The Effects of Serum Albumin on Degradation and Metabolism of S1P

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List of Abbreviations and Acronyms

ANOVA	Analysis of variance
AP	Alkaline phosphatase
APCI	Atmospheric Pressure Chemical Ionization
Apo M	Apolipoprotein M
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
DMSO	Dimethyl sulfoxide
E	Embryonic day
EC	Endothelial cells
ESI	Electrospray Ionization
FACS	Fluorescence-activated cell sorting
FAFBSA	Fatty-acid-free bovine serum albumin
FCS	Fetal calf serum
HA	Hemagglutinin
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HSA	Human serum albumin
HUVEC	Primary human umbilical vein endothelial cells
ICAM	Intercellular cell adhesion molecule
Kd	Dissociation constant
LC/MS/MS	Liquid chromatography coupled to triple-quadrupole mass spectrometry
LPA	Lysophosphatidate
LPP	Lipid phosphate phosphatase
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
NF-κB	Nuclear factor “kappa-light-chain-enhancer” of activated B-cells
NMR	Nuclear magnetic resonance
NP	Para-nitrophenol
NPBSA	Non-protein part of bovine serum albumin

NPP	4-Nitropheenyl phosphate
OVA	Oval albumin
RBC	Red blood cells
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SA	Serum albumin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGPP	Sphingosine-1-phosphate phosphatase
SOFA	The Sequential Organ Failure Assessment score
SOV	Sodium orthovanadate
Sph	Sphingosine
SphK	Sphingosine kinase
TNFα	Tumour necrosis factor
VE	Vascular endothelial

1 Summary

Sphingosine-1-phosphate (S1P) is a lipid metabolite with multiple biological functions. The regulation of vascular permeability and control of immune cell trafficking are two elemental functions of S1P-S1P receptor (S1PR) signaling, which is related to the regulation of inflammation. The gradient of S1P concentration in plasma> lymph > tissue is essential for the maintenance of endothelial barrier function and other S1P-dependent processes such as leukocyte trafficking. Therefore, the modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. It is still largely unclear how S1P is bound to its chaperone serum albumin (SA) and how the SA influences the S1P-S1PR signaling axis. As one of the important chaperones for S1P, the effects of SA on degradation and metabolism of S1P were investigated.

Cell culture experiments, liquid chromatography coupled to triple-quadrupole mass spectrometry (LC/MS/MS), spectrometric analyses, fluorescence-activated cell sorting (FACS) and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to investigate the effects of bovine serum albumin (BSA) and human serum albumin (HSA) on degradation and metabolism of S1P. Nuclear magnetic resonance (NMR) spectrometry was applied to determine the structure and interaction of S1P and SA in solution,

After overnight incubation with (HTC4) wild type cells, S1P was protected from metabolism by BSA and HSA, but ovalbumin (OVA) did not provide such a protective effect. BSA was effectively hydrolyzed by pepsin. The pepsinized BSA and the non-protein part of BSA (NPBSA) cold not protect S1P from the metabolism of HTC4 cells. Serum albumin (SA) inhibited the extracellular dephosphorylation of S1P but did not influence the uptake of sphigosine (Sph) by HTC4 cells. BSA and HSA increased S1P induced S1PR1 internalization after overnight incubation with HTC4 cells, but OVA did not. Meanwhile, in cell-free assay, BSA and HSA could inhibit S1P from dephosphorylation by alkaline phosphatase (AP). Fatty-acid-free BSA (FAFBSA) could prevent dephosphorylation of 4-nitrophenyl phosphate (NPP) by AP, but not the regular BSA used in all the experiments before. NMR spectrometry suggests that SA can bind S1P.

Our data suggest that the binding of SA to S1P enables SA to protect S1P from the metabolism of cells and the dephosphorylation of the phosphatases.

Zusammenfassung

Sphingosin-1-phosphat (S1P) ist ein Lipidmetabolit mit vielfältigen biologischen Funktionen. Die Regulation der vaskulären Permeabilität und die Kontrolle des Immunzelltransports sind zwei elementare Funktionen des S1P-S1P-Rezeptor (S1PR)-Signals, das mit der Entzündungsregulation in Zusammenhang steht. Der Gradient der S1P-Konzentration in Plasma> Lymph> Gewebe ist für die Aufrechterhaltung der endothelialen Barrierefunktion und anderer S1P-abhängiger Prozesse wie den Leukozytentransport essentiell. Daher ist die Modulation der S1P-Degradation ein kritisches Ereignis für den S1P-S1PR-Signalweg. Es ist noch weitgehend unklar, wie S1P an sein Chaperon-Serumalbumin (SA) gebunden wird und wie das SA die S1P-S1PR-Signalachse beeinflusst. Als eines der wichtigen Chaperone für S1P wurden die Auswirkungen von SA auf Abbau und Metabolismus von S1P untersucht.

Zellkulturexperimente, Flüssigchromatographie gekoppelt mit Triple-Quadrupol-Massenspektrometrie (LC/MS/MS), spektrometrische Analysen, fluoreszenzaktiviertes Zellsortierung (FACS) und Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese (SDS-PAGE) wurden verwendet, um die Auswirkungen von Rinderserumalbumin (BSA) und Humanserumalbumin (HSA) auf Abbau und Metabolismus von S1P zu untersuchen. Die Kernspinresonanzspektrometrie (NMR) wurde angewandt, um die Struktur und die Wechselwirkung von S1P und SA in Lösung zu bestimmen,

Nach nächtlicher Inkubation mit (HTC4)-Wildtypzellen war S1P durch BSA und HSA vor dem Metabolismus geschützt, ovariales Albumin (OVA) jedoch nicht. BSA wurde durch Pepsin wirksam hydrolysiert. Das pepsinisierte BSA und der Nicht-Protein-Teil des BSA (NPBSA) schützten das S1P nicht vor dem Metabolismus von HTC4-Zellen. Serumalbumin (SA) hemmte die extrazelluläre Dephosphorylierung von S1P, beeinflusste aber nicht die Aufnahme von Sphingosin (Sph) durch HTC_4-Zellen. BSA und HSA erhöhten die S1P-induzierte S1PR1-Internalisierung nach nächtlicher Inkubation mit HTC4-Zellen, OVA jedoch nicht. In der Zwischenzeit konnten BSA und HSA im zellfreien Assay die Dephosphorylierung von S1P durch alkalische Phosphatase (AP) hemmen. Fettsäurefreies BSA (FAFBSA) konnte die Dephosphorylierung von 4-Nitrophenylphosphat (NPP) durch AP verhindern, aber nicht das reguläre BSA, das in allen bisherigen Experimenten verwendet wurde.

Unsere Daten legen nahe, dass die Bindung von SA an S1P es SA ermöglicht, S1P vor dem Metabolismus der Zellen und der Dephosphorylierung der Phosphatasen zu schützen.

2 Introduction

2.1 Sphingosine-1-phosphate (S1P)

S1P is a lipid metabolite which has multiple biological functions including regulation of cell survival, migration, as well as the control of immune cell trafficking, angiogenesis and vascular integrity by activating 5 G-protein coupled S1P receptors (S1PR1-5) (Kunkel et al.2013). S1P belongs to sphingolipids which can be metabolized by almost all cells to build unites of cellular membranes and function as signaling mediators (Obinata and Hla 2019). Prior research confirms that serum albumin (SA), one of the S1P chaperones, can enhance S1PR signaling of S1P (Wilkerson et al. 2012). The molecular mechanism of this S1PR signaling enhancing effect of SA remains unclear. It was proposed that SA functions as a facilitator of lipid solubility for S1P. According to the idea that SA increased the availability of S1P to S1PRs, one would assume that SA could also boost the dephosphorylation and metabolism of S1P by S1P-phophatases. Therefore, it is of great interests to study the effects of SA on the dephosphorylation and metabolism of S1P by S1P-phosphatases.

2.1.1 The metabolism of S1P

S1P is intracellularly produced from the phosphorylation of sphingosine (Sph) by two isoforms of sphingosine kinase (SphK): sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) (Kunkel et al.2013). Sphingosine is derived from the deacylation of ceramide (generated from de novo synthesis pathway starting from serine and palmitoyl-CoA or alternatively from degradation of sphingolipids, such as sphingomyelin and glucosylceramide) by ceramidase (Obinata and Hla 2019). S1P can be either cleaved irreversibly by S1P lyase, which produces phosphoethanolamine and hexadecenal, or be dephosphorylated back to sphingosine by S1P phosphatase or lipid phosphatases (Grassi et al. 2019).

2.1.2 The vascular gradient of S1P

The concentration of S1P in different body compartments is different. S1P is more enriched in plasma (around 1μM) and in lymph (around 0.1μM) than in interstitial fluid of tissues (lower than 1 nM), which constitutes a sharp S1P gradient across endothelial barrier (Hla et al. 2008).

The activity of sphingosine kinases, the S1P-lyase, and S1P phosphatases as well as cellular uptake, storage and release of S1P are involved in S1P gradient maintenance.

Recent studies suggested that red blood cells (RBC) and endothelial cells (EC) are the main sources of S1P in blood plasma (Bode et al. 2010) (Andréani and Gräler 2010). Deletion of both SphK1 and SphK2 leads to total loss of S1P in plasma, and it was shown that transfer of wild-type RBC to a mouse strain lacking plasma S1P by conditional gene ablation of SK1 and SK2 restored S1P in plasma to the normal levels (Camerer et al. 2009). 30% decrease of S1P levels in plasma can be found in EC-specific SphK1 and SphK2 double knockout mice (Xiong 2014). Mice that lack platelets have normal S1P level in plasma and S1P release is increased by platelets activation. Additionally, serum S1P level is slightly higher than those in plasma (Hla et al. 2008). In human, S1P is present in plasma at concentration from 0.59 μM to 0.91 μM and in serum at concentration from 0.80 μM to 1.28 μM. S1P levels in human plasma are positively correlated with RBC counts but not platelets counts (Daum et al. 2020). These data show that platelets could not contribute to normal plasma S1P level in homeostasis, but it may participate in enhancement of plasma S1P level under non-homeostatic or its activated conditions.

SphK1 knockout RBC contain very little S1P, which shows that S1P in RBC is mainly produced by SphK1. SphK2 knockout mice have increased S1P and Sph in RBC (Urtz et al. 2015), which could be caused by the substrate (Sph) redistribution to SphK1 during inactivation of SphK2.

S1P is extracellularly hydrolyzed by phosphatases (Peest et al. 2008). Exogenously added S1P is cleared from blood in 15–30 min in vivo, and this process depends on the activity of cellular phosphatases but not on the S1P-lyase (Peest et al. 2008). The S1P concentration in blood ex vivo is stable for hours, which suggests that another tissue or organ removes S1P from blood. Liver is identified as a predominant place for clearance of S1P from plasma (Kharel et al. 2020).

2.1.3 The release of S1P in to plasma

RBC have high SpK activity which is able to synthesis S1P from sphingosine, but they lack the S1P-Lyase and S1P phosphatases which are S1P degrading enzyme systems (Ito et al. 2007) (Yang et al. 1999). Recently it has been found that erythrocytes express the specific S1P transporter Mfsd2b and Mfsd2b knockout mice have 42-54% less S1P levels in plasma compared with wild-type mice (Vu et al. 2017). Spns2 is a S1P transporter in EC and the

deficiency of Spns2 caused a drastic reduction in lymph S1P, but only around 23% decrease in plasma S1P (Mendoza et al. 2012). Shear stress can also stimulate S1P release from EC in vitro (Venkataraman et al. 2008).

2.1.4 The phosphatases

Phosphatases are critical for maintaining the vascular gradient of S1P. S1P is intracellularly dephosphorylated to sphingosine by specific S1P phosphatases (SGPP1 and SGPP2) which are localized in endoplasmic reticulum (ER) (Le Stunff et al. 2002). S1P can also be extracellularly hydrolyzed by lipid phosphate phosphatases LPP1, LPP2, and LPP3 (Bréart et al. 2011).

As mentioned in 2.1.2, exogenously added S1P is cleared from blood in 15–30 min in vivo, and this process depends on the activity of cellular phosphatases, but not on the S1P-lyasis (Peest et al. 2008). This shows that the LPPs could be essential regulators of plasma S1P concentrations.

LPPs are characterized as Mg^{2+}-independent phosphatases and can phosphohydrolyze a wide variety of lipid phosphates such as lysophosphatidate (LPA), S1P, ceramide 1-phosphate, diacylglycerol pyrophosphate and N-oleoylethanolamine phosphate (Tang et al. 2015). Mammalian LPP1, LPP2 and LPP3 are respectively encoded by three separate genes PPAP2A, PPAP2C and PPAP2B. The LPPs have six transmembrane α-helices domains and if it is located in the plasma membrane, the three catalytic domains are towards the luminal side and the C- and N-termini face the cytoplasmic side (Tang et al. 2015).

Detailed knowledge about how phosphatases contribute to the S1P gradient and which phosphatase is more important is still unknown. LPP3 was shown to be involved in the regulation of S1P gradient in the thymus and brain (Bréart et al. 2011) (López-Juárez et al. 2011). Furthermore, FTY720-phosphate (a S1P analog) is primarily dephosphorylated by LPP3 (Mechtcheriakova et al. 2007). Increased S1P level in blood and plasma was caused by the deletion of LPP3 expression in mice liver, which evidences that liver is a predominant place for the clearance of plasma-S1P (Kharel et al. 2020).

2.2 The receptors of S1P

S1P has pleiotropic biological functions by activating 5 G-protein coupled receptors (S1PR1-5) (Kunkel et al.2013). S1P can bind S1PR1-5 with the dissociation constants (Kd) within

2±30 nM (Hla et al. 2008). It activates these receptors which can transmit intracellular signals relying on the different coupled subunits of heterotrimeric G protein and the expression pattern of different receptors in different cells and tissues to induce various biological functions (Hla et al. 2008). S1PR1, S1PR2, and S1PR3 are highly expressed in cardiovascular and immune systems. They are widely distributed in brain, heart, lung and spleen, etc. S1PR4 and S1PR5 are relatively rare and limited to expression in lymphoid tissues and brain, respectively. The Gi/o alpha subunit of heterotrimeric G proteins is coupled with S1PR1. Gi/o and G12/13 are coupled with S1PR2, S1PR3, S1PR4 and S1PR5. Gq is coupled with S1PR2 and S1PR3 (Obinata and Hla 2019).

2.3 The pathophysiology of plasma S1P in inflammation

The regulation of vascular permeability and control of immune cell trafficking are two elemental functions of S1P-S1P receptor signaling related to the regulation of inflammation (Obinata and Hla 2019). S1P deficiency in blood causes severe leakage of the vasculature during development and in adult mice challenged with anaphylactic shock (Xiong et al. 2014) (Camerer et al. 2009). RBC-specific deletion of Sphk1 and Sphk2 in mice led to embryonic lethality between the embryonic day (E) 11.5 and E12.5 due to defects in vascular development (Xiong et al. 2014). S1PR1was indicated to play a pivotal role in maturation of vascular system, with S1PR2 and S1PR3 supportive or/and redundant roles (Obinata and Hla 2019). Selectively deficient S1P in plasma in mutant mice caused vascular leak and reduced survival after platelet-activating factor (PAF) or histamine induced anaphylaxis (Camerer et al. 2009). Rearrangements of cytoskeleton and assembly of adherent junctions in endothelial cells are elemental events to maintain vascular integrity. S1P can cause reorganization of the actin cytoskeleton and accumulation of vascular endothelial (VE)-cadherin and α-, β- and γ-catenin to the sites of cell–cell contact connected with adherent junction assembly (Obinata and Hla 2019).

Activation of small G proteins Rac and Rho downstream of the S1PR1 and S1PR3 signaling pathways are involved in the processes (Lee et al. 1999) (Garcia et al. 2001). While blood-borne S1P is a critical requirement for a functional vascular endothelial barrier, the regulatory pathways underlying its maintenance are still largely unknown.

S1P-S1PR1 signaling regulates the egress of lymphocytes from thymus and peripheral lymphoid organs (Obinata and Hla 2019). When S1PR1-deficient mature lymphocytes are

adoptively transferred into wild-type mice, they enter but cannot egress from peripheral lymphoid organs (Matloubian et al. 2004). The enrichment of S1P in blood and lymph compartment and low S1P levels in the interstitial fluid are fundamentally important for immune cell trafficking.

Disruption of the S1P gradient either by inhibition of SK 1/2 (responsible for S1P production) or by inhibition of S1P lyase (responsible for S1P degradation) leads to lymphopenia due to the loss of the lymphocyte egress (Pappu et al. 2007) (Schwab et al. 2005). The S1PR1 on the surface of lymphocytes easily gets internalized when bathing in the plasma with high concentration of S1P. The internalization/desensitization of S1PR1 is critical for circulating lymphocytes to enter lymphoid organs against the S1P gradient (Obinata and Hla 2019).

2.3.1 S1P and sepsis

In 2016, sepsis was defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection" and septic shock is termed as "a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality" by the Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine (ESICM) (Singer et al. 2016). Organ dysfunction was identified as an acute change in the Sequential Organ Failure Assessment score (SOFA) higher than 2 points consequent to the infection. SOFA score can estimate the neurological, pulmonary, cardiovascular, renal, haematological and hepatic dysfunctions of the patients (Singer et al. 2016). Septic shock was diagnosed with "sepsis with persisting hypotension requiring vasopressors to maintain mean arterial pressure higher than 65 mmHg and having a serum lactate level greater than 2 mmol/L (18 mg/dL) despite adequate volume resuscitation with the hospital mortality in excess of 40%" (Singer et al. 2016).

In 2017, it was estimated that around 48.9 million incident cases of sepsis were recorded around the globe and about 11.0 million sepsis-related deaths were reported, representing approximately 19.7% of all global deaths (Rudd et al. 2020). It was also reported that the age-standardized sepsis incidence reduced by about 37.0% and mortality fell by around 52.8% from 1990 to 2017. Whether the mortality from septic shock is on the descent is less clear at the moment.

Dysfunction of the vascular endothelial barrier is a major cause of cardiovascular collapse and resulting hypoperfusion, which is a hallmark of septic shock. The leaky capillary membranes

create massive loss of intravascular proteins, such as, albumin, and plasma fluids into the extravascular space, which is clinically known as edema and results in tissue and cellular hypoxia. Microcirculatory vasodilation changes capillary blood flow, which contributes to poor tissue perfusion and — ultimately — shock. Therefore, endothelial dysfunction is the key pathogenesis of sepsis and septic shock (Hotchkiss et al. 2016).

As mentioned before, S1P receptors are involved in the regulation of various physiological and pathophysiological phenomena. The endothelial dysfunction as a result of increased vascular permeability, increased pro-coagulant responses and increased pro-inflammatory pathways, which are able to be regulated and influenced by S1P, plays a crucial role in the organ failure caused by sepsis.

As mentioned in 2.3, the barrier enhancement is predominantly induced by S1PR1 (Wilkerson and Argraves 2014). The small GTPases Rac and Rho both of which are controlled by S1P are the key regulators of barrier function. S1P receptor activation was shown to enhance pulmonary barrier integrity in animal models of acute lung injury and acute respiratory distress syndrome and in the kidneys and myocardium after ischemia-reperfusion injury, and to enhance blood–brain barrier protection during experimental autoimmune encephalomyelitis (Wilkerson and Argraves 2014). The S1P pathway was shown to enhance the lymphatic endothelial barrier integrity in lymph nodes, leading to sequestration of lymphocytes in the lymph node. S1PR1 has been attributed anti-inflammatory properties regarding reduced expression of cell adhesion molecule (CAM) in EC and the release of pro-inflammatory cytokines (Wilkerson and Argraves 2014). S1PR1-dependent genes can provide gene signatures for predicting survival of sepsis patients (Feng et al. 2020).

FTY720 treatment can reduce levels of Evans blue leakage from blood into liver and lung in septic mice. The sphingosine analog FTY720 induces barrier protection in vivo, but it also down-regulates S1PR1 and acts as a functional antagonist of this receptor. The reduced left ventricular systolic contractility in experimental sepsis is attenuated by FTY720 treatment and activation of S1PR2 and the subsequent activation of the phosphoinositide 3-kinases (PI3K)-Akt survival pathway significantly contributes to the heart protective effect of FTY720 (Coldewey et al. 2016). Moreover, S1P deficiency in blood causes severe leakage of the vasculature during development and in adult mice challenged with anaphylactic shock (Oliver et al. 2010).

Autonomous Secretion of Sphingosine 1-Phosphate by Spns2 is also disrupted in inflammation and Spns2 is down-regulated in primary human umbilical vein endothelial cells (HUVEC) in inflammation condition, which contributed to destabilization of the EC barrier (Jeya Paul et al. 2020).

S1P is a potential sepsis biomarker. In sepsis, S1P levels in serum are dramatically decreased, especially in septic shock patients, and are inversely associated with SOFA scores or disease severity. S1P concentrations in serum can predict septic shock similar to SOFA score and with higher accuracy compared with IL-6, procalcitonin, lactate and C-reactive protein (Winkler et al. 2015).

While blood-borne S1P is a critical requirement for a functional vascular endothelial barrier, the regulatory pathways underlying its maintenance are largely unknown.

2.4 The chaperones of S1P in plasma

S1P is hydrophobic and it needs carrier proteins for circulation and transport in plasma. It is still largely unknown how S1P is bond to its chaperones and how the chaperones influence the S1P signaling. Around 54% S1P can be found in the high-density lipoprotein (HDL) fraction and about 35% S1P in the serum albumin fraction and much smaller composition in other lipoprotein fraction (Murata et al. 2000).

2.4.1 Serum albumin (SA)

SA, synthesized by the liver, is the most abundant blood protein in mammals.

The total body albumin pool is about 3.5–5.0 g/kg body weight. 42% of the pool is in the plasma compartment and the rest exists in extravascular compartment. In human, it comprises 50% protein content in plasma. The normal SA values of human are approximately 35–50 g/L (3.5–5.0 g/dL). SA can bind to its receptor, albondin (gp60), which was widely distributed on endothelial cells, and be discharged on the interstitial side (Nicholson et al. 2000). The Fc Receptor for IgG (FcRn) was also reported to bind SA, reduce its degradation in lysosomes and prolong its lifespan in vivo (Chaudhury et al. 2003).

In heathy young adult, albumin is synthesized around 194 mg/kg (body weight)/day in liver (Peterset 1996). Diseases and nutritional states can change the rate of albumin synthesis. The most synthetic machinery of hepatocytes is already used for SA at rest, so that the liver can only

increase albumin synthesis 2–2.7 times normal (Nicholson et al. 2000). The colloid osmotic pressure (COP) of the interstitial fluid around the liver cells is the most crucial regulating factor of SA synthesis (Nicholson et al. 2000).

Albumin is derived from pre-albumin which exists in hepatocytes. Albumin synthesis takes around 30 mins (Artigas et al. 2016). The turnover time of albumin in heathy humans is 25 days and the halftime of albumin is 17.3 days, which is balanced by liver synthesis (as mentioned before), renal (around 6%), gastrointestinal (around 10%) and catabolic (around 84%) clearance (Levitt and Levitt 2016). The total daily albumin degradation rate is around 5% of daily whole-protein turnover (Nicholson et al. 2000). Albumin degradation occurs in most organs. Free amino acids, which are added to the pool of amino acids within cells and plasma, are the final breakdown products of albumin (Nicholson et al. 2000).

2.4.1.1 The structure of SA

SA is a heart-shaped molecule in tertiary structure which consists of 585 amino acids with a molecular weight of 66.5 kDa (kilodaltons) (Nicholson et al. 2000). SA is abundant in charged residues, such as lysine, arginine, glutamic acid and aspartic acid and has a scarcity of tryptophan and methionine. It is ellipsoid in solution. Human serum albumin (HSA) has 35 cysteine residues and 34 of them form 17 disulfide bridges. The circulating molecule which is held and folded by 17 disulfide bridges, consists of a series of α-helices. The protein consists of 3 homologous domains (I-III). Each domain is composed of 2 sub-domains (A and B) and each sub-domain contains 4 and 6 α-helices respectively (Quinlan et al. 2005). This molecule is very flexible and its shape can vary with the changes of the environment and with the binding with endogenous and exogenous compounds (Peterset 1996). The denaturation of SA occurs in conditions where dramatic and non-physiological variances take place. The resiliency of the structure can enable it to regain the regular structure after the rupture because the disulfide bridges can be reestablished (Carter and Ho 1994). It also contains one free thiol (-SH) group (Cys-34), which is derived from the remaining cysteine residue.

2.4.1.2 The functions of SA

The most important function of SA is to regulate the oncotic pressure in the blood, because it constitutes 75%–80% normal plasma colloid oncotic pressure (Nicholson et al. 2000). The direct osmotic effect consists of 60% of the oncotic pressure of SA. The other 40% is provided

by Gibbs-Donnan effect that negatively charged albumin does not bind but holds sodium ions in its field (Levitt and Levitt 2016). Albumin can also bind to different endogenous and exogenous substances and has great influences on their distribution, metabolism, physiological or pharmacological actions and excretion. Examples of the compounds which can be bound by albumin are hydrophobic organic anions such as bilirubin, long-chain fatty acid, divalent cations, a wide variety of drugs, bile acid (Levitt and Levitt 2016).

Albumin provides also more than 50% of the total antioxidant of normal plasma, which could result from the abundance of sulfhydryl (-SH) groups of the albumin molecule (Nicholson et al. 2000). These are important scavengers of oxygen-free radicals.

2.4.1.3 SA and sepsis

SA level is associated with mortality from cardiovascular diseases, cancer and strongly correlated with surgical mortality (Phillips et al. 1989) (Levitt and Levitt 2016). The SA level on admission is an important prognostic indicator. Among patients in hospital, severer hypoalbuminemia correlates with an increased risk of short- and long-term morbidity and mortality (Amit et al. 2017) (Touma and Bisharat 2019). Lower serum albumin levels are associated with higher chances of abdominal/pelvic sources of infection, acute kidney or liver dysfunction, septic shock, and higher Acute Physical and Chronic Health Evaluation II (APACHE II) and SOFA scores. The 28-day survival rate of septic patients SA below 29.2 g/L was lower than that of the septic patients with SA at or above this level (Yin et al. 2018).

Circulatory dysfunction or shock correlated with a high mortality and characterized by hemodynamic changes that result in disrupted tissue perfusion. Fluid resuscitation is one of the most frequent medical interventions applied at the clinical bedside to enhance tissue perfusion, especially in septic shock (Vincent et al. 2016). Although there are still drastic debates about the best solution for fluid resuscitation, SA is suggested to treat septic patients when substantial amounts of crystalloids alone are insufficient (Rhodes et al. 2016).

2.4.2 High-density lipoprotein (HDL)

Lipoproteins are complicated particles composed of various proteins which transport all lipids around the body in blood circulation. HDL, one of five subtypes of lipoproteins, can protect against atherosclerosis (Rosenson et al. 2016). Lipoprotein typically consists of 80–100 proteins per particle and transporting up to hundreds of lipid molecules per particle.

Although the proteomics of HDL is very complex, the most majority of HDL particles contain apolipoprotein A-I (apoAI), which is the most abundant apolipoprotein in normal human plasma (Fisher et al. 2012). The HDL-cholesterol (HDL-C) level, i.e. the cholesterol associated with ApoA-1/HDL particles, is a substituted parameter for the direct and costly measurement of HDL value (AHA 1999). HDL-C that falls within the range of 0.40 to 0.59 g/L is considered normal.

Apolipoprotein M (Apo M) is a lipocalin which can be found mostly in HDL fraction in plasma. In HDL, S1P is mostly bind to ApoM and others binding partner of S1P in HDL may also exist. Apolipoprotein A4 has been recently identified as a S1P chaperone in ApoM and albumin deficiency mice (Obinata et al. 2019).

2.4.3 Chaperone-related S1P signaling

SA was postulated as a facilitator of lipid solubility for S1P and a minor chaperone for S1P-S1PR signaling axis compared with ApoM in HDL. The molecular mechanism of the functional differences between the albumin-S1P and ApoM-S1P remains to be cleared.

ApoM-S1P has been recently shown to be more dominant than albumin-S1P in chaperone-related S1P signaling. In endothelial culture system, HDL-S1P increased trans-endothelial electric resistance longer than albumin-S1P (Wilkerson et al. 2012). Exposed to ApoM+HDL-S1P, but not to albumin-S1P, HUVECs were shown to have the formation of a cell surface S1P1–β-arrestin 2 complex and the enhanced ability of the pro-inflammatory cytokine tumour necrosis factor (TNFα) to activate nuclear factor “kappa-light-chain-enhancer” of activated B-cells (NF-κB) and increased intercellular cell adhesion molecule-1 (ICAM-1) abundance. S1P bound to either chaperones could induce mitogen-activated protein kinase (MAPK) activation, but albumin-S1P triggered more Gi activation and S1PR1 receptor internalization (Galvani et al. 2015). ApoM-bound S1P, but not albumin-bound S1P regulated or inhibited lymphopoiesis in vitro (Blaho et al. 2015). ApoM-S1P seems to be more dominant than albumin-S1P in chaperone-related S1P signaling. Although there are only limited data concerning the functional and structural role of SA on S1P-S1PR axis, the researchers of molecular biology or sphingolipidologists incline to focus on ApoM-S1P, rather than albumin-S1P.

2.5 The aim of this thesis

The regulation of vascular permeability and control of immune cell trafficking are two elemental functions of S1P-S1P receptor signaling related to the regulation of inflammation. The gradient of S1P concentration in plasma> lymph> tissue is essential for the maintenance of endothelial barrier function and other S1P-dependent processes such as leukocyte trafficking. The activity of sphingosine kinases, the S1P-lyase, and S1P phosphatases as well as cellular uptake, storage and release of S1P are involved in S1P gradient maintenance. Therefore, the modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. It is still largely unknown how S1P is bound to its chaperone SA and how the SA influences the S1P-S1PR signaling axis. As one of the important chaperones for S1P, the effects of serum albumin on degradation and metabolism of S1P were investigated. To determine the molecular mechanism, incubations with the S1P-metabolizing alkaline phosphatase and cells, different spectrometric analyses for structural analyses and determinations of S1P-serum albumin-interactions were investigated.

3 Material and Methods

3.1 List of materials

3.1.1 Equipment

2 x 60 mm MultoHigh C18-RP column, 3 µm particle size	CS-Chromatographie Service GmbH, Langerwehe, Germany
Balance (max. 250 g)	PCB-Kern und Sohn GmbH, Balingen-Frommern, Germany
Balance AE124 (max. 120 g)	Sartorius AG, Göttingen, Deutschland
Binary pump 1100 series HPLC system	Hewlett Packard/Agilent, Waldbronn, Germany
Biosafety Cabinets, HS-12	Heraeus Group, Hanau, Germany
Centrifuge Heraeus Biofuge 15R	Heraeus Group, Hanau, Germany
Centrifuge Heraeus Megafuge 16	Heraeus Group, Hanau, Germany
Centrifuge Heraeus Varifuge 3.0R	Heraeus Group, Hanau, Germany
Centrifuge Hermle Z 233 MK-2	Hermle AG, Gosheim, Germany
Counting chamber type Neubaur	Hecht GmbH & Co KG, Sondheim vor der Rhön, Germany
Flowcytometer BD Accuri C6 plus	BD Franklin Lakes, USA
Freezer (-20°C) FS 18032	Quelle, Fürth, Deutschland
Freezer (-20°C) spezial 432	Kirsch, Willstätt-Sand, Germany
Freezer (-80°C) KM-DU53YE	Panasonic Corporation, Kadoma, Japan
Fridge (4°C) FKS 5000	Liebherr Group, Bulle, Switzerland
Fridge(4°C) UKS 5000	Liebherr Group, Bulle, Switzerland
LC/MS/MS system 2000 QTrap	AB Sciex, Darmstadt, Germany
Microplate Photometer, Multiskan FC Type 357	Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA
Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories, California, United States
pH-Meter	Mettler-Toledo GmbH, Gießen, Germany
PowerPac Basic Power Supply	Bio-Rad Laboratories, California, United States

RVC 2-25 CD plus vacuum rotator	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz
Spectrophotometer RF-5301 PC	Shimazu Corporation, Nakagyo-ku, Kyoto, Japan
Volumn pipets, adjustable	Eppendorf AG, Hamburg, Germany
Vortex G560E	Scientific Industries, Inc, New York , USA
Vortex VX2500	VWR International, Radnor, PA, USA
Water bath GFL 1083	GFL, Burgwedel, Germany

3.1.2 Consumables

6-well plate (for adherent cells)	Sarstedt, Nümbrecht, Germany
96-well plate (for inadherent cells)	Sarstedt, Nümbrecht, Germany
96-well plate (for adherent cells)	Sarstedt, Nümbrecht, Germany
96-well plate, U-Form	Sarstedt, Nümbrecht, Germany
Biosphere filter tips (various types)	Sarstedt, Nümbrecht, Germany
Cannula, 100 Sterican, 27 G	Braun, Melsungen, Germany
Cannula, BD microlane 3, 24 G	BD, Franklin Lakes, New Jersey, USA
Cellstar tubes, 15 ml	Greiner Bio One GmbH, Frickenhausen, Germany
Cellstar tubes, 50 ml	Greiner Bio One GmbH, Frickenhausen, Germany
Elisa micro-test plates 96-well C	Sarstedt, Nümbrecht, Germany
FACS-tubes	BD Falcon, Franklin Lakes, USA
Glass Pasteur pipettes	NeoLab, Heidelberg, Germany
Pipette tips (various types)	Starlab GmbH, Hamburg, Germany
Pyrex® glass centrifuge tubes	VWR International, Darmstadt, Germany
Reaction tubes, 1.5 ml	Greiner Bio One GmbH, Frickenhausen, Germany
Reaction tubes, 2 ml	Greiner Bio One GmbH, Frickenhausen, Germany
Reaction tubes, 5 ml	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Safe lock tubes, 0.5 ml	Eppendorf AG, Hamburg, Germany
Safe lock tubes, 1.5 ml	Eppendorf AG, Hamburg, Germany

Safe lock tubes, 5 ml	Eppendorf AG, Hamburg, Germany

3.1.3 Chemicals

4-Nitrophenyl phosphate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Albumin Faction V, biotin-free (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Albumin Human lyophilized	SERVA Electrophoresis GmbH, Heidelberg, Germany
Albumin, Bovine serum, Faction V, Fatty acid free (FAFBSA)	EMD Milipore Corp. Bilerica, M.A., USA
Alkaline Phosphatase (AP) (98000 U/ml)	Santa Cruz Biotechnology, Dallas, USA
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ATP	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Biotase Protease/EDTA solution 0,0042%/ 0,02% (w/v) in PBS w/o Ca^{2+} w/o Mg^{2+}	Biochrom, Berlin, Germany
Calcium chloride ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Diethanolamine	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Fetal calf serum (FCS)	Bionchrom AG, Berlin, Germany
FTY720-P	Biomol GmbH, Hamburg, Germany
Glucose	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
HBSS + Ca^{2+}	Life Technologies GmbH, Darmstadt, Germany

Hydrogen Chloride (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MEM	Biochrom, Berlin, Germany
Methanol	VWR International GmbH, Darmstadt, Germany
No-essential amino acid (NEA)	Biochrom, Berlin, Germany
Penicillin/Streptomycin, 10 000 U/ml / 10 000 µg/ml	Biochrom, Berlin, Germany
Pepsin, porcine stomach powder	Alfa Aesar, Massachusetts, USA
Potassium Chloride (KCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ROTIPHORESE®Gel 40 (29:1)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
S1P	Sigma-Aldrich Chemie GmbH, Hamburg, Germany
Sodium Bicarbonate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium Carbonate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium Hydroxide	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium orthovanadate (SOV)	Sigma-Aldrich Chemie GmbH, Hamburg, Germany
Sodium Pyruvate, 100 mM	Biochrom, Berlin, Germany
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
TMB solution	eBioscience Inc., San Diego, USA

Tris	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris-HCl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

3.1.4 Buffer and solutions

Buffer and solutions	Formula	Usage
Substrate solution	1mM 4-Nitrophenyl phosphate	pNPP assay
Diluent buffer	1M Diethanolamine, 0.5 mM $MgCl_2$, pH 9.8	
Enzyme solution	1u/ml Alkaline Phosphatase	
10x Electrophoresis buffer	250 mM Tris, 2M Glycin, 35 mM SDS, pH 8.7	SDS-PAGE Electrophoresis
Separating buffer	2 M Tris, pH 8.8	
Upper gel buffer	0.5 M Tris, pH 6.8	
RBC Buffer	150 mM NaCl, 5 mM KCl, 21 mM Tris-HCl, 5 mM Glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4	RBC Incubation
PBS	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH 7.4	Cell culture
FACS-Buffer	5% (v/v) FCS, PBS	FACS tests
Imperial™ Protein Stain	Not indicated (Prod # 24615, Thermo Fisher Scientific, Massachusetts, USA)	Coomassie dye R-250 staining

3.1.5 Antibodies for Flow Cytometry

Primary antibody	Manufacturer
Rat α-HA (RG1 3F10), Hybridoma supernatant	Roche, Penzberg, Germany

Secondary antibody	Manufacturer
α-rat-Cy5, 0,75 mg/ml	Jackson ImmunoResearch, Hamburg, Germany

3.1.6 Media

Name	Formula	Manufacturer
MEM (for cell culture)	10% Fetal bovine serum (FCS); 1% Penicillin/Streptomycin; 1% Sodium Pyruvate; 2% Non-essential amino acid	Biochrom, Berlin, Germany
MEM (for cell-free experiments)	1% Penicillin/Streptomycin; 1% Sodium Pyruvate; 2% Non-essential amino acid	Biochrom, Berlin, Germany

3.1.7 Cell line

Cell line	Organism	Origin	Cell type	Reference
Hepatoma HTC_4 cells, wild type	Rat	Liver cancer cell	Parenchymal cells	Lab collection
Hemagglutinin (HA) epitope-tagged S1P1 Hepatoma HTC_4 cells				

3.1.8 Gel recipes for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

3.1.8.1 Separating gel (12.5%)

AB 30%	Separating buffer	SDS 20%	Water	APS 20%	TEMED
6.25 ml	2.85 ml	75 µl	5.9 ml	60 µl	12 µl

3.1.8.2 Collecting gel (4%)

AB 30%	Upper gel buffer	SDS 20%	Water	APS 20%	TEMED
0.66 ml	1.25 ml	25 µl	3.1 ml	30 µl	4 µl

3.1.9 Software

Analyst 1.4	Applied Biosystems, Darmstadt, Germany
BD Accuri C6 Plus	Becton, Dickinson and Company, Franklin Lakes, USA
GraphpAD Prism 6.0	GraphPad Software, Inc., USA
Microsoft Office Excel 2010	Microsoft corporation, Washington, USA
Super Ion Probe Software	Shimazu Corporation, Nakagyo-ku, Kyoto, Japan

3.1.10 Statistical analysis

For testing the data on normal distribution a Kolmogorov-Smirnov test was used and for testing the data with an equal variance F-test was used. A Two Way Analysis of variance (ANOVA) with a post-hoc Bonferroni t-test was performed to analyze two or more than two experimental groups with the change of time. Level of significance: * $p \leq 0.01$. If the data are normally distributed, One Way ANOVA on Ranks with a post-hoc Holm-Sidak's Methods Test was performed to analyze more than two experimental groups. If the data are not normally distributed, Mann-Whitney test was performed to analyze two groups. One Way ANOVA on Ranks with a post-hoc Dunn's Methods Test was performed to analyze more than two experimental groups. Level of significance: * $p \leq 0.05$.

3.2 Experimental design

The modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. As one of the important chaperones for S1P, the effects of SA on degradation and metabolism of S1P were investigated. To determine the molecular mechanism, incubations with the S1P-metabolizing alkaline phosphatase and cells, different spectrometric analyses for structural analyses, and determinations of S1P-serum albumin-interactions were investigated.

3.2.1 Characterization of the serum albumin on degradation and metabolism of S1P in cell culture experiments

3.2.1.1.1 S1P extraction from RBC by fraction V bovine serum albumin (BSA)

Recent studies suggested that RBC are the main source of S1P in blood plasma. S1P-binding molecules can extract S1P from the outer cell membrane of erythrocytes (Bode et al. 2010). 8 ml of human blood was obtained from volunteers in University Hospital Jena and was collected in three sterile S-monovettes (3 x 6 ml; anticoagulant: lithium-heparin; 35 I.E. heparin/ml blood). In order to obtain the RBC, the whole blood was centrifuged (3000 rpm, 5 min, 4 °C) and the plasma was removed. The pelleted RBC was washed and centrifuged (3000 rpm, 5 min, 4 °C) once by calcium and magnesium-supplemented RBC-buffer (150 mM NaCl, 5 mM KCl, 21 mM Tris-HCl, and 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). RBC were incubated with 20 µM sphingosine in RBC-buffer for 2 h at 37 °C with constant agitation. After sphingosine loading, RBC were washed and centrifuged (3000 rpm, 5 min, 4 °C) twice by RBC-buffer. In order to estimate the ability of BSA to extract S1P from RBC, 100 µl of pelleted RBC (about 5×10^8 cells) was incubated in 100 µl RBC-buffer with and without 50 mg/ml BSA (around 751 µM) for 2 h at 37 °C with constant agitation (samples are shown in Table 5). After centrifugation of the samples for 5 min at 3000 rpm, 100 µl of the supernatant was removed to a new glass tube. 50 µl of the RBC pellet was obtained after a wash and centrifugation (3000 rpm, 5 min, 4 °C) step by RBC-buffer. Supernatant and RBC samples were analyzed by liquid chromatography coupled to triple-quadrupole mass spectrometry (LC/MS/MS) for their S1P concentrations after lipid extraction.

3.2.1.1.2 Lipid extraction

Samples (50 µl of RBC pellet, or 100 µl of plasma) were adjusted to 1 ml sample volume with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4, pH 7.4) and transferred into a glass centrifuge tube. 10 µl of an internal standard (Table 1) was added to each sample for the quantification in ESI LC/MS/MS analysis. After addition of 200 ml of 6 M HCl, 1 ml of methanol, 2 ml of chloroform to each sample, the sample with the other reagents were mixed by vortex mixer at maximum speed. Since lipids are water insoluble organic molecules, they can be extracted with nonpolar solvents (e.g., chloroform). After centrifugation at 1372 x g for 3 min, the lower organic phase was transferred to another glass centrifuge tube. Chloroform (2 ml) was added again, and the samples were vigorously re-vortexed for 10 min. After the samples were centrifuged for 3 min at 1372 x g, the lower phase was carefully removed again and combined with the previously removed phase. After the lipid extraction was repeated with 2 ml of the organic phases, the organic phases were combined and vacuum dried in a speed-vac for 45 min at 65 ° C. At the end, 80 µl methanol and 20 µl chloroform was added in each glass tube. After mixed by a vortex mixer for 1 min, the 100 µl solution was transferred into a glass bottle and taken to ESI LC/MS/MS analysis. Before mass spectrometric analysis, the samples were stored at -20 °C.

Table 1: Composition of the internal standard for mass spectrometric analysis

Internal standard from 22 Dec. 2017	
Component	**Concentration**
C15-Ceramide	30 µM
C17-Sphingosine	30 µM
C17-Lysophosphatidylcholine	30 µM
C17-Sphingomyelin	30 µM
C17-Sphingosine-1-phosphate	10 µM
C17-phosphatidylcholine	30 µM

3.2.1.1.3 ESI LC/MS/MS analysis

Mass spectrometry (MS) is an analytical technique, which can produce, separate and subsequently identify charged species. Liquid Chromatography-Mass Spectrometry (LCMS) combines the separating ability of High Performance Liquid Chromatography with the

detection capacity of mass spectrometry. Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) are ionization methods in LC-MS which can separate charged species. The charged species are produced as gas phase ions under atmospheric pressure conditions and the separation of the gaseous ions is achieved within the mass spectrometer using electrical and magnetic fields to differentiate ions. ESI was used in this case and is able to use condensed phase charge separation and ion evaporation techniques to produce vapour phase analyte ions. The targeted molecules must be in the ionized form prior to spraying into the Electrospray interface to achieve a desirable response. In quadrupole and Ion Trap Mass analyzers, ions are filtered using electrostatic potentials applied to the elements of the mass analyzers which are able to separate ions according to their mass to charge ratio. Tandem mass spectrometry (MS/MS) is the combination of two or more MS experiments, in order to get structural information by fragmenting the ions isolated during first experiments or to achieve better selectivity and sensitivity for quantitative analysis by selecting representative ion transitions using both the first and second analyzers.

QTrap triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) interfaced with a Merck-Hitachi Elite LaChrom series 3.1.3 chromatograph and autosampler (VWR, Darmstadt, Germany) was used for ESI LC/MS/MS analysis. Positive ion ESI LC/MS/MS analysis was employed for detection of all analytes. The ion source conditions and gas settings are as follows: ion spray voltage = 5,500 V, ion source heater temperature = 450 °C, collision gas setting = low, ion source gas 1 setting = 30 psi, ion source gas 2 setting = 60 psi, and curtain gas setting = 45 psi. MRM transitions for the detection are given in Table 2. Major fragment ions of the analytes are shown in Fig. 1. Liquid chromatographic resolution of all analytes was achieved using a Zorbax Extend-C18 column (2.1 mm 50 mm, 3.5 mm; Agilent Technologies). The elution protocol was composed of a 10-min column equilibration with 10% solvent A (methanol) and 90% solvent D (1 % formic acid) followed by sample injection and a 20-min period with 100% solvent A. Samples were infused into ESI through an electrode tube at a rate of 300 ml/min. Standard curves, with C17-S1P, C17-Sph as the internal standard, were constructed by adding increasing concentrations of the analytes from 1 pmol to 100 pmol of C17-S1P, C17-Sph (internal standard). Linearity of the standard curves and correlation coefficients were obtained by linear regression analyses. Typical retention times are given in Table 2. Linearity of the standard curves and correlation coefficients were obtained by linear regression analysis. Typical standard curves are shown in Fig. 2. All mass spectrometry analyses were performed with Analyst 1.4 (Applied Biosystems, Darmstadt, Germany).

Table 2: Mass transitions and the typical retention time for analyzed sphingolipid (data from Bode C, Gräler MH, Quantification of sphingosine-1-phosphate and related sphingolipids by liquid chromatography coupled to tandem mass spectrometry. Methods in Molecular Biology. 2012.)

Sphingolipid	Mass transition (m/z)	Retention time (min)
C17-Sph	286/268	7.2
Sph	300/282	7.5
C17-S1P	366/250	8.7
S1P	380/264	8.2

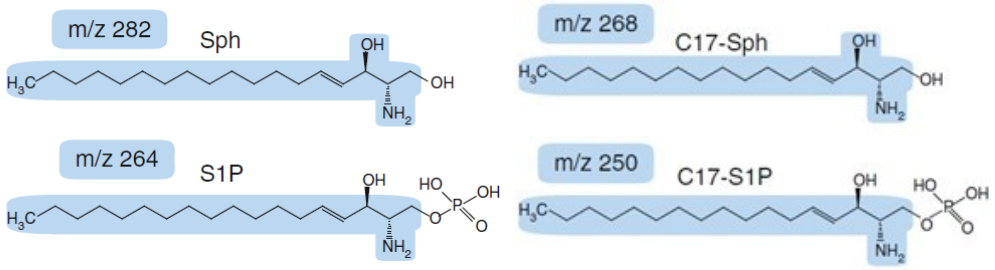

Fig. 1: MS/MS fragmentation patterns with the most prominent fragment ions generated in positive ESI mode. (The graph adjusted from Bode C, Gräler MH, Quantification of sphingosine-1-phosphate and related sphingolipids by liquid chromatography coupled to tandem mass spectrometry. Methods in Molecular Biology. 2012.)

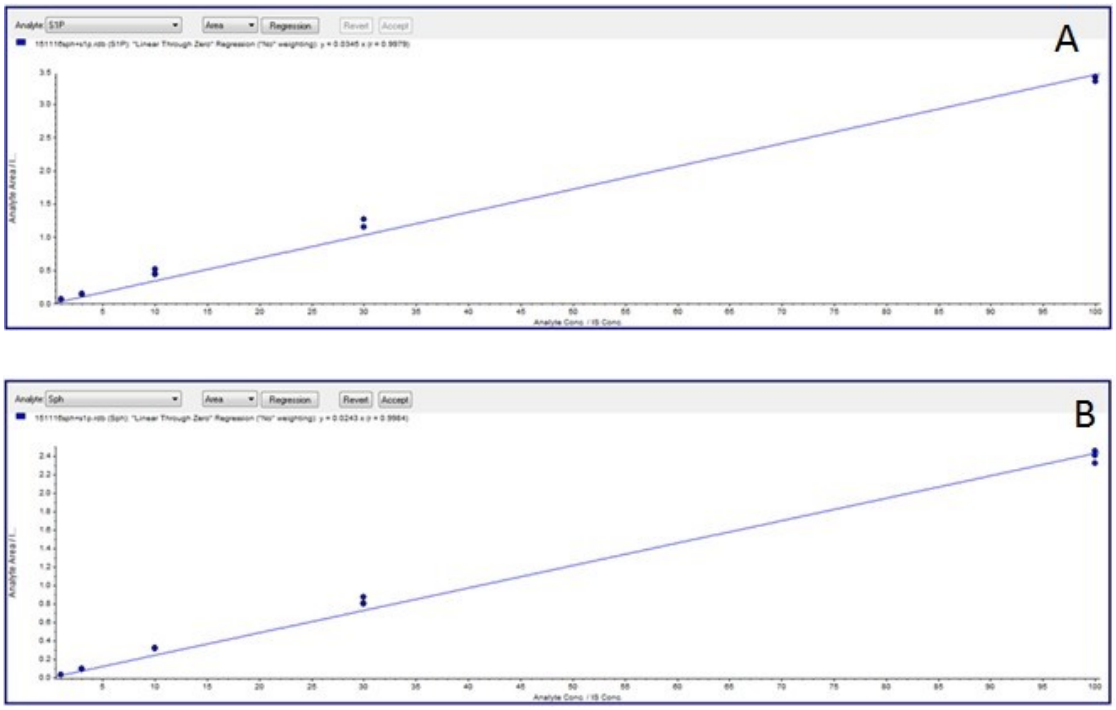

Fig. 2: Generation of typical standard curves for S1P and Sph quantification as a demonstration. (A) C17-S1P was used as internal standard. (B) C17-Sph was used as internal standard.

3.2.1.2.1 Cell culture

All work related to the cultivation of eukaryotic cells was performed under the class II safety cabinet (Biosafety Cabinets, HS-12, Hanau, Germany). The eukaryotic cells were cultured under sterile conditions at 37 °C in a water vapor saturated atmosphere with 5% carbon dioxide in the cell culture incubator. All cell culture media and solutions were warmed to 37 °C in the water bath for at least 10 min before use.

Adherent cells were split every 2 to 3 days at a confluence of 80 to 90% in order to avoid overgrowth. Cell culture medium was discarded and the cell layer was gently rinsed with 5 ml sterile PBS and aspirated to remove all traces of calcium ion and serum, which contains trypsin inhibitor. Then, 1 ml of fresh Trypsin/EDTA-solution were added again to the cell layer and removed subsequently except a small rest. After incubating cells at 37 °C for 3 min in the incubator, the complete detachment of cells was checked by the light microscope and facilitated by slightly hitting or shaking the culture dishes, if necessary. To block the effects of trypsin, pre-warmed fresh cell culture medium was added and cells were dispersed by pipetting several times with a filtered 5-ml-glass pipette. Appropriate aliquots of cell suspension were put into new cell culture dishes according to the required split ratio from 1:10 to 1:5. For this purpose,

the required amount of cell suspension was transferred into a Falcon-tube and centrifuged at 312 × g for 5 min. After discarding the supernatant, cells were taken up in fresh pre-warmed medium and returned to a 37 °C incubator.

Long-term storage of cells over several years is only possible in liquid nitrogen at -196 °C. In order to ensure that cell keep viable as much as possible as a result of the formation of ice crystals in the cell interior in the freezing process, they must be mixed with dimethyl sulfoxide (DMSO). The adherent cells were washed, trypsinized and re-suspended with fresh complete medium. It was then centrifuged for 5 min at 312 × g and 4 °C, the supernatant aspirated and the cell pellet re-suspended and frozen in 90% cell culture medium supplemented with FBS and 10% DMSO in a 2 ml cryotube at a density of about 2×10^6 cells /ml. For a slow cooling process, freezing vials were placed in a styrofoam container in a -80 °C freezer for 24–48 h. The cryotubes were finally transferred to permanent storage in -196 °C liquid nitrogen.

The further cultivation of the frozen cells was carried out by rapid thawing at 37 °C in a water bath. Immediately after thawing, the cells were mixed in 10 ml of the appropriate culture medium and centrifuged for 5 min at 312 × g. The cell pellet was finally re-suspended in 10 ml of complete medium, the entire volume transferred to a 100 mm cell culture dish and further cultured at 37 °C and 5% CO_2 in the cell incubator.

3.2.1.2.2 Cultivation of HTC_4-S1PR1 wild type cells

The immortalized rat hepatoma HTC_4 cell line is an adherent cell line which was cultivated on culture dishes (Ø 100 mm) for adherent cells in MEM medium, with further additives.

Table 3: Composition of HTC_4 culture medium

Component	**Company**
MEM Earles Medium (500 ml)	Biochrom, Berlin, Germany
supplemented with:	
FCS, 10%	Biochrom, Berlin, Germany
Penicillin / Streptavidin (10000 µg / ml), 1%	
Sodium pyruvate (100 mM), 1%	
nonessential amino acid solution (100x), 2%	

3.2.1.2.3 Determination of vitality and cell number

Trypan blue vital staining makes it possible to differentiate between vital and dead cells, since trypan blue is a high molecular weight dye that can penetrate cells only through damaged membranes and thus turns them into deep blue. The trypan blue test and the cell count were done in a Neubauer counting chamber. The chamber and coverslip were rinsed with 70% ethanol prior to testing before being applied to the counting chamber. From the previously prepared single cell suspension, approximately 10 µl were mixed with 10 µl of a 0.1% trypan blue solution. After 1 to 2 min of incubation of the cells with the dye, the counting chamber was filled with 10 µl of the stained cell suspension by means of a pipette and the number of cells was determined with the aid of a light microscope.

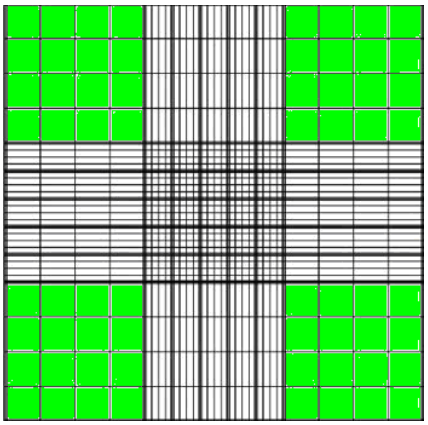

Fig 3: Structure of the counting field of a Neubauer counting chamber. In each case, the four green corner squares for cell number determination were counted.

Cell concentration (/ml) = total cell number in the four green squares×2×10×1000/4

3.2.1.3 Serum albumin's preventing extracellular S1P from the metabolism by HTC_4 wild type cells

3.2.1.3.1 A direct way to detect S1P concentration by LC/MS/MS

To demonstrate the ability of serum albumin to prevent extracellular S1P from metabolism by HTC_4 wild type cells, 1 µM of extracellularly added S1P with or without ovalbumin (OVA), BSA, or HSA were incubated overnight with HTC_4 cells (the compositions of S1P, OVA, BSA, and HSA of each sample are listed in Table 6). The OVA of chickens consists of 385 amino acids with a molecular mass of 42.7 kDa (Nisbet et al. 1988). The OVA was used as a protein control for BSA and HSA in this case. 500 000 cells / 2 ml cell culture medium were put in each well of 6-well plate (for adherent cells). Cell culture medium was discarded at the second day and 1 µM S1P with or without OVA, BSA, or HSA in 750 µl cell culture medium (after incubation, 500 µl cell culture medium would be used for lipid extraction and the other 200 µl would be used to stimulate S1PR1 overexpressing HTC_4 cells as mentioned in 3.2.1.3.2.2) was

put into different well of a 6-well plate. Sample 14 (as listed in Table 6) was used as a 100% control and incubated in a well without cells overnight. After overnight incubation, supernatant was harvested and centrifuged for 5 min at 312 × g and 20 °C. 500 µl of the supernatant with 500 µl PBS (1 ml end volume) would be analyzed by LC/MS/MS for their S1P concentrations after lipid extraction. Lipid extraction and mass spectrometry analysis are the same as described in 3.2.1.1.2 and 3.2.1.1.3. Cell culture and cell counting are the same as described in 3.2.1.2.1, 3.2.1.2.2 and 3.2.1.2.3.

Sodium orthovanadate (SOV) is a phosphatase inhibitor. 1 µM of extracellularly added S1P with 1 mM SOV was also incubated overnight with HTC_4 cells (the same setting as mentioned above). In order to demonstrate this effect of serum albumin derived from the protein part of serum albumin, methanol precipitation was used to extract the non-protein part of BSA (as mentioned in 3.2.1.3.1.2). 1 µM of extracellularly added S1P with non-protein part of BSA (NPBSA) extracted from 100 mg/ml BSA was also incubated overnight with HTC_4 cells (the same setting as mentioned above).

3.2.1.3.1.1 Preparation of 100 mM Sodium orthovanadate (SOV)

1.839 mg SOV was put in a 10 ml Falcon, to which 8 ml of distilled water was added. Then pH of the solution was adjusted to 10. Afterwards, it was placed in boiling water until the solution became clear. It was stored at -20 °C.

3.2.1.3.1.2 Methanol precipitation of BSA

To extract non-protein part from serum albumin, 2 g regular fraction V BSA was put into a 50 ml Falcon, to which 5 ml of distilled water was added. After all the BSA was dissolved in the water, 45 ml of methanol was added to the BSA solution and the solution was mixed by vortex mixer at maximum speed for 5 min. Then it was kept in -80 °C for one hour. Afterwards, the precipitated protein was discarded and the rest solution was transferred to 1.5 ml tubes which were centrifuged at 15 000 g for 10 min. The precipitated protein was discarded and the transparent solution was transferred to glass wells which were dried in a vacuum speed-vac for 45 min at 65 °C. At the end, 100 µl of methanol was added in each glass tube. After mixed by a vortex mixer for 1 min, the 100 µl of solution was transferred into a glass bottle and taken to -20 °C.

3.2.1.3.2 An indirect way to show relative S1P concentration by S1P elicited S1PR1 internalization

3.2.1.3.2.1 Cell culture and cell counting

This part is similar as mentioned in 3.2.1.2.1 and 3.2.1.2.2. Surface expression of hemagglutinin (HA) epitope-tagged S1PR1 (S1PR1-HA) on rat hepatoma HTC_4 cells was analyzed by fluorescence-activated cell sorting (FACS). One 10 cm plate of HA epitope-tagged S1PR1 HTC_4 cells was taken from cell incubator to the class II safety cabinet. For cell harvesting, cells were washed once with 5 ml PBS which was aspirated later and 1 ml Biotase (Biotase Protease/EDTA solution 0,0042% / 0,02% (w/v) in PBS w/o Ca^{2+} w/o Mg^{2+}; Biochrom, Berlin, Germany) was added to the cells which was incubated in the incubator for 3 min. The complete detachment of cells was checked by the light microscope and facilitated by slightly hitting or shaking the culture dishes, if necessary. To inhibit the Biotase activity, 9 ml growth medium was added to the plate. The full 10 ml was transferred to a 15 ml Falcon and 200 µl medium was transferred in a 1.5 ml Eppendorf tube for cell counting.

10 µl growth medium with cells were mixed with 10 µl of a 0.1% trypan blue solution in 96-well plate. The counting chamber was filled with 10 µl of the stained cell suspension and the number of cells was determined with the aid of a light microscope, as mentioned in 3.2.1.2.3. For stimulation and incubation, 300 000 cells per stain were needed in each well of 96-well plate (for inadherent cells). After calculation, an appropriate amount of cells was removed and transferred to each well in growth medium.

3.2.1.3.2.2 S1P elicited S1PR1 internalization

After centrifuged for 5 min at 312 × g and 4 °C, the supernatant of the cell suspension was discarded and the pellet re-suspended in 200 µl solutions (200 µl solutions were taken from cell culture medium as shown in Table 6 after overnight incubation with HTC_4 wild type cells, and listed in Table 11). Steps are summarized in 3.2.1.3.1. After overnight incubation, the samples were subsequently applied to stimulate HTC_4 S1PR1 HA cells in the cell incubator for 15 min, the cell suspension was centrifuged for 5 min at 204 × g and 4 °C and the supernatant was discarded.

3.2.1.3.2.3 Flow cytometry

In fluorescence-activated cell sorting (FACS) measurement, cells or particles are transported in a fluid stream by the fluidic system, illuminated by lasers in the optic system which also consists of optical filters to direct resulting light signals to appropriate detectors and finally analyzed by computer. For each cell or particle, the scattered light is measured in the forward scatter in the forward direction, which is for the measurement of the cell or particle size. Meanwhile, the scattered light, which propagated perpendicular to the laser axis, is detected in the side scatter and proportional to the complexity or granularity of the cells or particles. In addition, at right angles to the laser axis, fluorescence signals are also detected via corresponding mirrors and filters, which are arranged upstream of the respective detectors in a specific arrangement. By labeling cells with fluorochromes, either directly or coupled via an antibody, various parameters can be examined. Fluorochromes can absorb light energy in a characteristic wavelength range (absorption spectrum). Therefore, the electrons are raised to a higher energy level and when falling back to the ground level, the electron emits a photon (fluorescence). When multiple fluorochromes are measured at the same time, the emission spectra must be different. Sometimes, however, partial overlays of the emission spectra occur. In this case, compensation must be made to avoid false positive signals.

The following fluorochromes were used in the studies using the BD AccuriC6 Plus Flow Cytometer:

Table 4: Fluorochromes used for flow cytometric analysis laser.

Laser (wavelength)	Detector	Optical filter	Fluorochromes
Blue laser (488 nm)	FL 2	585/40	PE
	FL 3	670	PI
Red laser (640 nm)	FL 4	675/25	Cy5

The calculation and analysis of the data were carried out with the BD Accuri C6 Plus software. The results were shown graphically either as a histogram, a simple frequency distribution in which the strength of the fluorescence signal versus the number of events, or two-dimensional as a dot plot, in which each cell is represented as a point on the graph.

In order to control whether HTC_4 cells express the transfected plasmid with the S1PR1, antibodies capable of recognizing and labeling a hemagglutinin tag (HA tag) were used. Rat α-HA (Rat α-HA, RG1 3F110, hybridoma supernatant) was used as the primary antibody, 50 µl of this antibody supernatant was used after diluted 10 times by PBS with 5% FCS to resuspend the cell pellet. Subsequently, the cells were incubated for 30 min on ice and at 4 °C. It was then centrifuged again for 5 min at 204 × g and 4 °C, the supernatant discarded and the cells resuspended in 200 µl PBS with 5% FCS. After re-centrifugation for 5 min at 204 × g and 4 °C, the supernatant was discarded and the cells stained with the detection antibody. Depending on the primary antibody used, α-rat-Cy5 (0.75 mg/ml) was chosen to be used as a secondary antibody in this case. The secondary antibody was diluted 1:100 in PBS with 5% FCS and 50 µl of this solution were resuspended for each cell pellet. After incubation on ice and at 4 °C for 30 min, centrifugation was continued for 5 min at 204 × g and 4 °C, the supernatant was discarded and the cells were washed in 200 µl PBS with 5% FCS. After another centrifugation step, the supernatant was discarded, the cell pellet finally resuspended in 200 µl PBS with 5% FCS and transferred to FACS tubes. For a subsequent live / death analysis, 100 µl each were stained with 5 µl propidium iodide solution (c = 50 µg/ml). Propidium iodide (PI) can penetrate perfused cell membranes from dead cells and intercalate with the DNA. The measurement was subsequently carried out in the BD AccuriTM C6 Flow Cytometer.

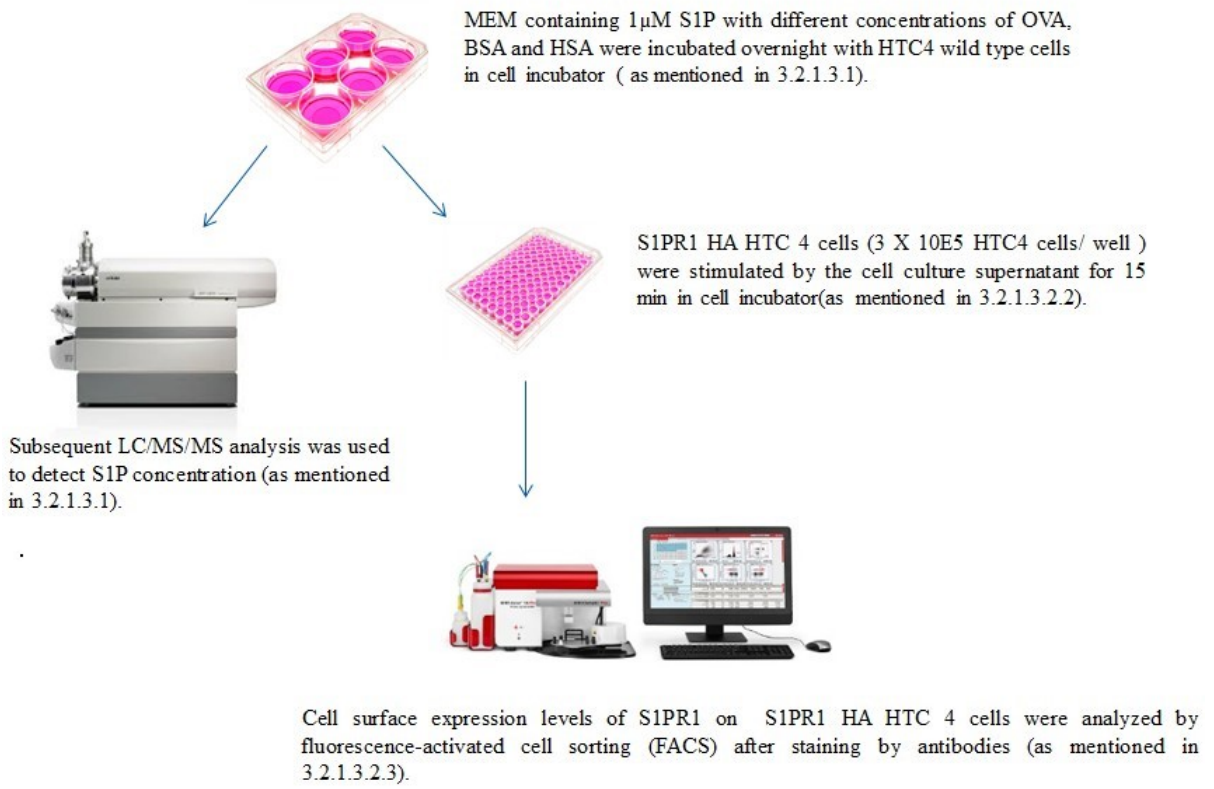

Fig 4: Overview of the technical steps in 3.2.1.3 (Serum albumin's preventing extracellular S1P from the metabolism by HTC$_4$ wild type cells). 1 µM S1P in 750 µl MEM containing different concentrations of OVA, BSA, or HSA were incubated overnight with 5 × 10^5 HTC$_4$ cells in cell incubator (as mentioned in 3.2.1.3.1). 500 µl from the supernatant of each sample was used to detect S1P concentration directly by subsequent LC/MS/MS analysis (as mentioned in 3.2.1.3.1). The other 200 µl from the supernatant of each sample was used to stimulate HTC$_4$ S1PR1 HA cells (as mentioned in 3.2.1.3.2.2) and cell surface expression levels of S1PR1 on HTC$_4$ S1PR1 HA cells were analyzed by fluorescence-activated cell sorting (FACS) as an indirect way to show the relative S1P concentration in the supernatant of each sample (as mentioned in 3.2.1.3.2.3).

3.2.1.4 Effects of serum albumin on degradation kinetics of extracellular S1P and Sph with HTC$_4$ wild type cells

To demonstrate that serum albumin can prevent S1P instead of Sph from metabolism by HTC$_4$ wild type cells, 1 µM S1P with or without 50 mg/ml BSA and 1 µM Sph with or without 50 mg/ml BSA were incubated certain time (as indicated in Table 9) with adherently grown HTC$_4$ cells (samples are listed in Table 9). 500 000 cells / 2 ml cell culture medium were added to each well of 6-well plate (for adherent cells). The cell culture medium was discarded at the second day and afterwards 1 µM S1P with or without 50 mg/ml BSA and 1 µM Sph with or

without 50 mg/ml BSA in 750 µl cell culture medium was put into different wells of a 6-well plate. After certain incubation time (as shown in Table 9), 500 µl cell culture medium (supernatant) of a sample with 500 µl PBS (end volume: 1 ml) were analyzed by LC/MS/MS for their S1P or Sph concentrations after lipid extraction. Lipid extraction and mass spectrometry analysis are the same as described in 3.2.1.1.2 and 3.2.1.1.3. Cell culture and cell counting are the same as described in 3.2.1.2.1, 3.2.1.2.2 and 3.2.1.2.3.

3.2.1.5 The effect of pepsinized BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells

3.2.1.5.1 The proteolysis of BSA by pepsin

BSA at concentration of 50 mg/ml in MEM (pH was changed to 1.9 by HCl) without or with pepsin at different concentration as indicated was incubated overnight in the water bath at 37 °C (as listed in Table 7). Pepsin is an endopeptidase and can digest peptide bonds. The activity of pepsin is dependent on an acidic environment for it to digest protein. Pepsin is most active around a pH of 1.5 to 2. Pepsin is inactive at pH above 4 (Samloff 1989).

3.2.1.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

All the samples normally were diluted 1000 times (if not indicated) by MEM before denaturing process. Then, 15 µl of each diluted sample was incubated with 5 µl ROTI®Load 1 for 5 min at 95°C.

The gel cassette and comb were carefully removed and the gel cassette was rinsed with 1x electrophoresis buffer. The SDS-electrophoresis chamber with the gel cassette was assembled and placed in the chamber system. 200 mL 1× electrophoresis buffer was filled in the inner cathode chamber and subsequently filled the outer anode chamber with 550mL 1x electrophoresis buffer. 15 µL per sample or 5 µL protein size marker (ROTI®Mark 10-150 PLUS) was loaded in each gel bags. Then, the lid was placed on the chamber and the system was connected to the power supply. The gel ran for approx. 90 min at 120 V (observe running buffer). At the end of the electrophoresis, the power supply was switched off, the lid was removed and the electrophoresis chamber was taken out. Gel was carefully taken out and used for the staining process.

Imperial™ Protein Stain (Thermo Fisher, Massachusetts, U.S.) was used for the gel staining. The procedure was done according to the user guide from Thermo Fisher.

3.2.1.5.3 Cell culture and ESI LC/MS/MS analysis of S1P concentration

To determine the effect of pepsinized BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells, 1 µM S1P with or without 50 mg/ml pepsinized BSA were incubated for 2 hours with adherently grown HTC_4 cells (samples are listed in Table 8). 500 000 cells / 2 ml cell culture medium were added to each well of 6-well plate (for adherent cells). The cell culture medium was discarded at the second day and afterwards 1 µM S1P with or without 50 mg/ml pepsinized BSA in 750 µl cell culture medium was put into different wells of a 6-well plate (sample as mentioned in Table 8; after changing pH of the previous medium from 1.9 to 7.4, the sample B in Table 7 was used as sample C in Table 8 and the sample C in Table 7 was used as sample B in Table 8). After 2 h incubation, 500 µl cell culture medium (supernatant) of a sample with 500 µl PBS (end volume: 1 ml) were analyzed by LC/MS/MS for their S1P and Sph concentrations after lipid extraction. Lipid extraction and mass spectrometry analysis are the same as described in 3.2.1.1.2 and 3.2.1.1.3. Cell culture and cell counting are the same as described in 3.2.1.2.1, 3.2.1.2.2 and 3.2.1.2.3.

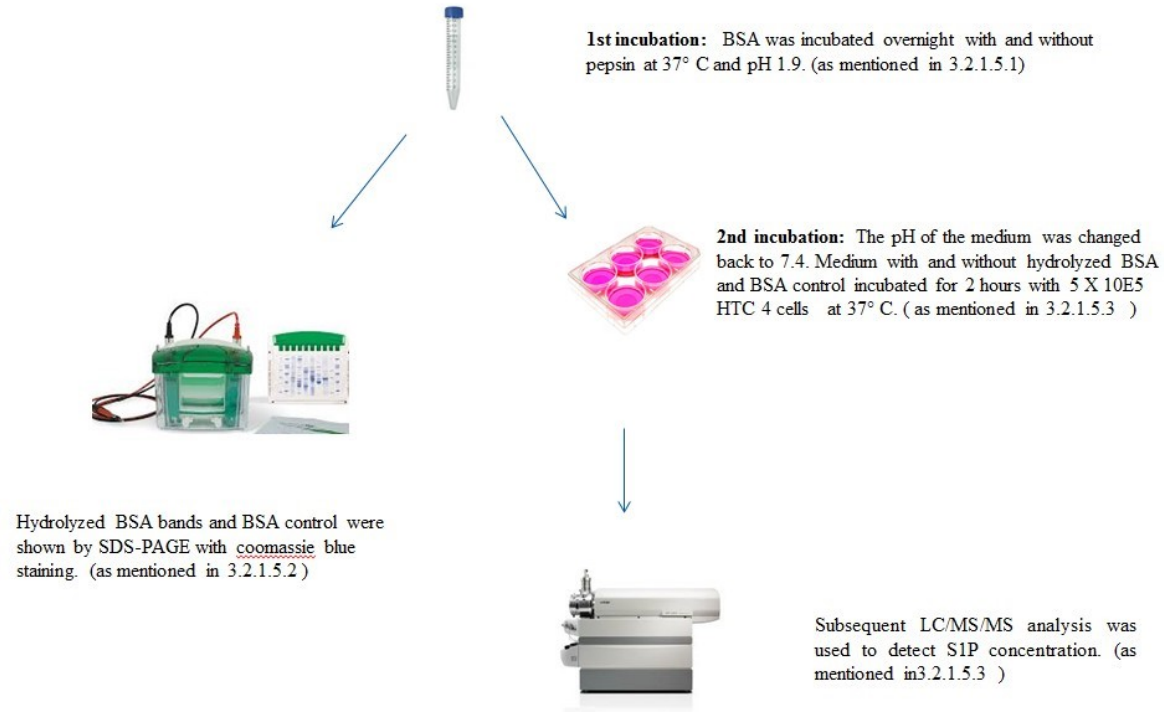

Fig. 5: Overview of the technical steps in 3.2.1.5 (the effect of pepsinized BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells). 50 mg/ml BSA was incubated overnight with and without pepsin at 37 °C and pH 1.9 (1st incubation) (as mentioned in 3.2.1.5.1). The pH of the medium was changed back to 7.4. Medium with and without hydrolyzed BSA and BSA control incubated for 2 h with 5×10^5 HTC_4 cells at 37 °C. Subsequent LC/MS/MS analysis was used to detect S1P concentration in the cell culture medium (as mentioned in 3.2.1.5.3). Hydrolyzed BSA bands were shown by SDS-PAGE with coomassie blue staining (as mentioned in 3.2.1.5.2).

3.2.2 Characterization of the serum albumin on dephosphorylation of S1P in cell-free experiments

3.2.2.1 S1P overnight incubation with alkaline phosphatase with or without serum albumin

Extracellular S1P was shown to be dephosphorylated by phosphatase without the involvement of S1P-lyase and ceramide synthase (Peest et al. 2008). In order to prove that serum albumin can prevent S1P from dephosphorylation by phosphatase, 1 µM S1P and 3 u alkaline phosphatase (AP) with or without 50 mg/ml BSA or 50 mg/ml HSA were incubated overnight. 1 µM S1P and 3 u alkaline phosphatase (AP) were added to 1 ml MEM (without FCS). The samples (listed in Table 10) were incubated overnight at 37 °C in a water vapor saturated

atmosphere with 5% carbon dioxide in the cell culture incubator. After overnight incubation, these samples (1 ml) were analyzed by liquid chromatography coupled to triple-quadrupole mass spectrometry (LC/MS/MS) for their S1P and Sph concentrations after lipid extraction (5 mg BSA was added to each sample before lipid extraction). Lipid extraction and mass spectrometry analysis are the same as described in 3.2.1.1.2 and 3.2.1.1.3.

3.2.2.2 4-Nitropheenyl phosphate (NPP) phosphatase assay

NPP phosphatase assay can be applied to detect phosphatase activity in samples using 4-nitropheenyl phosphate (NPP) as a colorimetric substrate for alkaline phosphatase. Alkaline phosphatase can catalyze the hydrolysis of NPP producing inorganic phosphate and the conjugate base of para-nitrophenol (NP). NP (a yellow product with a strong absorption at 405 nm) can be detected at 405 nm by using a Microplate Photometer Multiskan FC Type 357 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Diluent buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) with or without 50 mg/ml BSA or fatty-acid-free BSA (FAFBSA) were prepared at the same day. 98 µl of diluent buffer, 1 µl of enzyme solution (3 u/ml alkaline phosphatase) and 1 µl of substrate solution (1 mM 4-nitrophenyl phosphate) were put to 96-well plate (Sarstedt, Nümbrecht, Germany) on ice (sample as listed in Table 12). The wavelength of Microplate Photometer Multiskan FC Type 357 was set at 405 nm and the mode was set at fast. The absorbance of each well at 405 nm was measured before the experiment started (0 h) and at 1 h, 2 h, 4 h, 6 h and 24 h after incubated in the incubator at 37 °C.

3.2.2.3 Nuclear magnetic resonance (NMR) spectrometry

To determine the structure and interaction of S1P and albumin in solution, NMR spectrometry was applied. NMR spectrometry is a technique to observe local magnetic fields around atomic nuclei. The sample is placed in a magnetic field and the NMR signal is produced by excitation of the nuclei sample with radio waves into nuclear magnetic resonance, which is detected with a sensitive radio receiver. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule and its individual functional groups.

The samples were prepared as listed in Table 13 and Table 14 and stored at -20 °C.

These experiments were measured in cooperation with Dr. Peter Bellstedt from the NMR instrument platform (Friedrich Schiller University Jena) and data were provided by Prof. Dr. Markus Gräler.

4 Results

The modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. It was proved that serum albumin (SA), one of the S1P chaperones, can enhance S1PR signaling of S1P (Wilkerson et al. 2012). The molecular mechanism of this S1PR signaling enhancing effect of SA remains unclear. It was proposed that SA functions as a facilitator of lipid solubility for S1P. According to the idea that SA increased the availability of S1P to S1PRs, one would assume that SA could also boost the dephosphorylation and metabolism of S1P by S1P-phosphatases. Therefore, it is of great interest to study the effects of SA on the dephosphorylation and metabolism of S1P by S1P-phosphatases. As one of the important chaperones for S1P, the effects of BSA and HSA on degradation and metabolism of S1P were investigated. To determine the molecular mechanism, incubations with the S1P-metabolizing alkaline phosphatase and cells, different spectrometric analyses for structural analyses and determinations of S1P-SA interactions were investigated.

4.1 S1P extraction ability of BSA from the outer RBC membrane in RBC incubation experiments

In order to investigate the capability of BSA to extract S1P from the outer membrane of RBC, 100 µl of pelleted RBC (about 5×10^8 cells) were incubated in 100 µl RBC-buffer with and without 50 mg/ml BSA for 2 h at 37 °C with constant agitation after incubation with 20 µM sphingosine in RBC-buffer for 2 h at 37 °C with constant agitation. As shown in Fig. 6, 751.9 µM BSA can increase S1P concentration by 5.8 µM in 100 µl RBC buffer from 100 µl the pelleted RBC. After 2 h incubation with 100 µl RBC, 100 µl supernatant without BSA contained 0.9 ± 0.8 µM S1P while 6.7 ± 1.6 µM S1P was found in 100 µl supernatant with 751.9 µM BSA. The molecular ratio between S1P and BSA in the supernatant after incubation is 1:129.

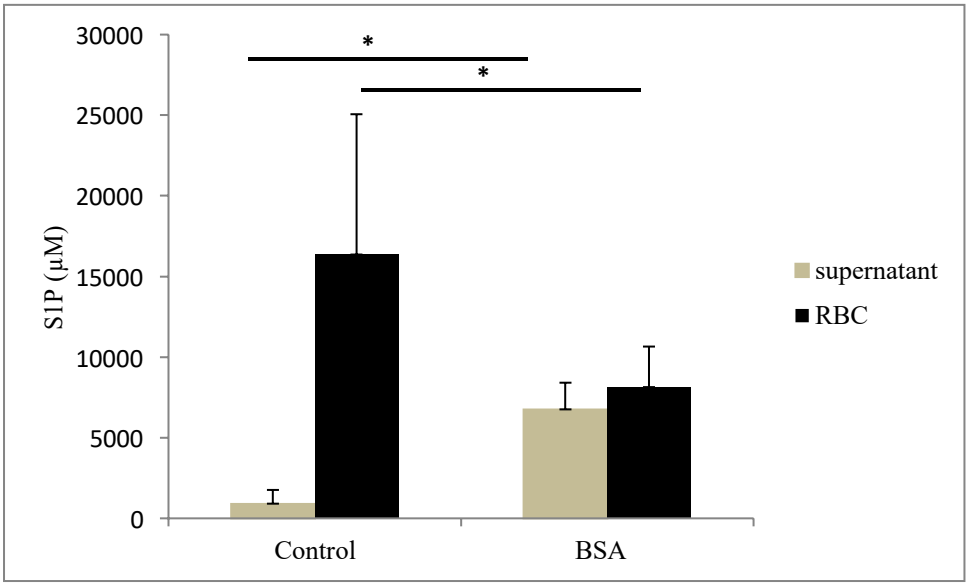

Fig. 6: S1P extraction ability of BSA from the outer RBC membrane in RBC incubation experiments. Incubations and subsequent LC/MS/MS analysis of S1P preloaded RBC (about 5×10^8 cells) at 37 °C for 2 h without and with 50 mg/ml BSA. Shown are mean+SD (n=3). Mann-Whitney test was performed to determine significances. *: $p \leq 0.05$. (Samples are listed in Table 5)

Table 5: Composition of samples for S1P extraction.

Samples	RBC	RBC-buffer plus 50 mg/ml BSA	RBC-buffer
Control	100 µl	0 µl	100 µl
BSA	100 µl	100 µl	0 µl

4.2 The ability of serum albumin to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells

FAF-BSA binds S1P with a Kd of 41 µM (Fleming et al. 2016). BSA extracted S1P from RBC with a molecular ratio of 129:1 (shown in 4.1). Both results indicate that SA does not bind S1P very strongly. To demonstrate the effect of SA on extracellular metabolism of S1P by cells, 1 µM S1P without or with different concentration of OVA, BSA, and HSA (as listed in Table 6) was extracellularly added to the HTC_4 wild type cells (about 5×10^5 cells) and incubated overnight. OVA is an albumin form from chicken egg and was expected to serve here as a functional control to BSA and HSA. SOV is a phosphatase inhibitor. To demonstrate the effect of phosphatase on extracellular metabolism of S1P, 1 µM of extracellularly added S1P with

1 mM SOV was also incubated overnight with HTC_4 cells (the same setting as mentioned above). Methanol precipitation was used to extract the NPBSA (as mentioned in 3.2.1.3.1.2) from the regular fraction V BSA. To show this effect of SA deriving from protein part, 1 μM of extracellularly added S1P with NPBSA extracted from 100 mg/ml BSA was also incubated overnight with HTC_4 cells (the same setting as mentioned above). After overnight incubation, supernatant was harvested and centrifuged. Lipid extraction and LC/MS/MS were performed to quantify the S1P and Sph concentration in the cell culture medium after overnight incubation. The S1P concentration of the cell culture medium to which 1 μM S1P was added and which was incubated in a well without cells overnight was used as a 100% control. Very low levels of Sph were found in all samples (Fig. 7, A, B, C, and D). Very low levels of S1P and Sph were found in the supernatant to which 50 mg/ml OVA, BSA, or HSA was added without S1P (Fig. 7, A, B, and C), which suggests that OVA, BSA, and HSA contain very little amount of S1P. It was proved that extracellular S1P is dephosphorylated by phosphatases of cells to Sph and Sph is further metabolized by cells (Peest et al. 2008), which is also supported by Fig. 7 D. It is shown that 70 ± 15% S1P could still be found in cell culture medium with 1 mM SOV after overnight incubation with HTC_4 cells, compared with that 1 μM S1P was almost all degraded by HTC_4 cells data shown in Fig. 7 A, B, and C. The OVA consists of 385 amino acids, its relative molecular mass is 42.7 kDa and it was applied as a protein control for serum albumin. As shown in Fig. 7 A, 1 μM S1P in the supernatant was almost all metabolized by HTC_4 cells in samples without the addition of OVA and with 15 mg/ml OVA, 30 mg/ml OVA and 50 mg/ml OVA and the statistical differences among the S1P levels of samples in Fig. 7 A were not significant. These data show that OVA had no effect on S1P metabolism by HTC_4 cells. As shown in Fig. 7 B, compared with 5.9 ± 4.2% S1P found in the supernatant without BSA after overnight incubation, 17.6 ± 4.3% S1P was found in the supernatant with 15 mg/ml BSA, 33.7 ± 5.6% S1P was found in the supernatant with 30 mg/ml BSA and 64.0 ± 9.3% S1P was found in the supernatant with 50 mg/ml BSA and differences among these samples are statistically significant. These data suggested that BSA could prevent extracellularly added S1P from metabolism by HTC_4 wild type cells, which could derive from the effect of BSA to inhibit phosphatase of S1P. From 0 mg/ml to 50 mg/ml, the higher the BSA concentration was added to the cell culture medium, the more S1P could be found in the supernatant after overnight incubation, which showed that BSA had a dose dependent effect to inhibit the metabolism of S1P by HTC_4 cells. As shown in Fig. 7 D, NPBSA, the amount of which equals to 100 mg/ml BSA, could not prevent S1P from metabolism by HTC_4 wild type cells. Hence, the effect of

BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells is suggested to derive from the protein part of BSA. As shown in Fig. 7 C, compared with 6.3 ± 5.3% S1P found in the supernatant without HSA after overnight incubation, 26.5 ± 9.74% S1P was found in the supernatant with 15 mg/ml HSA, 66.8 ± 21.2% S1P was found in the supernatant with 30 mg/ml HSA and 56.5 ± 21.9% S1P was found in the supernatant with 50 mg/ml HSA. The S1P concentration of samples with 50 mg/ml HSA or 30 mg/ml HSA was significantly higher than that without addition of HSA and with 15 mg/ml HSA. The S1P concentration of samples with 15 mg/ml HSA was higher than that without addition of HSA but the difference is not significantly different. The S1P concentration of samples with 50 mg/ml HSA was slightly lower than that with 50 mg/ml HSA but the difference is not significantly different. These data suggested that HSA could prevent extracellularly added S1P from metabolism by HTC_4 wild type cells, but this effect was similar in the range from 30 mg/ml to 50 mg/ml HSA.

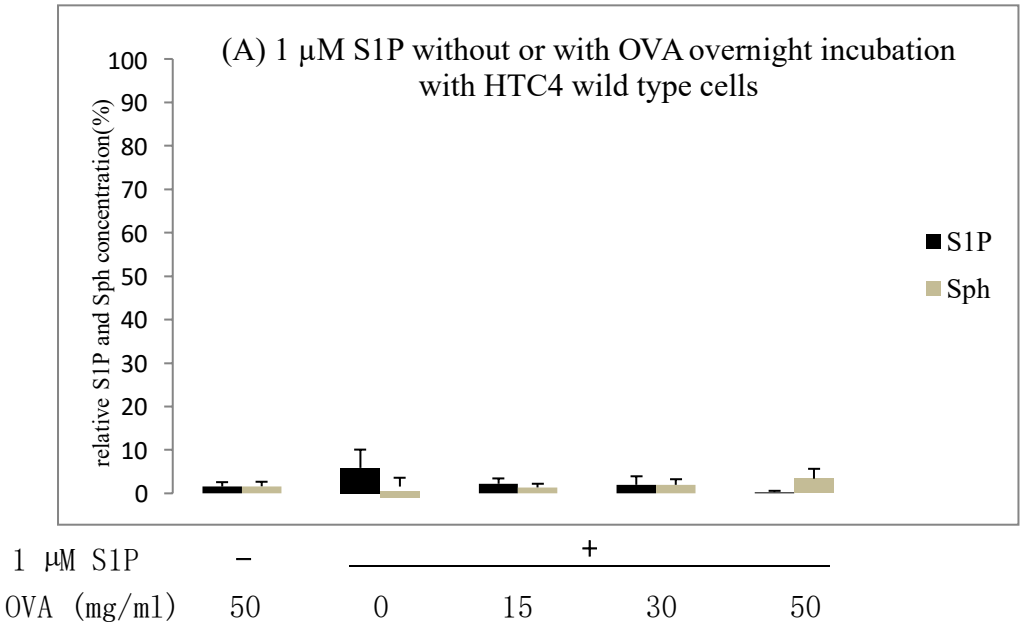

Fig. 7 A: S1P and Sph concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with extracellularly added 1 µM S1P and the indicated concentration of OVA.

1 µM S1P with the indicated concentration of OVA, in cell culture medium (supernatant) was incubated with 5×10^5 HTC4 wild type cells (adherently grown) overnight. The S1P concentration of cell culture medium containing 1 µM S1P and incubated in a well without cells overnight was used as a 100% control. After overnight incubation, subsequent LC/MS/MS analysis was performed. Shown are mean+SD of triplicate (n=6). One-way ANOVA with a post-hoc Holm-Sidak's t-test was performed to determine significances. *: $p \leq 0.05$.

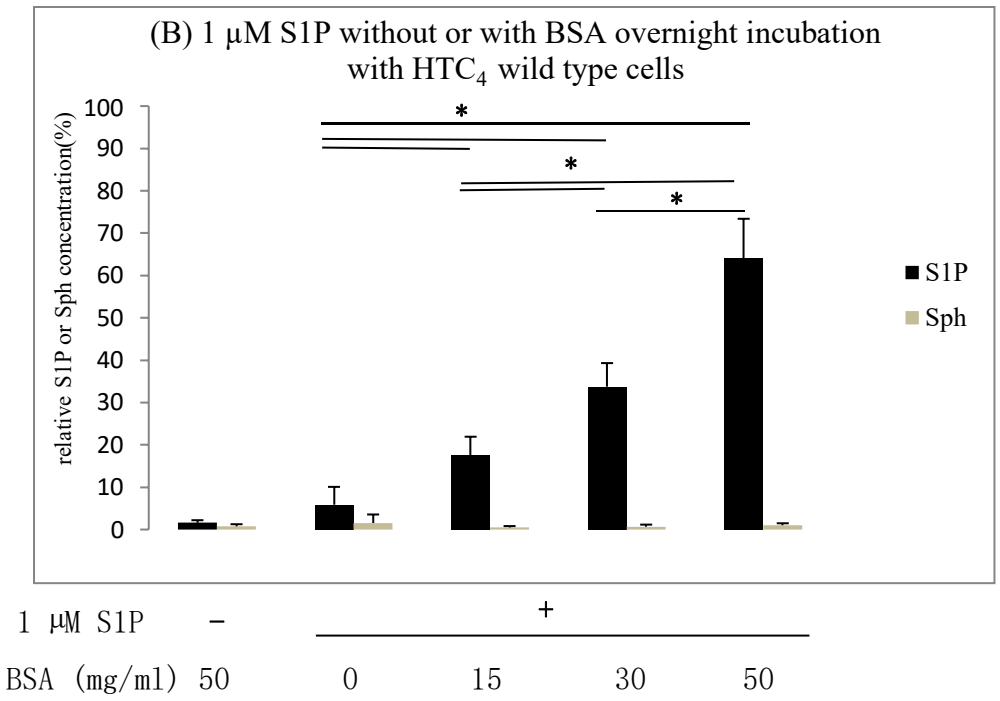

Fig. 7 B: S1P and Sph concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with extracellularly added 1 µM S1P and the indicated concentration of BSA:

1 µM S1P with the indicated concentration of BSA in cell culture medium (supernatant) was incubated with 5×10^5 HTC4 wild type cells (adherently grown) overnight. The S1P concentration of cell culture medium containing 1 µM S1P and incubated in a well without cells overnight was used as a 100% control. After overnight incubation, subsequent LC/MS/MS analysis was performed. Shown are mean+SD of triplicate (n=6). One-way ANOVA with a post-hoc Holm-Sidak's t-test was performed to determine significances. *: $p \leq 0.05$.

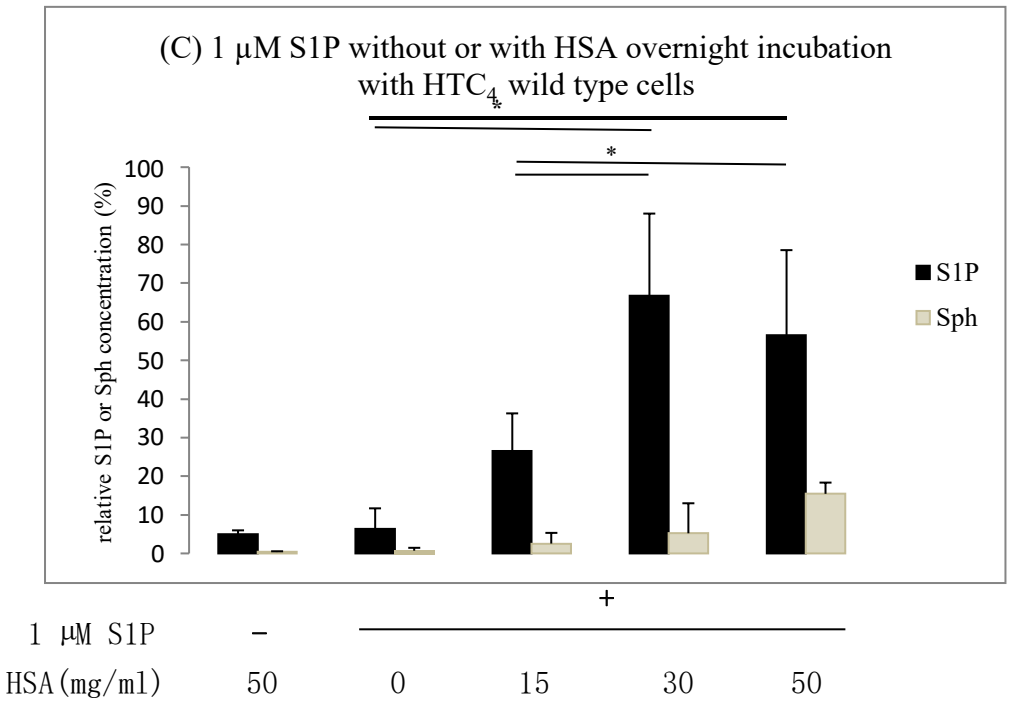

Fig. 7 C: S1P and Sph concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with extracellularly added 1 µM S1P and the indicated concentration of HSA.

1 µM S1P with the indicated concentration of HSA in cell culture medium (supernatant) was incubated with 5×10^5 HTC4 wild type cells (adherently grown) overnight. The S1P concentration of cell culture medium containing 1 µM S1P and incubated in a well without cells overnight was used as a 100% control. After overnight incubation, subsequent LC/MS/MS analysis was performed. Shown are mean+SD of triplicate (n=6). One-way ANOVA with a post-hoc Holm-Sidak's t-test was performed to determine significances. *: $p \leq 0.05$.

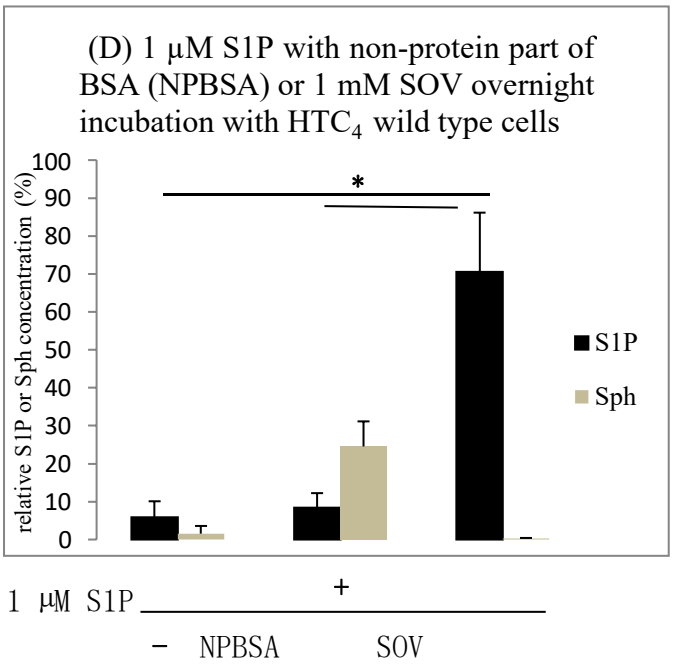

Fig. 7 D: S1P and Sph concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with extracellularly added 1 µM S1P and the indicated concentration of SOV, or NPBSA. 1 µM S1P with the indicated concentration of 1 mM SOV, or non-protein part of BSA (NPBSA) extracted from 100 mg/ml BSA in cell culture medium (supernatant) was incubated with 5×10^5 HTC4 wild type cells (adherently grown) overnight. The S1P concentration of cell culture medium containing 1 µM S1P and incubated in a well without cells overnight was used as a 100% control. After overnight incubation, subsequent LC/MS/MS analysis was performed. Shown are mean+SD of triplicate (n=6). One-way ANOVA with a post-hoc Holm-Sidak's t-test was performed to determine significances. *: $p \leq 0.05$.

Table 6: Addition of S1P, OVA, BSA, HSA, SOV, and non-protein part of BSA (NPBSA) extracted by methanol precipitation to the cell culture medium of HTC_4 wild type cells.

Sample	S1P	OVA	BSA	HSA	Others	Incubation with HTC_4 cells
1	-	50 mg/ml	-	-	-	+
2	1 µM	15 mg/ml	-	-	-	+
3	1 µM	30 mg/ml	-	-	-	+
4	1 µM	50 mg/ml	-	-	-	+
5	-	-	50 mg/ml	-	-	+
6	1 µM	-	-	-	-	+
7	1 µM	-	15 mg/ml	-	-	+
8	1 µM	-	30 mg/ml	-	-	+
9	1 µM	-	50 mg/ml	-	-	+
10	-	-	-	50 mg/ml	-	+
11	1 µM	-	-	15 mg/ml	-	+
12	1 µM	-	-	30 mg/ml	-	+
13	1 µM	-	-	50 mg/ml	-	+
14	1 µM	-	-	-	-	-
15	1 µM	-	-	-	NPBSA extracted from 100 mg/ml BSA	+
16	1 µM	-	-	-	1 mM SOV	+

4.3 Inability of pepsinized BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells

BSA and HSA could prevent extracellularly added S1P from metabolism by HTC_4 cells, but NPBSA could not, so that it was crucial to demonstrate that this effect was derived from the protein part of BSA.

4.3.1 The effectiveness of pepsin to digest BSA

Pepsin is an endopeptidase and can digest peptide bonds. The activity of pepsin is dependent on an acidic environment for it to digest protein. Pepsin is most active around a pH of 1.5 to 2. Pepsin is inactive at pH above 4 (Samloff 1989). BSA at concentration of 50 mg/ml in MEM (pH was changed to 1.9 by HCl) without or with pepsin at different concentration as indicated was incubated overnight in the water bath at 37 °C (as listed in Table 7). As shown in Fig. 8 A, for native BSA without overnight incubation, a main band was obtained at a molecular mass (Mm) of 67 kDa, which could also be seen in BSA overnight incubated with no pepsin, 0.1 µg/ml pepsin, and 0.01 µg/ml pepsin at pH 1.9. But this band could not be seen in BSA overnight incubated with 1 µg/ml pepsin at pH 1.9.

To show the fragmented BSA caused by the digestion of pepsin, the dilution of samples after incubated with 1 µg/ml pepsin in MEM was done before they were denatured (all the samples normally were diluted 1000 times by MEM before denaturing in Fig. 8 A). As shown in Fig. 8 B, several bands (below 66.5 kDa) corresponding to hydrolyzed BSA were found. 50 mg/ml BSA can be digested by overnight incubation with 1 µg/ml pepsin in MEM at pH 1.9.

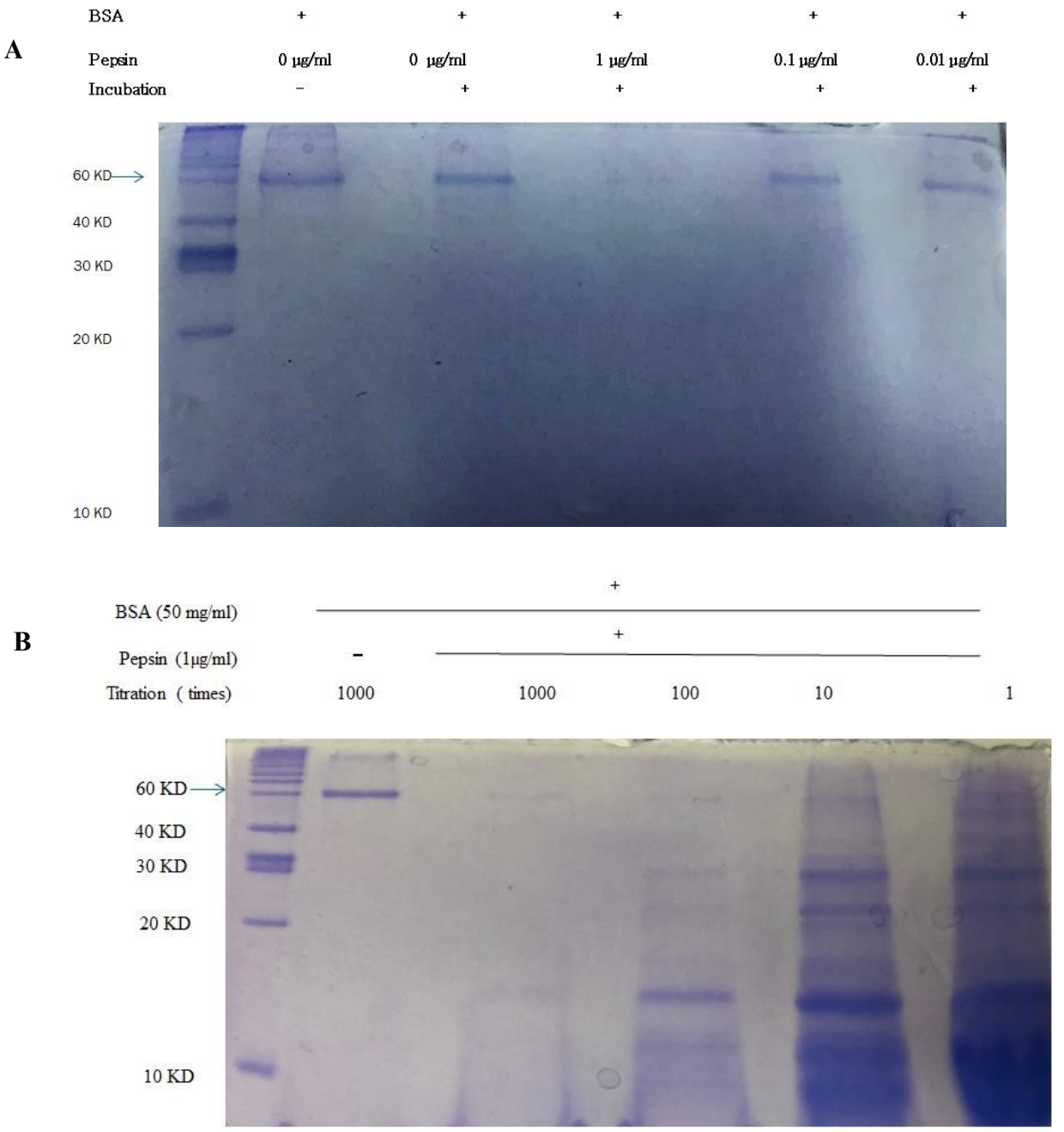

Fig. 8: SDS-PAGE patterns of pepsin treated BSA during proteolysis. (**A**) 50 mg/ml BSA was incubated overnight with and without pepsin at indicated concentration at 37 °C and pH 1.9. (**B**) The sample of 50 mg/ml BSA incubated overnight with 1 µg/ml pepsin at indicated concentration at 37 °C and pH 1.9 was shown in different dilution by MEM. Each experiment was done at least three times and shown results are one of the representatives.

Table 7: The proteolysis of BSA by pepsin.

Sample	BSA (50 mg/ml)	Pepsin (µg/ml)	1st overnight incubation (37 °C)
A	+	-	-
B	+	-	+
C	+	1	+
D	+	0.1	+
E	+	0.01	+

4.3.2 S1P and Sph concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells without or with S1P, BSA, or pepsinized BSA

To investigate whether the effect of serum albumin on the extracellular metabolism of S1P by cells is derived from the protein part of BSA, 1 µM S1P without or with 50 mg/ml BSA, and 50 mg/ml pepsinized BSA (as listed in Table 8) was extracellularly added to the HTC_4 wild type cells (5×10^5 cells) and incubated for 2 h. After the incubation, supernatant was harvested and centrifuged. Lipid extraction and LC/MS/MS were performed to quantify the S1P and Sph concentration in the cell culture medium after the incubation. The S1P concentration of the cell culture medium to which 1 µM S1P was added and which was incubated in a well without cells for 2 h was used as a 100% control (Sample D in Table 8). Very low levels of Sph were found in all samples (Fig. 9). As shown in Fig. 11, 17.3 ± 1.8% S1P could be found in the supernatant after 2 h incubation with HTC_4 wild type cells. As shown in Fig. 9, compared with 17.3 ± 1.8% S1P found in the supernatant without BSA after 2 h incubation, 24.1 ± 5.9% S1P was found in the supernatant with 50 mg/ml pepsinized BSA and the difference was not statistically significant. 63.1 ± 4.0% S1P could be found in the supernatant with 50 mg/ml BSA, which underwent pH change and the 1st incubation but without pepsin, after 2 h incubation with HTC_4 wild type cells. The S1P levels in the supernatant with 50 mg/ml BSA is statistically significantly higher than that in the supernatant without BSA and that in the supernatant with 50 mg/ml pepsinized BSA. These data together with data in Fig. 7 D suggest that the effect of BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cell derives from the protein part of BSA, instead of the non-protein part of BSA.

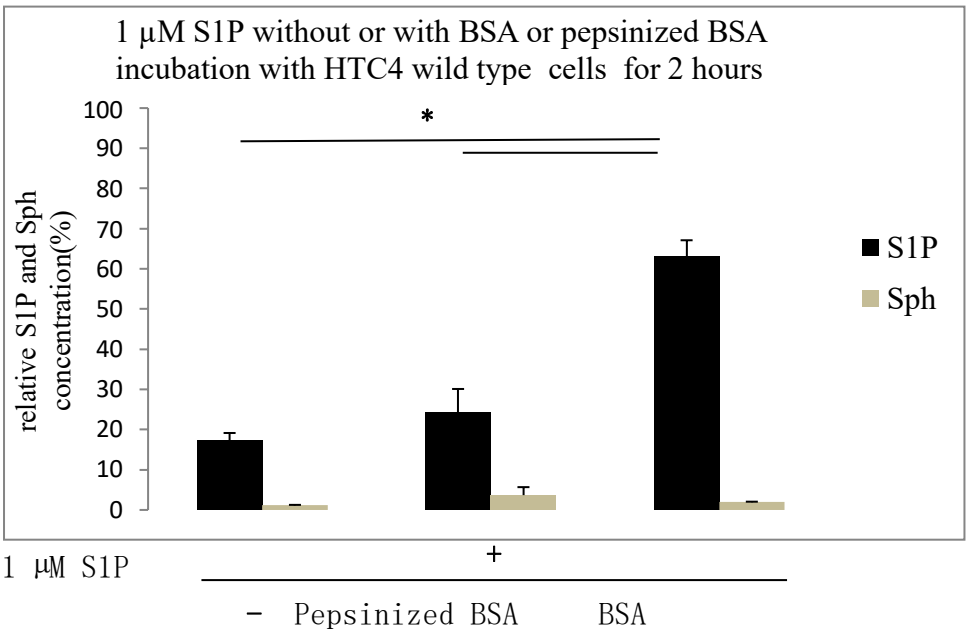

Fig. 9: S1P and Sph concentration in the supernatant of cell culture medium after 2h incubation with HTC_4 cells with extracellularly added 1 µM S1P and 50 mg/ml BSA (B), 50 mg/ml pepsinized BSA. 1 µM S1P with 50 mg/ml BSA or 50 mg/ml pepsinized BSA in cell culture medium (supernatant) was incubated with 5×10^5 HTC4 wild type cells (adherently grown) for 2 h. The S1P concentration of cell culture medium containing 1 µM S1P and incubated in a well without cells for 2 h was used as a 100% control. After 2h incubation, subsequent LC/MS/MS analysis was performed. Shown are mean+SD of triplicate (n=6). One-way ANOVA with a post-hoc Holm-Sidak's t-test was performed to determine significances. *: $p \leq 0.05$.

Table 8: Addition of S1P, BSA, and pepsinized BSA to the cell culture medium of HTC_4 wild type cells.

Sample	S1P	BSA (50 mg/ml)	Pepsin (µg/ml)	1st incubation	2nd incubation with HTC_4 cells
A	+	-	-	-	+
B	+	+	+	+	+
C	+	+	-	+	+
D	+	-	-	-	-

4.4 Effects of serum albumin on degradation kinetics of extracellular S1P and Sph with HTC_4 wild type cells

Since SA could inhibit extracellular metabolism of S1P by cells, the next question was whether SA inhibited the extracellular dephosphorylation of S1P or/and the take-in of Sph by cells. To demonstrate the effect of serum albumin on extracellular metabolism of S1P and Sph, 1 μM of extracellularly added S1P (as shown in Fig. 10 A) or 1 μM of extracellularly added Sph (as shown in Fig. 10 B) in cell culture medium with or without 50 mg/ml BSA was incubated with 5×10^5 HTC_4 wild type wells. After a certain time point of incubation as indicated, the supernatant was harvested and centrifuged. Lipid extraction and LC/MS/MS were performed to quantify the S1P or Sph concentration in the cell culture medium (supernatant) after the incubation. The S1P concentration of the cell culture medium to which 1 μM S1P was added and which was incubated in a well without cells and without incubation (0 h) was used as a 100% control for S1P levels in samples with extracellular added S1P. As shown in Fig. 10 A, 1 μM S1P in the supernatant without 50 mg/ml BSA was almost all metabolized by HTC_4 cells after 24 h, while 42.5 ± 8.2% S1P could still be found in the supernatant with 50 mg/ml BSA after 24 h. This statistically significant difference is in accord with the result shown in 4.1.2.1. 38.5 ± 9.3% S1P could be detected in the supernatant without 50 mg/ml BSA after 4 h incubation. In contrast, 42.5 ± 8.2% S1P could be still found in the supernatant with 50 mg/ml BSA after 24 h incubation.

The Sph concentration of the cell culture medium to which 1 μM Sph was added and which was incubated in a well without cells and without incubation (0 h) was used as a 100% control for Sph levels in samples with extracellular added Sph. As shown in Fig. 10 B, 1 μM Sph in the supernatant without and with 50 mg/ml BSA were almost all metabolized by HTC_4 cells after 24 h. No significant difference was between the relative Sph concentration in the supernatant without and with 50 mg/ml BSA after 24 h.

The difference of the S1P concentrations between the supernatant to which 1 μM S1P without BSA was added and with 50 mg/ml BSA was added was statistically significant. The difference of the Sph concentrations between the supernatant to which 1 μM Sph without BSA was added and with 50 mg/ml BSA was added was found not statistically significant. These data show that the clearance of extracellularly added S1P and Sph by HTC_4 cells shared the similar decay curve and serum albumin do not change the decay curve of extracellularly added Sph but slow down the decay of extracellularly added S1P.

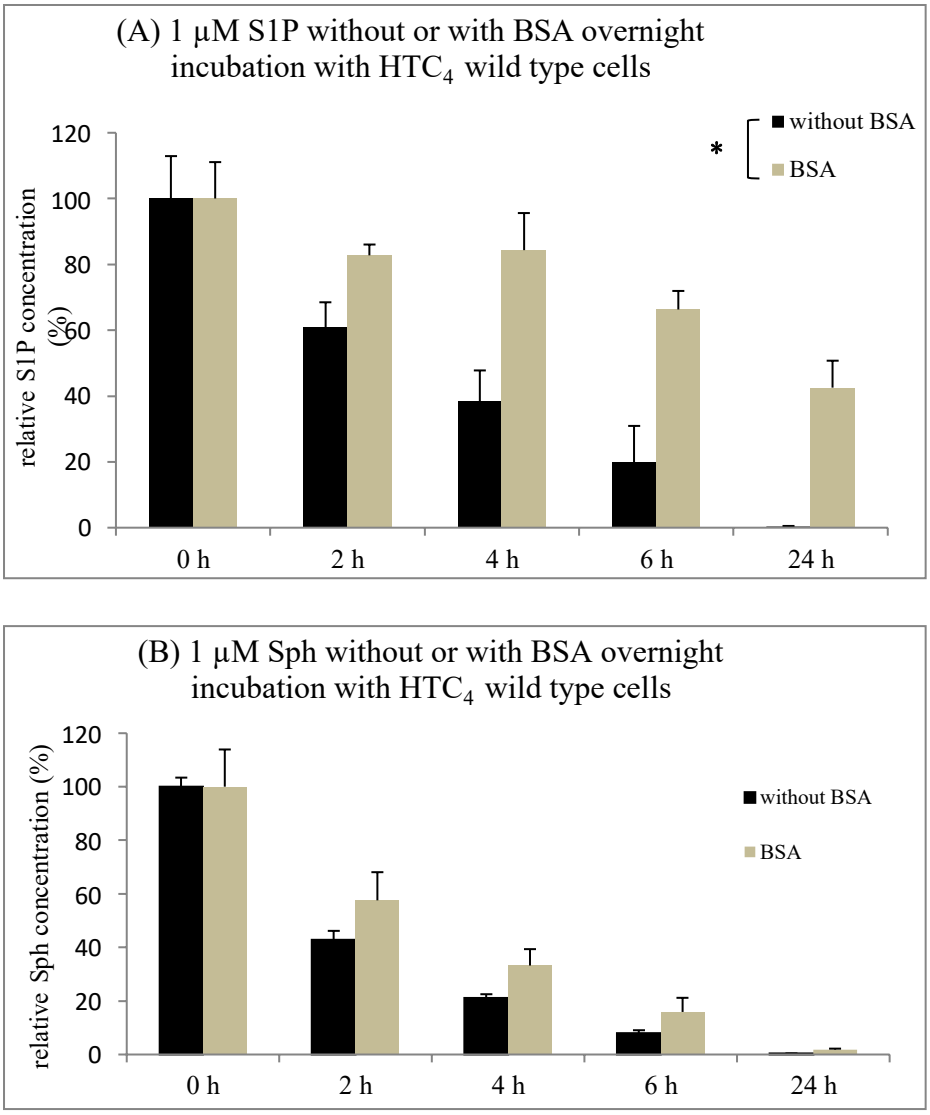

Fig. 10: The effect of serum albumin on degradation kinetics of extracellular added S1P (A) and Sph (B) with HTC_4 wild type cells.

1 µM of extracellularly added S1P (A) or Sph (B) in cell culture medium with or without 50 mg/ml BSA was incubated with 5×10^5 HTC_4 wild type wells after a certain incubation time as indicated. The S1P (A) or Sph (B) concentration of cell culture medium containing 1 µM S1P (A) or Sph (B) incubated in a well without cells and without overnight (0 h) was respectively used as a 100% control. After a 24 h period, subsequent LC/MS/MS analysis was performed to detect the extracellular concentrations (supernatant) of added sphingolipids. Shown are mean+SD of triplicate. Two-way ANOVA with a post-hoc Bonferroni t-test was performed to determine significances. *: $p \leq 0.05$.

Table 9: Incubation time and extracellular addition of S1P or Sph and with or without addition of BSA to the cell culture medium of HTC_4 wild type cells.

Compositions of samples	**Time point (hours)**
1 µM S1P	0
1 µM S1P	2
1 µM S1P	4
1 µM S1P	6
1 µM S1P	24
1 µM S1P and 50 mg/ml BSA	0
1 µM S1P and 50 mg/ml BSA	2
1 µM S1P and 50 mg/ml BSA	4
1 µM S1P and 50 mg/ml BSA	6
1 µM S1P and 50 mg/ml BSA	24
1 µM Sph	0
1 µM Sph	2
1 µM Sph	4
1 µM Sph	6
1 µM Sph	24
1 µM Sph and 50 mg/ml BSA	0
1 µM Sph and 50 mg/ml BSA	2
1 µM Sph and 50 mg/ml BSA	4
1 µM Sph and 50 mg/ml BSA	6
1 µM Sph and 50 mg/ml BSA	24

4.5 The capability of SA to prevent S1P from dephosphorylation by alkaline phosphatase (AP)

The results of 4.1, 4.2 and 4.3 suggest that SA prevent S1P from dephosphorylation in cell culture experiments. Therefore, it was necessary to demonstrate this effect in cell-free experiments. In order to investigate the ability of SA to prevent S1P from dephosphorylation by AP, 1 µM S1P and 3 u AP were added to 1 ml MEM (without FCS) with or without 50 mg/ml BSA or 50 mg/ml HSA. After overnight incubation at 37 °C in a water vapor

saturated atmosphere with 5% carbon dioxide in the cell culture incubator, the S1P and Sph concentrations of the samples were determined by LC/MS/MS. The S1P concentration of the solution to which 1 µM S1P, 50 mg/ml BSA or 50 mg/ml HSA was added and which was incubated in a glass tube without AP overnight was respectively used as a 100% control to the samples with the same amount of S1P, BSA, or HSA but without AP (as listed in Table 10), respectively. As shown in Fig. 11, after overnight incubation, 43.1 ± 19.5% Sph was found in the samples to which 1 µM S1P and 3 u AP was added and which was incubated overnight, while 6.1 ± 1.7 % Sph was detected in the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA was added, and 2.8 ± 1.3% Sph was detected in the samples to which 1 µM S1P, 3 u AP and 50 mg/ml HSA was added. The differences of the Sph concentrations were statistically significant between the samples to which 1 µM S1P and 3 u AP was added and the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA or 50 mg/ml HSA was added. The differences of the Sph concentrations were not statistically significant between the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA was added and the samples to which 1 µM S1P, 3 u AP and 50 mg/ml HSA was added. 44.3 ± 7.1% S1P was found in the samples to which 1 µM S1P and 3 u AP was added and which was incubated overnight, while 86.1 ± 5.2% S1P could be still detected in the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA was added, and 101.4 ± 28.7% S1P could be detected in the samples to which 1 µM S1P, 3 u AP and 50 mg/ml HSA was added. The differences of the S1P concentrations were statistically significant between the samples to which 1 µM S1P and 3 u AP was added and the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA or 50 mg/ml HSA was added. The S1P concentrations were not statistically different between the samples to which 1 µM S1P, 3 u AP and 50 mg/ml BSA was added and the samples to which 1 µM S1P, 3 u AP and 50 mg/ml HSA was added. These suggested that both BSA and HSA can prevent S1P from dephosphorylation by AP at physiological pH.

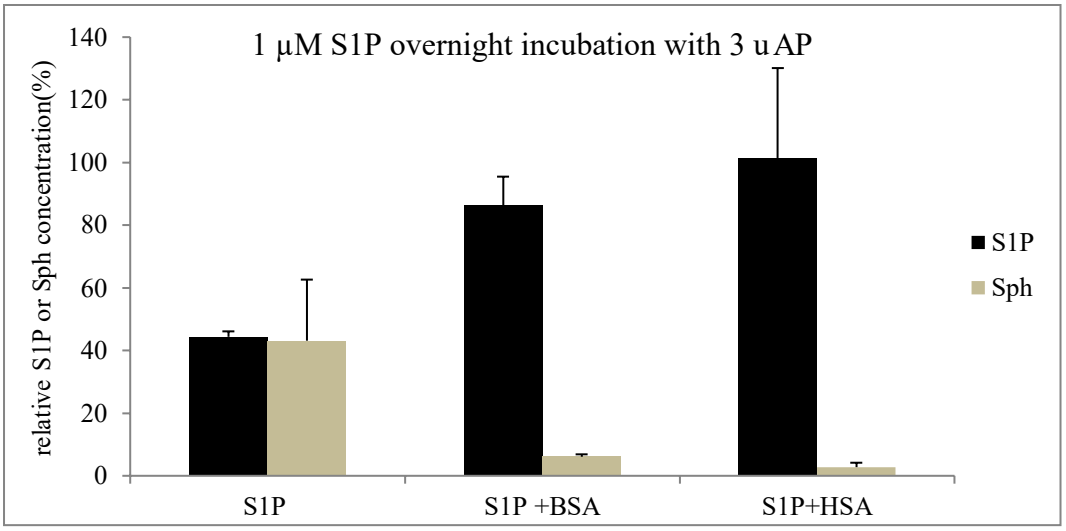

Fig. 11: The capability of serum albumin to prevent S1P from dephosphorylation by alkaline phosphatase.

1 µM S1P and 3 u AP were added to 1 ml MEM (without FCS) with or without 50 mg/ml BSA or 50 mg/ml HSA. The S1P concentration of the solution to which 1 µM S1P, 50 mg/ml BSA or 50 mg/ml HSA was added and which was incubated in a glass tube without AP overnight was respectively used as a 100% control to the samples incubated with AP. Shown are mean+SD of triplicate (n=6). One Way ANOVA with a post-hoc Holm-Sidak's test was performed. *: $p \leq 0.05$.

Table 10: Addition of S1P, AP, BSA, or HSA to the MEM.

Component / Sample	S1P	AP	HSA	BSA
S1P control	1 µM	-	-	-
S1P+BSA control	1 µM	-	-	50 mg/ml
S1P+HSA control	1 µM	-	50 mg/ml	-
S1P	1 µM	3 u	-	-
S1P+BSA	1 µM	3 u	-	50 mg/ml
S1P+HSA	1 µM	3 u	50 mg/ml	-

4.6 The effect of SA on S1P metabolism by HTC_4 cells shown by S1P elicited S1PR1 internalization

SA prevents S1P from dephosphorylation and metabolism. It is interesting to investigate how this effect plays a role in S1PR expression.

Upon stimulation with S1P, S1PR1 expression on cell surface is down-regulated by agonist-induced receptor internalization. Surface expression of S1PR1 after incubation with different reagents (samples are as shown in Table 11, which were overnight incubated with 5×10^5 HTC_4 wild type cells as mentioned in 3.2.1.3.2.2 and Fig. 4) for 15 min was measured by FACS on HTC_4 S1PR1 HA cells after cell staining. After incubation, Cell staining was performed in phosphate-buffered saline (PBS with Ca^{2+} and Mg^{2+}) with 5% FCS for 60 min on ice. The N-terminal HA-epitope (peptide sequence: MGYPYDVPDYAGGP) was detected with a rat anti-HA primary antibody (Rat α-HA, RG1 3F110, hybridoma supernatant) and an anti-rat fluorescence-labelled secondary antibody (α-rat-Cy5).

The linear mean values of fluorescence intensity at FL4 of HTC_4 S1PR1 HA cells from each group (as shown in Table 11) were calculated after gating (gating strategy is shown in 6.1). The linear mean of HTC_4 S1PR1 HA cells which were stimulated with 1 μM S1P (sample 14 as shown in Table 11) was set as 0% for all groups, while the linear mean of control cells which received both Rat α-HA (the primary antibody) and α-rat-Cy5 (the second antibody) was set as 100%.

As shown in Fig. 12 A, the relative S1P1 surface expression level of HTC_4 S1P1 HA cells was 101.7 ± 8.6% after 15 mins' stimulation with the cell culture medium without the addition of OVA, BSA, and HSA after pre-incubation overnight with HTC_4 wild type cells (as mentioned in 3.2.1.3.2.2), compared with 100% control. The relative S1P1 surface expression level was 112.2 ± 9.2%, when the cell culture medium contained 1 μM S1P and 15 mg/ml OVA and it was 111.2 ± 23.5% when the cell culture medium contained 1 μM S1P and 30 mg/ml OVA. When the cell culture medium contained 1 μM S1P and 50 mg/ml OVA, the relative S1P1 surface expression level stayed at 134.3 ± 23.9%. There were no significant differences in statistical analyses found among the relative S1P1 surface expression levels of these groups. No S1P were in the cell culture medium of groups shown in Fig. 12 A. It confirmed the data shown in Fig. 7 A. S1P was extracellularly metabolized by HTC_4 wild type cells and OVA could not prevent S1P from metabolism by HTC_4 wild type cells.

The relative S1P1 surface expression level decreased to 44.2 ± 4.8%, when the cell culture medium contained 1 µM S1P and 15 mg/ml BSA and it reduced to 34.0± 2.8% when the cell culture medium contained 1 µM S1P and 30 mg/ml BSA. When the cell culture medium contained 1 µM S1P and 50 mg/ml BSA, the relative S1P1 surface expression level decreased to 30.8 ± 12.6%. A clear tendency of dose dependent effect of BSA on S1PR1 expression level can be seen in Fig. 12 A. Significant difference between the S1PR expression levels of the HTC_4 S1PR1 HA cells stimulated with samples in which 1 µM S1P were overnight incubated with HTC_4 wild type cells and that in which1 µM S1P with 50 mg/ml BSA were overnight incubated with HTC_4 wild type cells was found. The S1PR expression levels of the HTC_4 S1PR1 HA cells were also significantly different between the samples in which 1 µM S1P were overnight incubated with HTC_4 wild type cells and that in which 1 µM S1P with 30 mg/ml BSA were overnight incubated with HTC_4 wild type cells.

The relative S1P1 surface expression level decreased to 52.6 ± 17.5%, when the cell culture medium contained 1 µM S1P and 15 mg/ml HSA and it reduced to 43.6 ± 10.1% when the cell culture medium contained 1 µM S1P and 30 mg/ml HSA. When the cell culture medium contained 1 µM S1P and 50 mg/ml HSA, the relative S1P1 surface expression level decreased to 35.6 ± 8.4%. A clear tendency of dose dependent effect of HSA on S1PR1 expression level can be seen in Fig. 12 A. The S1PR expression levels of the HTC_4 S1PR1 HA cells were significantly different between the samples in which 1 µM S1P were overnight incubated with HTC_4 wild type cells and that in which 1 µM S1P with 50 mg/ml HSA were overnight incubated with HTC_4 wild type cells.

BSA and HSA can increase S1P induced S1PR1 internalization after overnight incubation with cells, but OVA cannot.

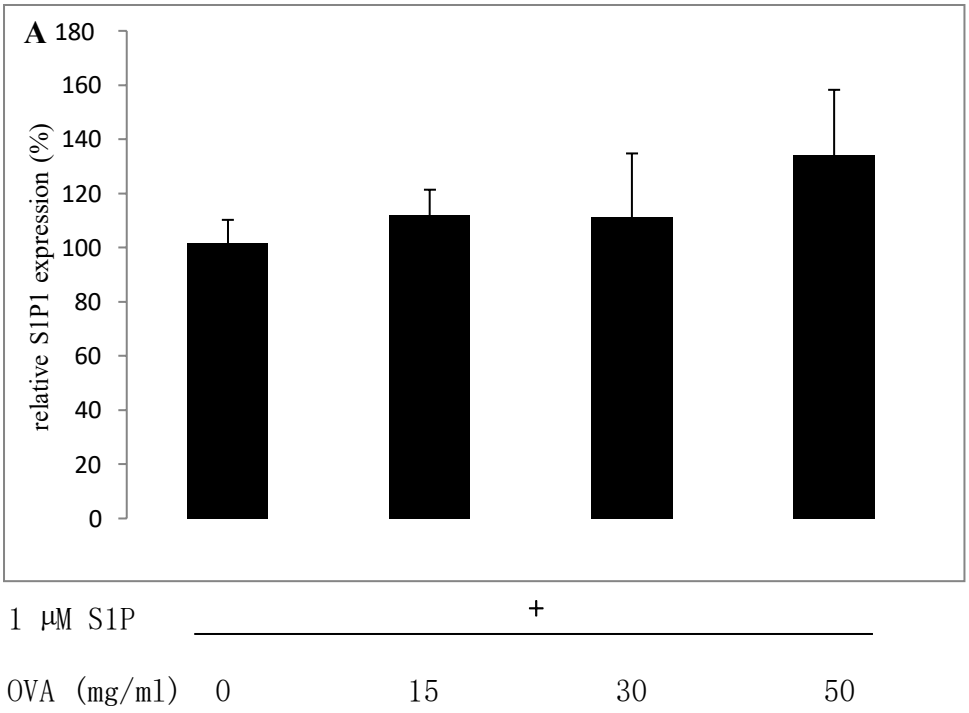

Fig. 12 A: Relative S1P level shown by S1P elicited S1PR1 internalization to indirectly show S1P concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with the indicated concentration of OVA. 1 μM S1P with the indicated concentration of OVA in cell culture medium was incubated with 5×10^5 HTC_4 wild type wells overnight. After this pre-incubation, the supernatant was subsequently used to stimulate HTC_4 S1PR1 HA cells. The cell surface expression levels of S1PR1 on HTC_4 S1PR1 HA cells were analyzed by fluorescence-activated cell sorting (FACS) as an indirect way to show the relative S1P concentration in the supernatant of each sample. Shown are mean+SD of duplicates (n=4). Kruskal-Walis test was performed to determine significances. *: $p \leq 0.05$.

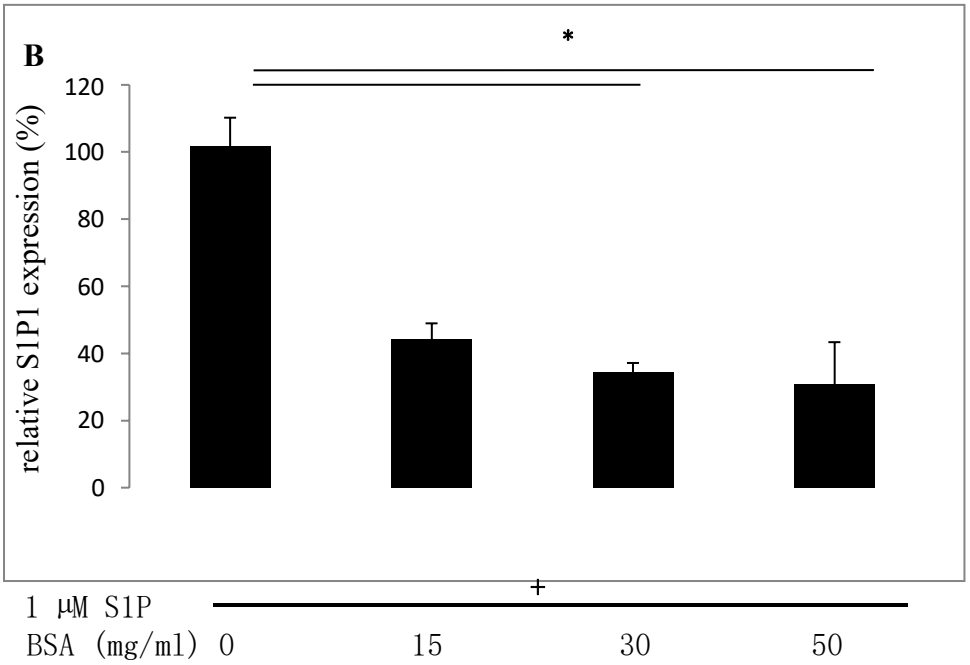

Fig. 12 B: Relative S1P level shown by S1P elicited S1PR1 internalization to indirectly show S1P concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with the indicated concentration of BSA. 1 μM S1P with the indicated concentration of BSA in cell culture medium was incubated with 5×10^5 HTC_4 wild type wells overnight. After this pre-incubation, the supernatant was subsequently used to stimulate HTC_4 S1PR1 HA cells. The cell surface expression levels of S1PR1 on HTC_4 S1PR1 HA cells were analyzed by fluorescence-activated cell sorting (FACS) as an indirect way to show the relative S1P concentration in the supernatant of each sample. Shown are mean+SD of duplicates (n=4). Kruskal-Walis test was performed to determine significances. *: $p \leq 0.05$.

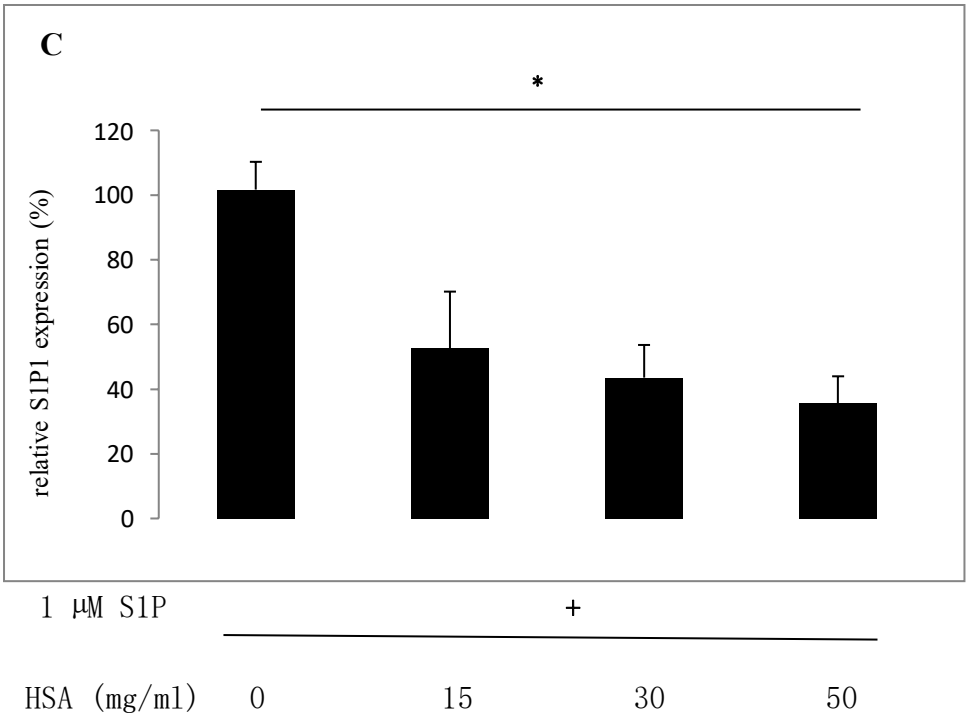

Fig. 12 C: Relative S1P level shown by S1P elicited S1PR1 internalization to indirectly show S1P concentration in the supernatant of cell culture medium after overnight incubation with HTC_4 cells with the indicated concentration of HSA. 1 μM S1P with the indicated concentration of HSA in cell culture medium was incubated with 5×10^5 HTC_4 wild type wells overnight. After this pre-incubation, the supernatant was subsequently used to stimulate HTC_4 S1PR1 HA cells. The cell surface expression levels of S1PR1 on HTC_4 S1PR1 HA cells were analyzed by fluorescence-activated cell sorting (FACS) as an indirect way to show the relative S1P concentration in the supernatant of each sample. Shown are mean+SD of duplicates (n=4). Kruskal-Walis test was performed to determine significances. *: $p \leq 0.05$.

Table 11: Addition of S1P, OVA, BSA, or HSA to the cell culture medium overnight incubation with HTC_4 wild type cells and subsequent stimulation for 15 mins to HTC_4 S1PR1 HA cells to determine S1PR1 expression levels.

Sample	1 µM S1P	OVA	BSA	HSA	1st overnight incubation with HTC_4 wild type cells
1	+	-	-	-	+
2	+	15 mg/ml	-	-	+
3	+	30 mg/ml	-	-	+
4	+	50 mg/ml	-	-	+
5	+	-	-	-	+
6	+	-	15 mg/ml	-	+
7	+	-	30 mg/ml	-	+
8	+	-	50 mg/ml	-	+
9	+	-	-	-	+
10	+	-	-	15 mg/ml	+
11	+	-	-	30 mg/ml	+
12	+	-	-	50 mg/ml	+

4.7 The effects of Fatty-acid-free BSA (FAFBSA) and fraction V BSA on the dephosphorylation of 4-nitrophenyl phosphate (NPP) by AP

The regular fraction V BSA could inhibit S1P dephosphorylation in cell culture and cell-free experiments (as mentioned in 4.2, 4.3 and 4.4). NPP phosphatase assay was implemented to detect phosphatase activity or to observe the ability of BSA to prevent NPP from dephosphorylation. 4-nitrophenyl (NP) which is the dephosphorylated product of NPP and can be detected at 405 nm by a Microplate Photometer Multiskan FC Type 357 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Beer–Lambert law was applied to the analysis of the concentration of NP in the samples by spectrophotometry during a 24-hour period. 3 u/ml AP and 10 µM NPP with or without BSA or FAFBSA in diluent buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) were put to 96-well plate (Sarstedt, Nümbrecht, Germany) on ice

(sample as listed in Table 12). The wavelength of Microplate Photometer Multiskan FC Type 357 was set at 405 nm and the mode was set at fast. The absorbance of each well at 405 nm was measured before the experiment started (0 h) and at 1 h, 2 h, 4 h, 6 h and 24 h after incubated in the incubator at 37 °C. The absorbance of each sample at 405 nm at 0 h was set as 0.

As shown in Fig. 13 A, 10 μM NPP was hydrolyzed to NP by AP. The absorbance of the NPP group reached 0.07595 ± 0.01280 at 1 h, and 0.15681 ± 0.02711 at 2 h with the highest speed, compared with later period. At 6 h, the absorbance of the NPP group reached 0.31003 ± 0.06934 and then increased at the slowest rate and reached 0.39613 ± 0.06494 at 24 h. The absorbance of the NPP 100 mg/ml BSA group reached 0.04678 ± 0.01608 at 1 h and 0.13315 ± 0.0115 at 2 h with the highest speed during this period, compared with later. At 6 h, the absorbance of the NPP 100 mg/ml BSA group reached 0.29020 ± 0.05221 and then increased at the slowest rate of the reaction and reached 0.42135 ± 0.03340 at 24 h. The NPP group, NPP 100 mg/ml BSA group, NPP 50 mg/ml BSA group, NPP 25 mg/ml BSA group and NPP 12.5 mg/ml BSA group shared a similar shape of curve and the statistical differences among these groups were not significant (as shown in Fig. 13 B). BSA had no effects on the dephosphrylation of NPP by AP.

The NPP 100 mg/ml FAFBSA group had the lowest absorbance value at 405 nm among all groups after 0 h. The absorbance of the NPP 100 mg/ml FAFBSA group reached 0.00553 ± 0.01528 at 1 h and 0.01300 ± 0.01435 at 2 h. At 6 h, the absorbance of the NPP 100 mg/ml FAFBSA group reached 0.01618 ± 0.01286 and then stayed at 0.01255 ± 0.00976 at 24 h. The NPP 12.5 mg/ml FAFBSA group had the highest absorbance value at 405 nm among all groups with FAFBSA after 0 h. The absorbance of the NPP 12.5 mg/ml FAFBSA group reached 0.03738 ± 0.01642 at 1 h, and 0.07635 ± 0.02818 at 2 h with the highest speed, compared with later. At 6 h, the absorbance of the NPP 12.5 mg/ml FAFBSA group reached 0.15343 ± 0.05612 and then increased at the slowest rate and reached 0.18440 ± 0.06040 at 24 h. The NPP 50 mg/ml FAFBSA group had the second lowest absorbance value at 405 nm among all groups after 0 h. The absorbance of the NPP 50 mg/ml FAFBSA group reached 0.01453 ± 0.00684 at 1 h and 0.02765 ± 0.00766 at 2 h. At 6 h, the absorbance of the NPP 50 mg/ml FAFBSA group reached 0.04430 ± 0.01138 and then stayed at 0.0592 ± 0.00760 at 24 h. The differences between the NPP 12.5 mg/ml and 100 mg/ml FAFBSA group and between the NPP 12.5 mg/ml and 50 mg/ml FAFBSA group were statistically significant (as shown in Fig. 13 B). No significant differences were found in multiple comparisons of statistics among other groups with FAFBSA. The differences between the NPP group and the NPP 12.5 mg/ml FAFBSA

group, the NPP group and the NPP 25 mg/ml FAFBSA group, the NPP group and the NPP 50 mg/ml FAFBSA group, as well as the NPP group and the NPP 12.5 mg/ml FAFBSA group were significant in statistical analyses (as shown in Fig. 13 B). FAFBSA can prevent dephosphrylation of NPP by AP, but not the regular fraction V BSA (used in all the experiments mentioned before).

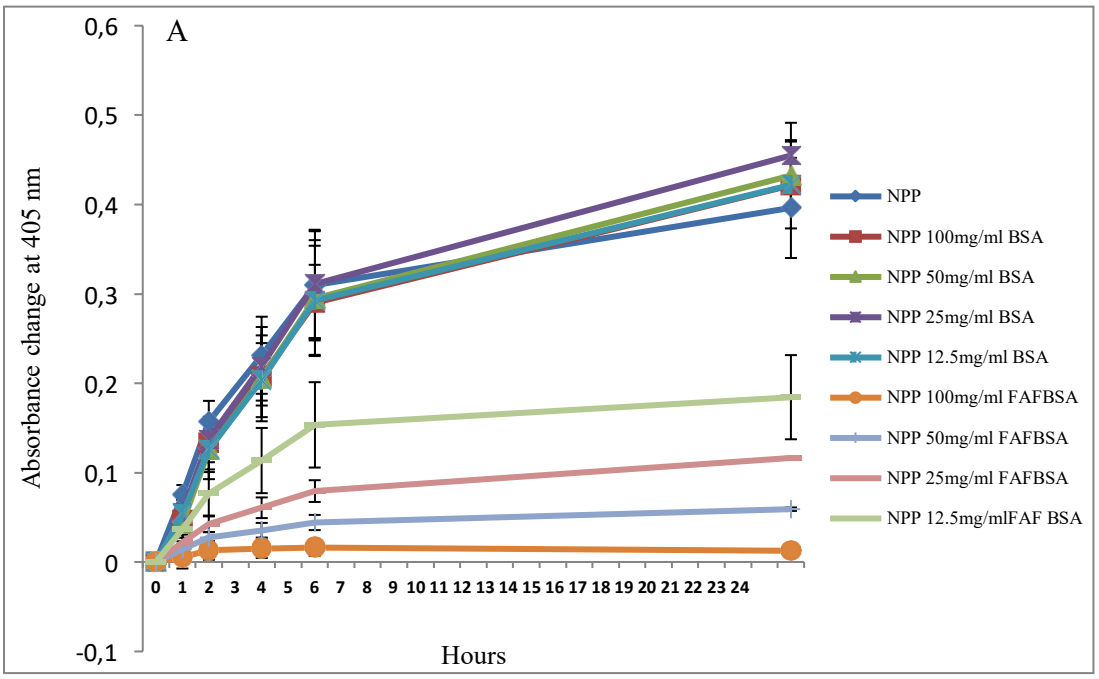

Fig. 13 A: The capability of FAFBSA to prevent 4-nitrophenyl phosphate (NPP) from dephosphorylation. (Samples are listed in Table 12)

3 u/ml AP and 10 µM NPP with or without BSA or FAFBSA in diluent buffer were put to 96-well plate. The absorbance of each well at 405 nm was measured before the experiment started (0 h) and at 1 h, 2 h, 4 h, 6 h and 24 h after incubated in the incubator at 37 °C. The absorbance change of each sample at 405 nm was shown in (**A**), compared with the absorbance change of each sample at 405 nm before the incubation (0 h). Shown are mean ± SD of duplicates (n = 4). (The multiple comparison is shown in Fig. 13 B.)

B

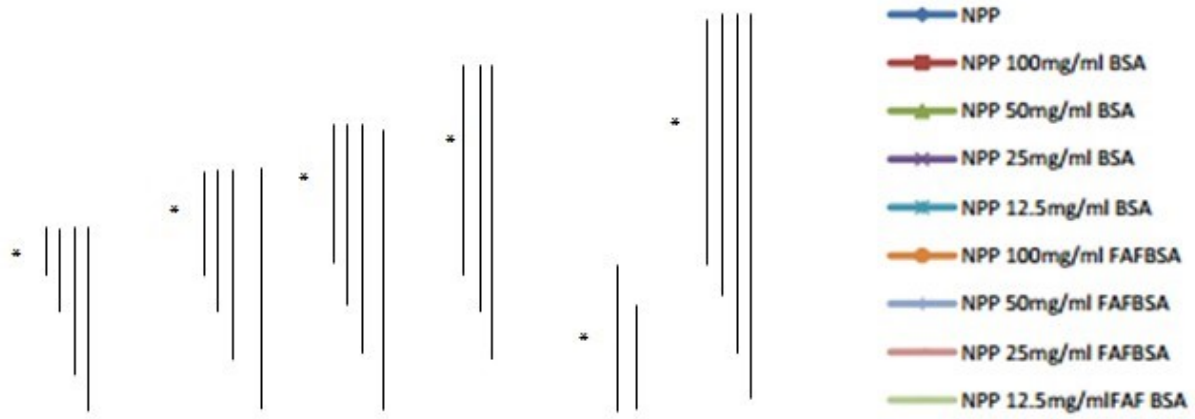

Fig. 13 B: The multiple comparison of each group from NPP dephosphorylation experiment (4.7) Two-way ANOVA with post-hoc Bonferroni Multiple Comparison test was performed to determine significances. *: ≤0.05.

Table 12: Composition of samples for NPP phosphatase assay.

Groups	**NPP**	**AP**	**BSA**	**FAFBSA**
NPP	10 µM	30 mu/ml	-	-
NPP 100 mg/ml BSA	10 µM	30 mu/ml	100 mg/ml	-
NPP 50 mg/ml BSA	10 µM	30 mu/ml	50 mg/ml	-
NPP 25 mg/ml BSA	10 µM	30 mu/ml	25 mg/ml	-
NPP 12.5 mg/ml BSA	10 µM	30 mu/ml	12.5 mg/ml	-
NPP 100 mg/ml FAFBSA	10 µM	30 mu/ml	-	100 mg/ml
NPP 50 mg/ml FAFBSA	10 µM	30 mu/ml	-	50 mg/ml
NPP 25 mg/ml FAFBSA	10 µM	30 mu/ml	-	25 mg/ml
NPP 12.5 mg/ml FAFBSA	10 µM	30 mu/ml	-	12.5 mg/ml

4.8 The ability of FAFBSA to bind NPP

As mentioned in 4.6, FAFBSA can prevent dephosphrylation of NPP by AP, but not the regular BSA (fraction V). NMR spectra of 1 mM NPP with 50 mg/ml regular BSA or 50 mg/ml FAFBSA in diluent buffer (the same as in NPP assay) were compared. As shown in Fig. 14, the left higher peaks in both blue and red line are phosphate control. The right peak in red shows the NPP with 50 mg/ml regular BSA, but the right peak in blue is decreased with 50 mg/ml FAFBSA. These data highly suggest that FAFBSA can bind NPP but the regular BSA (fraction V) cannot.

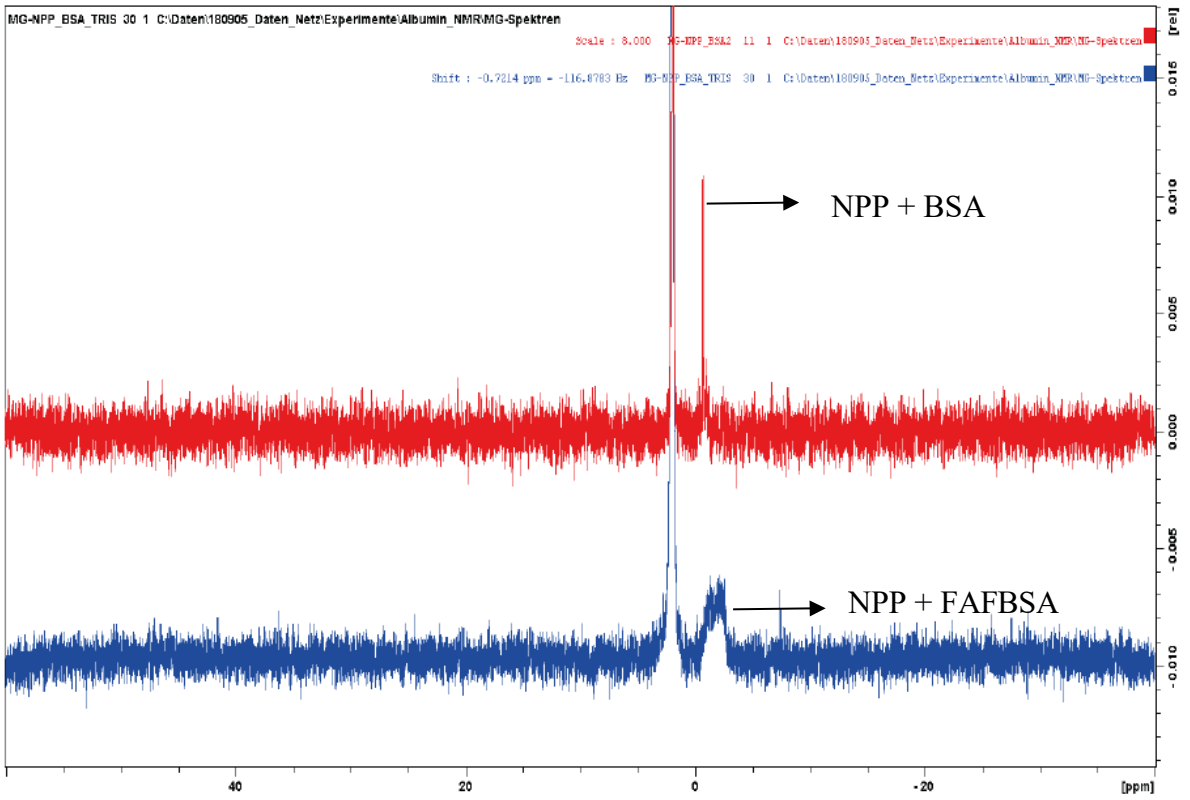

Fig. 14: The capability of FAFBSA to bind 4-nitrophenyl phosphate (NPP). The NMR spectra was measured in sample A (red) and B (blue) (as listed in Table 13). The left higher peaks in both blue and red line are phosphate control. The right peak in red shows the NPP with 50 mg/ml regular BSA, but the right peak in blue is decreased with 50 mg/ml FAFBSA.

Table 13: NPP comparison in fraction V BSA and FAFBSA.

Sample	Composition
A	1 mM NPP in diluent buffer with 50 mg/ml regular BSA (fraction V)
B	1 mM NPP in diluent buffer with 50 mg/ml FAFBSA

4.9 The ability of BSA to bind S1P

On one hand, SA can prevent S1P from dephosphorylation by phosphatases. On the other hand, SA can enhance S1P signaling. Whether BSA can bind S1P in physiological condition becomes an interesting question. As shown in Fig.15, a narrow high peak can be seen in blue line (S1P at pH 11). Compared with the broad peak in red line (S1P in BSA at pH 7.4), the peak in blue line is higher and left shifted (due to the high pH and double-deprotonation of the phosphate group), which highly suggests that the regular BSA can bind S1P.

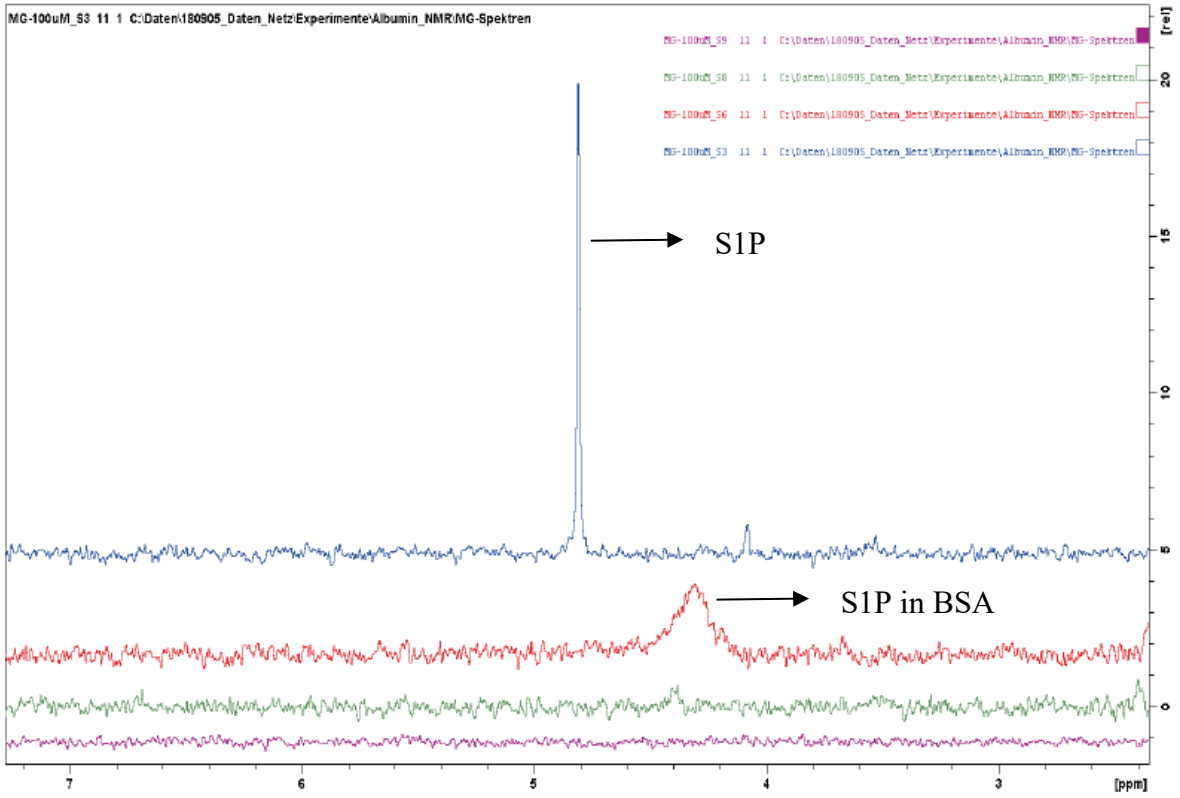

Fig. 15: The capability of BSA to bind S1P. The NMR spectra was measured in sample A (blue), B (red), sample C (green), and sample D (purple) (as listed in Table 14). A narrow high peak can be seen in blue line (S1P at pH 11). Compared with the broad peak in red line (S1P in BSA at pH 7.4), the peak in blue line is higher and left shifted (due to the high pH and double-deprotonation of the phosphate group).

Table 14: S1P comparison in fraction V BSA.

Sample	Composition
A	1 mM S1P in water at pH 11 (pH was adjusted by NaOH)
B	1 mM S1P with regular 50 mg/ml BSA in 1 mM Tris buffer at pH 7.4
C	regular 50 mg/ml BSA in 1 mM Tris buffer at pH 7.4
D	1 mM Tris buffer at pH 7.4

5 Discussion

The modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. As one of the important chaperones for S1P, the effects of serum albumin on degradation and metabolism of S1P were investigated. To explore the molecular mechanism, incubations with the S1P-metabolizing alkaline phosphatase and cells, different spectrometric analyses for structural analyses and determinations of S1P-SA interactions were investigated. It is still largely unclear how S1P is bond to its chaperone SA and how the SA influences the S1P-S1PR signaling axis. Although there are only limited data concerning the functional and structural role of albumin on S1P-S1PR axis, the researchers of molecular biology or sphingolipidologists incline to focus on ApoM-S1P, rather than albumin-S1P.

5.1 Functional characterization of the SA on S1P metabolism in cell culture experiments

5.1.1 SA can extract S1P from the outer RBC membrane.

As mentioned in 2.1.2, blood has the highest concentration of S1P, compared with lymph and interstitial fluids of tissues. Recent studies suggested that RBC are the main source of S1P in blood plasma (Bode et al. 2010) (Andréani and Gräler 2010). The ablation of both SphK1 and SphK2 caused total loss of S1P in plasma and the transfer of wild-type RBC to a mouse strain lacking plasma S1P by conditional gene deletion of SK1 and SK2 restored S1P in plasma to the normal levels (Camerer et al. 2009). Despite of being unable to synthesize Sph from ceramide, RBC have high SpK activity which is able to synthesis S1P from Sph, and lack the S1P-lyase and S1P phosphatases which belong to the S1P degrading enzyme system (Ito et al. 2007) (Yang et al.1999). After RBC incubation with 20μM sphingosine in RBC-buffer for 2 h at 37 °C with constant agitation, the S1P concentration in pelleted RBC was increased from about 2-4 μM (data not shown) to around 16 μM in Fig. 6. This result demonstrates that RBC are able to phosphorylate exogenously added Sph into S1P.

Erythrocytes can release S1P into plasma but not in serum- or plasma-free medium (Bode et al. 2010). Erythrocytes express the specific S1P transporter Mfsd2b and Mfsd2b knockout mice had 42-54% less S1P levels in plasma compared with wild-type mice (Vu et al. 2017). It was shown that S1P-binding molecules, such as Anti-S1P antibody Sphingomab, are able to extract S1P from the outer cell membrane of RBC (Bode et al. 2010). It is possible that albumin, other

S1P chaperones, or S1P binding molecules could interact with S1P transporter Mfsd2b to facilitate the egress of S1P from RBC to plasma.

Around 54% S1P can be found in the HDL fraction, about 35% S1P in the SA fraction, and much smaller composition in other lipoprotein fraction (Murata et al. 2000). It is still largely unknown how S1P is bound to its chaperone SA and how the SA influences the S1P signaling.

To investigate the effect of SA to extract S1P from the outer RBC membrane, Sph-preloaded RBC (about 5×10^8 cells) was incubated in 100 µl RBC-buffer with and without 50 mg/ml BSA (751 µM) for 2 h at 37 °C. As shown in Fig. 6, after 2 hours incubation with 100 µl RBC, 100 µl supernatant without BSA contained about 0.9 µM S1P while around 6.7 µM S1P was found in 100 µl supernatant with 751 µM BSA (50 mg/ml). The molecular ratio between S1P and BSA in the supernatant after incubation is 1:129. Although the incubation time could be too short to show the ability of BSA to extract S1P from RBC, our data show that BSA is able to extract S1P from the outer RBC membrane at molecular ratio of 129:1 between BSA and S1P.

S1P is present in human plasma at concentration from 0.59 µM to 0.91 µM and in human serum at concentration from 0.80 µM to 1.28 µM (Daum et al. 2020). The normal serum albumin values of human are approximately 35–50 mg/mL (526-751 µM). If 35% S1P is in the serum albumin fraction, one can assume that the ratio between HSA and S1P is much larger in physiological situation than in supernatant which contains only BSA and S1P (as shown in Fig. 6).

Monoclonal anti-S1P antibodies were employed to compete with chaperones of S1P to determine the equilibrium Kd for these carrier proteins binding S1P. It was evidenced that fatty FAF-BSA bound S1P with 41 µM, and the Kd values for S1P binding ApoM in the context of human HDL and LDL particles were 21 nM and 2.4 nM respectively according to this experiment (Fleming et al. 2016). The result that the ratio between SA and S1P is much larger than 129:1 between BSA and S1P, and the Kd values for S1P binding FAF-BSA is 41 µM (much higher than physiological S1P concentration in human plasma), illustrates that SA has a very low affinity to S1P compared with ApoM-HDL. Hence, one would tend to surmise that the majority of serum albumin cannot bind S1P in human plasma in physiological condition.

The one of the drawbacks of the S1P extracting experiment with BSA (4.1) is that HSA was not used due to limited amount of RBC. Moreover the incubation time could be too short to show the precise ability of BSA to extract S1P from RBC. One can also argue that the S1P

levels in RBC could be too low to show the ability of BSA to extract S1P from RBC. The S1P levels in RBC can be much lower without the Sph-loading procedure after incubation with BSA (data not shown).

5.1.2.1 Serum albumin can prevent extracellularly added S1P from metabolism by HTC_4 wild type cells.

To determine the effect of serum albumin on extracellular metabolism of S1P by cells, extracellularly added 1 μM S1P without or with different concentration of OVA, BSA, and HSA was incubated overnight with HTC_4 wild type cells. After overnight incubation, supernatant was harvested and centrifuged. Lipid extraction and LC/MS/MS were applied to determine the S1P and Sph concentration in the cell culture medium after overnight incubation.

The concentration levels of SA were chosen at 0 mg/ml, 15 mg/ml, 30 mg/ml, and 50 mg/ml, because the normal serum albumin range of human is approximately between 35 and 50 mg/ml. When SA concentration is below 34 mg/ml is diagnosed as hypoalbuminemia. Low serum albumin level is a risk factor and predictor of morbidity and mortality despite of implicated diseases (Gatta et al. 2012). Patients with low albumin on admission to hospital have lower survival rate, longer stays in hospital, and more likely to stay in hospital again after discharge (Herrmann et al. 1992). A drop of serum albumin can be resulted from a decreasing energy and amino acid supply, reduced liver synthesis, vascular leak, increased tissue catabolism, and the change of albumin distribution (Gatta et al. 2012). Albumin is a negative acute-phase protein. Hypoalbuminenia is associated with inflammation state, for example sepsis. Symptoms include edema in the lower parts of the body and ascites in the abdomen are typical for hypoalbuminenia. Therefore, the concentration setting of serum albumin and S1P in this experiment is of physiopathological meaning. It is possible to show the effects of SA in physiological range and pathological range on the metabolism of S1P.

Compared with the result that OVA had no effect on S1P metabolism by HTC_4 cells, BSA and HSA could prevent extracellularly added S1P from metabolism by HTC_4 wild type cells. From 0 mg/ml to 50 mg/ml, the higher the BSA concentration was added to the cell culture medium, the more S1P could be found in the supernatant after overnight incubation, which shows that BSA has a dose dependent effect to inhibit the metabolism of S1P by HTC_4 cells. Although from 0 mg/ml to 30 mg/ml the HSA had also a dose dependent effect to inhibit the metabolism of S1P by HTC_4 cells, from 30 mg/ml to 50 mg/ml HSA, similar S1P levels were found in the

supernatant of these samples. It is likely that from 30 mg/ml HSA (slightly below the normal serum albumin level in human blood) to 50 mg/ml (the maximum normal range of serum albumin in human blood), the ability of HSA to prevent S1P from extracellular metabolism stays at the similar level or reaches the upper platform of the efficacy curve (Daum et al. 2020), which becomes a possible reason why the levels of plasma S1P in a healthy group were not influenced by albumin concentration. But when albumin level is lower than 30 mg/ml, this effect is weaker and less S1P can be found in the supernatant, supporting the fact that septic patients have lower S1P and albumin levels in plasma (Winkler et al. 2015) (Yin et al. 2018). Meanwhile, data from our lab also showed that HDL at physiological level had no effect as SA on degradation of S1P by HTC_4 cells (data are not published). These data usher us in a new idea that S1P chaperones play different roles in the modulation of S1P degradation, thus SA is critical in maintaining S1P concentration in plasma.

The drawback of this experiment is that more detailed titration of HSA between 30 mg/ml and 50 mg/ml could be needed and the level of BSA and HSA higher than 50 mg/ml could also be interesting to be applied to observe this effect but of less pathophysiological significance compared with hypoalbuminemia, because high albumin or hyperalbuminemia is almost always the result of dehydration in clinical medicine.

Our lab is the first to show this effect of SA on the metabolism of S1P. As mentioned before, the modulation of S1P degradation is a critical event for the S1P-S1PR signaling pathway. The idea that extracellular S1P is dephosphorylated by phosphatases of cells to Sph, and Sph is further metabolized by cells was advanced (Peest et al. 2008). Deletion of LPP3 expression in mice liver resulted in increased S1P level in blood and plasma, which strengthens the predominant position of liver for the clearance of plasma-S1P (Kharel et al. 2020). Therefore, HTC_4 cells could be an ideal cell line to study LPP3 and the interactions between LLP3 and the carriers of S1P. Since very low levels of Sph were found in all samples (Fig. 7, A, B, and C), albumin seems to have little effect on Sph metabolism. SOV is a phosphatase inhibitor. Around 70% S1P could still be found in cell culture medium with 1 mM SOV after overnight incubation with HTC_4 cells, compared with the result that 1 µM S1P was almost all degraded by HTC_4 cells. These data suggested that the effect of SA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells could derive from the effect of BSA and HSA to inhibit the phosphatases of S1P. The drawback of this experiment is that 1 mM SOV did not totally

block the dephosphorylation of S1P. It is likely that the amount of SOV at 1 mM was too little to show the maximum blocking effect of SOV.

Methanol precipitation was used to extract the NPBSA (as mentioned in 3.2.1.3.1.2). NPBSA, the amount of which equals to 100 mg/ml BSA, could not prevent S1P from metabolism by HTC_4 wild type cells. These data imply that the effect of SA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cells could depend on its protein part. The disadvantage of this experiment is that NPBSA should have been extracted from higher than 100 mg/ml BSA to exclude loss in the extraction procedure, but the cells tended to die very fast when very high concentration of NPBSA was added (data not shown).

5.1.2.2 A dose dependent effect of BSA and HSA to protect S1P from degradation and thus induce S1PR1 surface internalization

A clear tendency of dose dependent effect of BSA and HSA on S1PR1 expression level can be seen in Fig. 7 B and C. There were no significant differences in statistical analyses found among the relative S1P1 surface expression levels of the groups in Fig. 12 A. It is reflected that very low levels of S1P were in the cell culture medium of groups shown in Fig. 12 A. It confirmed the data shown in Fig 7. A. S1P was extracellularly metabolized by HTC_4 wild type cells and OVA could not prevent S1P from metabolism by HTC_4 wild type cells.

The S1PR expression levels of the HTC_4 S1PR1 HA cells were not significantly different between the samples in which 1 µM S1P were overnight incubated with HTC_4 wild type cells and that in which1 µM S1P with 15 mg/ml BSA were overnight incubated with HTC_4 wild type cells. The explanation could be that the linear S1P levels in the samples are reflected as a logarithmic S1PR1 expression level on HTC_4 S1PR1 HA cells. The same explanation could also find in the fact that the S1PR expression levels of the HTC_4 S1PR1 HA cells were not significantly different between the samples in which 1 µM S1P were overnight incubated with HTC_4 wild type cells and that in which 1 µM S1P with 30 mg/ml HSA were overnight incubated with HTC_4 wild type cells.

Our data demonstrate that BSA and HSA increased S1P induced S1PR1 internalization after overnight incubation with HTC_4 cells, but OVA could not. The similar scenario could also happen in the blood vessel. Less S1PR1 might express on the surface of endothelial cells because of normal S1P and albumin level in health people, while more S1PR1 might express

on the surface of endothelial cells because of less albumin and resultant less S1P level in patient with pathological state, such as sepsis.

5.1.3 Serum albumin can prevent extracellularly added S1P, but not Sph, from metabolism by HTC_4 wild type cells

To demonstrate the effect of serum albumin on extracellular metabolism of S1P and Sph, extracellularly added S1P or Sph in cell culture medium with or without 50 mg/ml BSA was incubated with HTC_4 wild type wells. The difference of the S1P concentrations between the supernatant to which 1 μM S1P without BSA was added and with 50 mg/ml BSA was added was statistically significant. The difference of the Sph concentrations between the supernatant to which 1 μM Sph without BSA was added and with 50 mg/ml BSA was added was found not statistically significant. These data show that the clearance of extracellularly added S1P and Sph by HTC_4 cells shared the similar decay curve and serum albumin do not change the decay curve of extracellularly added Sph but slow down the decay of extracellularly added S1P. Radiolabel sphingosine was applied to human fibroblasts, rat neurons, and murine neuroblastoma cells, which showed that extracellularly added sphingolipids directly reached the lysosomes and then entirely catabolized (Chigorno et al. 2005).

These data also suggested that serum albumin can inhibit the extracellular dephosphorylation of S1P but cannot influence the taking-in of Sph by cells. According to the exponential decreasing curve of S1P and Sph without BSA, the degradation of these sphingolipids was relatively slow in this setting, because around 42% S1P and 60% Sph could still be found in the supernatant after 2 hours without BSA, while exogenously added S1P is cleared from blood in 15–30 min in vivo.

It is also extrapolated that the different effects of BSA on S1P and Sph also will also apply to HSA.

5.1.4 Pepsinized BSA cannot prevent extracellularly added S1P from metabolism by HTC_4 wild type cells

As mentioned in 4.3, the data corroborate that the effect of BSA to prevent extracellularly added S1P from metabolism by HTC_4 wild type cell derives from the protein part of BSA, instead of the non-protein part of BSA.

These data are of vital importance for the future study on the structural and functional interactions of SA and S1P. When the effective part of SA on S1P is located and identified, we may can pharmaceutically modulate the degradation of S1P in vivo, so as to increase the concentration of S1P in plasma in some pathological conditions where low plasma S1P is a contributor or one of the initiators of these diseases, such as, sepsis. Serum albumin is a heart-shaped molecule in tertiary structure which consists of 585 amino acids with a molecular weight of 66.5 kDa (Nicholson et al. 2000). Human serum albumin (HSA) has 35 cysteine residues and 34 of them form 17 disulfide bridges. The circulating molecule, which is held and folded by 17 disulfide bridges, consists of a series of α-helices. The protein consists of 3 homologous domains (I-III), each composed of 2 sub-domains (A and B), containing 4 and 6 α-helices respectively (Quinlan et al. 2005). As mentioned before, albumin can bind various drugs and change the distribution, clearance and increase the half-life of the drugs. Most compounds bind to one of the two major binding sites (site I and II) (Simard et al. 2006). It is also difficult to determine interactions between different ligands binding to albumin, because there are multiple fatty acid (FA) binding sites on HSA. Despite well adaption of the binding pockets to embrace FA, most of the binding sites can bind other ligands (Simard et al. 2006).

5.2 Functional characterization of the SA on S1P metabolism in cell-free experiments

5.2.1 The serum albumin (SA) can prevent S1P from dephosphorylation by alkaline phosphatase (AP)

AP is a homodimeric protein enzyme of 86 kDa. Each monomer has five cysteine residues. Zinc and magnesium are essential to its catalytic function, and it is optimally active at alkaline pH conditions. Although AP is not considered as a natural phosphatase for S1P, as shown that only around half of the 1 μM S1P could be dephosphorylated overnight incubated with 3 u AP, serum albumin is shown to have the capability of inhibiting dephosphorylation of S1P by AP.

It would be better if S1P-specific phosphatases, for example LLP3, are applied in this condition to substantiate this effect of SA.

Our lab is the first to show this effect of SA on the dephosphorylation of S1P. Does this effect of albumin play a role in disease like sepsis or septic shock, wherein both plasma-S1P and plasma-albumin drops and are positively associated with survival rates? There are still drastic debates on which kind of the resuscitation fluids is the best for sepsis even after large-scale

clinical trials. In experimental settings, albumin was shown to have more beneficial influences on the microcirculations than crystalloids, which are aqueous solutions of mineral salts or other water-soluble molecules (Vincent et al 2016). These impacts were connected with decreased cellular damage, decreased platelet and leukocyte adhesion. In severe sepsis, the mortality in albumin treated group was lower than that in saline treated group (Finfer et al.). In the Albumin Italian Outcome Sepsis (ALBIOS) trial where 1818 patients were included, although there were no significant differences between with and without albumin administration in survival after 28 or 90 days, a significant survival advantage was seen in the large subgroup of septic shock (more than 1300 subjects) after 90 days (Caironi et al. 2014). In this subgroup, albumin treatment caused also a favorable negative fluid balance at earlier stage, a longer hemodynamic stabilization in the first 24 hours, and a less dependence on vasopressor. Serum albumin is also now suggested to treat septic patients when substantial amounts of crystalloids alone are insufficient (Rhodes et al. 2016). Therefore, our results may offer a molecular mechanism or biochemical explanation for the clinical significance of the usage of albumin in septic shock or other severe diseases, in which the descent of albumin in plasma might cause the decrease of S1P in plasma and S1P or both of them could be the contributor or enhancer in the pathophysiological mechanism.

5.2.2 Fatty-acid-free BSA (FAFBSA) can prevent the dephosphorylation of 4-nitrophenyl phosphate (NPP) by AP, but not BSA (fraction V BSA).

NPP phosphatase assay can be used to detect phosphatase activity. 4-nitrophenyl (NP) which is the dephosphorylated product of NPP and can be detected at 405 nm by a Microplate Photometer Multiskan FC Type 357 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Beer–Lambert law was applied to the analysis of the concentration of NP in the samples by spectrophotometry during a 24-hour period. An absorbance value below 2 is recommended for colorimetric assays since 1% transmitted light is the maximum limit to fulfill the prerequisites of Beer–Lambert law for colorimetric measurements and the theoretical absorbance value of 1% transmitted light is 2. All the data of absorbance in this experiment are below 2.

It seems that the different results between FAFBSA and regular BSA are due to the binding of the NPP to FAFBSA. The reason why regular BSA could not inhibit the dephosphorylation of NPP in this case could be that it is saturated with fatty acids. This regular BSA was used in all the experiments mentioned before. The mechanism of SA to inhibit dephosphorylation of S1P

is still not clear. It could be the binding of SA to S1P, alteration of S1P ionization to changing the interaction between S1P and phosphatases, and so on. The NMR studies are needed to study the interactions between SA and S1P.

5.2.3 SA can bind S1P

FAFBSA can inhibit the dephosphorylation of NPP by phosphatases, but the regular BSA (fraction V) cannot, very likely due to the saturation of the fatty acid binding parts of BSA. This picture is different in the interaction of S1P and SA.

On one hand, SA can prevent S1P from dephosphorylation by phosphatases. On the other hand, SA can enhance S1P signaling. NMR studies strongly suggest that the regular BSA can bind S1P. Although this result may not be the real physiological situation (1 mM S1P was used in 4.9 due to the sensitivity of NMR spectrometry), 50 mg/ml SA can still bind 1 μM S1P in physiological situation. In spite of the evidence that the Kd of FAF-BSA to bind S1P is 41 μM, 50 mg/ml BSA (around 751 μM) can still bind around 95% of S1P after the calculation by the Kd equation. Meanwhile, in septic condition, the less S1P could be bound by SA due to decreased SA level. It is also interesting to investigate how SA enhances the S1P signaling when 95% of S1P is bound to SA.

5.3 Conclusion and Outlook

After overnight incubation with HTC_4 wild type cells, S1P was protected from metabolism by BSA and HSA. OVA, pepsinized BSA and NPBSA did not have this effect. SA inhibited the extracellular dephosphorylation of S1P but did not influence the uptake of Sph by cells. BSA and HSA increased S1P induced S1PR1 internalization after overnight incubation with HTC_4 cells, but OVA could not. In cell-free experiments, BSA and HSA can inhibit dephosphorylation of S1P by AP. FAFBSA can prevent dephosphrylation of NPP by AP, but not the regular BSA used in all the experiments before. Our data suggest that the binding of SA to S1P enables SA to have the above mentioned effects.

These data are of vital importance for the future studies on the structural and functional interactions of SA and S1P. The alteration of S1P degradation is a crucial event for the S1P-S1PR signaling pathway. Both plasma S1P and albumin are involved in various important pathophysiological events.

For the treatment of lots of clinical diseases, the major obstacle to use albumin is still its cost. When the effective part of SA on S1P is located and identified, we may pharmaceutically modulate the degradation of S1P in vivo, so as to increase the concentration of S1P in plasma in some pathological conditions where low plasma S1P is a contributor or one of the initiators of these diseases, for example, sepsis. And the costs of albumin could also be reduced.

Since the structure and function of albumin could influence the S1P-S1PR signaling pathway by having impacts on plasma S1P concentration, the post-translational modification on HSA could also play a role on plasma S1P concentration. Glycation, cysteinylation, S-nitrosylation, S-transnitrosylation, and S-guanylation are the most discussed modification of HSA now (Lee and Wu 2015). For example, glycation is binding a sugar molecule to some sites of a protein. It was reported that glycation can change HSA structure (Lee and Wu 2015). The glycation of HSA seems to have some impacts on the binding of some drugs (Lee and Wu 2015). Since the increased glycation of HSA correlates with blood glucose level and it is rising as a monitor for blood glucose level for diabetic patients. It would be of pathophysiological importance to study the effects of the modification of HSA in certain diseases on the metabolism and degradation of S1P.

6 Appendix

6.1 Gating strategy of FACS

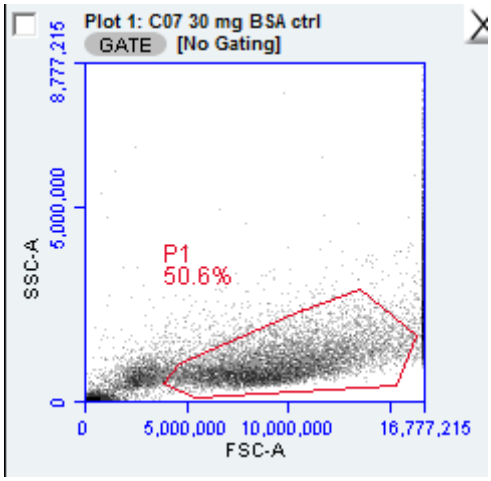

Fig. 14: Forward-scatter light (FSC) is proportional to cell-surface area or size, and side-scatter light (SSC) is proportional to cell granularity or internal complexity. The samples were first presented on FCS vs SSC plot to allow differentiation of different cell types after the flow cytometry measurement. Depending on size and granularity, the HTC_4 S1P1 population was labeled as the P1 gate.

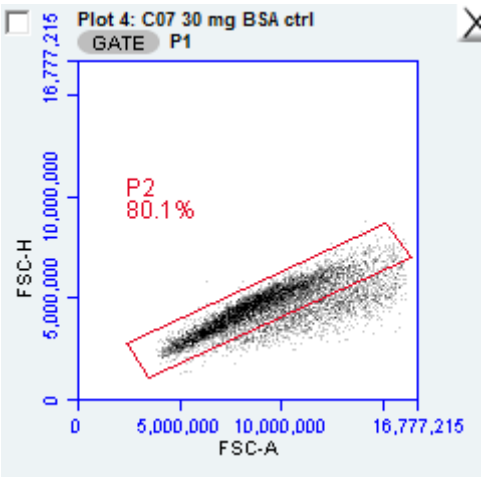

Fig. 15: To exclude doublets, FSC-A and FSC-H were applied in the P1 gate, because cells of the same size show similar A and H. Singlets are clustered diagonally and can be distinguished from doublets/multiplets. Diagonally clustered singlets were labeled as the P2 gate.

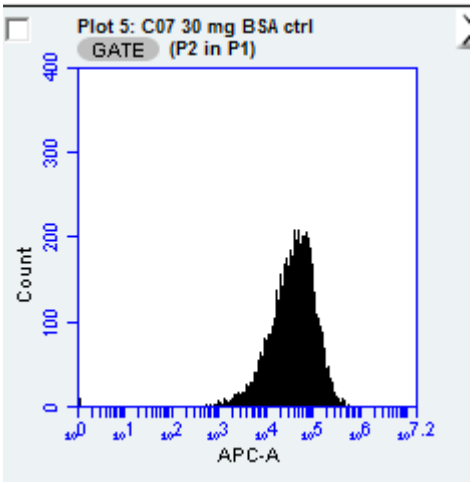

Fig. 16: Since Cy5 is excited at red laser (488 nm) and registered at the FL4 detector (optical filter 675/25, named also as APC channel), the next step considered the P2 population in the FL-4 histogram after dead cell exclusion. The Cy5 stained positive cells in the FL-4 histogram were marked as V1-R.

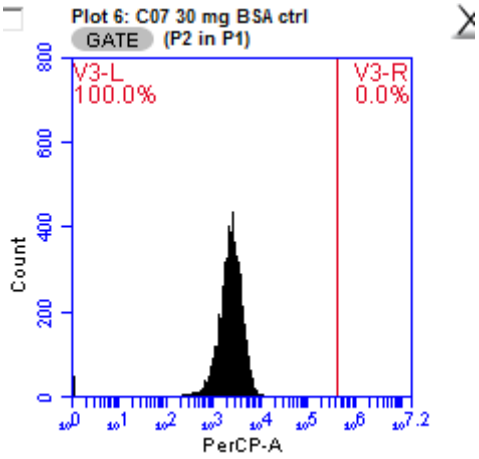

Fig. 17: To exclude dead HTC_4 S1P1 cells from analysis, a propidium iodide (PI) staining was performed. PI is detected in FL-3 (optical filter 670 LP). By visualizing a PI stained and unstained sample in the FL-3 histogram, the dead cell population can be labeled V3-R and excluded from further analysis.

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6.3 Sworn statement

I declare in lieu of oath that I have researched and written the doctoral thesis myself (statement of authorship), no passages of text have been taken from third parties or own exam papers without having been identified as such and that all tools, personal notifications, and sources used by the applicant have been indicated in the doctoral thesis.

The persons, who have supported the applicant in selecting and analyzing the material and preparing the manuscript are declared completely.

The assistance of a professional consultant has not been utilized and no third parties have either directly or indirectly received monetary benefits from the candidate for work related to the contents of the submitted doctoral thesis.

The doctoral thesis has not yet been submitted as an examination paper for state or other academic examinations.

Location, date signature, full name

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