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High density lipoprotein (HDL)-associated sphingosine 1-phosphate (S1P) inhibits macrophage apoptosis by stimulating STAT3 activity and survivin expression

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11 May 2024

HIGH DENSITY LIPOPROTEIN (HDL)-ASSOCIATED SPHINGOSINE 1-PHOSPHATE (S1P) INHIBITS MACROPHAGE APOPTOSIS BY STIMULATING STAT3 ACTIVITY AND SURVIVIN EXPRESSION

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Short running title: HDL, S1P and macrophage apoptosis

Main text word count: 4290

Abstract word count: 250

Number of references: 58

Number of figures and tables: 5

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ABSTRACT

Background and aims: Macrophage apoptosis is critically involved in atherosclerosis. We here examined the effect of anti-atherogenic high density lipoprotein (HDL) and its component sphingosine-1-phosphate (S1P) on apoptosis in RAW264.7 murine macrophages. **Methods and Results**: Mitochondrial or endoplasmic reticulum-dependent apoptosis was induced by exposure of macrophages to etoposide or thapsigargin/fukoidan, respectively. Cell death induced by these compounds was inhibited by S1P as inferred from reduced annexin V binding, TUNEL staining, and caspase 3, 9 and 12 activities. S1P induced expression of the inhibitor of apoptosis protein (IAP) family proteins cIAP1, cIAP2 and survivin, but only the inhibitor of survivin expression YM155 and not the cIAP1/2 blocker GDC0152 reversed the inhibitory effect of S1P on apoptosis. Moreover, S1P activated signal transducer and activator of transcription 3 (STAT3) and Janus kinase 2 (JAK2) and the stimulatory effect of S1P on survivin expression and inhibitory effects on apoptosis were attenuated by STAT3 or JAK2 inhibitors, S3I-201 or AG490, respectively. The effects of S1P on STAT3 activation, survivin expression and macrophage apoptosis were emulated by HDL, HDL lipids, and apolipoprotein (apo) M-containing HDL, but not by apoA-I or HDL deprived of S1P or apoM. In addition, JTE013 and CAY10444, S1P receptor 2 and 3 antagonists, respectively, compromised the S1P and HDL capacities to stimulate STAT3 activation and survivin expression, and to inhibit apoptosis. **Conclusion**: HDL-associated S1P inhibits macrophage apoptosis by stimulating STAT3 activity and survivin expression. The suppression of macrophage apoptosis may represent a novel mechanism utilized by HDL to exert its anti-atherogenic effects.

Key Words: High density lipoproteins (HDL), sphingosine 1-phosphate (S1P), macrophage, apoptosis, atherosclerosis.

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INTRODUCTION

Atherosclerosis is an inflammatory vascular disease characterized by accumulation of lipid-loaded macrophages within arterial wall [1,2]. The number of macrophages in atherosclerotic lesions is determined by the monocyte recruitment and differentiation, the macrophage egress, as well as the apoptotic death, which is an important feature of atherosclerotic plaque development [1,2]. Studies directed at understanding the functional consequences of macrophage apoptosis in atherosclerosis revealed its opposing roles at various stages of disease [3-5]. In early lesions, apoptosis may help to eliminate superfluous macrophages and thereby limit the progression of atherosclerotic plaque. In advanced lesions, however, macrophage apoptosis is thought to promote the development of necrotic core and to increase the plaque vulnerability to disruption. Distinct molecular mechanisms were postulated to underlie the apoptotic death of macrophages in early and late atherosclerosis. Mitochondrial dysfunction characterized by the loss of mitochondrial membrane potential and brought about by the imbalance between pro- and anti-apoptotic members of Bcl family was found to prevail in the initial phase of plaque development [4-9]. By contrast, endoplasmic reticulum (ER) stress arising primarily as a consequence of unrestricted free cholesterol accumulation in macrophages was strongly correlated with advanced lesional macrophage apoptosis and plaque necrosis [3,5,10-13]. Irrespective of the mitochondrial or endoplasmic origin of cell death signals, apoptosis in atherosclerosis is accompanied by activation of executor caspases such as caspase-3, the active form of which was repeatedly identified both in early and late atherosclerotic lesions [9,11,12].

Sphingosine 1-phosphate (S1P) is a bioactive lysosphingolipid produced by phosphorylation of sphingosine by sphingosine kinases 1 and 2 [14,15]. S1P interacts with five cognate G protein-coupled receptors termed $S1P_{1-5}$, which modulate a

number of cellular functions including proliferation, survival, cytoskeletal rearrangements, cell motility, and exert potent cytoprotective effects [14,15]. In the vasculature, S1P receptors are present on endothelial and smooth muscle cells, where they play a prominent role in the regulation of angiogenesis and vascular barrier integrity [15,16]. In addition, S1P was found to interfere with migration and activation of monocytes/macrophages and to control their recruitment to sites of inflammation [17,18]. The major interest in S1P in relationship to atherosclerosis arose after discovery that it is associated with an apolipoprotein M (apoM)-containing subfraction of high density lipoprotein (HDL) - a potent plasma-borne anti-atherogenic factor [19]. There is substantial evidence suggesting that S1P may account for atheroprotective effects attributed to HDL. For instance, S1P correlates with HDL in plasma in a concentration range, in which HDL most effectively protects against atherosclerosis, and decreased HDL-bound S1P levels were noted in patients with cardiovascular disease [20-22]. In addition, S1P was found to emulate *in vitro* several atheroprotective effects attributed to HDL, including inhibition of endothelial and smooth muscle cell apoptosis [23]. However, less is known about the effect exerted by free or HDL-bound S1P on the apoptotic death of macrophages. In the present study we demonstrate that S1P inhibits macrophage apoptosis originating from both mitochondrial and ER-dependent pathways by stimulating signal transducer and activator of transcription (STAT3) activation and survivin expression.

MATERIALS AND METHODS

Cell culture - RAW 264.7 murine and THP-1 human macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) or Roswell Park memorial Institute (RPMI) medium, respectively, supplemented with 10% fetal calf serum (FCS). Cells were allowed resting in FCS-free medium for 18 h before stimulation.

Lipoprotein isolation and modification - HDL and low density lipoproteins (LDL) were isolated from human plasma by isopycnic ultracentrifugation. S1P-depleted HDL (HDL^{-S1P}) was prepared by swirling native HDL for 24 h with charcoal. HDL lipid fraction (HDL-L) was extracted according to Bligh and Dyer [24] and used for cell stimulation in amounts indexed to original HDL concentration. ApoM-containing HDL (HDL^{+apoM}) and apoM-deprived HDL (HDL^{-apoM}) were isolated by anti-apoM immunoaffinity chromatography as described elsewhere [25]. S1P was reconstituted with albumin as described by Nguyen-Tran et al. [26].

Lipoprotein composition – Apolipoprotein and S1P contents in native HDL, HDL^{-S1P}, HDL-L, HDL^{+apoM}, HDL^{-apoM}, and LDL were deremined by micro-liquid chromatography (microLC) coupled to quadrupole-linear ion trap mass spectrometry (QTRAP) or hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry as described previously [27,28].

Fluorescence-activated cell sorter (FACS) analysis - For determination of apoptosis macrophages were incubated for 30 min with annexin V-FITC. For TUNEL assay, fixed and permeabilized macrophages were labeled with Br-dUTP and stained with FITC-conjugated anti-BrdU antibodies. Necrotic cells were detected by counterstaining with propidium iodide.

Assays for caspases 3, 9 and 12 – Caspase activities were determined in lysed macrophages by fluorescence spectroscopy using Ac-DEVD-AFC, LEHD-AFC and ATAD-AFC as substrates for caspases 3, 9 and 12, respectively.

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Real-time quantitative RT-PCR - The entire cDNA was synthesized from total RNA isolated from macrophages by mean of reversed transcription. Fully automated RT-PCR set-up was done on a Genesis 150 workstation (TECAN, Creilsheim, Germany) and PCR products were detected using ABI7900ht sequence detection system (Applied Biosystems, Darmstadt, Germany). Relative gene expression was calculated by applying the $2^{-\Delta\Delta Ct}$ method.

Western Blotting – RAW264.7 cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to nitrocellulose membranes, which were blocked overnight in saline containing non-fat dry milk prior to incubations with antibodies.

General Procedures - Data are presented as means ± S.D. from at least three separate experiments or as results representative for at least three repetitions unless indicated otherwise. Comparisons between the groups were performed with two-tailed Student t-test. p values less than 0.05 were considered significant. Detailed Methods can be found in Supplementary Appendix online (http://www.atherosclerosis-journal.com).

RESULTS

S1P inhibits mitochondrial and ER stress-dependent apoptosis in macrophages – The

phosphatidylserine translocation from endo- to exofacial leaflets of plasma membrane

and the genomic DNA fragmentation represent measurable features of early and late

apoptosis, respectively. To assess the effect of S1P on macrophage apoptosis, we

exposed RAW264.7 cells to etoposide or thapsigargin/fukoidan, which trigger apoptotic

death through mitochondrial or ER stress-dependent pathways [29-32], respectively,

and quantified phosphatidylserine-positive cells using annexin V staining. In parallel,

the DNA fragments appearing in apoptotic cells were evaluated using the TUNEL

assay. As shown in Fig. 1A and B (left and center panels), incubation of RAW264.7

macrophages with etoposide or thapsigargin/fukoidan for 24h increased the number of

annexin V- and BrdU-positive cells and this effect was attenuated in the presence of

S1P (5.0 μmol/L, added 24h prior to apoptotic trigger). The inhibitory effect of S1P on

macrophage apoptosis was concentration-dependent, with a maximal suppression

observed at 1.0 - 5.0 μmol/L S1P (Fig. 1A and B, right panels).

The execution of both mitochondrial and ER stress-triggered apoptotic programs

requires the induction of effector caspases. Therefore, we next investigated the effect

of S1P on the activation of caspase-3 by measuring cleavage of its fluorogenic

substrate, Ac-DEVD-AFC. Pre-incubation with S1P suppressed the activity of caspase-

3 measured 24 h after addition of etoposide or thapsigargin/fukoidan in a

concentration-dependent manner with a ~75 % inhibition seen at S1P concentration

of 5.0 μmol/L (Fig. 1C, upper panel). As a biochemical marker for caspase activation,

we also determined the effect of S1P on the proteolytic processing of pro-caspase-3,

which is required for the full caspase activity. As shown in Fig. 1C (lower panel), S1P

at a concentration of 5.0 μmol/L reduced the generation of the active 23 kDa caspase-

3 subunit in RAW264.7 cells exposed to etoposide or thapsigargin/fukoidan. Since pro-

caspase-3 proteolysis is executed by upstream caspases 9 and 12, the activation of which is initiated in course of mitochondrial and ER stress-triggered apoptotosis [31,32], respectively, we additionally examined the effect of S1P on the cleavage of caspase-9 and -12 fluorogenic substrates LEHD-AFC and ATAD-AFC. As shown in Fig. 1D, exposing RAW264.7 macrophages for 24 h to etoposide stimulated only the activity of caspase-9, whereas thapsigargin/fukoidan induced the activation of caspase-9 and caspase-12. The activation of both caspases could be effectively suppressed by pre-incubation of cells for 24h with S1P (5.0 µmol/L) prior to addition of etoposide or thapsigargin/fukoidan.

Plasma S1P is transported in blood bound to albumin, which may serve both as S1P acceptor and donor for cells. Therefore, we next examined the effect of S1P reconstituted with albumin on the etoposide- or thapsigargin/fukoidan-induced annexin V-binding and caspase 3 activation. As shown in Fig. 1E, albumin-free and albumin-bound S1P (5.0 µmol/L) were equally potent in inhibiting apoptosis in RAW264.7 cells. As unrestricted cholesterol accumulation is typical for macrophages encountered within atherosclerotic lesions, we tested the anti-apoptotic effect of S1P in RAW264.7 cells loaded with cholesterol by overnight incubation with AcLDL. As shown in Fig.1F, the effects of etoposide- or thapsigargin/fukoidan on annexin V-binding and caspase 3 activation were blunted in cholesterol-laden macrophages, but significantly inhibited by pre-incubation of cells for 24h with S1P (5.0 µmol/L).

Finally, to substantiate the potential anti-apoptotic role of S1P in humans, we examined the effect of this lysosphngolipid on the the etoposide- or thapsigargin/fukoidan-induced annexin V-binding and caspase 3 activation in THP-1 macrophages. Fig. 1G demonstrates that both effects were inhibited in the concentration-dependent fashion after pre-incubating THP-1 cells for 24h with S1P (5.0 µmol/L).

Anti-apoptotic effect of S1P in macrophages is survivin-dependent – The inhibitory effect of S1P on RAW264.7 cell death triggered by etoposide and thapsigargin/fukoidan suggested that this lysosphingolipid might interfere with a common terminal mechanism utilized by both mitochondrial and ER stress-dependent apoptotic pathways. Therefore, we next examined the effect of S1P on the expression of inhibitor of apoptosis protein (IAP) family members, which are known to inhibit caspase activation in both the mitochondrial and the ER stress setting [33-36]. As shown in Fig. 2A, incubation of RAW264.7 macrophages with S1P (5.0 µmol/L) for 24 h failed to affect the expression of X-linked IAP (xIAP), neuronal apoptosis inhibitory protein (NAIP), IAP-like protein 2 (ILP2) and livin, but markedly up-regulated survivin and cIAPs 1 and 2. Further analysis on mRNA and protein levels revealed that S1P promoted survivin (*Birc5*) as well as cIAP 1 (*Birc2*) and 2 (*Birc3*) expression in a time- and concentration-dependent manner with the maxima seen between 6 h and 24 h incubation and at a concentration of 5.0 µmol/L (Fig. 2B and C). To investigate, whether the inhibitory effect of S1P on macrophage apoptosis causally depends on the elevated expression of survivin and/or cIAPs, we took the advantage of the compound YM155, which targets the interleukin enhancer-binding factor 3 (ILF3/NF110) and thereby blocks the survivin promoter activity, and the compound GDC0152, which directly blocks the activation of cIAP1, cIAP2 and xIAP [37,38]. As shown in Fig. 2D (left upper panel), co-treatment of RAW264.7 cells with YM155 (0.1 µmol/L) abolished survivin expression induced by incubation with S1P. Fig. 2D (lower panels) demonstrates that the anti-apoptotic effect of S1P (5.0 µmol/L) as assessed by annexin V translocation was abrogated in RAW264.7 macrophages co-incubated for 24 h with YM155 prior to exposure to etoposide or thapsigargin/fukoidan. Likewise, YM155 neutralized the inhibitory effect exerted by S1P (5.0 µmol/L) on the etoposide- or thapsigargin/fukoidan-induced caspase-3 activation (Fig. 2D). By contrast, GDC0152

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(1.0 µmol/L) failed to reverse the inhibitory effect of S1P on the etoposide- or thapsigargin/fukoidan-induced annexin V translocation and the caspase-3 activation in RAW264.7 cells (Fig. 2D).

STAT3 activation is required for S1P-mediated survivin expression and suppression of apoptosis - Survivin expression is controlled by protein kinase STAT3, the activation of which was previously observed in cardiomyocytes and prostate cancer cells in the presence of S1P or HDL [39,40]. Therefore, we next investigated, whether similar signaling pathways operate in macrophages. To this purpose, RAW264.7 cells were exposed to S1P and the activation state of STAT3 as well as the upstream-localized Janus kinases (JAK) 1 and 2 and the tyrosine kinase 2 (Tyk2) was assessed using phosphospecific antibodies. We found JAK1, JAK2 and Tyk2 to be phosphorylated 5 – 10 min after exposure to S1P (5.0 µmol/L) and these responses remained evident after prolonged incubation (Fig. 3A). In addition, S1P induced a time-dependent phosphorylation of STAT3 with the maximal response occurring 90 – 120 min after exposure to S1P. Moreover, JAK1, JAK2, Tyk2 and STAT3 phosphorylations in response to S1P were concentration-dependent with maximal stimulations seen at a concentration of 5.0 µmol/L (Fig. 3A). To test the involvement of the JAK2/STAT3 pathway in the S1P-mediated stimulation of survivin expression and inhibition of macrophage apoptosis, we used ZM39923, AG490 and S3I-201, three selective and structurally unrelated inhibitors of JAK1, JAK2 and STAT3, respectively. As shown in Fig. 3B, co-incubation of RAW264.7 cells with AG490 (10.0 µmol/L) or S3I-201 (100 µmol/L), but not ZM39923 (10.0 µmol/L) abrogated the expression of survivin brought about by stimulation with S1P (5.0 µmol/L, 24 h). Furthermore, AG490 but not ZM39923 fully reversed the inhibitory effect of pre-incubation for 24h with S1P (5.0 µmol/L) on annexin-V binding and caspase-3 activation during etoposide- or thapsigargin/fukoidan-induced apoptosis (Fig. 3C). The unexpected stimulatory effect

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of S3I-201 on caspase-3 activity (not shown) prevented us from testing the effect of this compound on the S1P-mediated apoptosis inhibition.

HDL emulates anti-apoptotic effects of free S1P in macrophages – In the final step we tested, whether S1P exerts anti-apoptotic effects as a component of HDL. As shown in Fig.4A (left panel), exposure of RAW264.7 cells to HDL (1.0 g/L) produced STAT3 phosphorylation with the time-course resembling that of free S1P. In addition, HDL (1.0 g/L) induced time-dependent expression of survivin in RAW264.7 macrophages (Fig. 4A, left panel). Both, STAT3 phosphorylation and survivin expression in response to HDL were concentration-dependent with the maxima seen at HDL levels close to physiological (Fig. 4A, center panel). Moreover, HDL inhibited caspase-3 activation in RAW264.7 cells exposed to etoposide or thapsigargin/fukoidan in a concentration-dependent manner (Fig. 4A, right panel).

To discern HDL entities responsible for the stimulation of STAT3/survivin signaling pathway, we characterized native HDL, HDL^{-S1P}, HDL^{-apoM}, HDL^{+apoM}, HDL-L and LDL with respect to the apolipoprotein composition and the S1P content, and compared their capacity to stimulate STAT3 phosphorylation and survivin expression and to inhibit etoposide- or thapsigargin/fukoidan-stimulated caspase-3 activation. As shown in Table 1, all HDL fractions showed similar composition with respect to major apolipoproteins (A-I, A-II, B, C-I, C-II, C-III, D, E, M) except that HDL^{+apoM} was relatively enriched in apoM at the expense of apoA-I and apoA-II. Both HDL^{-S1P} and HDL^{-apoM} were entirely deprived of apoM. In these two fractions S1P content was reduced approx. four to fivefold in comparison to native HDL, whereas it was increased fivefold in HDL^{+apoM}. The S1P content in HDL-L was comparable to native HDL (94.1 vs. 101.7 ng/mL). The S1P content in LDL was similar to that seen in HDL^{-apoM}. In further experiments we observed that both STAT3 phosphorylation and survivin expression were induced by HDL-L added to RAW264.7 cells in the amount corresponding to 1.0

g/L native HDL, while etoposide- and thapsigargin/fukoidan-stimulated caspase-3 activation was inhibited (Fig. 4B). By contrast, these cellular responses were diminished or absent in macrophages exposed to HDL^{-S1P} (1.0 g/L), apoA-I (0,25 g/L) or LDL (0,5 g/L, Fig.4B). Moreover, the HDL capacity to stimulate STAT3 phosphorylation and survivin expression and to inhibit caspase-3 activation was preserved by HDL^{+apoM} added to RAW264.7 cells in concentrations corresponding to its plasma level (~0.1 g/L), but failed to appear, when HDL^{-apoM} was used as agonist (Fig. 4C).

To further strengthen the evidence pointing to S1P as an anti-apoptotic component of HDL, we examined the expression of S1P receptors in RAW264.7 cells during apoptosis and determined the effect of HDL and S1P on the STAT3/survivin signaling pathway as well as the mitochondrial and ER stress-induced apoptosis in the presence of pharmacological S1P receptor antagonists. RT-PCR quantification of S1P receptors revealed that *S1pr1* was most abundantly expressed in RAW264.7 cells followed by *S1pr2* (76% of *S1pr1* expression) and *S1pr3* (<1.0% of *S1pr1* expression). Similar expression pattern was observed in THP-1 macrophages. Etoposide-induced apoptosis exerted no effect on the expression of *S1pr1* and *S1pr2* in RAW264.7 macrophages, whereas exposure of cells to thapsigargin/fukoidan reduced *S1pr1* and *S1pr2* expression by 27% and 64%, respectively. By contrast, exposure of RAW264.7 macrophages to etoposide or thapsigargin/fukoidan increased the expression of *S1pr3* by at least 2 orders of magnitude. Fig. 4D (left panel) demonstrates that stimulating effects of S1P (5.0 µmol/L) and HDL (1.0 g/L) on the STAT3 phosphorylation and the survivin expression were attenuated by JTE013 (1.0 µmol/L) and CAY1044 (10.0 µmol/L), selective antagonists of $S1P_2$ and $S1P_3$, respectively, and to lesser extent by W123 (10.0 µmol/L), the antagonist of $S1P_1$. Moreover, these compounds partly reversed the inhibitory effect of S1P and HDL on caspase-3 activation during

etoposide- or thapsigargin/fukoidan-induced apoptosis (Fig. 4D, right panel). To additionally narrow the identity of S1P receptors responsible for the HDL- and S1P-mediated induction of the STAT3/survivin signaling and inhibition of apoptosis we also tested the effects of S1P receptor agonists (Fig. 4E). These experiments revealed that the STAT3 phosphorylation and the survivin expression are induced by FTY720P, a synthetic S1P mimetic activating receptors 1, 3, 4 and 5 and – less potently – by KRP203P and SEW2871, two agonist of $S1P_1$. In addition, all three compounds emulated the inhibitory effect of S1P or HDL on etoposide- or thapsigargin/fukoidan-induced caspase-3 activation (Fig. 4E).

DISCUSSION

S1P exerts protective effects in the cardiovascular system and this has been partly attributed to its capacity to prevent apoptosis in endothelial cells and cardiomyocytes [23]. However, despite the crucial contribution of macrophages to the development of atherosclerotic lesions the influence of S1P on the macrophage survival has not been systematically examined to date. In the present study, we provide the evidence that S1P inhibits apoptosis produced by mitochondrial or ER stress both in human and murine macrophages regardless of their cholesterol-loading status. Although the maximal anti-apoptotic effect of S1P was observed in a supraphysiological concentration (5.0 µmol/L), this compound remained effective at 1.0 µmol/L, which is close to its physiological level in plasma [23]. In addition, macrophage apoptosis was inhibited by physiological concentrations of both total and apoM-containing HDL, which serves as a principal S1P carrier in plasma.

The induction of apoptotic cell death in macrophages proceeds along diverse signaling pathways. In the mitochondrial pathway the increase of the mitochondrial membrane permeability leads to the release of cytochrome C to the cytoplasm and induction of caspases 9 and 3 [41]. The ER stress triggers activation of endoplasmic stress sensors (PKR)-like ER kinase, activating transcription factor 6 and inositol-requiring enzyme 1, the concerted action of which culminates in the expression of pro-apoptotic CCAAT/-enhancer-binding protein homologous protein (CHOP), activation of caspases 12 and 3, and cross-activation of caspase 9 [41,42]. The activation of executioner caspases constitutes a common distal path of apoptotic signaling and its inhibition by members of the IAP family was shown to suppress apoptosis originating from both mitochondrial and ER stress [33-36]. The present study demonstrates that S1P promotes the expression of IAP protein survivin, which is known to block caspase-3, caspase-9, and caspase-12 activity either directly or by interfering with cytochrome C [34,36,43,44]. In

addition, our results suggest that the up-regulation of survivin is instrumental for the inhibitory effect of S1P on macrophage death, as the reduced apoptosis and caspase-3 activity seen in cells exposed to activators of mitochondrial or ER stress in the presence of S1P were reversed by YM155, which disables the survivin promoter. To our knowledge, this is the first demonstration that S1P affords protection against apoptosis in macrophages by inducing the IAP family member. The regulatory effect of S1P on survivin expression was previously noticed in neuroblastoma cell lines [45]. The activity of survivin promoter is under tight control of the STAT3 transcription factor. Actually, the permanent activation of STAT3 followed by the survivin-dependent suppression of apoptosis was identified as a key event supporting malignant tumor progression [46-48]. A couple of investigations documented the direct induction of JAK2-mediated STAT3 phosphorylation and activation by S1P. For instance, free S1P was found to elicit STAT3-dependent inflammatory responses in intestinal cells and to link STAT3 to the development of chronic colitis and colitis-associated cancer [49]. In addition, S1P-induced STAT3 activation has been observed in several tumor lines and cells of hematopoietic origin [50-52]. There is also more limited evidence suggesting that STAT3 is activated in response to HDL-bound S1P. Frias et al. demonstrated that both native and reconstituted HDL containing S1P promote STAT3 activation in cardiomyocytes and thereby protect these cells from the doxorubicin-induced apoptosis [39,53]. Sekine et al. reported that the HDL-bound S1P promotes STAT3-dependent prostate cancer cell migration and invasion [40]. Our present study extends these findings to show that both S1P and HDL induce time- and concentration-dependent STAT3 activation in macrophages and that this signaling event leads to enhanced survivin expression. In addition, the STAT3 activation is required for the anti-apoptotic effect of S1P, as its reversal could be observed in macrophages pre-incubated with the inhibitor of JAK2-dependent STAT3 phophorylation. By contrast,

JAK1 inhibition promoted S1P-induced survivin expression, which may indicate a compensation derived from the enhanced JAK2/STAT3 signalling once the activity of other JAK kinases is attenuated. Taken together, our findings establish the S1P/HDL→STAT3→survivin signaling pathway as a novel cytoprotective mechanism in macrophages. Future research will clarify, whether similar mechanism accounts for cytoprotective effects of HDL observed in other atherosclerosis-relevant cells such as endothelial and smooth muscle cells.

While HDL-bound S1P was previously shown to attenuate apoptosis in cardiomyocytes and endothelial cells, the capacity of HDL to promote endothelial survival correlated not only with its S1P content, but also with other lipid species including several odd-chain phosphatidylcholines (=plasmalogens), two even-chain phosphatidylcholines (PC32:0, PC34:2) and one sphingomyelin (SM42:2) [23,54]. The present study provides several pieces of evidence firmly linking anti-apoptotic effects of HDL in macrophages to its S1P content. First, the HDL-mediated stimulation of STAT3 phosphorylation and survivin expression as well as the inhibition of etoposide- and thapsigargin/fukoidan-induced caspase-3 activation were emulated by lipids isolated from HDL, but not by purified apoA-I or charcoal-treated HDL, which has been stripped of both S1P and its binding partner apoM. In addition, LDL particles, which contain minor S1P amounts and no apoM, were incapable to produce anti-apoptotic effects in macrophages. Second, the STAT3/survivin-mediated anti-apoptotic pathway could be activated only by S1P-rich apoM-containing, but not by S1P-poor apoM-deprived HDL. Third, pharmacological antagonists of S1P receptors substantially attenuated anti-apoptotic signaling produced by HDL in macrophages. While consistent with the notion that S1P represents a major determinant of the anti-apoptotic activity exerted by HDL, these results do not preclude the possibility that other lipid species with anti-apoptotic properties additionally contribute to survival-promoting effects seen in macrophages in

the presence of HDL. Additional studies using HDL with defined lipid composition will be necessary to identify further components of this lipoprotein that might help to attenuate macrophage apoptosis.

Extracellular S1P engages in the interaction with a family of plasma membrane receptors designated $S1P_1$ to $S1P_5$ [15]. To date, the S1P receptor subtypes 1, 2 and 3 were found to preponderate in the vasculature and to be involved in the development of atherosclerotic lesions [23]. In the present study we show that $S1P_1$, $S1P_2$, $S1P_3$ are expressed in murine macrophages, albeit the expression levels vary considerably in course of apoptosis. Moreover, using various S1P receptor agonists or antagonists with well-characterized pharmacological properties, we demonstrate that both $S1P_2$ and $S1P_3$ are primarily mediating the stimulation of STAT3 activation and survivin expression and thereby the inhibitory effect of S1P and HDL on macrophage apoptosis. Our present results are in agreement with findings of previous studies, which uncovered the contribution of $S1P_2$, $S1P_3$ or both receptors to the S1P-induced temporary STAT3 activation in cardiomyocytes, intestinal smooth muscle cells, mesenchymal skeletal cells, and prostate cancer cells [39,40,55,56]. By contrast, the persistent STAT3 activation required for the proliferation of hepatocellular carcinoma, urothelial carcinoma and melanoma cells as well as the oncogenic transformation of intestinal cells was found to be mediated by $S1P_1$ [50,51], which in the present study seemed to be less active in protection against the macrophage death. The involvement of more than one S1P receptor subtype in the initiation of STAT3-depndent signaling cascade may be attributed to the marked redundancy of S1P signaling. Actually, $S1P_2$ and $S1P_3$ depend on the same trimeric G proteins (Gi, Gq, $G_{12/13}$) and downstream signaling cascades for eliciting cellular responses [15,23]. Though $S1P_1$ also operates through Gi, the degree of overlap between this and other S1P receptors with respect to activation of intracellular signaling pathways is less pronounced, which might help

explaining weaker anti-apoptotic effects exerted by $S1P_1$ agonists KRP203P and SEW2871.

The relevance of present findings to anti-atherogenic effects exerted by HDL-associated lysosphingolipids remains uncertain. Previous studies demonstrated that the reduced susceptibility of macrophages to apoptosis in early atherosclerotic lesions was associated with increased plaque burden. Actually, several factors triggering mitochondrial apoptosis were found to accelerate murine atherosclerosis [5-9]. Conversely, sustained induction of apoptosis due to cholesterol accumulation and the ensuing ER stress in late lesional macrophages went along with increased lesion size [10-13]. Acting as an inhibitor of both mitochondrial and ER stress-induced macrophage death S1P might be predicted to exert a biphasic effect in atherosclerotic disease depending on the lesion development stage. In this context, however, it is worth noticing that the enhanced expression of survivin – the principal mediator of S1P-dependent anti-apoptotic effect – was observed mainly in advanced plaques, in which it coincided with caspase-3 [57,58]. The upregulation of survivin in advanced lesions was postulated to reflect the endogenous defense mechanism protecting against the pro-apoptotic effect of inflammatory stimuli released in the plaque. Conversely, oxidized LDL was found to downregulate survivin and thereby to promote cell death in advanced plaques and plaqe vulnerability [58]. Taking this into account, it would be tempting to hypothesize that the activation of the S1P/HDL→STAT3→survivin signaling pathway counteracting apoptosis in macrophages might represent a novel atheroprotective mechanism exerted by HDL within late atherosclerotic lesions.

CONFLICT OF INTEREST

None.

FINANCIAL SUPPORT

ACKNOWLEDGEMENTS

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Figure 1. **S1P inhibits mitochondrial and ER stress-dependent apoptosis in macrophages.** RAW264.7 cells were incubated for 24 h with S1P (5.0 µmol/L) or with indicated concentrations of S1P prior to addition of etoposide (Etp, 250 µmol/L) or thapsigargin/fukoidan (Tp/Fk, 0.5 µmol/L and 25.0 µg/mL) for further 24 h. **(A)** and **(B)**. *Left panels*: Flow cytometric analysis of annexin (Anx)-V-positive (**A**) or Br-dUTP-positive (TUNEL-positive) (**B**) apoptotic RAW264.7 cells incubated with etoposide or thapsigargin/fukoidan. Macrophages not exposed to apoptotic stimuli served as control (Ctrl). Original histograms were superimposed for comparison. *Right panels:* Percentages of Anx-V- or Br-dUTP-positive cells after incubation with S1P. (△) - etoposide, (▽) - thapsigargin/fukoidan. **(C)**. *Upper panel:* Caspase-3 activity in RAW264.7 cells exposed to etoposide (△) or thapsigargin/fukoidan (▽) after pre-treatment with S1P. *Lower panel:* Western blot identification of the caspase-3 active form (cleaved caspase) in RAW264.7 cells pre-treated with S1P prior to stimulation with etoposide or thapsigargin/fukoidan. **(D)**. Caspase-9 (*left panel*) and caspase-12 (*right panel*) activities in RAW264.7 cells pre-treated with S1P and exposed to etoposide or thapsigargin/fukoidan. **(E)** to **(G)**. Effect of S1P (5.0 µmil/L) on etoposide- or thapsigargin/fukoidan-induced caspase-3 activity in RAW264.7 cells after pre-treatment with S1P reconstituted with albumin (Alb) (**E**), RAW264.7 cells loaded for 18 h with acetylated LDL (AcLDL) (**F**) or in THP-1 cells (**G**) * - p<0.05 ** - p<0.01 *** - p<0.001 (with vs. without S1P treatment). n.s. – not significant.

Figure 2. **Anti-apoptotic effect of S1P in macrophages is survivin-dependent. (A)**. Western blot demonstration of IAP family members in RAW264.7 cells incubated for 24 h with S1P (5.0 µmol/L). **(B).** Survivin, cIAP 1 and 2 mRNA quantification in RAW264.7 cells exposed to increasing S1P concentrations for 6h or to 5.0 µmol/L S1P

at different time points. (□) - survivin, (Δ) - cIAP1, (∇) - cIAP2. **(C)**. Western blot demonstration of survivin or cIAPs1/2 in RAW264.7 cells exposed to S1P (5.0 μmol/L) or to increasing S1P concentrations for 6h. **(D)**. *Upper left panel*: Western blot demonstration of survivin in RAW264.7 cells exposed for 24h to S1P (5.0 μmol/L) with or without YM155 (0.1 μmol/L). *Lower panels:* Percentages of annexin-V-positive cells (*left panels*) or caspase-3 activity (*right panels*) in RAW264.7 cells pre-incubated for 24 h with S1P (5.0 μmol/L) with or without YM155 (0.1 μmol/L) or GD0152 (1.0 μmol/L) and subsequently exposed for 24 h to etoposide (Etp, 250 μmol/L) or thapsigargin/fukoidan (Fk/Tp, 0.5 μmol/L and 25.0 μg/mL). * - $p < 0.05$ ** - $p < 0.01$ *** - $p < 0.001$ (S1P vs. S1P+YM155 or S1P+GD0152).

Figure 3. **STAT3 activation is required for S1P-mediated survivin expression and suppression of apoptosis. (A).** Western Blot visualization of phosphorylated JAK1, JAK2, TYK2 and STAT3 after incubation of RAW264.7 cells with S1P (5.0 μmol/L) for indicated times (*left*) or with increasing concentrations of S1P for 120 min (*right*). **(B).** Western blot evaluation of survivin expression in RAW264.7 cells exposed for 24 h to S1P (5.0 μmol/L) with or without ZM39923 (10.0 μmol/L), AG490 (20.0 μmol/L) or S3I-201 (300 μmol/L). **(C).** Percentages of annexin-V-positive cells (*left panels*) or caspase-3 activity (*right panels*) in RAW264.7 cells pre-incubated for 24 h with S1P (5.0 μmol/L) with or without ZM39923 (10.0 μmol/L) or AG490 (20.0 μmol/L) and subsequently exposed for 24 h to etoposide (Etp, 250 μmol/L) or thapsigargin/fukoidan (Tp/Fk, 0.5 μmol/L and 25.0 μg/mL). ** - $p < 0.01$ *** - $p < 0.001$ (S1P vs. S1P+ZM39923 or S1P+AG490).

Figure 4. **HDL emulates anti-apoptotic effects of S1P in macrophages. (A).** Western blot demonstration of survivin expression and STAT3 phosphorylation after incubation of RAW264.7 cells with HDL (1.0 g/L) for indicated times (*left*) or with

increasing HDL concentrations (*center*). *Right panel*: Caspase-3 activity in RAW264.7 cells exposed to etoposide (Etp, 250 µmol/L, Δ) or thapsigargin/fukoidan (Tp/Fk, 0.5 µmol/L and 25.0 µg/mL, ∇) after pre-treatment for 24 h with increasing concentrations of HDL. **(B)** and **(C).** Survivin expression, STAT3 phosphorylation or etoposide- or thapsigargin/fukoidan-induced caspase-3 activity in RAW264.7 cells incubated for 24 h or 120 min (STAT3 phosphorylation) with purified HDL constituents or LDL (**B**) or HDL fractions with or without apoM (**C**). Ctrl: vehicle, HDL: native HDL (1.0 g/L), HDL-L: HDL- lipids, HDL^{-S1P}: S1P-depleted HDL (1.0 g/L), ApoA-I: apolipoprotein A-I (0.25 g/L), LDL: low density lipoprotein (0.50 g/L), HDL^{+apoM}: apoM-containing HDL (0.1 g/L), HDL^{-apoM}: apoM-deprived HDL (0.1 g/L) * - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$ (absence vs. presence of HDL constituents or fractions). **(D)**. *Left panels:* Survivin expression and STAT3 phosphorylation in RAW264.7 cells exposed to S1P (5.0 µmol/L) or HDL (1.0 g/L) with or without S1P receptor antagonists W123 (10.0 µmol/L), JTE013 (1.0 µmol/L), or CAY10444 (10.0 µmol/L). *Right panels:* Caspase-3 activity in RAW264.7 cells pre-incubated for 24 h with S1P or HDL with or without W123, JTE013, or CAY10444 and subsequently exposed for 24 h to Etp or Tp/Fk. * - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$ (S1P vs S1P+W123 or S1P+JTE013 or S1P+CAY10444). **(E)**. Effect of S1P receptor agonists on STAT3/survivin signaling and inhibition of apoptosis. *Left panel:* Survivin expression and STAT3 phosphorylation in RAW264.7 cells exposed to S1P, FTY720P, KRP203P or SEW2871 (each 5.0 µmol/L) for 24 h (survivin expression) or 120 min (STAT3 phosphorylation). *Right panel:* Caspase-3 activity in RAW264.7 cells pre-incubated for 24 h with S1P, FTY720P, KRP203P or SEW2871 prior to stimulation with etoposide or thapsigargin/fukoidan. ** - $p<0.01$ *** - $p<0.001$ (absence vs. presence of S1P or S1P receptor agonist).
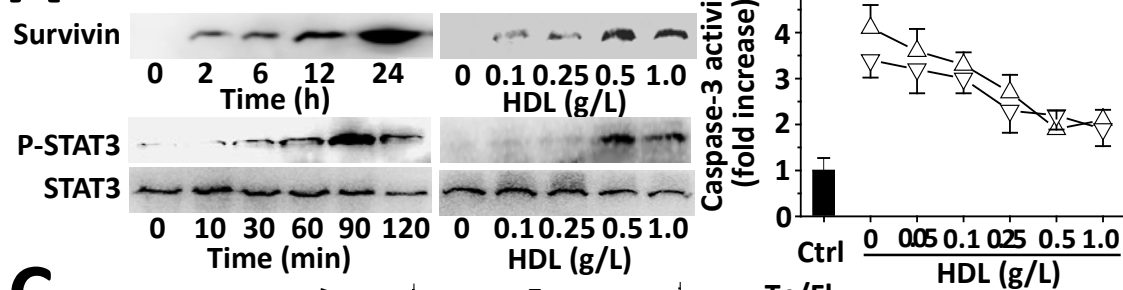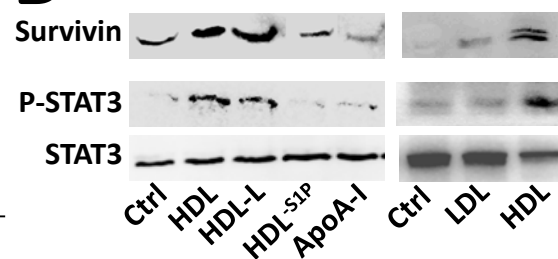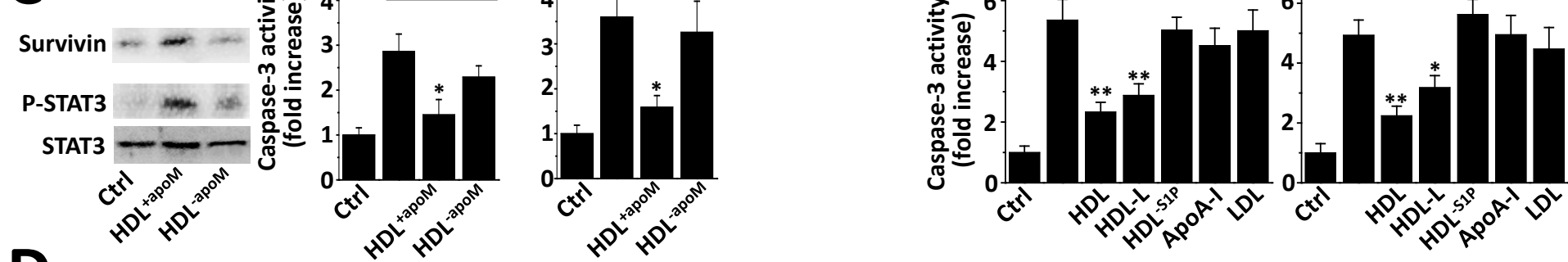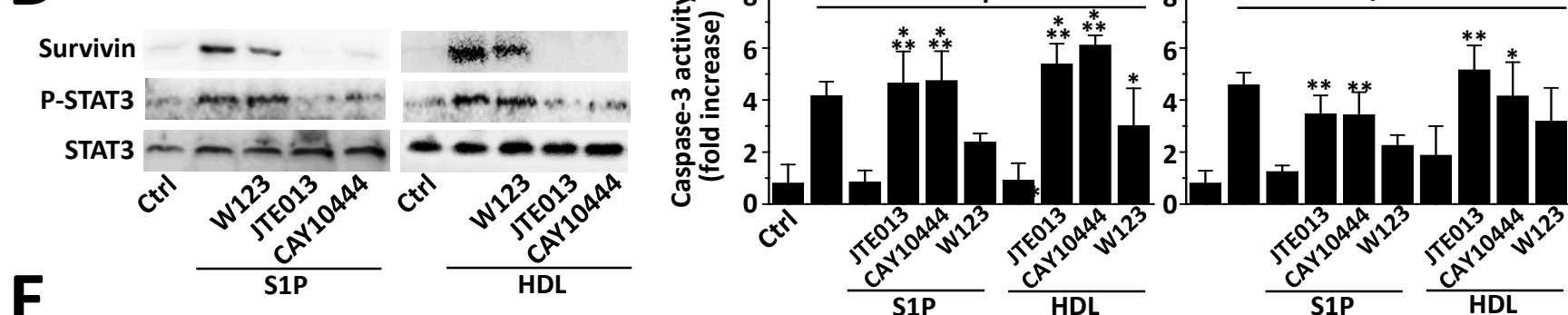
Feuerborn et al. Figure 1

Feuerborn et al. Figure 4
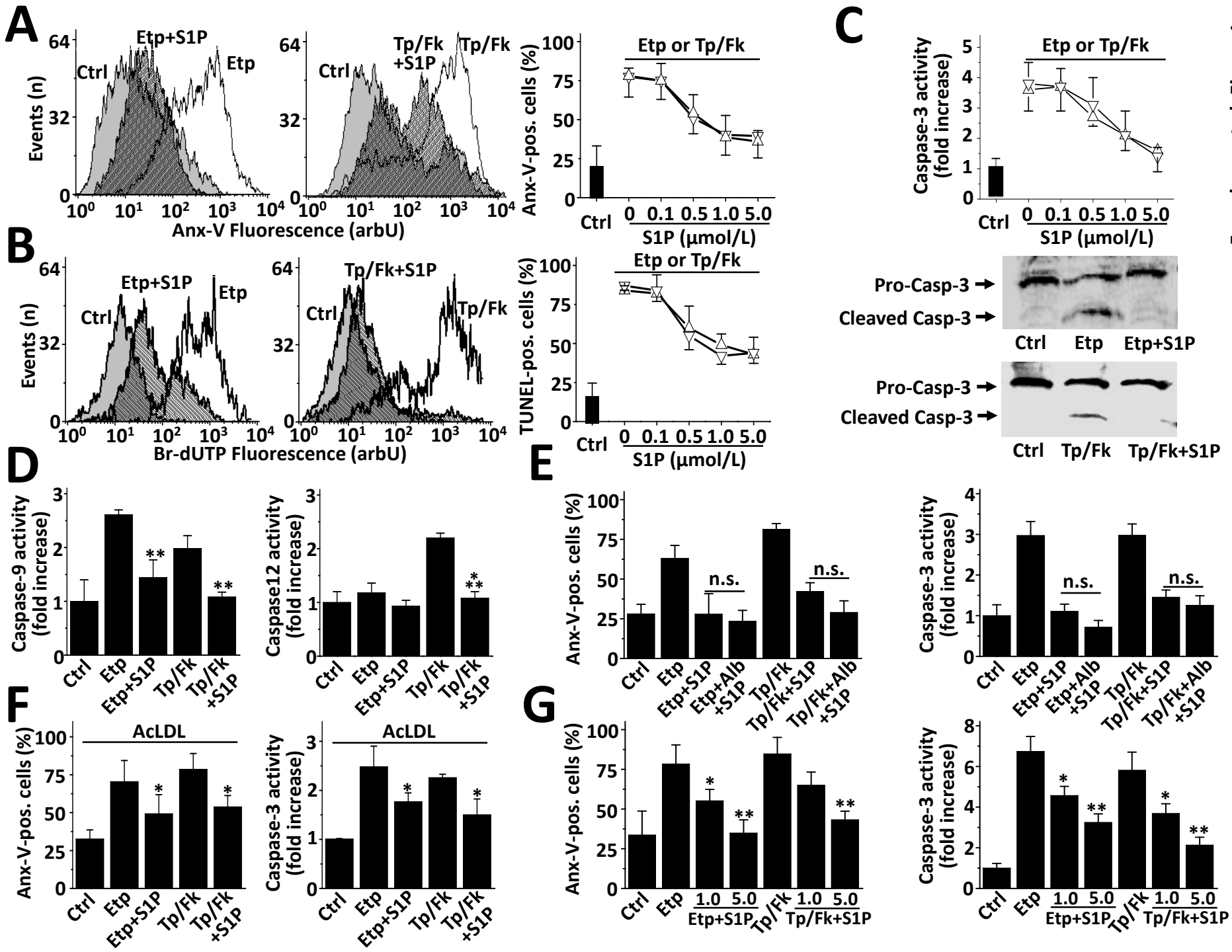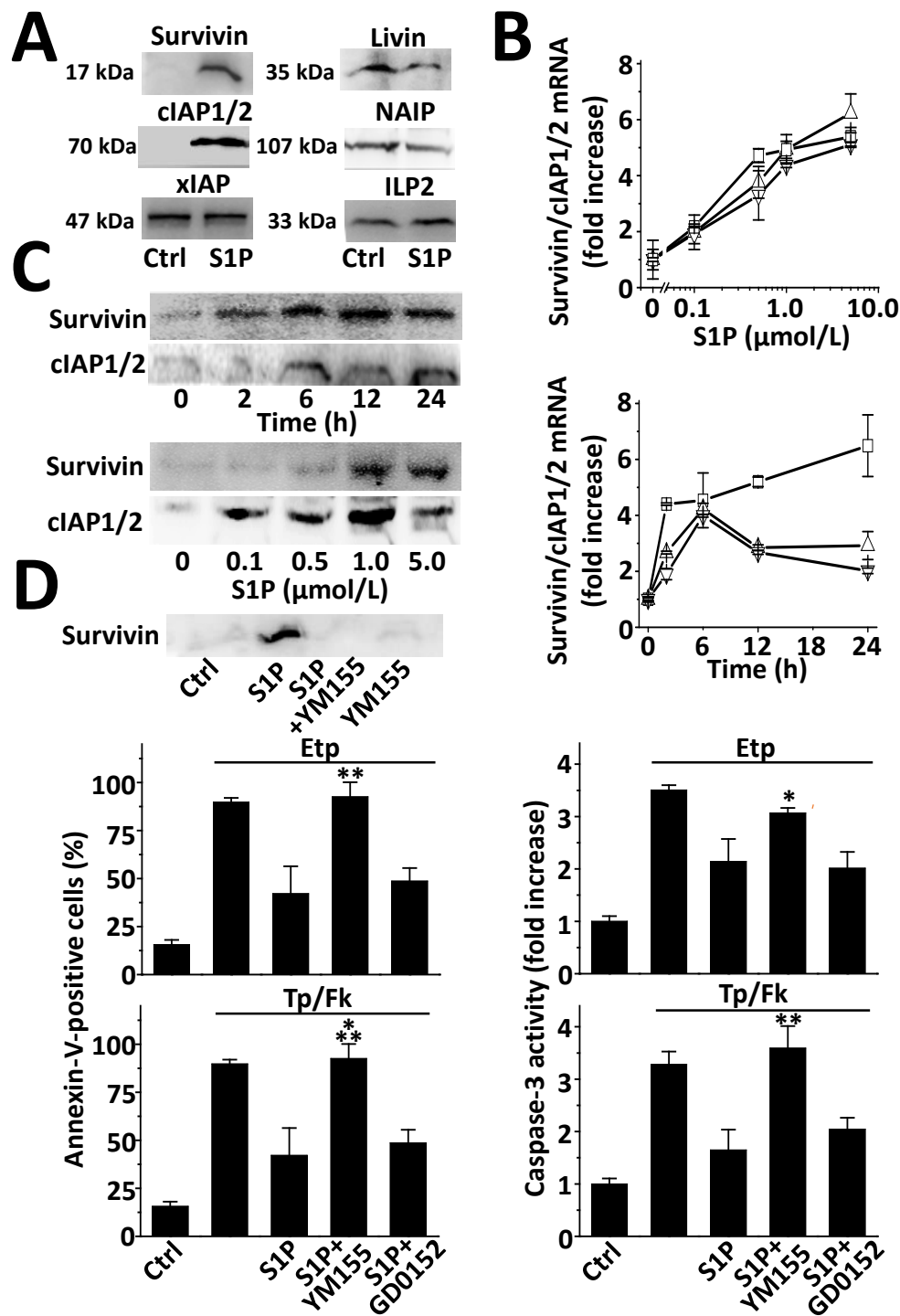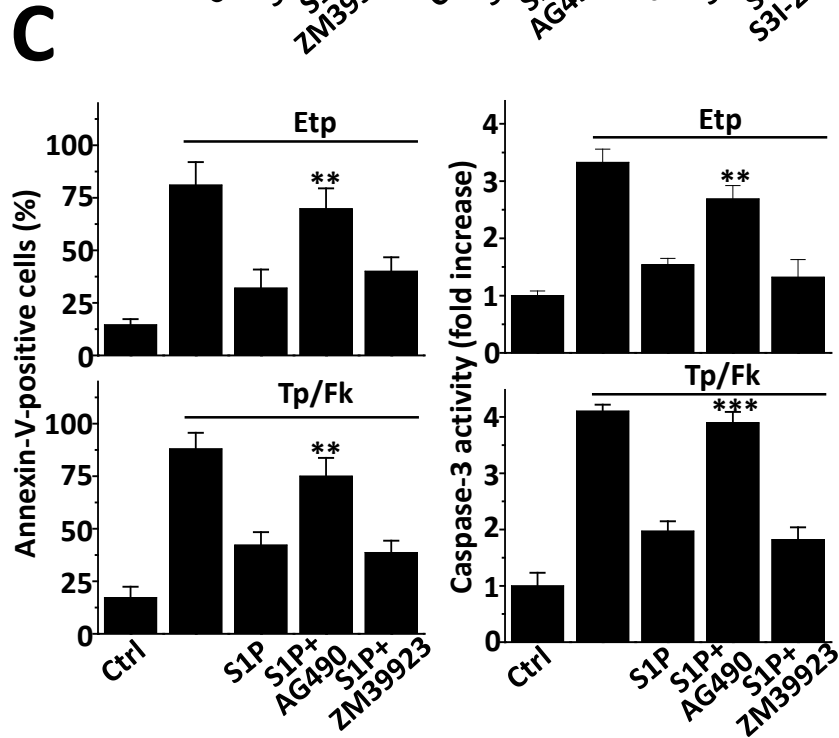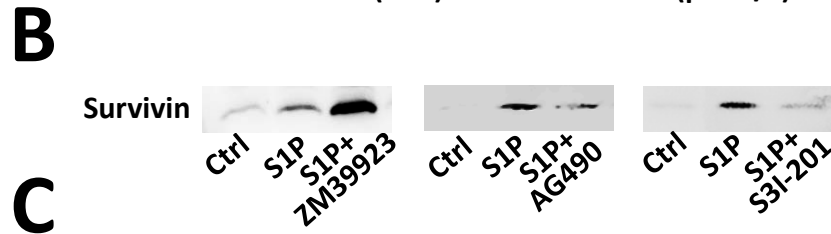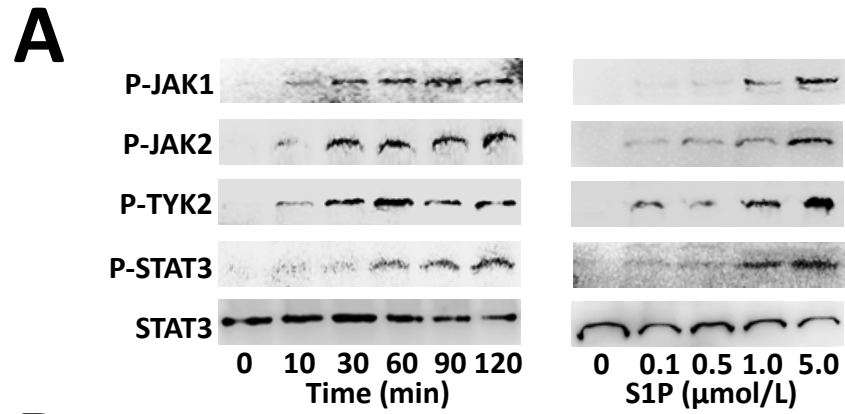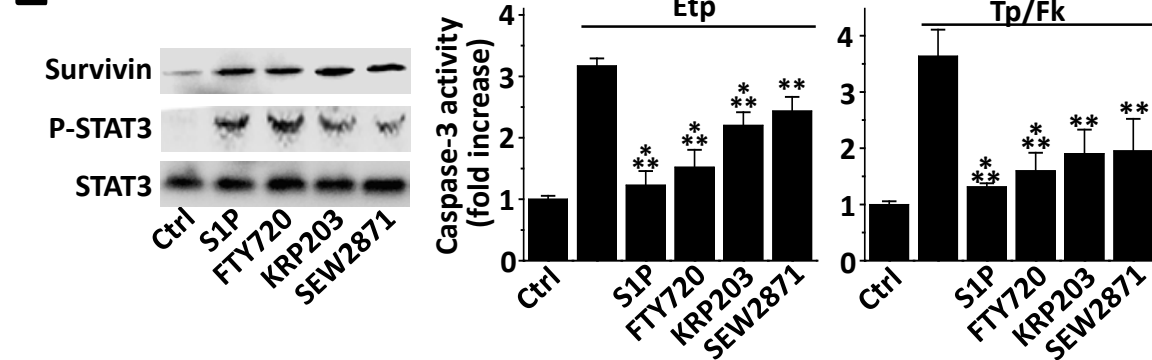
Table 1. Protein and lipid composition of lipoprotein fractions

	Apolipoprotein (% mol)									S1P[b] (ng/mg)
	A-I	**A-II**	**B**	**C-I**	**C-II**	**C-III**	**D**	**E**	**M**	
HDL[a]	32,5 ± 1,5	52,3 ± 3,9	n.d.[c]	5,0 ± 1,4	0,9 ± 0,2	5,5 ± 1,9	2,5 ± 0,6	0,3 ± 0,1	0,8 ± 0,2	70,4 ± 23,6
HDL[-S1P]	31,4 ± 0,3	57,2 ± 1,9	n.d.	2,4 ± 1,6	0,2 ± 0,1	6,9 ± 0,7	2,1 ± 0,2	0,2 ± 0,1	n.d.	18,1 ± 3.2
HDL[+apoM]	27,5	33,8	n.d.	5,3	n.d.	13,08	1,9	0,4	19,1	354,1
HDL[-apoM]	32,2	54,9	n.d.	4,6	0,9	4,5	2,6	0,2	n.d.	17,2
LDL	5,1	5,3	42,1	6,0	3,5	27,9	4,6	3,4	n.d.	16,7

[a]Data represent means ± S.D. from three separate preparations (HDL, HDL[-S1P]) or values in single preparations (HDL[-apoM], HDL[-+apoM], LDL). [b]S1P concentration is expressed in relation to total protein. [c]n.d. – not detectable.

HIGHLIGHTS

- S1P inhibits mitochondrial and endoplasmic reticulum-dependent apoptosis in macrophages

- Inhibitory effects of S1P on apoptosis depend on the increased expression of survivin

- Survivin upregulation by S1P is mediated through activation of JAK2 and STAT3

- High density lipoprotein emulates anti-apoptotic effect of S1P in macrophages
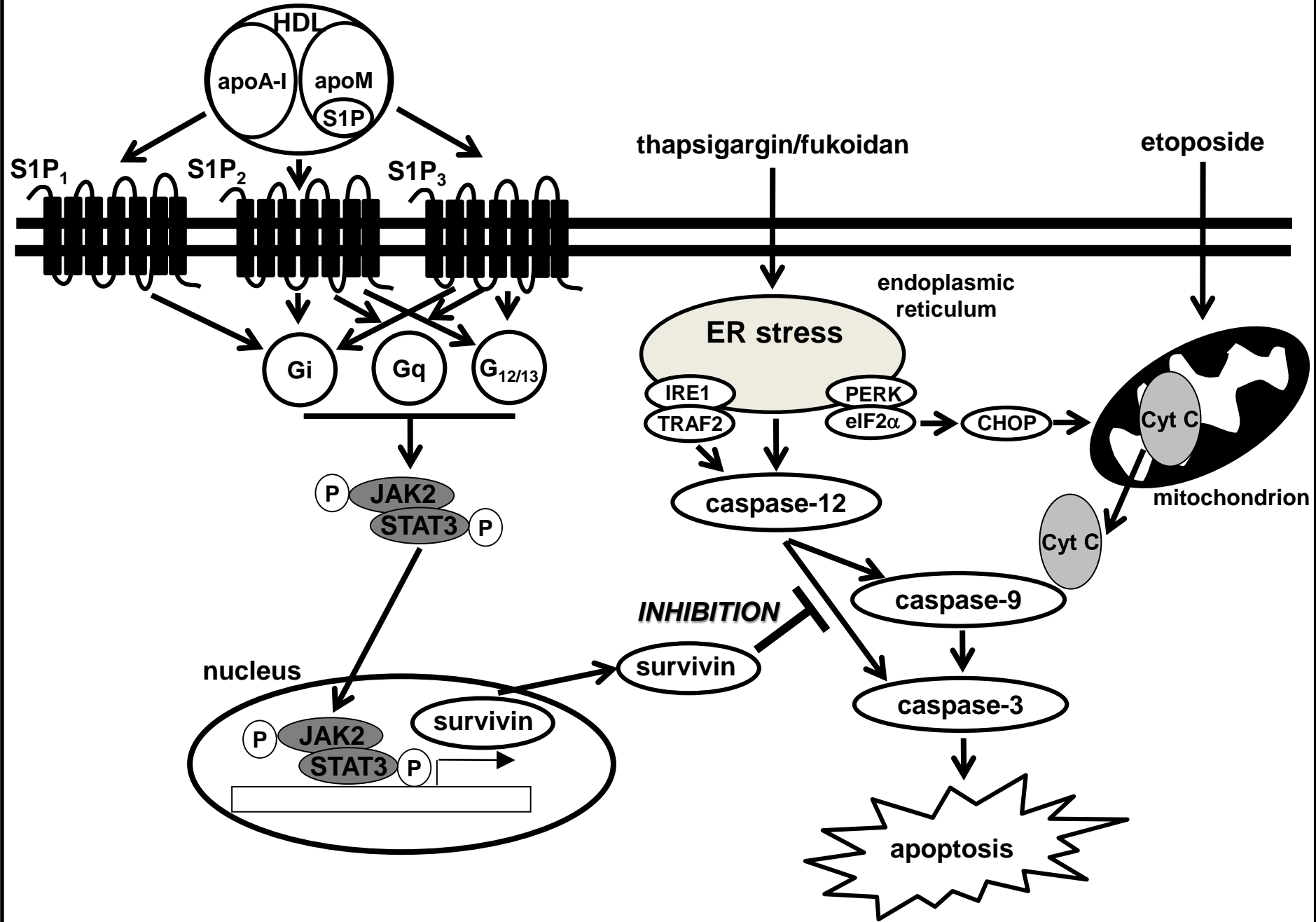
Feuerborn et al.: High density lipoprotein (HDL)-associated sphingosine 1-phosphate (S1P) inhibits macrophage apoptosis by stimulating STAT3 activity and survivin expression.

MATERIAL AND METHODS

<u>Reagents</u> – Apoptosis inducers etoposide, thapsigargin and fukoidan as well as S1P were obtained from Sigma, Deisenhofen, Germany. S1P receptor anta- and agonists W123, JTE013, CAY1044, FTY720 phosphate (FTY720P), KRP203 phosphate (KRP203P) and SEW2871 were from IBL-International, Hamburg, Germany. Compounds YM155 (Sepantronium bromide), GDC0152 as well as kinase inhibitors AG490 and S1155 were obtained from Selleck Chemicals (München, Germany). Norit A™ was purchased from Serva Electrophoresis (Heidelberg, Germany). Annexin V-FITC apoptosis detection kit was obtained from Bender Med-Systems Diagnostics, Vienna, Austria. APO-BRDU™ TUNEL kit was purchased from AbD Serotec, Oxford, UK. Sensolyte® homogenous AFC caspase-3 assay kit was from Anaspec Inc., Fremont, C, whereas caspase-9 and caspase-12 fluorimetric assay kits were obtained from BioVision, Milpitas, CA. Antibodies were from following suppliers: Janus kinase (JAK2), phosphoJAK2(Tyr1007) and signal transducer and activator of transcription 3 (STAT3) – CellSignlling, Frankfurt, Germany; phospho STAT3(Tyr705) - Acris, Herford, Germany or Abcam, Cambridge, UK; phosphoJAK1(Tyr1022), phosphotyrosine kinase 2 (Tyk2, Tyr1054) – BiorByt, Cambridge, UK; survivin – Novus, Cambridge, UK; cellular inhibitor of apoptosis 1/2 (cIAP1/2), X-linked IAP (xIAP), livin, neuronal apoptosis inhibitory protein (NAIP), IAP-like protein 2 (ILP2) – ProSci, Poway, CA. Chemicals used for chromatographic/mass spectroscopic analyses were from the following suppliers: acetonitrile, methanol, isopropanol and formic acid - Biosolve (Valkenswaard, The Netherlands), hydrochloric acid (37%) -

Merck (Darmstadt, Germany), ammonium formate, formic acid, iodoacetate, trifluoroacetate - Fluka (Buchs, Switzerland), dimethyl sulfoxide - Sigma. Ultra-pure water was from a Barnstead NANOpure water purification system (Thermo Fisher Scientific, Waltham, MA). All other chemicals were from Sigma and were of highest available purity.

Cell culture – The murine macrophage-like RAW 264.7 cell line and the human monocyte THP-1 cell line were obtained from American Tissue Cell Collection (ATCC, Manassas, VA) and incubated in Dulbecco's modified Eagle's medium (DMEM) or Rosell Park Memorial Institute (RPMI) medium, respectively, supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 U/mL penicillin G, 100 µg/mL streptomycin and 2 mmol/L L-glutamine. THP-1 monocytic cells (1×10^6 cells) were differentiated into macrophages by incubating in RPMI medium containing 100 ng/ml phorbol myristate acetate (PMA) over 48 h. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For stimulation cells were plated in either 6-, 12-, or 24-well culture plates (5, 1 or 0.25×10^6 cells/well, respectively) and allowed resting in FCS-free DMEM for 18 h before stimulation.

Lipoprotein isolation, modification, and fractionation – High density lipoproteins (HDL) and low density lipoproteins (LDL) were isolated from human plasma by a discontinuous KBr gradient centrifugation (d=1.125-1.210 g/mL and 1.006-1.063 g/mL, respectively).

For LDL acetylation equal volumes of native LDL and saturated sodium acetate were mixed in ice-cold water bath. Next, acetic anhydrate in the amount corresponding to 1.5 fold of total LDL protein content was added to the mixture in 1,5 µL aliquots every 5 minutes under continuous stirring. The mixture was stirred for 30 min, dialyzed for 24 h at 4°C against phosphate-buffered saline (PBS, pH 7.4), and stored at 4°C for

further experiments. Macrophages were loaded with cholesterol by incubating them with acetylated LDL (100.0 µg/mL) for 18 h.

For preparation of S1P-depleted HDL, lipoprotein solution was swirled with charcoal (20% Norit A) overnight at 4°C, centrifuged at 2300 rpm to eliminate resting charcoal, and dialyzed against PBS. To obtain HDL lipid fraction, ~0.5 mL of native HDL (~5.0 mg) were extracted according to Bligh and Dyer [1]. The lower organic phase arbitrarily called HDL lipid fraction was dried, dissolved in ethanol, and rapidly injected into PBS while vortexing. HDL lipid fraction was used for cell stimulation in amounts indexed to original HDL concentration.

Apolipoprotein M (apoM)-containing and apoM-deprived HDL were isolated by immunoaffinity chromatography as described previously [2]. Briefly, anti-human apoM-specific antibody (19-01) was raised in mice and used for generation of an affinity column. The antibody was immobilized by coupling to 1.0 mL HiTrap N-hydroxy-succinimide (NHS)-activated agarose resin (GE Healthcare, Brondby, Denmark). Purified HDL (10 mL, ~42 mg total protein) was passed over the antibody-coupled resin column. ApoM-containing HDL particles were subsequently eluted with glycine (0.1 mol/L, pH 2.2). The column was washed with water followed by Tris-buffered saline. The procedure was repeated until apoM was undetectable in the flow-through (six times in total). Total protein concentration in the eluate and flow-through was determined by Pierce BCA Protein Assay Kit (Thermo-Fischer Scientific, Naerup, Denmark).

ApoA-I isolated from human plasma was obtained from Calbiochem, (Merck Group, Darmstadt, Germany).

S1P reconstitution with albumin – S1P was reconstituted with albumin as described by Nguyen-Tran et al. [3]. Briefly, S1P dissolved in methanol (1.0 mg/mL) was evaporated under a stream of nitrogen to deposit a thin film on the inside of the glass tube.

Afterwards, S1P was resuspended in DMEM containing bovine serum albumin (BSA, fatty acid free, 4 mg/mL) to the initial concentration.

Mass spectrometric determination of apolipoproteins, S1P, or phosphatidylcholine (PC) in lipoprotein fractions - Apolipoprotein content in lipoprotein fractions was determined by micro-liquid chromatography (microLC) coupled to quadrupole-linear ion trap mass spectrometry (QTRAP) as described previously [4]. Micro-LC equipment consisted of an Ultimate 3000RSLCnano System, an Ultimate 3000TCC-3000SD column oven and an Ultimate 3000RS Autosampler (Thermo Scientific Dionex, Sunnyvale, CA). Chromatographic conditions were controlled by CHROMELEON 6.80 software. A QTRAP5500 LC/MS/MS System equipped with a Turbo VTM ion spray source operating in positive ESI mode and controlled by Analyst 1.5.1 software was used for detection (Sciex, Darmstadt, Germany). The chromatographic separation was performed on a ZORBAX 300SB-C18 column (150×1.0 mm id, 3.5 µm particle size)

with corresponding guard column (Agilent Technologies, Santa Clara, CA). The autosampler temperature was set at 10°C. The injection volume was 1.0 µL. The chromatographic separation was performed at 40°C. The mobile phase consisted of 0.1% formic acid in water/methanol (90:10, v/v) as eluent A and 0.1% formic acid in water/methanol (10:90, v/v) as eluent B. Gradient elution was performed with a linear increase from 20 to 100% B in 3.5 min, 100% B until 6.5 min, and re-equilibration from 6.6 to 7.6 min with 20% B. The flow rate was set to 50 µL/min. The Turbo VTM ion spray source was operated in positive ionization mode using the following settings: ion spray voltage = 5500 V, ion source heater temperature = 400°C, source gas 1 = 20 psi, source gas 2 = 50 psi, curtain gas = 35 psi, unit resolution on Q1 and Q3. Data processing was performed by use of Sciex software MultiQuantTM 2.0.

For sample preparation, lipoprotein fractions (15 µL) were diluted 1:2 with 100 mmol/L ammonium bicarbonate. Denaturation was performed with 6.9 mol/L 2,2,2-trifluoroethanol (TFE, Sigma) at 90°C for 60 min. Disulfide bonds were reduced using 5 mmol/L *tris*(2-carboxyethyl)phosphine (TCEP, Sigma) for 30 min at 60°C and alkylated with 10 mmol/L iodoacetamide for 30 min at room temperature in the dark. Digestion was performed with 40 µg of trypsin in 50 mmol/L acetic acid (1:30 enzyme-to-protein (E/P) ratio) overnight at 37°C after a tenfold dilution with 100 mmol/L ammonium bicarbonate. Digestion was stopped by addition of 2% formic acid to a final concentration of 0.1%. Samples were stored at −80°C until further clean-up that included desalting using 10 mg Oasis HLB 1cc Flangless Vac Cartridges (Waters, Milford, MA). The cartridges were conditioned with 1 mL 0.1% trifluoroacetate (TFA) in water/acetonitrile (20:80, v/v) and equilibrated with 1.5 mL 0.1% TFA in water. After sample loading, washing with 1.5 mL 0.1% TFA in water was performed. 400 µL of 0.1% TFA in water/acetonitrile (20:80, v/v) were used for elution. Samples were dried in a stream of nitrogen, reconstituted with 500 µL of 0.1% formic acid in water/methanol (90:10, v/v), and filtered using Costar Spin-X Centrifuge Tube Filters (Corning, NY) at 13 000 rpm for 2 min. After a tenfold dilution with 0.1% formic acid in water/methanol (90:10, v/v), an internal standard (IS) mix containing IS Apo A-I, IS Apo B-100, and IS Apo E (25 µmol/L, 5 µmol/L, and 10 µmol/L, respectively, (all from Biozol Diagnostica, Eching, Germany) in eluent A was added in a ratio of 1:100 to obtain final concentrations of 250 nmol/L IS Apo A-I, 50 nmol/L IS Apo B-100, and 100 nmol/L IS Apo E.

S1P content in lipoprotein fraction was determined by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry as described previously [5]. The HPLC equipment consisted of two Series 200Micro Pumps, a Series

200 Column Oven and a Series 200 Autosampler (Perkin Elmer, Waltham, USA). An API 4000™ LC/MS/MS system equipped with a Turbo V™ ion spray source operating in positive ESI mode was used for detection (Sciex, Darmstadt, Germany). The Turbo V™ ion spray source was operated using the following settings: ion spray voltage = 1500 V, ion source heater temperature = 300 °C, source gas 1 = 40 psi, source gas 2 = 50 psi, and curtain gas= 20 psi. The analytes were quantified using multiple reaction monitoring (MRM). Data analysis was performed with MultiQuant™ 2.0 (Sciex, Toronto, ON, Canada). The chromatographic separation was performed on a SeQuant™ (Merck, Darmstadt, Germany) ZIC®-HILIC column (50 mm × 2.1 mm, 3.5 µm particle size). The column was maintained at 50°C and the injection volume was 5 µL. The flow rate was set to 500 µL/min. The mobile phase consisted of 50 mmol/L ammonium formate in water/formic acid (100/0.2, v/v) as eluent A and acetonitril/eluent A/formic acid (95/5/0.2, v/v/v) as eluent B. Gradient elution was performed with 0% A for 1.0 min, a linear increase to 50% A until 1.9min, 50% A until 4.0 min and re-equilibration from 4.1 to 6.0 min with 0% A.

For sample preparation, 15 µL of lipoprotein fractions and 85 µL of an internal standard solution containing 11.8 ng/mL C17-sphingosine and 588 ng/mL C17-S1P in methanol (both from Avanti Polar Lipids, Alabaster, AL) were mixed to yield final concentrations of 10 ng/mL and 500 ng/mL, respectively. The mixture was vortex-mixed and centrifuged at 12,000 ×g for 5 min. The supernatant was transferred into glass vials prior to injection into the LC–ESI–MS/MS system.

Fluorescence-activated cell sorter (FACS) analysis of apoptotic cells - For determination of the annexin V binding RAW264.7 cells or THP-1 cells (5×10^5/mL) were resuspended in 140 mmol/liter NaCl, 10 mmol/liter Hepes, and 2.5 mmol/L CaCl$_2$. Annexin V-FITC was added for 30 min at room temperature according to the supplier instruction. For TUNEL assay, RAW264.7 macrophage suspension (1×10^6 cells/mL)

was fixed in paraformaldehyde (1%, w/v) and permeabilized with ice-cold ethanol (70%, v/v). DNA was labeled with Br-dUTP as indicated by the supplier and cells were stained with FITC-conjugated anti-BrdU antibodies. Flow cytometric measurements of annexin V binding and TUNEL assay were performed on FACScalibur flow cytometer (BD Bioscience, San Jose, CA) equipped with a 488 nm argon laser. Minimum of 5000 events was collected for each sample. Necrotic cells were detected by counterstaining with propidium iodide (PI).

Assays for caspases 3, 9 and 12 **-** RAW264.7 or THP-1 macrophages were resuspended in a hypotonic cell lysis buffer, subjected to three freeze/thaw cycles, and centrifuged. Caspase activities were measured in the supernatant according to the manufacturer protocols using Ac-DEVD-AFC, LEHD-AFC and ATAD-AFC (380(ex)/500(em) nm), as substrates for caspases 3, 9 and 12, respectively. Fluorescence was determined using a LS70 spectral fluorimeter (PerkinElmer, Rodgau, Germany). Data were expressed as relative fluorescence units (RFU) adjusted for the sample protein content.

Analysis of gene expression by real-time quantitative RT-PCR - Total RNA was isolated from RAW264.7 cells using RNAeasy Plus Purification Kit (Qiagen, Hilden, Germany) according to manufacturer protocol. RNA was eluted in water and quantified using BioPhotometer (Eppendorf, Hamburg, Germany). The entire cDNA was synthesized from 1.0 µg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fischer Scientific, Schwerte, Germany). Fully automated RT-PCR set-up was done on a Genesis 150 workstation (TECAN, Creilsheim, Germany) and PCR products were detected using ABI7900ht sequence detection system (Applied Biosystems, Darmstadt, Germany) in a 384-well format. PCR primer sequences were as follows: *Birc5* (survivin) - forward primer: GGTTTTGTGGCTTTGCTCTA, reverse primer: CTGCATTAGCAGCCCTGTAT; *Birc2* (cIAP1) - forward primer:

TGCTTGCTGTATGTCCTTCA, reverse primer: GATGCACGCCTTTAAAAGAA; *Birc3* (cIAP2) - forward primer: TTTCCAACATTTGACGTCTG, reverse primer: GTTGCTGCAGTGTTTCCTTT. Following primer sequences were used for human and murine S1P receptors: *S1PR1/S1pr1* – forward primer: TTCTCATCTGCTGCTTCATCATCC, reverse primer: GGTCCGAGAGGGCTAGGTTG; *S1PR2/S1pr2* – forward primer: GCCATCGCCATCGAGAGA, reverse primer: TGTCACTGCCGTAGAGCTTGA; *S1PR3/S1pr3* – forward primer: GCCAGTCTTGGGAAATGACACT, reverse primer: TGCCAGTTTCCCCACGTAA. Relative gene expression was calculated by applying the $2^{-\Delta\Delta Ct}$ method. Briefly, the threshold cycle number (Ct) of target genes was subtracted from the Ct of GAPDH ($Ct_{housekeeping}$) and raised to the 2nd power of this difference.

Western Blotting – RAW264.7 macrophages were lysed in a buffer containing 50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1.0 % (v/v) Nonidet P-40, 5.0 % (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 300 mmol/L NaF, 1.0 mmol/L EGTA, 1.0 mmol/L orthovanadate, and protease inhibitors (Complete, Roche). Cell lysates (30 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to nitrocellulose membranes, which were blocked overnight in Tris-buffered saline containing 5.0 % non-fat dry milk prior to incubations with antibodies.

General Procedures - Data are presented as means ± S.D. from at least three separate experiments or as results representative for at least three repetitions unless indicated otherwise. Comparisons between the groups were performed with two-tailed Student t-test using the MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium). *p* values less than 0.05 were considered significant.

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