumor hypoxia, the interaction of tumor-associated macrophages with dying tumor cells could then promote VEGFA release to stimulate tumor angiogenesis. This proposal is corroborated by the finding that treatment of macrophages with AC-CM stimulated differentiation of embryonic stem cells through the induction of CD31

expression, which is an established marker for endothelial cell differentiation (227). Differentiation was inhibited in hypoxia inducible factor-1α knockout mice and was ascribed to VEGF, which is a target gene of hypoxia inducible factor-1α.

When I observed that VEGF expression was induced by S1P via STAT1, it was rather an extraordinary finding, as S1P receptor activation had not been linked to STAT signaling previously. I considered that Src kinase activation downstream of a GPC receptor such as $S1P_1$ might be a missing communication link between S1P and STAT (228), although mechanisms of Src activation in response to GPC receptor agonists are not fully understood (229). Src kinase activation occurs upstream of STAT signaling in human monocytes (230), which led me to the speculation that Src kinase might link S1P receptor activation to STAT activation in my system. Strikingly, in rat aortic vascular smooth muscle cells, the S1P-stimulated transactivation of STAT-coupled epidermal growth factor receptor and PDGF-β receptor were Src-dependent (231). Recently, it was shown that S1P activates STAT in cardiomyocytes (232). Indeed, in this report Src kinase activation was shown to connect S1P to STAT3 activation.

STAT3 was necessary for the autocrine induction of HO-1 by VEGF, which was observed previously in chronic lymphocytic leukemia B cells (212). Furthermore, STAT1 and STAT3 were involved in hyperoxia-induced gene transcription of HO-1 in RAW 264.7 macrophages (233). Activation of STATs presumably plays an important role in establishing an alternative macrophage activation phenotype in the tumor setting, because STAT1 is constitutively active in tumor-associated macrophages (234) and its enhanced signaling properties mediate T cell deletion (235). Furthermore, STAT3 and STAT6 are believed to contribute to alternative macrophage polarization (236).

My work reveals that HO-1 in macrophages, besides accomplishing anti-apoptotic functions by enhancing the expression of survival promoting proteins such as Bcl-2 and Bcl-X_L, also conveys an anti-inflammatory potential, which is exemplified by the expression of Adora A_{2A} in response to AC-CM. Adora A_{2A} agonists are capable of not only blocking the inflammatory potential of human macrophages, such as pathogen-stimulated NO, TNF-α, or IL-12 production

(237), but also promoting wound-healing in disease states such as diabetes (238). Likely, in an inflammatory environment, the presence of AC might help to promote healing once an inflammatory stimulus is eliminated by modulating adaptive immune responses. Thus, successful resolution and repair of tissue damage, rather than persistence of the inflammatory response, which can promote scarring and loss of organ function, might be provided by AC. In an inflammatory environment, neutrophils as a first line of immune defense, are rapidly recruited to the site of infection in response to a variety of inflammatory stimuli and engulf the invading infectious agents, after which neutrophils undergo apoptosis (37). Macrophages, which phagocytose apoptotic neutrophils during inflammation, might be protected against apoptosis and the expression of Adora A_{2A} could prelude the resolution of inflammation. Today it is recognized that at least 15% of tumors worldwide can directly be referred to an infectious origin (239) and cancer was considered as an ‘overhealing wound’ (240).Thus, the induction of HO-1 by AC could also play an important role during tumor development, as it seems to play a crucial role in inflammation as well as wound-healing. This suggestion is further reinforced by the finding that inhibition of HO-1 reduced tumor growth of LL/2 lung cancer in C57BL mice (241). Furthermore, sphingolipid metabolism was suggested to be often dysregulated in cancer (86, 88) and it was proposed that SphK may not only protect tumors from apoptosis, but may also increase their vascularization, further enhancing growth (88). As discussed before, in my system this could be performed by VEGF, which is secreted by macrophages in response to AC-derived S1P.

During the clearance of invading pathogens, pro-inflammatory processes ensure maximal immunological activation in the early phase but are followed by resolution and active suppression of inflammation (72). The notion that Deta-NO induced Adora A_{2A} rather weakly compared to AC-CM implies that HO-1-dependent anti-inflammatory effects were only partially mimicked by Deta-NO. This may point to the induction of HO-1 by different signaling pathways and transcriptional regulators. The induction of HO-1 by NO could be mediated by Nrf2 (242), instead of p38 MAPK, which is involved in AC-CM-mediated HO-1 induction. Likely, Adora A_{2A} is not only induced by HO-1, as I observed some expression also with a knockdown of HO-1. Thus, an anti-inflammatory

response achieved with AC-CM is rather unique and differs from a situation with only a pro-inflammatory stimulus such as LPS or NO to induce HO-1 expression. Consequently, in an inflammatory environment, pro-inflammatory stimuli foster the initiation phase of inflammation. However, they might at the same time prime for the resolution phase by induction of HO-1, but to a lesser extent than AC do and possibly also other target genes of HO-1 are involved. Thus, the presence of AC subsequent to or as a result of a pro-inflammatory stimulus might be required for the resolution of inflammation.

According to the suggestions by Zhang and co-workers, enhanced Bcl-2 and Bcl-X_L expression by HO-1 could be attributed to CO, as shown in a murine model of ischemia-reperfusion (243). Generally, several anti-apoptotic effects attributed to HO-1 are thought to be CO-mediated (244). This might also apply to Adora A_{2A}, because overexpression of HO-1 as well as exposure of RAW264.7 macrophages to CO augmented Adora A_{2A} mRNA as well as protein expression (245). The rather marginal effect observed with CO and bilirubin in my experiments may open a further possibility that HO-1 translocates to the nucleus to bind to a transcription factor or a protein complex, resulting in enhanced transcription of Adora A_{2A} (246).

Interestingly, the expression of the anti-inflammatory marker IDO was, at least in human macrophages exposed to AC-CM, HO-1-independent. Nevertheless, the principle finding that AC-CM augments the expression of IDO is exciting. IDO catalyzes the rate-limiting step of tryptophane degradation. Kynurenine, one of the products of this reaction, affects proliferation as well as differentiation of Th cells (247, 248). As IDO was shown to be upregulated by PGE_2 (249) and COX-2 is also induced in macrophages by AC (80), the induction of IDO by COX-derived PGE_2 appears likely.

The development of a macrophage phenotype, which dampens T cell immune responses, was further corroborated by my finding that macrophages downregulate HLA-DMB in response to AC. Although it was reported that bilirubin, a degradation product of biliverdin, suppressed MHC II expression in endothelial cells (250), I could not confirm that HLA-DMB expression was HO-1-dependent in macrophages. This could probably be referred to cell type differences. However, IL-10 being produced either by AC or by macrophages in response to AC could inhibit MHC II expression (75, 251).

Reduced expression of HLA-DMB and increased abundance of IDO favor an attenuated response of Th1 cells, which is important to progress from inflammation towards healing. In this way Adora A_{2A} also plays an important role as it is known to convey immunosuppression by inhibiting IL-12 formation (237). IL-12 is essential for the induction of Th1 infammatory responses, provoking maturation of naïve $CD4^+$ T cells to Th1 effector cells and engendering IFN-γ secretion by $CD8^+$ T cells as well as natural killer cells (252).
Together, AC supernatants provoked alternative activation of human macrophages, characterized by the upregulation of Bcl-2, Bcl-X_L, Adora A_{2A}, and IDO, but downregulation of MHC II expression. The establishment of this anti-inflammatory phenotype was in part dependent on the induction of HO-1 by AC-derived S1P. Thus, targeting HO-1 and/or its downstream effectors could be a therapeutic approach to treat patients suffering from diseases linked to anti-inflammatory macrophage polarization during, e.g. the late immunosuppressive phase of sepsis (253) or in cancer (52), because this would influence the viability of alternative macrophages as well as their polarization. This is of special importance during cancer therapy as unfortunately, HO-1 can be induced in response to chemotherapy, radiation or photodynamic therapy, which then negatively affects the outcome of the respective therapy (189, 190). As the induction of HO-1 during these therapies might be referred to AC-derived S1P, it might be a therapeutic advance to drive cancer cells into necrotic cell death rather than into apoptosis. Possibly, also interference with the clearance of AC could provide a therapeutic approach. Lowering the rate and/or capacity of phagocytosis of macrophages could result in the generation of secondary necrotic cells, and thus in impaired HO-1 induction. However, problems of these approaches could be the development of chronic inflammation and autoimmunity (252). Furthermore, also scavenging of ROS, which constitute potential HO-1 inducers (149) and might be generated during anti-cancer therapies, could prove to be beneficial.
Interestingly, HO-1 knockdown mice showed an accelerated and more advanced atherosclerotic lesion formation (254). HO-1 gene expression was demonstrated to be enhanced in human arteries with advanced atherosclerosis and was considered as an adaptive response to injurious stimuli, such as oxidized lipoproteins, cytokines and hemodynamic changes (255). HO-1

induction in the artery wall scavenged ROS, which lead to the attenuation of monocyte adhesion and chemotaxis (256). Furthermore, HO-1 was shown to inhibit signaling of TLR 4, the LPS receptor required for classical macrophage activation (257). However, the induction of HO-1 during atherosclerosis seems not to be sufficient to provide beneficial effects and should be targeted pharmacologically. Statins, whose effects were suggested to be mediated indirectly through HO-1 induction (164), form already one therapeutic approach to treat atherosclerosis by interfering with HO-1.

It is accepted nowadays that the presence of AC is vital for the successful resolution of inflammation. Although dampening of T cell immune responses might not be attributed to HO-1, HO-1 seems to play a central role in the resolution of inflammation. However, HO-1 induction by AC can be beneficial in one disease, whereas it proves to be deleterious during another disease, e.g. in the case of cancer. Therefore, therapies targeting HO-1 have to be balanced thoroughly.

6.2 $S1P_1$ is involved in enhanced motility of macrophages induced by apoptotic cell supernatants

Following treatment with supernatants from AC, $S1P_1$ was rapidly upregulated in macrophages. In particular, mRNA was induced after 1 h and protein after 3 h. This fits well to the observations of Hla and Maciag, who identified $S1P_1$ as an immediate-early gene product, which was induced by phorbol 12-myristate 13-acetate in human endothelial cells (119). As also $S1P_1$ promoter activity was enhanced after incubation with AC-CM, a fast transcriptional process seems to be responsible for the induction of $S1P_1$.

Among the potential candidates, which are possible as transcription factor mediating the induction of $S1P_1$, KLF seems to be the most likely one (Figure 36). KLF2 was considered as a negative regulator of monocyte activation, as it inhibited pro-inflammatory activation of monocytes and the transcriptional activity of both NF-κB and AP-1 (258). Since I observed that $S1P_1$ mRNA was already induced after incubation of macrophages with AC-CM for 1 h, activation

of KLF appears more likely than regulation of KLF at the transcriptional level. In this way, post-translational modifications serve as a switch for KLF proteins to function as activators or repressors. Acetylation of fetal KLF2 by CBP/p300 and p300/CBP-associated factor enhanced fetal KLF2 transcriptional activity in K562 myelogenous leukemia cells (259). Besides acetylation, modification of KLF5 with SUMO proteins was also proposed as a means to regulate KLF activity (260). Under basal conditions, SUMOylated KLF5 and unligated PPAR-δ were supposed to interact with co-repressors to form transcriptionally repressive complexes. Ligand binding to PPAR-δ was suggested to initiate rapid local deSUMOylation, which was followed by the exchange of co-regulators, chromatin remodelling and activation of transcription. As PPAR was shown to be activated in macrophages in response to treatment with AC (73), EMSA analysis should be performed to prove the involvement of KLF and/or PPAR isoforms in the induction of $S1P_1$ following treatment with AC-CM.

I attributed the unexpected observation of elevated $S1P_1$ expression following treatment with AC-CM first to autocrine VEGF, because $S1P_1$ expression was increased in bovine aortic endothelial cells after their exposure to authentic VEGF (120). However, mRNA analysis of $S1P_1$ revealed that it was increased already after 1 h of incubation with AC-CM. Although VEGF mRNA expression was also induced following treatment with AC-CM for 1 h, it is unlikely to act as an autocrine factor to induce $S1P_1$ expression, as autocrine signaling would require more time. Rather, an AC-derived factor might be important for the induction of $S1P_1$ expression. In this way, S1P can be ruled out, as $S1P_1$ mRNA expression was unaffected following treatment with authentic S1P. Besides AC-CM, also IL-4 and IL-10, which might be produced by AC (75, 261), provoked induction of $S1P_1$ mRNA expression in primary human macrophages. However, IL-4 and IL-10 activate STAT signaling rather than KLF (262). As the induction of $S1P_1$ mRNA expression was more pronounced in the presence of AC-CM than in the presence of IL-4 or IL-10, an additional factor being secreted by AC, which induces $S1P_1$ via KLF, seems more likely. To identify this factor, a first approach would be to treat AC-CM with proteinase K, DNAse and RNAse and to perform a lipid extraction. After identifying the principal nature of this factor, these procedures should be followed by more detailed analyses.

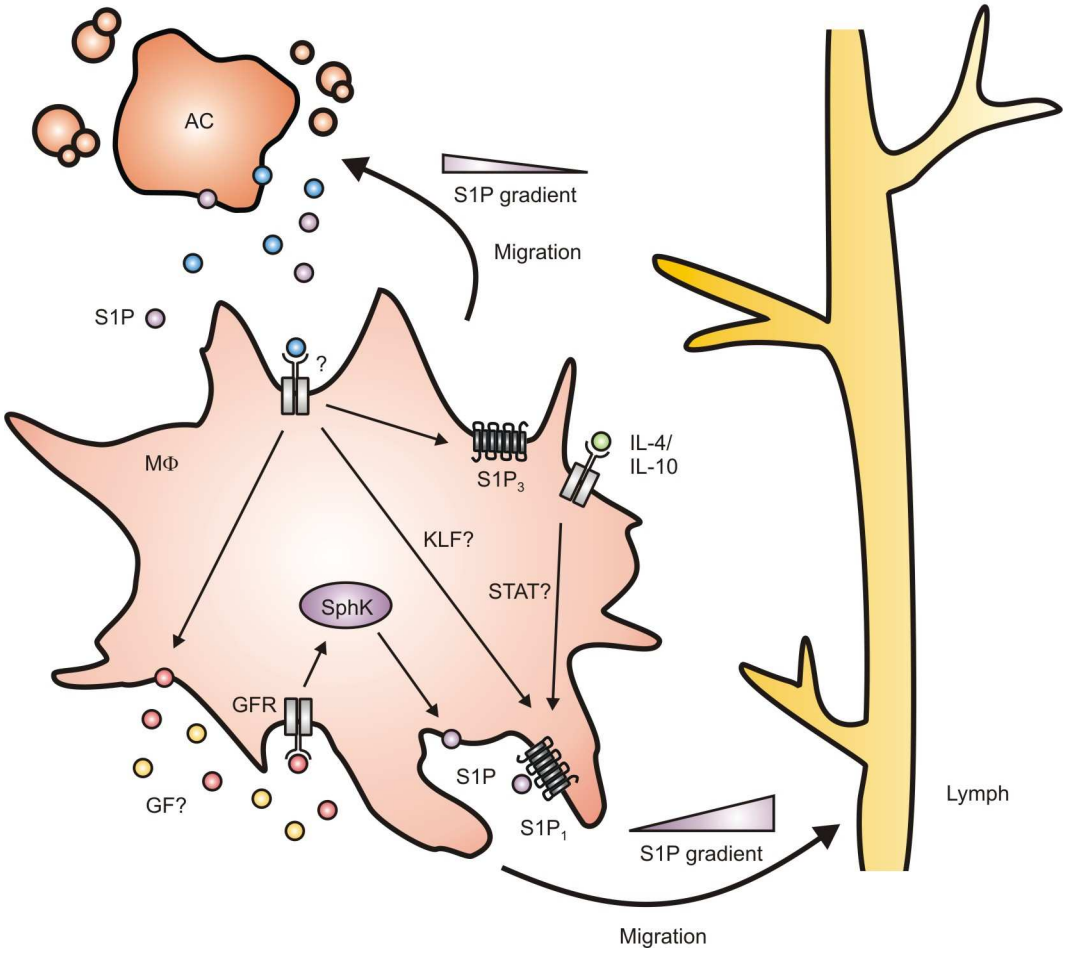

Figure 36. S1P receptor 1 is involved in migration of macrophages induced by apoptotic cell supernatants. S1P receptor 1 ($S1P_1$) and S1P receptor 3 ($S1P_3$) are upregulated in macrophages (MΦ) following incubation with supernatants from apoptotic cells (AC). One potential candidate, which is possible as transcription factor mediating $S1P_1$ induction is krüppel-like factor (KLF). $S1P_1$ is predominatly localized to pseudopodia-like structures of macrophages. Autocrine growth-factor (GF) signaling might cross-activate $S1P_1$ via SphK, which generates 'inside-out' acting S1P, to induce migration of macrophages into the lymph and towards AC along S1P gradients. This step could be crucial for the resolution of inflammation. Induction of $S1P_1$ by IL-4 or IL-10 might involve STAT signaling. Factors/pathways, whose identification require further studies, are marked with '?'.

Activation of $S1P_1$ and $S1P_3$ by S1P is generally associated with stimulation of migration, whereas activation of $S1P_2$ attenuates this process (263). These differences are predominantly referred to the divergent activation of the small GTPase Rac (263). Phosphorylation of $S1P_1$ was demonstrated to be essential for Rac activation, cortical actin assembly, lamellipodia formation and chemotaxis (217). $S1P_2$ and $S1P_3$ are also coupled to Rac signaling (112).

However, $S1P_2$ is considered to inhibit Rac as well as growth-factor induced chemotaxis (83). Rho activation occurs downstream of $S1P_2$ as well as $S1P_3$, but is considered to be more prominent downstream of $S1P_2$, where it markedly inhibits Rac activation (112). In contrast to Rac activation, Rho activation was associated with the assembly of contractile actin-myosin filaments, so-called stress fibers, and associated focal adhesion complexes and thus the inhibition of cell migration (264). These observations are also true for my studies. I observed formation of pseudopodia-like structures by macrophages following treatment with AC-CM. Interestingly, $S1P_1$ was located in these structures and thus on the cell surface. Consequentely, besides being upregulated, $S1P_1$ was located on the plasma membrane to be responsive to S1P and therefore to prepare macrophages for further signaling through S1P.

Gude *et al.* speculated that recruitment signal gradients sensed by $S1P_1$ might regulate phagocyte migration towards AC (36). Consequently, I pre-treated macrophages with AC-CM for 1 h, washed them with PBS and incubated macrophages in fresh medium with or without the addition of the $S1P_{1/3}$ antagonist VPC 23019 to avoid blocking of AC-CM-mediated autocrine signaling.

As S1P derived from AC-CM might rapidly be ingested by macrophages via receptor internalization and degraded by S1P lyase or S1P phosphohydrolase, it can be excluded as a crucial factor directly mediating migration of macrophages. However, also other AC-derived factors can be excluded to directly affect migration, as migration was inhibited by VPC 23019 after removal of AC-CM. Rather, AC-derived factors might prime macrophages for migration, which then could be mediated by the upregulation of $S1P_1$ and autocrine factors.

As a gradient was not present in my system, macrophages might show enhanced motility as a response to a growth factor contained in the culture medium. One potential candidate as a growth factor provoking migration of macrophages could be PDGF. Transactivation of $S1P_1$ by PDGF was shown to mediate motility of human embryonic kidney cells (216). This involved stimulation of SphK by PDGF. Intracellularly accumulated S1P was suggested to signal ‘inside-out’ to occupy its cell surface receptors and to modulate chemotaxis. Another study revealed that cytoskeletal rearrangements,

lamellipodia extensions, and cell motility induced by PDGF were abrogated in $S1P_1$ null fiboblasts (265). In this study, PDGF was shown to stimulate SphK, inducing translocation of SphK to membrane ruffles. Furthermore, immunoprecipitation experiments suggested possible protein interactions between the PDGF receptor and $S1P_1$ (266). Indeed, PDGF was upregulated in a whole genome array performed in human macrophages following treatment with AC-CM (unpublished data). Thus, PDGF could be secreted by macrophages and signal in an autocrine manner to transactivate $S1P_1$ and to induce migration of macrophages.

VEGF, which was secreted by macrophages after incubation with AC-CM, can also be taken into account as a possible factor being responsible for the migration of macrophages following treatment with AC-CM. There is evidence that VEGF can activate SphK1 (86). Nevertheless, it is still not known whether S1P, that is generated intracellularly due to VEGF, can occupy cell surface S1P receptors to regulate cell movement (86). Another potential factor for cross-activation might be insulin-like growth factor-1, which trans-activated $S1P_1$ through phosphorylation (217). However, it remains elusive, whether insulin-like growth factor-1 is also secreted by macrophages after treatment with AC-CM.

In an inflammatory environment, AC could induce $S1P_1$ expression, which might enable macrophages to migrate towards the vascular S1P gradient and thus to contribute to the resolution of inflammation by recruiting macrophages back into the lymph. According to the so-called 'S1P-$S1P_1$ control-of-lymphocytes model' this process could be ensured by constitutively open endothelial cell junctions (267). However, S1P secreted by AC could also serve as a chemoattractant to direct macrophages towards AC and thus to ensure efficient clearance of AC. Nevertheless, lymph levels of S1P are much higher compared to those in inflamed tissues. Thus, migration into the lymph might be dominant, although migration towards AC-derived S1P might simultaneously occur. In an inflammatory environment, $S1P_1$ will be internalized due to the continuous presence of AC and their associated production of S1P. However, as internalization in response to S1P is a reversible process (118) and furthermore also AC-derived factors are present to induce $S1P_1$ continuously, $S1P_1$ will nearly constantly be present on the cell surface.

Besides clarifying its role in cross-activation of $S1P_1$, investigating SphK expression and activity in macrophages after treatment with AC-CM would also be very interesting considering the phagocytic capacity of macrophages. Recently, it was indicated that regulatory macrophages, which were induced by TLR agonists and immune complexes, showed enhanced SphK1 expression (56). SphK1 was also reported to be recruited to phagosomes in human macrophages (89). In phagosomes, SphK1 can phosphorylate sphingosine, which is produced in the salvage pathway, by hydrolysis of membrane sphingomyelin. I observed that $S1P_1$ was located in pseudopodia-like structures of macrophages treated with AC-CM. However, $S1P_1$ could be internalized into phagosomes following phagocytosis of AC. Simultaneously, SphK1 expression and/or activity could be induced, which might provide high intraphagosomal levels of S1P. Ligation of $S1P_1$ could then enhance the phagocytosis rate of macrophages through activation of Rac, which is crucial during phagocytosis (1, 268), thus fostering the resolution of inflammation. In this case, VEGF could also have enhancing potential, as VEGF was also shown to stimulate SphK1 (86). Thus, S1P and VEGF seem not to be involved in the upregulation of $S1P_1$, but may be involved in enhancing the phagocytosis rate and migratory potential of macrophages.

Following incubation with LPS or IFN-γ, $S1P_1$ was not induced. This fits well to the observations provided by literature, as it was demonstrated that LPS mediated downregulation of $S1P_1$ in marginal zone B cells (269). Despite the potential of LPS to induce HO-1, migration of macrophages would be inhibited, further scutinizing that the macrophage phenotype induced by pro-inflammatory stimuli markly differers from the phenotype induced by AC-CM.

Investigating the phenomenon of enhanced migration after encounter of AC in vascular endothelial cells would also be very interesting. In a tumor environment there is a continuous existence of AC, which could secrete factors that foster migration of vascular endothelial cells directly or through the ‘macrophage relay’, and thus promote angiogenesis. $S1P_1$ is critically involved in endothelial cell biology, since $S1P_1$ knockout mice died *in utero* owing to vascular abnormalities that were caused by the defective migration of vascular smooth muscle cells and pericytes around nascent blood vessels (86).

Together, the induction of $S1P_1$ following treatment of macrophages with AC-CM was associated with enhanced motility of macrophages. This could be of importance in an inflammatory environment, in which AC could promote the migration of macrophages into the lymph, thus contributing to the resolution of inflammation.

6.3 Concluding remarks

$S1P_1$ can be considered as a central key player during alternative activation of macrophages by provoking increased HO-1 expression, thereby triggering its associated anti-apoptotic as well as anti-inflammatory signaling in response to AC-derived S1P. Furthermore, $S1P_1$ itself can be upregulated following treatment with supernatants from AC and thereupon enhance the migratory potential of macrophages.

This may be vital for the resolution of inflammation by eliciting anti-inflammatory responses of macrophages, which at the same time are protected against apoptosis and show enhanced migratory potential towards AC and the lymph. Thus, efficient clearance of AC and trafficking of macrophages out of the inflammatory environment is ensured. In this way, the presence of AC and/or other alternative macrophage activating stimuli prove to be vital, as classical macrophage activating factors elicite only a marginal anti-inflammatory response and are also not associated with increased $S1P_1$ expression.

The macrophage phenotype, which is obtained by interaction with AC, is characterized by anti-inflammatory signaling, which is mediated by Adora A_{2A} as well as the inhibition of pro-inflammatory cytokine production in response to LPS treatment (57). Furthermore, this phenotype shows a reduction of MHC II expression and enhanced arginase expression (70). Thus, this macrophage phenotype can be considered as a hybrid-type between alternatively activated wound-healing macrophages and regulatory macrophages. Since macrophage phenotypes are extremely versatile and depend on the exact composition of the respective microenvironment, a detailed understanding of the mechanisms of macrophage programming could provide therapeutic strategies to successfully

treat inflammatory disorders. In this regard, interference with $S1P_1$ and/or HO-1 induction could provide therapeutical benefits in controlling inflammation, autoimmunity, cancer and graft rejection.

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8 PUBLICATIONS

Papers

Weis, N., Weigert, A., von Knethen, A., and Brüne B., Heme Oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. Molecular Biology of the Cell, 2009. 20(5): p. 1280-1288.

Weigert, A., **Weis, N.**, and Brüne B., Regulation of macrophage function by sphingosine-1-phosphate. Immunobiology, 2009. 214(9-10): p. 748-760.

Weis, N., Weigert, A., and Brüne B., Apoptotic cell supernatants provoke enhanced migration of macrophages due to upregulation of sphingosine-1-phosphate receptor 1. In preparation.

Sola, A., Weigert, A., Jung, M., Vinuesa, E., Rosenberger, K., **Weis, N.**, Brüne, B., and Hotter, G., Sphingosine-1-phosphate signalling induces the production of neutrophil gelatinase-associated lipocalin from macrophages and promotes kidney regeneration. In preparation.

Von Knethen, A., Neb, H., Meilladec-Jullig, V., Schmidt, M.V., Kuhn, A.-M., **Weis, N.** and Brüne, B., ROS-driven PPARγ activation increased HO-1 mRNA stability in monocytes/macrophages. In preparation.

Meeting abstracts

Weis, N., Weigert, A., von Knethen, A., and Brüne B., The induction of an alternatively activated macrophage phenotype by apoptotic cell-derived sphingosine-1-phosphate partly depends on heme oxygenase 1. Leopoldina Symposium on Lipid Signalling (2008), Abstract book, p. 19.

Weis, N., Weigert, A., von Knethen, A., and Brüne B., Heme Oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. 23rd Annual Meeting of the European Macrophage & Dendritic Cell Society (2009), Abstract book, p. 67.

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03.2006	Diplom im Fach Biologie am Institut für Molekulare Zellbiologie der Technischen Universität Kaiserslautern mit dem Thema: „Charakterisierung anti-apoptotischer Faktoren aus apoptotischen Zellen“
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11.2009	Voraussichtliches Ende der Promotion

11 ERKLÄRUNG

Ich erkläre, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

“Role of Sphingosine-1-Phosphate Receptor 1 and Downstream Heme Oxygenase-1 Induction in Alternative Macrophage Activation Induced by Apoptotic Cells”

im Institut für Biochemie I - Pathobiochemie unter Betreuung und Anleitung von Prof. Dr. Bernhard Brüne mit Unterstützung durch Dr. Andreas Weigert ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Teile der vorliegenden Arbeit wurden im folgenden Publikationsorgan veröffentlicht:
Weis, N., Weigert, A., von Knethen, A., and Brüne B., Heme Oxygenase-1 contributes to an alternative macrophage activation profile induced by apoptotic cell supernatants. Molecular Biology of the Cell, 2009. 20(5): p.1280-1288.

Frankfurt am Main, den 30.09.2009