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CD95/Fas downregulation in lymphoma cells through acquired alkyl-lysophospholipid resistance; partial role of associated sphingomyelin deficiency

Wim J. VAN BLITTERSWIJK*[1], Jeffrey B. KLARENBEEK[*], Arnold H. VAN DER LUIT[*2], Maaike C. ALDERLIESTEN[*], Menno VAN LUMMEL[*3] and Marcel VERHEIJ[†]

[*]Division of Cell Biology (B5) and [†]Department of Radiotherapy, The Netherlands Cancer Institute/ Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Short title: CD95/Fas downregulation through acquired resistance to alkyl-lysophospholipid

Abbreviations used: SM, sphingomyelin; SMS, sphingomyelin synthase; C12-SM, lauroyl-SM; ALP, alkyl-lysophospholipid; MβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GlcCer. glucosylceramide; DISC, death-inducing signalling complex; FasL, Fas ligand.

[1] To whom correspondence should be addressed (Tel: +31-205121976; Fax: +31-20-5121944; E-mail: w.v.blitterswijk@nki.nl)

[2]Present address: Oncodesign, 20, rue Jean Mazen, BP 27627, 21076 Dijon Cedex, France

[3]Present address: Leiden University Medical Center, Dept. of Immunehematology and Blood Transfusion (E3-Q), Albinusdreef 2, 2333 ZA Leiden, The Netherlands

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Synopsis

The alkyl-lysophospholipid (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine induces apoptosis in S49 mouse lymphoma cells. A variant cell line, $S49^{AR}$, made resistant to ALP, was previously found impaired in ALP uptake via lipid raft-mediated endocytosis. Here, we report that these cells display cross-resistance to Fas/CD95 ligation (FasL), and can be gradually resensitized by prolonged culturing in the absence of ALP. Fas and ALP activate distinct apoptotic pathways, since ALP-induced apoptosis was not abrogated by dominant-negative FADD, cFLIP or the caspase 8 inhibitor IETD. ALP-resistant cells showed decreased Fas expression, both at the mRNA and protein level, in a proteasome-dependent fashion. The proteasome inhibitor MG132 partially restored Fas expression and resensitized the cells to FasL but not to ALP. Resistant cells completely lacked sphingomyelin (SM) synthesis, which seems a unique feature of the S49 cell system, having very low SM levels in parental cells. Lack of SM synthesis did not affect cell growth in serum-containing medium, but retarded growth under serum-free (SM-free) conditions. SM deficiency determined in part the resistance to ALP and FasL. Exogenous short-chain (C12-)SM partially restored cell surface expression of Fas in lipid rafts and FasL sensitivity but did not affect Fas mRNA levels or ALP sensitivity. We conclude that acquired resistance of S49 cells to ALP is associated with downregulated SM synthesis and Fas gene transcription and that SM in lipid rafts stabilizes Fas expression at the cell-surface.

Keywords: alkyl-lysophospholipid; apoptosis resistance; Fas/CD95; lipid raft; sphingomyelin synthase; proteasome

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INTRODUCTION

Synthetic alkylphospholipids such as edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; alkyl-lysophospholipid; ALP) and perifosine (D-21266) selectively induce apoptosis in tumor cells [1-3], have anti-angiogenic properties [4], are enhancers of radiation-induced apoptosis [1,3,5-7] and are currently being tested as novel anticancer agents in clinical trials [1,7]. These single-chain alkylphospholipids accumulate in cell membranes [8], resist cellular lipases and interfere with lipid-based signal transduction [1,3,9] and phospholipid biosynthesis, as we have previously studied a.o. in S49 mouse lymphoma cell [10,11]. A variant cell line, $S49^{AR}$, made resistant to edelfosine (ALP), showed impaired uptake of this compound as well as other alkylphospholipids by lipid raft-mediated endocytosis [10-12]. Interestingly, these $S49^{AR}$ cells lacked the biosynthesis of the raft constituent sphingomyelin (SM) due to complete downregulation of SM synthase 1 (SMS1) [13]. We furthermore demonstrated that the downregulation of SMS1 in parental S49 cells by siRNA evoked cellular resistance to multiple structurally related alkylphospholipids [11]. This SM deficiency and impaired raft-dependent uptake associated with alkylphospholipid resistance was specific for lymphoma cells, since carcinoma cells made resistant to these compounds showed normal SM synthesis/levels and displayed a different uptake mechanism [12].

Two mammalian SMS isotypes exist: SMS1 residing in the *trans*-Golgi network, and SMS2 located both at the Golgi and the plasma membrane [14,15]. Only the former isotype is expressed in the S49 lymphoma system studied here [13]. SMS1 and its two enzymatic products, SM and diacylglycerol, play a major role in membrane microdomain (lipid raft) formation and vesicle biogenesis at the *trans*-Golgi network, respectively [14,16]. Diacylglycerol recruits and co-activates protein kinase D [17], which is required for efficient formation of Golgi-derived secretory vesicles [16]. This SMS1-protein kinase D-mediated process is critical for transport of proteins from the *trans*-Golgi to the plasma membrane. Cells that lack SMS1 (and SMS2) activity reportedly have a severe growth defect [15,18,19].

Since SMS1-deficient $S49^{AR}$ cells have a defect in raft-mediated endocytosis of ALP [10,13], and since lipid rafts have been implicated in apoptosis induction via the death receptor Fas/CD95/APO-1 [20-25], we questioned if the SMS1/raft defect in the $S49^{AR}$ cells could have consequences for the sensitivity of these cells to Fas ligation. The interaction between Fas and its ligand (FasL) triggers receptor oligomerization in lipid rafts at the plasma membrane, death domain-mediated recruitment of the adaptor protein FADD, and binding and activation of pro-caspase-8 and/or –10 to FADD through their death-effector domains in a death-inducing signaling complex (DISC) [26]. Complete DISC assembly requires Fas to move into endosomal compartments [26,27], further initiating a caspase cascade responsible for the apoptotic process. In certain cells (type II cells; see Discussion), ALP was found to trigger Fas signaling independent of ligation [22,28,29].

Here we report that, in S49 cells, ALP induces apoptosis independent of the Fas receptor, and that the ALP-resistant $S49^{AR}$ cells are cross-resistant to Fas ligation. We find that the lack of SM synthesis in these resistant cells retarded cell growth under serum-free conditions and is associated with reduced, proteasome-dependent Fas mRNA and protein levels.

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EXPERIMENTAL

Materials

ALP (edelfosine;Et-18-OCH_3;1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) was purchased from BioMol (Plymouth Meeting, PA). [^{3}H]edelfosine (^{3}H-ALP; 58 Ci/mmol), was synthesized by Moravek Biochemicals (Brea, CA). [*Methyl*-^{14}C]choline chloride (58 mCi/mmol) and L-[3-^{14}C]serine (54 mCi/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). [^{3}H]1-sphingosine was synthesized by Piet Weber (DSM, Delft, The Netherlands). $C_{12:0}$-sphingomyelin (C12-SM) was from Avanti Polar Lipids. Tween-20 and Silica 60 TLC plates were from Merck (Darmstadt, Germany). Anti-Fas monoclonal antibody 7C10 was from CAMPRO Scientific BV (Veenendaal, The Netherlands). Anti-Fas monoclonal antibody Jo2 (hamster IgG2) and its fluorescein isothiocyanate (FITC) conjugate, as well as FITC-conjugated anti-CD90/Thy-1.1 monoclonal antibody were from BD Biosciences Pharmingen (Erembodegem, Belgium). Rabbit anti-β-actin was from Cell Signaling Technology; Rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (HRP) and swine anti-rabbit immunoglobulin-HRP were from DAKO A/S (Glostrup, Denmark), Protein G sepharose Fast flow beads were from Amersham Biosciences (Roosendaal, The Netherlands); Soluble recombinant human Fas ligand (FasL; APO-1L) was from Alexis (Leiden, The Netherlands); Z-IETD-fmk was from Calbiochem (Breda, The Netherlands). MG132, lactacysteine en MβCD were from Sigma (Zwijndrecht, The Netherlands).

Cells and culture conditions

Mouse S49.1 lymphoma cells (S49) were cultivated in Dulbecco's modified Eagle's medium (Gibco-Invitrogen), containing high glucose and pyruvate, supplemented with 8% fetal calf serum, 2 mM L-glutamine and antibiotics. ALP-resistant variants (S49^{AR}) were isolated in two selection rounds of growth in 15 µM ALP (edelfosine) for 72 h, followed by plating in semi-solid medium and isolation of colonies of surviving cells [30]. S49^{AR} cells could be grown continuously in 15 µM ALP. All experiments with S49^{AR} cells were performed with cells grown without the selection agent for at least one week. Loading of S49^{AR} cells with exogenous C12-SM was performed by injecting the ethanol-dissolved lipid into the complete culture medium (final concentration 20 µM C12-SM; 0.02% ethanol) and culturing the cells in this medium for three days. To estimate growth rate in the absence of (exogenous) SM, cells were grown in a serum-free medium [31].

Plasmids, quantitative PCR and RNA interference

Human dominant-negative FADD (dnFADD), lacking amino acids 2-77, and mouse cellular FLIP$_L$ were cloned into the retroviral vector LZRS-MS-IRES-eGFP as described by Werner et al. [32]. Transduced cells were selected for enhanced green fluorescent protein (eGFP) expression using a MoFlo high speed cell sorter (Cytomation, Fort Collins, CO). For quantitative PCR, we used for Fas/CD95 the forward primer GCGATTCTCCTGGCTGTGAA, and the reverse primer

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CACGGCTCAAGGGTTCCAT. The amplification was related to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), using the forward primer TGCACCACCAACTGCTTAG, and the reverse primer GGATGCAGGGATGATGTTC.

SMS1 was downregulated in S49 cells by retroviral transduction of short interfering RNAs (siRNAs), yielding $S49^{siSMS1}$ cells, as published previously [13]. Control cells ($S49^{mock}$) were transduced with scrambled siRNA [13]. Retroviral transduction of HA-tagged SMS1 in $S49^{AR}$ cells, yielding AR-SMS1 cells, was done after cloning SMS1 (from S49 cDNA) in the pBabe vector, using forward primer GATCGGATCCTACGATGTTCCAGATTACGCGATGTTGTCTGCCAGGACCATGA AGG and reverse primer GAATTCTTATGTGTCGTTTACCAGCCG.

Immunoblotting and immunoprecipitation

S49 cells were washed once with PBS and lysed in lysis buffer (10 mM Tris/HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P40, protease inhibitor cocktail (Roche, Almere, The Netherlands), 1 mM Na-vanadate and 20 mM NaF. Lysates were incubated for 30 min at 4˚C and spun to remove cellular debris (12,000 x *g* for 20 min), before being normalized for protein content. Prior to Western blot analysis, samples were heated for 10 min at 70˚C in reducing SDS sample buffer from Invitrogen Life Technologies (Breda, The Netherlands) containing 1 mM DTT and run on a Novex mini-gel in NuPage MES/SDS running buffer (Invitrogen Life Technologies, Breda, The Netherlands). Separated proteins were transferred onto nitrocellulose membranes and blocked with 3% (w/v) BSA for 1 h in TBS-T buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20) containing 1 in 50 dilution of Roche Blocking reagent (Roche, Mannheim, Germany). Blots were incubated overnight at 4˚C with 7C10 MoAb (1:1000 dilution), followed by incubation with horseradish-peroxidase-conjugated rabbit anti-rat immunoglobulin (1:2000 dilution), and immunoreactive proteins were visualized by ECL.

For immunoprecipitation experiments, equal protein contents from pooled raft fractions (fractions 3-5) and non-raft fraction (fractions 8-10) from S49, $S49^{AR}$, $S49^{mock}$ or $S49^{siSMS1}$ cells [11,13] were added to a final volume of 500 µl lysis buffer. Anti-Jo2 mAb was added to the samples and incubated overnight at 4˚C under continuous rotation. Immune complexes were incubated with Protein G-Sepharose beads for an additional 1 h. Precipitated proteins were washed three times with IP washing buffer (50 mM Tris, pH 7,5, 5 mM EDTA, 150 mM NaCl, 0.1% Tween-20. Samples were subjected to gel electrophoresis for immunoblotting as described above.

Confocal microscopy and flow-cytometry

Cells were seeded onto RetroNectin-coated coverslips, washed three times with PBS and fixed in 4% (v/v) formaldehyde (30 min). Cells were washed with PBS, blocked in TBS-T buffer containing 1% bovine serum albumin, washed 5 times in TBS-T, incubated with anti-Jo2 mAb, washed three times with TBS-T buffer and then incubated with FITC-anti-hamster-antibody. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingham, CA) and viewed under a Leica TCS NT confocal laser-scanning microscope (Leica Microsystems, Germany). For flow cytometry, cells were washed three times with 0.5% BSA in PBS containing 0.01% sodium azide. Cells were incubated with FITC-

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conjugated Jo2 mAb, or with FITC-anti-CD90/Thy-1.1 mAb. FACScan analysis was performed using FCS express 2.0.

Apoptosis assay

Cells were seeded at 1 x 10^6 cells/ml, cultured overnight and incubated for indicated time periods with various concentrations of ALP or FasL. Cells were stained with propidium iodide and the percentage of sub-diploid apoptotic nuclei was determined on a FACScan (Becton Dickinson) [10,33].

Lipid analysis

Cells at 1 x 10^6 cells/ml were labeled with [*methyl*-^{14}C]choline chloride (1 µCi/ml), or L-[3-^{14}C]serine (0.4 µCi/ml), or [^{3}H]1-sphingosine (1 µCi/ml). At given time points, aliquots of cells were taken, washed and resuspended in 200 µl PBS. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in methanol/H_2O/chloroform, 47:49:3 (v/v/v), and separated by silica TLC, using chloroform:methanol:acetic acid:water, 60:30:8:5 (v/v/v/v) or, in the case of sphingosine labeling, using chloroform:methanol:0.2% (w/v) $CaCl_2$, 60:40:9 (v/v/v). Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR Phosphor-Imager and identified using iodine stained standards. Tritiated lipids were visualized after dipping the TLC plate in 12.5% diphenyloxazole dissolved in diethylether, drying and subsequent autoradiography. Unlabeled lipids (e.g. C12-SM) were visualized by spraying the plate with sulfuric acid, followed by heating (charring) in an oven at 180 ^{o}C for 30 min, and (semi-)quantified by densitometry.

Mass sphingolipid analyses were conducted by liquid chromatography, electrospray tandem mass spectrometry as described by Merrill et al. [34].

Isolation of lipid rafts

A lipid raft fraction was prepared by detergent extraction of cells and sucrose gradient centrifugation [10]. Briefly, 2 x 10^8 cells were solubilized into 1 ml of ice-cold 25 mM MES, 150 mM NaCl, 1% Triton X-100, homogenized using a Dounce homogenizer and fractionated on a discontinuous sucrose gradient at 39,000 rpm in a SW41 rotor for 20 hours at 4°C.

Statistical analysis

Results are expressed as means ± S.D. Mean values were compared using the Student's *t*-test. Significant differences were expressed as * $p<0.01$ and ** $p<0.001$.

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RESULTS

S49 lymphoma cells made resistant to edelfosine (ALP; S49^{AR} cells) are cross-resistant to Fas-induced apoptosis; edelfosine does not initiate Fas signaling

Previously, we reported that a synthetic alkyl-lysophospholipid (ALP; edelfosine) induces apoptosis in S49 lymphoma cells, in a dose- and time-dependent fashion [10,12]. The onset of apoptosis was already apparent after 3 h (supplementary Figure 1). In the present study, we used 15 μM ALP for 6 h to induce 50-90 % apoptosis (variation among different experiments) in these cells An ALP-resistant variant cell line, S49^{AR} was generated by culturing S49 cells in the presence of this ether-lipid and repeated selection of surviving cells [10,29]. Figure 1A shows that these ALP-resistant S49^{AR} cells were also largely resistant to Fas-induced apoptosis, whereas the parental S49 cells underwent apoptosis by ALP as well as by Fas ligation.

To explain this cross-resistance of S49^{AR} cells to ALP and FasL, we tested the obvious possibility that the two apoptotic stimuli would initiate the same signaling pathway, that is via the Fas receptor, as has been suggested for HL-60 and Jurkat T cells [28,29]. Fas signaling depends on recruitment of the Fas adapter protein FADD and on recruitment and activation of caspase 8, whereas this process is inhibited by c-FLIP$_L$ [26,32,35]. We therefore retrovirally transduced S49 cells with a dominant-negative form of FADD (FADDdn) or with c-FLIP$_L$ (transduced cells were sorted on base of eGFP expression) and found that the resulting S49^{FADDdn} and S49^{FLIP} cells were fully resistant to FasL, but not to ALP (Figures 1B and 1D). The same was true for S49 cells that were treated with the caspase 8-specific inhibitor z-IETD-fmk [26,27] (Figure 1D). In addition, we tested the combination of (suboptimal dose of) ALP and FasL together, which gave, at most, an additive but no synergistic effect on apoptosis, whereas FADDdn in this case reduced apoptosis to the level induced by ALP alone (Figure 1B). These data indicate that, in S49 cells, ALP does not induce apoptosis via the Fas pathway, neither is there cross-talk between ALP- and FasL-induced apoptotic signaling. In agreement with this notion, ALP- and FasL-induced apoptosis were differentially sensitive to the lipid raft-disrupting agent methyl-β-cyclodextrin (MβCD, extracting cholesterol from rafts) (Figure 1C). MβCD did not affect FasL-induced apoptosis in S49 cells, but inhibited ALP-induced apoptosis in these cells, as we reported previously [10,11].

From these collective data, we conclude that ALP does not activate the Fas signaling pathway. Thus, the cross-resistance of S49^{AR} cells to ALP and FasL has to be explained by a mechanism that differs from a common defect in Fas signaling.

Apoptosis-resistant S49^{AR} cells show SM deficiency as a result of downregulated SMS1; effect on cell proliferation

We recently reported [13] that the ALP-resistance of S49^{AR} cells was accompanied by a lack of sphingomyelin (SM) synthesis (supplementary Figure 2) as a result of an almost complete downregulation of SMS1 expression, whereas SMS2 is not present at all. To investigate a possible causal relationship between SMS1/SM deficiency and the resistance of S49^{AR} cells to Fas ligation, we first excluded possible involvement of secondary changes in lipid synthesis/composition: The S49^{AR} cells displayed no altered rates of biosynthesis of lipids other than SM (supplementary Figure 2). We next measured the mass levels of individual species of sphingolipids in S49 and S49^{AR} cells

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and we determined how SMS1 knockdown by siRNA, yielding $S49^{siSMS1}$ cells [13], would affect the mass levels of SM and its metabolic precursor ceramide (commonly associated with apoptosis). We also measured GlcCer, precursor for the more complex glycolipids. Figure 2A shows the palmitoyl (C16:0) species to be most prominent in cellular SM and GlcCer. Ceramide contained, in addition, substantial amounts of other acyl species (C16DH, C24:0, C24:1). Although $S49^{AR}$ cells completely lack SMS activity, they still contained 20% SM relative to S49 cells. $S49^{siSMS1}$ cells even contained 40% of the SM content of S49 cells. Clearly, these SM molecules in SMS1-deficient cells were mostly derived from the serum in the culture medium (compare the two upper panels in Figure 2A). The contents and compositions of ceramide and GlcCer showed no significant differences between the three cell types.

In light of several reports suggesting that SM synthesis would be essential for cell growth [15,18,19], we compared the growth of $S49^{AR}$ cells with S49 cells. We found that, in serum-containing medium, SMS-deficient $S49^{AR}$ cells have almost the same growth rate as the parental S49 cells. However, in serum-free (SM-free) conditioned medium [31], S49 cells grew slower and growth of $S49^{AR}$ cells was even more retarded, particularly after two days (Figure 2B), but were not completely growth arrested.

The data thus suggest that SM is the only sphingolipid downregulated in the $S49^{AR}$ and $S49^{siSMS1}$ cells, and that SMS1 deficiency does not substantially affect the (mass) levels of ceramide or GlcCer, so that these lipids are not likely related to the mechanism of apoptosis resistance. Furthermore, we find growth retardation (no complete arrest) of cells that are SMS-deficient, but only if the culture medium is deprived of SM as well.

Deficient SM synthesis is not the sole cause of resistance to apoptosis induction

We previously demonstrated that $S49^{AR}$ cells are resistant to ALP because, contrary to S49 cells, they are unable to internalize ALP via their SM-deficient lipid rafts [10,11,13]. Figure 3 illustrates that $S49^{siSMS1}$ cells in which SMS1 is downregulated [13], SM synthesis is barely visible and ALP-induced apoptosis is decreased from 55% to 15%, whereas $S49^{AR}$ cells are completely devoid of SM synthesis and completely resistant to ALP. Intriguingly, we find that, together with SMS1 downregulation, FasL-induced apoptosis also dropped from 54% to 28% in $S49^{siSMS1}$ cells, and to 14% in $S49^{AR}$ cells (Figure 3B).

While apoptosis resistance was induced by prolonged culturing of S49 cells in the continuous presence of ALP, we found that when the resistant $S49^{AR}$ cells were cultured in the absence of ALP for more than 4 weeks, they gradually lost their resistance (yielding resensitized $S49^{ARS}$ cells) and, strikingly, regained SM synthesis (Figure 3C). This finding would argue against a possible 'clone' effect of $S49^{AR}$ cells, rather suggesting a reversible selection pressure of ALP. So far, we conclude that SM deficiency in $S49^{AR}$ and $S49^{siSMS1}$ cells and SM reappearance in $S49^{ARS}$ cells closely correlate with cross-resistance/ resensitization to ALP- and FasL-induced apoptosis.

To confirm the causal link between SM synthesis and the sensitivity to ALP and FasL, we reintroduced SMS1 into $S49^{AR}$ cells by retroviral transduction of HA-tagged SMS1, yielding cells designated as AR-SMS1. HA-SMS1 was properly expressed (Western blot in Figure 3B) and active (appearance of SM spot on TLC; Figure 3A, right panel), and the SM produced was recovered in the detergent-resistant membrane (lipid

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raft) fraction, like in the S49 parental cells (Supplementary Figure 3). Yet, these cells failed to undergo apoptosis by ALP or FasL (Figure 3B). So, by simple reappearance of SM the cells did not regain their original (S49-like), ALP-sensitive phenotype. This negative result excludes SMS1 as direct and sole determinant of apoptosis sensitivity. The apparent discrepancy with the $S49^{siSMS1}$ data can be explained by proposing the causal involvement of yet another (additional) unknown factor/protein, the expression of which is modulated concomitantly with downregulated SMS1 in $S49^{AR}$ and $S49^{siSMS1}$ cells. Thus, unlike our earlier suggestion [13], SMS1 deficiency is not the sole determinant of the apoptosis resistance.

Fas is downregulated in ALP-resistant $S49^{AR}$ and $S49^{siSMS1}$ cells

Both SM and the Fas receptor are located in lipid raft domains at the cell surface [14,20-25]. The first step in Fas signaling is ligand-induced formation of Fas microaggregates at the cell surface [26]. We therefore compared the surface expression of Fas in the FasL-resistant, SM-deficient $S49^{AR}$ and $S49^{siSMS1}$ cells with the FasL-sensitive S49 cells. Confocal microscopic images of Fas (Figure 4A) revealed that the expression at the cell surface was significantly reduced in $S49^{siSMS1}$ cells and, even more so, in $S49^{AR}$ cells. Figure 4B shows a quantification of this reduced Fas surface expression, as determined by FACS analysis.

Since Fas is often localized in lipid raft domains, even before stimulation [20,21,24,25], we measured Fas in detergent-resistant lipid raft fractions from FasL-sensitive and -resistant cells. It was already apparent in immunoblots of the total lysates of these cells, that Fas expression was much reduced in $S49^{siSMS1}$ cells and almost undetectable in $S49^{AR}$ cells (Figure 4C). Lipid raft fractions (with SM as a marker) were isolated and pooled as shown in Supplementary Figure 3. In the FasL-sensitive S49 and $S49^{mock}$ cells, Fas was immunoprecipitable from the combined lipid raft fractions but not from the non-raft fractions (Figure 4D). Furthermore, Fas was low or virtually absent in raft fractions from FasL-resistant $S49^{siSMS1}$ and $S49^{AR}$ cells, respectively. Reduced cell surface expression seemed specific for Fas, since expression of other receptors, the transferrin receptor [10] and raft-localized Thy-1 (a glycosyl-phosphatidylinositol-linked protein) (Figure 4B, lower panel), were not decreased in the resistant cells.

We next compared the Fas mRNA levels of $S49^{siSMS1}$ and $S49^{AR}$ cells with those of the parental S49 cells, using quantitative PCR. Figure 5A shows that these levels are dramatically decreased in the resistant cell. Artificial loading of cells with short-chain (C12-)SM (see below) had no effect, so that Fas mRNA expression is not regulated by SM content

SM stabilizes Fas expression at the cell surface

We questioned if SM would determine Fas expression at the cell surface and we therefore reconstituted $S49^{AR}$ cells with exogenous SM. However, substantial loading of the plasma membrane with exogenous natural SM (a rigid molecule with two long hydrophobic chains) is notoriously difficult and, eventually, we were only successful when we preloaded the serum in the culture medium with the less hydrophobic, shorter-chain C12-SM, as published and discussed before [13]. Upon subsequent cell culturing, sufficient C12-SM was incorporated in cell membranes (Figure 5A, inset), also in the lipid raft fractions [13], to an estimated amount of 500 pmol/10^6 cells (i.e. 3-fold higher

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than endogenous SM in S49 cells). This SM loading of $S49^{AR}$ cells enhanced the surface expression of Fas significantly and consistently, although not to the high levels found in the S49 cells (Figure 6A). SM-loaded $S49^{AR}$ cells became partly resensitized to FasL - induced apoptosis (dose-dependently), but notably not to apoptosis induction by ALP (Figure 5B). Total Fas underwent no change at the mRNA level (Figure 5A), and there was only a minor elevation at the total Fas protein level, but this elevation was amplified in the isolated lipid raft fraction (Figure 5C). Collectively, these data would suggest that SM promotes or stabilizes Fas localization in lipid rafts at the cell surface.

Since SM apparently shifted Fas partitioning from the cell interior towards the cell surface without substantial changes in total Fas protein, it is conceivable that nascent Fas protein follows the same vesicular route as newly synthesized SM, which originates in the Golgi (where SMS1 resides [15]) and ends up in the outer leaflet of the plasma membrane lipid bilayer [14-16,36]. To support this idea, we treated the cells with brefeldin A, commonly employed to induce an *in vivo* collapse of the cis/medial Golgi onto the ER [37], and known to prevent SM vesicular trafficking to the plasma membrane [38]. Figure 6C shows the rate of SM synthesis, using [^{14}C]choline as a precursor, and the effect of brefeldin A. (Similar results were obtained with [^{14}C]serine as a SM precursor (data not shown)). Figure 6C (right panel) confirms that newly synthesized SM indeed ends up in the outer plasma membrane leaflet, as it is accessible/ hydrolysible by exogenous (bacterial) sphingomyelinase. Figure 6C (middle panel) shows that brefeldin A precludes SM synthesis in the S49 cell system, whereas Figure 6B shows that such SM deprivation of the plasma membrane is associated with a decreased expression of Fas at the cell surface. Brefeldin A had no effect on the very low Fas surface expression in $S49^{AR}$ cells (data not shown). The inhibitory effect of Brefeldin A on FasL-induced apoptosis in S49 cells was about 30% (after subtracting some background apoptosis; data not shown).

We conclude that SMS1 deficiency in the ALP-resistant $S49^{siSMS1}$ and $S49^{AR}$ cells is associated with downregulated CD95/Fas, both at the mRNA and protein level. In ALP-sensitive S49 cells, the SMS1 product SM, while undergoing vesicular transport to the cell surface, may (co-)determine (stabilize) Fas partitioning at the cell surface. Failure to do so may to some extent (but not fully) explain cellular resistance to FasL-induced apoptosis.

Fas downregulation is proteasome-dependent

We further searched for the mechanism by which Fas was downregulated in ALP-resistant cells. Since SM stabilized Fas at the cell surface, we considered the possibility that, in the absence of SM, Fas protein might be cleaved from the cell surface by matrix metalloproteinase(s) [36,39]. However a broad-spectrum inhibitor of these proteases, GM 6001 (from BIOMOL; up to 500 nM), had no effect on Fas surface expression or FasL-induced apoptosis (data not shown).

Since Fas has been found associated with proteins implicated in the ubiquitination pathway [36], and proteasome inhibitors reportedly enhanced the expression of Fas in smooth muscle cells [40], we added the well-known proteasome inhibitor MG132 and found a gradual restoration of Fas protein expression in $S49^{AR}$ and $S49^{siSMS1}$ cells, in a 4 h time period (Figure 7A). Similar results were obtained with another proteasome inhibitor, lactacysteine (5 μM) (data not shown). Proteasome inhibition also enhanced

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Fas mRNA levels (about 5-fold), both in resistant and sensitive cells (data not shown) but did not affect the prominent Fas protein expression in parental S49 or in $S49^{mock}$ cells (Figure 7A), indicating that that proteasomal regulation of Fas expression is particularly apparent in resistant, SMS1-deficient cells. We found neither an effect of MG132 on SM synthesis in S49 cells, nor on the lack of SM synthesis in $S49^{AR}$ or $S49^{siSMS1}$ cells (data not shown). We found no evidence for Fas ubiquitination (using anti-ubiquitin antibodies).

We next tested if MG132-induced recovery of Fas could restore FasL sensitivity in the $S49^{AR}$ and $S49^{siSMS1}$ cells. Cells were preincubated with MG132 for various times (up to 3h) and then stimulated with FasL or ALP (for comparison). Figure 7B shows that, indeed, FasL-induced apoptosis in these MG132-treated SMS1-deficient cells was time-dependently increased, whereas MG132 by itself did not induce apoptosis, neither did it restore ALP-induced apoptosis in these cells. MG132 thus selectively enhanced FasL-induced apoptosis, in agreement with the enhanced Fas expression (Figure 7A).

In conclusion, continuous culturing of S49 cells in the presence of ALP causes resistance to ALP as well as FasL. The resistance to FasL, but not to ALP, is to a large extent due to enhanced proteasome-dependent degradation of (an) unknown factor(s) that determine(s) Fas transcription. The observation that the resistance to ALP was not reversed by the proteasome inhibitor is in line with our earlier conclusion that ALP-induced apoptosis does not proceed via the Fas receptor.

DISCUSSION

In this paper, we have shown that S49 lymphoma cells that have acquired resistance to ALP and related anti-tumor alkylphospholipids ($S49^{AR}$ cells) are cross-resistant to FasL-induced apoptosis. We excluded the possibility that the cross-resistance might be due to a common defect in Fas-mediated death signaling, since ALP induced S49 apoptotic death independent from Fas engagement: Unlike Fas signaling, ALP-induced apoptosis was not dependent on FADD, not inhibited by $FLIP_L$ or the caspase-8 inhibitor z-IETD-fmk, and was blocked by MβCD sequestration of cholesterol. Furthermore, the proteasome inhibitor MG132 alleviated the resistance to Fas ligation but not to ALP. Our conclusion that ALP induces apoptosis independent from Fas does not agree with reports by Mollinedo's group [2,22,28], who showed ALP-induced clustering/redistribution of Fas into lipid rafts. However, this discrepancy can be explained by the different cell types used. In so-called type-1 cells (such as JY, HuT78, SKW6, H9), Fas is located in lipid rafts [21,25] and DISC formation is fast and efficient, with high amounts of active caspase-8 formed, which directly activates the effector caspase-3. In type-2 cells (such as CEM, Jurkat, multiple myeloma [2]), Fas is largely located outside rafts and redistributes into rafts upon ligation [21,25], DISC formation being delayed and insufficient to activate caspase-3 directly. For apoptosis induction, these type-2 cells need an amplification pathway via mitochondria, which can be inhibited by overexpression of Bcl-2 or $Bcl\text{-}X_L$ [41]. In S49 cells, these Bcl proteins do not block apoptosis induction by ALP [30] or FasL [42], and Fas was found localized in lipid raft fractions (Figure 4D), consistent with the type-1 cell category. Accordingly and similar to other type-1 cells [21,26], we found no effect of cholesterol sequestration by MβCD on FasL-induced apoptosis in S49 cells

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(Figure 1C). Collectively, our present and previous data suggest that, in S49 lymphoma cells, ALP does not induce Fas signaling but induces apoptosis mainly by its raft-mediated internalization and subsequent inhibition of PC synthesis in the endoplasmic reticulum [1,10,11,43].

We reported that the apoptosis resistance of $S49^{AR}$ cells was associated with SMS1 downregulation and consequent deficiency of the raft constituent SM. Such lack of SM may impair (Fas-) receptor clustering in rafts and subsequent signaling, as was shown for the T cell receptor in Jurkat cells [44]. Our reconstitution experiments with exogenous C12-SM in $S49^{AR}$ cells revealed that SM can stabilize Fas expression in rafts at the cell surface. It is conceivable that SMS1 may deliver (endogenous) SM to nascent lipid rafts in Golgi vesicles [14,15], thus stabilizing newly made or recycling Fas in these microdomains. SMS1/SM-deficiency in $S49^{AR}$ cells would then disable such stabilization of Fas, leading to its degradation or redirected intracellular location [36]. Our brefeldin A data support the idea that in the absence of SM synthesis and anterograde vesicular trafficking, Fas translocation to the cell surface is reduced. Similar observations have been reported for the nicotinic acetylcholine receptor [45].

Lack of Fas stabilization, however, provides only part of the explanation why these SM-deficient, ALP-resistant cells are cross-resistant to FasL. $S49^{AR}$ and $S49^{siSMS1}$ cells showed severely downregulated expression of total Fas, largely already at the mRNA level. How SMS1 deficiency in these cells relates to decreased Fas mRNA remains unknown. The mere SMS1 product, SM seems not involved in this Fas mRNA regulation, as loading of the SMS1-deficient cells with exogenous SM failed to enhance these mRNA levels. This artificial SM supplementation, however, elevated Fas exposure at the $S49^{AR}$ cell surface, as discussed, and hence FasL sensitisation (despite the low levels of total Fas) (Figures 5, 6A). Reduced Fas expression in SMS1-deficient cells was proteasome dependent, because proteasome inhibitors (MG132, lactacysteine) prevented this downregulation. Proteasome inhibition has previously been reported to upregulate molecules implicated in pro-apoptotic cascades, including a Fas-dependent pathway [36,40]. However, proteasome involvement was not seen in Fas expressed in the SMS1-proficient S49 cells, nor in ALP (in)sensitivity. We ruled out the possibility that proteasome inhibition would restore SM synthesis in $S49^{AR}$ cells (data not shown), so that other, yet unknown factor(s) regulating Fas expression are subject to proteasomal degradation in the resistant cells.

SMS1 downregulation is not the only factor that determines the cellular cross-resistance to ALP and FasL. Although SMS1 siRNA-downregulated cells ($S49^{siSMS1}$) were indeed resistant to both ALP and FasL, and long-term spontaneous resensitization (in the absence of ALP; $S49^{ARS}$ cells) was accompanied by regained SM synthesis, re-expression of SMS1 in $S49^{AR}$ cells by retroviral transduction failed to resensitize the cells to these apoptotic stimuli. We therefore conclude that the lack of SM synthesis in $S49^{AR}$ cells is neither a 'clone' effect, nor is it (solely) responsible for the ALP and FasL resistance, but that other factor(s)/protein(s) are co-modulated together with SMS1 in the $S49^{AR}$ and $S49^{siSMS1}$ cells that (co-)determine apoptosis sensitivity in these lymphoma cells. In this regard, we should also note that we found no reduced SM synthesis in other T cells, such as Jurkat and CEM, that we made (partly) ALP-resistant (and also turned out to be partly cross-resistant to FasL) (data not shown). Our results might therefore be specific for the S49 cell system. The reason why ALP resistance in $S49^{AR}$ cells seems so

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uniquely associated with SM deficiency is not clear, but might be related to the fact that SM content in the parental S49 cells is already very low (Figure 2A) compared to other T cells.

In the literature, there is no consensus on the possible role of SMS in apoptosis sensitivity. While SMS1 alone did not determine apoptosis sensitivity in our S49 cell system, overexpression of SMS1 or -2 in CHO cells increased TNFα-induced apoptosis [46]. Conversely, siRNA-induced downregulation of SMS1 or -2 in macrophages reduced lipopolysaccharide-mediated apoptosis [46]. SMS1 was suggested to play a role in Fas-mediated apoptosis in WR19L/Fas mouse lymphoid cells, in which human Fas was artificially expressed [24]. In these cells, however, unlike $S49^{AR}$ cells, SMS1 deficiency was not associated with reduced Fas expression at the cell surface, and SMS1 reintroduction facilitated ligand-induced Fas redistribution from non-raft into raft domains and the subsequent DISC formation (typically type-2 cells). Furthermore, ceramide generation shortly after Fas ligation was suggested to be responsible for an increase in apoptosis induction in SMS1-expressing cells, in line with the concept that small amounts of ceramide generated (by an acid sphingomyelinase) would facilitate clustering of Fas in lipid rafts [47]. However, we (unpublished data in S49 cells) and others [22] have not been able to detect rapid ceramide formation after ALP addition or Fas ligation, as opposed to abundant ceramide formation after hours, in the effector phase of apoptosis [48,49]. Thus, S49 cells differ from WR19L/Fas cells in that SM synthesis relates to Fas expression and stabilization in lipid rafts, rather than to SM-derived ceramide formation in apoptosis induction.

Contrary to the above, there are also reports where SMS expression seems to play an anti-apoptotic role: SMS1 was a suppressor of Bax-mediated cell death in yeast [50]. Further, SMS1 overexpression protected Jurkat cells from photodamage-induced apoptosis [51], whereas SMS1 knockdown sensitized the cells [52]. Finally, SMS1/2 overexpression was also somewhat protective against apoptosis induction in oligodendrocytes [53].

Unlike current belief [15,18,19], the SM deficiency in $S49^{AR}$ cells did not affect cell growth, at least not in normal, serum containing medium. The cells showed normal phospho- and glycolipid profiles, except for decreased SM, which was essentially taken up from the serum. In serum-free (SM-free) medium, growth of $S49^{AR}$ cells was retarded but not completely growth arrested.

In conclusion, we found that, in S49 cells, ALP induces apoptosis independent of Fas engagement and that the ALP-resistant $S49^{AR}$ cells are cross-resistant to Fas ligation. We find that the lack of SM synthesis in these resistant cells, although leading to destabilization and reduction of Fas at the cell surface, is not the only factor that causes resistance. Proteasome-dependent reduction of Fas mRNA and protein levels is a major determinant in the resistance to FasL. How this relates to the acquired ALP resistance and SM deficiency in these cells needs to be resolved in future studies.

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LEGENDS TO FIGURES

Figure 1. ALP-resistant S49^{AR} cells are cross-resistant to Fas ligation, but ALP does not induce Fas signaling. (**A**) ALP-sensitive S49 cells (closed bars) and ALP-resistant S49^{AR} cells (open bars) were left untreated (contr) or were treated with ALP (edelfosine; 15 μM) or FasL (500 ng/ml) for 6 hours. Apoptotic nuclear fragmentation was measured by FACScan analysis (see Materials and Methods). Data are means of eight experiments ± SD. (**B**) ALP-induced apoptosis in S49 cells is not inhibited by expression of FADDdn (grey bars), while this FADDdn inhibits Fas-induced apoptosis. Unlike cells in panel **A,** cells in panel **B** were retrovirally transduced with the GFP vector (see Materials and Methods) and were treated with a suboptimal dose (5 μM) of ALP (where indicated) to enable possible visualization of synergism. Data are means of eight experiments ± SD. (**C**) Methyl-β-cyclodextrin (MβCD, 1 mg/ml, preincubation for 30 min) inhibits induction of apoptosis in S49 cells by ALP (15 μM) but not by FasL (500 ng/ml). Cells were stimulated for 4 h, after which apoptosis was determined by nuclear fragmentation. (**D**) ALP-induced apoptosis in S49 cells is not inhibited by retroviral transduction of of c-FLIP$_L$ (S49^{FLIP} cells; grey bars) or by the caspase 8 inhibitor z-IETD-fmk (10 μM; 30 min preincubation), while these treatments lead to inhibition of FasL-induced apoptosis (measured at 6 h). Data in panels C and D are means of three experiments ± SD.

Figure 2. Reduced SM mass in ALP-resistant S49^{AR} cells and SMS1-downregulated S49^{siSMS1} cells; effect on cell growth. (**A**) Individual sphingomyelins, glucosylceramides and ceramides in lipid extracts from cells (left panel) and from the fetal calf serum in the culture medium (right panel) were assayed by liquid chromatography, electrospray ionization tandem MS [34]. Sphingolipid patterns of S49 cells transduced with scrambled siRNA (S49^{mock}, not shown) [13] were identical to parental S49 cells. Data are means of triplicates ± SD. SMS-deficient cells appear to have taken up some SM (predominantly of C16:0 acyl chain length) from the culture medium. Length and (un)saturation of N-acyl groups in the sphingolipid species are indicated. C16DH, sphingolipid containing an N-palmitoyl-dihydrosphingosine moiety. C20:0, C22:0, C26:0 and C26:1 were also present, but in minor amounts (for cells, less than 1.7, 3.4 and 3.8 pmol/cell x 10^6 for SM, GlcCer and ceramide, respectively; for serum, less than 60, 0.5 and 4.4 pmol/mg protein for SM, GlcCer and ceramide, respectively; data not shown). (**B**) Proliferation of S49 and SMS-deficient S49^{AR} cells in serum-containing and serum- (SM-) free medium [31]. The number of cells were counted during 3-4 consecutive days.

Figure 3. Apoptosis-resistance (S49^{AR} and S49^{siSMS1} cells) and time-dependent spontaneous resensitization (S49^{ARS} cells) correlate with down-/upregulated SM synthesis; however SMS1 re-expression in S49^{AR} (AR-SMS1 cells) fails to resensitize cells. (**A**) Lack of SM synthesis in SMS1-deficient S49^{AR} and S49^{siSMS1} cells in comparison with parental S49 cells and S49^{AR} cells in which HA-SMS1 was transduced (AR-SMS1 cells). Cells were labeled with [^{3}H]1-sphingosine for 4 h. Lipids were extracted and separated by TLC (see "Materials and methods"). The location of SM and other sphingolipids, ceramide (Cer), glucosylceramide (GlcCer) and lactosylceramide (LacCer) is indicated. Phosphatidylethanolamine (PE, indicated) is a catabolic end-product of sphingosine degradation. So, residual sphingosine. (**B**) S49 cells (closed bars),

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SMS1-downregulated cells ($S49^{siSMS1}$; open bars), ALP-resistant, SMS1-deficient $S49^{AR}$ cells (gray bars) and AR-SMS1 cells (hatched bars) were left untreated (control) or were treated with ALP (edelfosine; 15 μM) or FasL (500 ng/ml) for 6 hours. Apoptotic nuclear fragmentation was measured by FACScan analysis. Data are means of six experiments ± SD. Percentages of apoptosis induced in S49 cells that were transduced with scrambled siRNA ($S49^{mock}$, not shown) [13] were identical to parental S49 cells. Right: Immmunoblot showing the expression of HA-tagged SMS1 (+; arrow head) in AR-SMS1 cells, compared to empty vector-transduced $S49^{AR}$ cells (-), using anti-HA antibody. (**C**) Prolonged culturing of $S49^{AR}$ cells in the absence of ALP results after 4 weeks in spontaneous resensitization of the cells ($S49^{ARS}$ cells) towards ALP and FasL and in regained SM synthesis. Assays were done in triplicate ± S.D. as described above.

Figure 4. Fas deficiency in $S49^{AR}$ and $S49^{siSMS1}$ cells. (**A**) Cells were fixed and Fas is visualized by confocal microscopy, using Jo2 monoclonal antibody and secondary FITC-labeled antibody staining. (**B**) Cell surface expression of Fas analysed by flow cytometry using Jo2 antibody (against mouse Fas) conjugated to FITC (Jo2-FITC). Surface expression of CD90/Thy-1 (lower panel, as a control) was measured using FITC-conjugated anti-Thy-1 monoclonal antibody. Shown are the fluorescence distributions of S49 cells (black), $S49^{AR}$ cells (dark grey), $S49^{siSMS1}$ cells (light grey), and control cells (no antibody; open curves). Flow-cytometric traces of Fas in S49 cells that were transduced with scrambled siRNA ($S49^{mock}$) completely overlapped those of parental S49 cells. (**C**) Fas protein expression in lysates from indicated cells, determined by Western blotting using anti-Fas antibodies. Western blot of β-actin served as loading control (**D**) Fas associates to a lipid raft fraction from parental S49 cells or mock-transduced $S49^{mock}$ cells but not (or less) from SMS1-deficient $S49^{AR}$ and $S49^{siSMS1}$ cells. Lipid rafts were isolated by Triton X-100/sucrose density gradient fractionation and pooled as shown in Supplementary figure 3. Fas was immunoprecipitated using Jo2 antibody and visualized by Western blotting using 7C10 antibody. R, lipid raft fractions pooled; NR, non-raft fractions pooled (equal amounts of protein used for i.p.); h.c., immunoglobulin heavy chain.

Figure 5. $S49^{AR}$ and $S49^{siSMS1}$ cells show reduced Fas mRNA levels. Loading of these SM-deficient cells with exogenous C12-SM partly restores FasL sensitivity and Fas expression in lipid rafts. (**A**) Real-time PCR analysis of Fas in S49, $S49^{AR}$, $S49^{siSMS1}$ and $S49^{mock}$ cells in untreated (black bars) and SM-treated cells (hatched bars). Gene transcription is expressed as percentage of that in untreated S49 cells. Loading of cells with exogenous SM (C12-SM, 20 μM, in complete culture medium) was for 3 days. Inset: Appearance of the SM spot on TLC in lipid extract from C12-SM-loaded $S49^{AR}$ cells, as detetected by charring of the TLC plate. (**B**) Restoration of FasL apoptosis sensitivity (but not ALP sensitivity) in $S49^{AR}$ cells after loading of C12-SM. S49 cells and $S49^{AR}$ cells, preloaded with C12-SM-loaded where indicated, were left untreated (open bars, controls) or were treated with ALP (edelfosine; 15 μM; hatched bars) or FasL (200 ng/ml; grey bars; or 500 ng/ml; black bars)) for 6 hours. Apoptotic nuclear fragmentation was measured by FACScan analysis. Data are means of seven experiments ± SD. (**C**) Western blots showing Fas protein expression in cell lysates and (pooled) lipid raft fractions of $S49^{AR}$ cells without or after C12-SM loading (indicated). Western blot of

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β-actin (indicated) or Ponceau-S red staining (not shown) served as equal loading control of cell lysates and rafts, respectively.

Figure 6. Exogenous C12-SM enhances Fas surface expression in S49^{AR} cells, whereas brefeldin A blocks appearance of SM and Fas at the S49 cell surface. (**A**, **B**) Cell surface expression of Fas was analysed by flow cytometry using Jo2-FITC antibody. FACS curves of Fas fluorescence in untreated S49 and S49^{AR} control cells are depicted in black and dark grey, respectively. Open curves represent cells without antibody. **(A)** S49^{AR} cells were loaded with C12-SM for 3 days (as in Fig. 5) (light grey) (**B**) S49 cells were treated with brefeldin A (BFA, 5 μM, for 1h); light grey). (C) BFA (5 μM) inhibits SM synthesis in the Golgi, while bacterial sphingomyelinase (bSMase; 300 mU) hydrolyses SM in the outer leaflet of the plasma membrane. S49 cells were labeled in time with [*methyl*-^{14}C]choline. In parallel incubation samples, BFA or bSMase were added at 4 hours (indicated by arrow). Lipids were extracted and separated by TLC. Positions of (lyso)phosphatidylcholine (LPC and PC), and SM are indicated.

Figure 7. Proteasome inhibition partially restores Fas expression and FasL sensitivity in S49^{AR} and S49^{siSMS1} cells. (**A**) Equal numbers of cells were treated with the proteasome inhibitor MG132 (10 μM) for the times indicated, and Fas protein content was determined by Western blotting, β-actin serving as loading control. (**B**) MG132 treatment of S49^{AR} cells (left panel; squares) and S49^{siSMS1} cells (right panel; triangles) leads to resensitization to FasL but not to ALP. S49 cells (circles), S49^{mock} cells (diamonds), S49^{AR} cells (squares) and S49^{siSMS1} cells (triangles) were pretreated with MG132 (10 μM) for the times indicated and were subsequently left unstimulated (open symbols; controls) or were stimulated for 4 h with FasL (500 ng/ml) (black symbols) or ALP (15 μM) (grey symbols) in the continuous presence of MG132 (10 μM). The first data points on the X-axis (-) represent control samples without MG132. Apoptotic nuclear fragmentation was measured by FACScan analysis. Data are means of three experiments ± SD. No error bar means SD within the size of the symbol.

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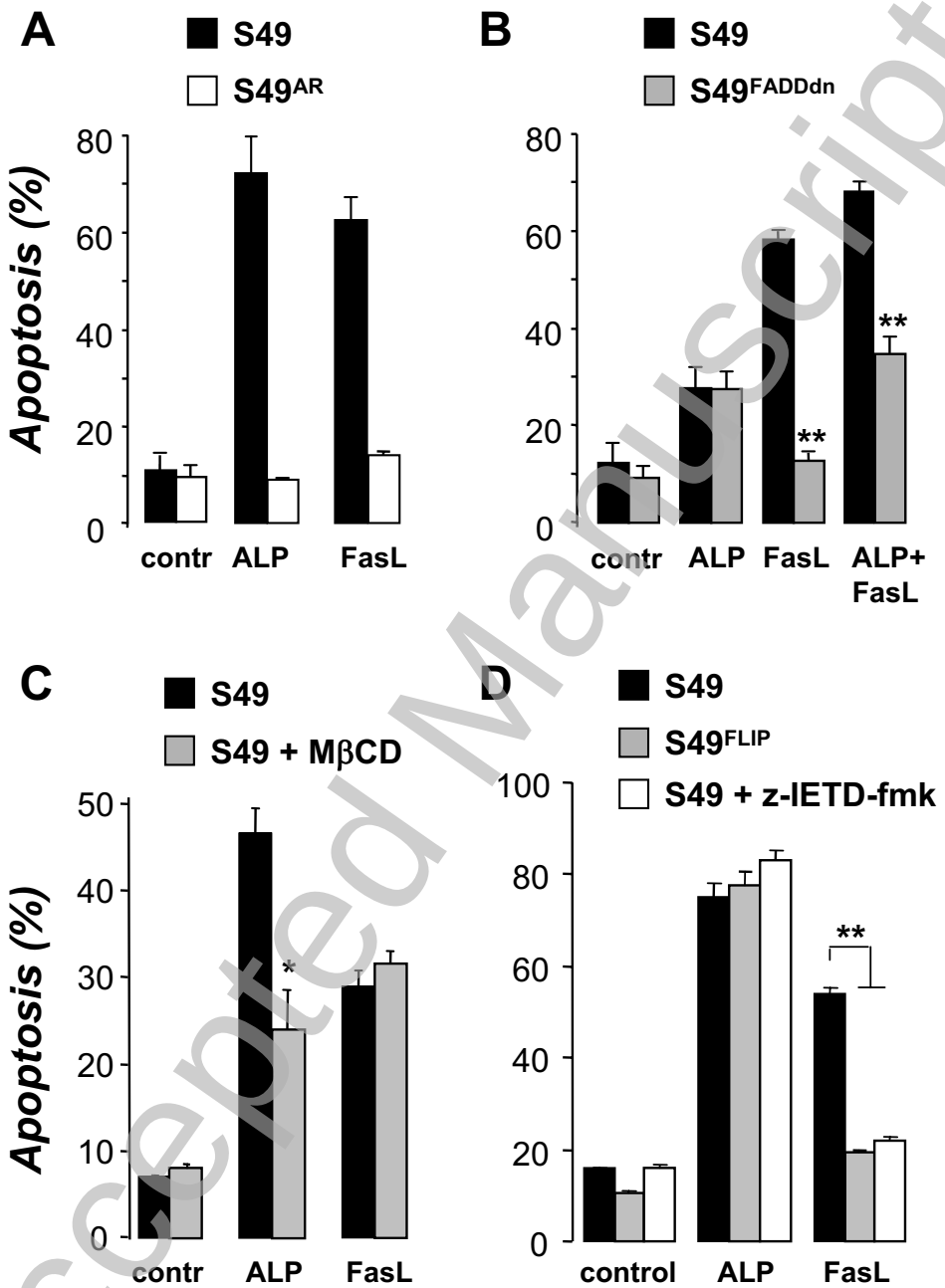

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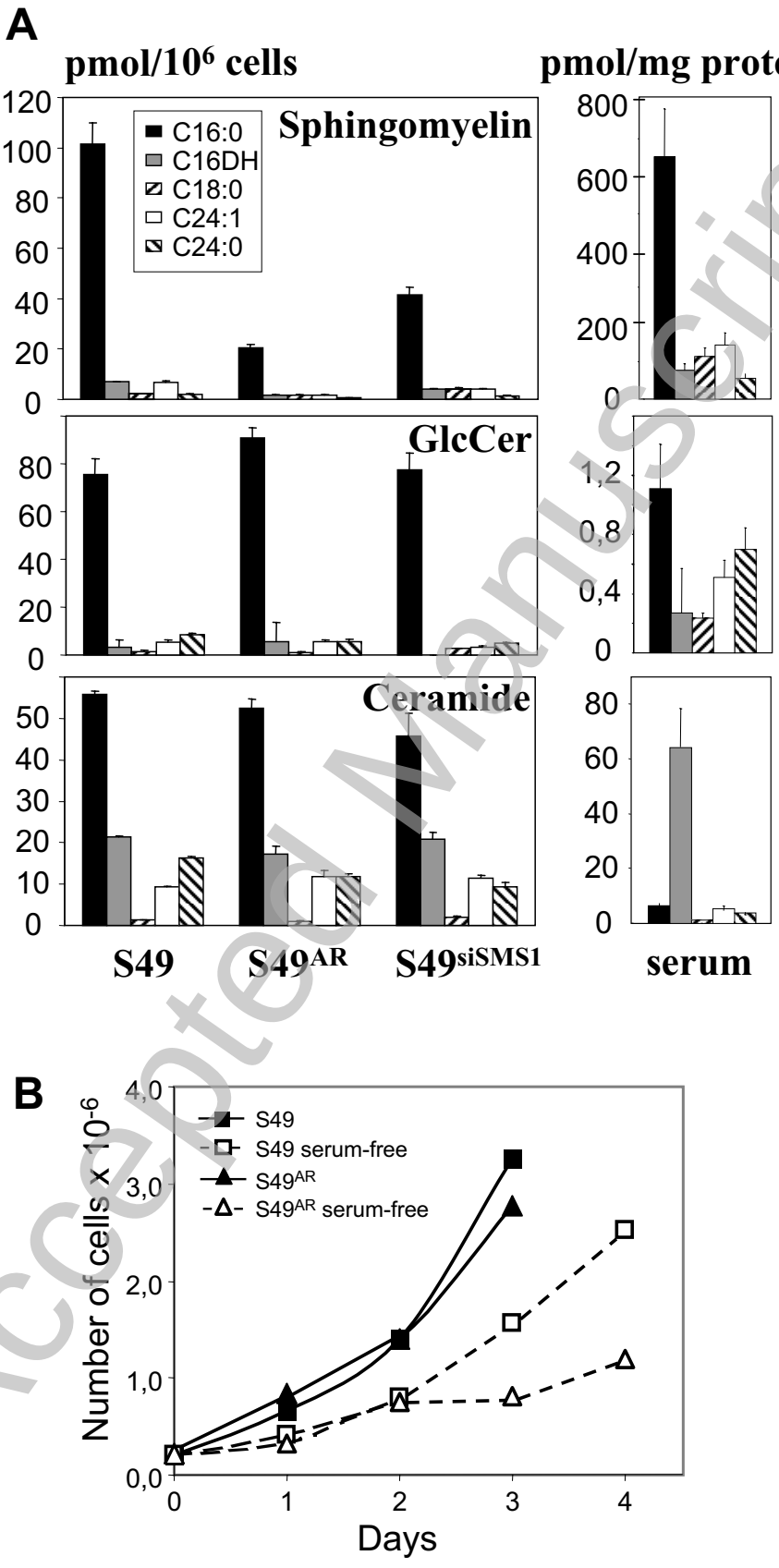

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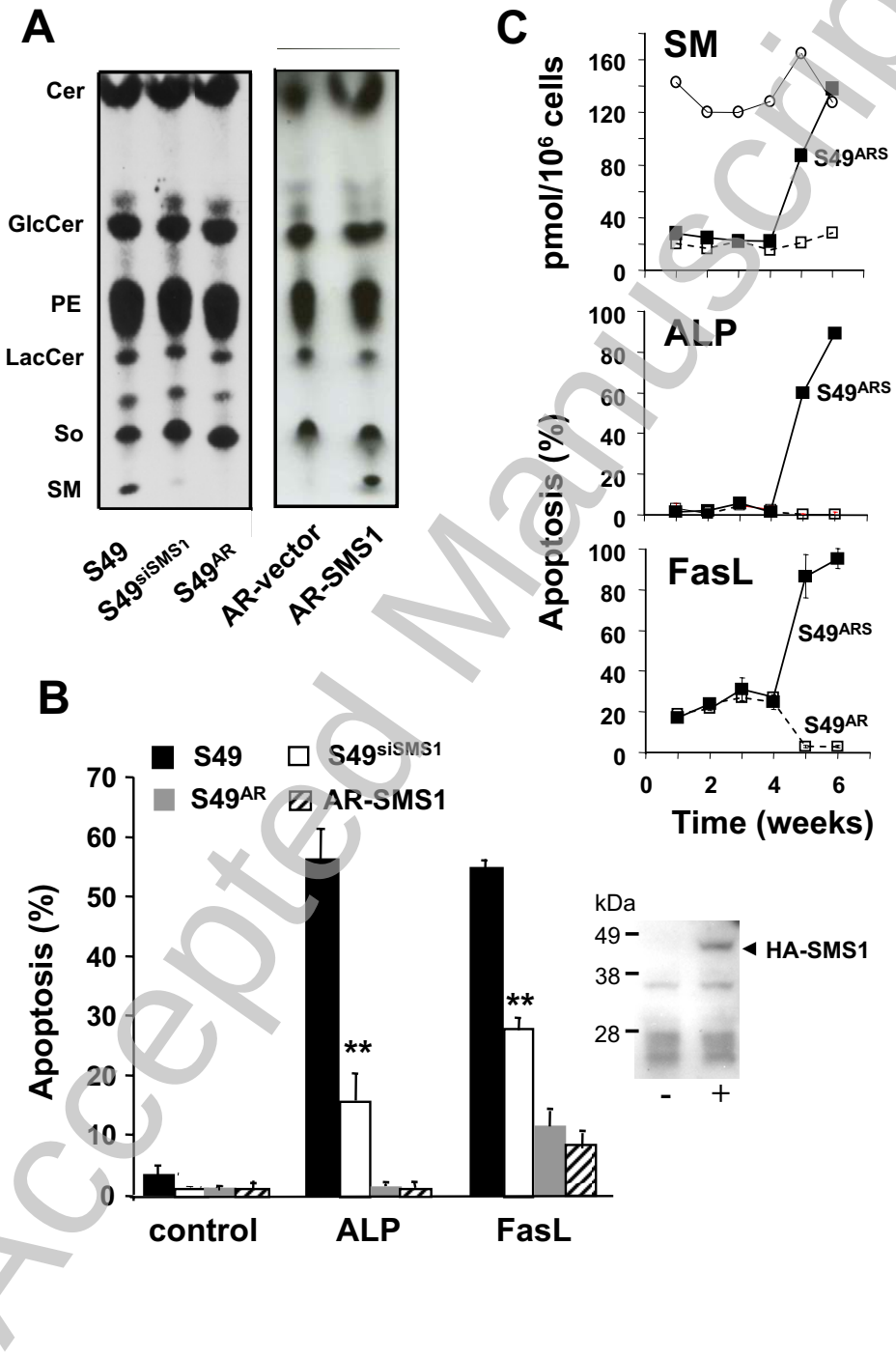

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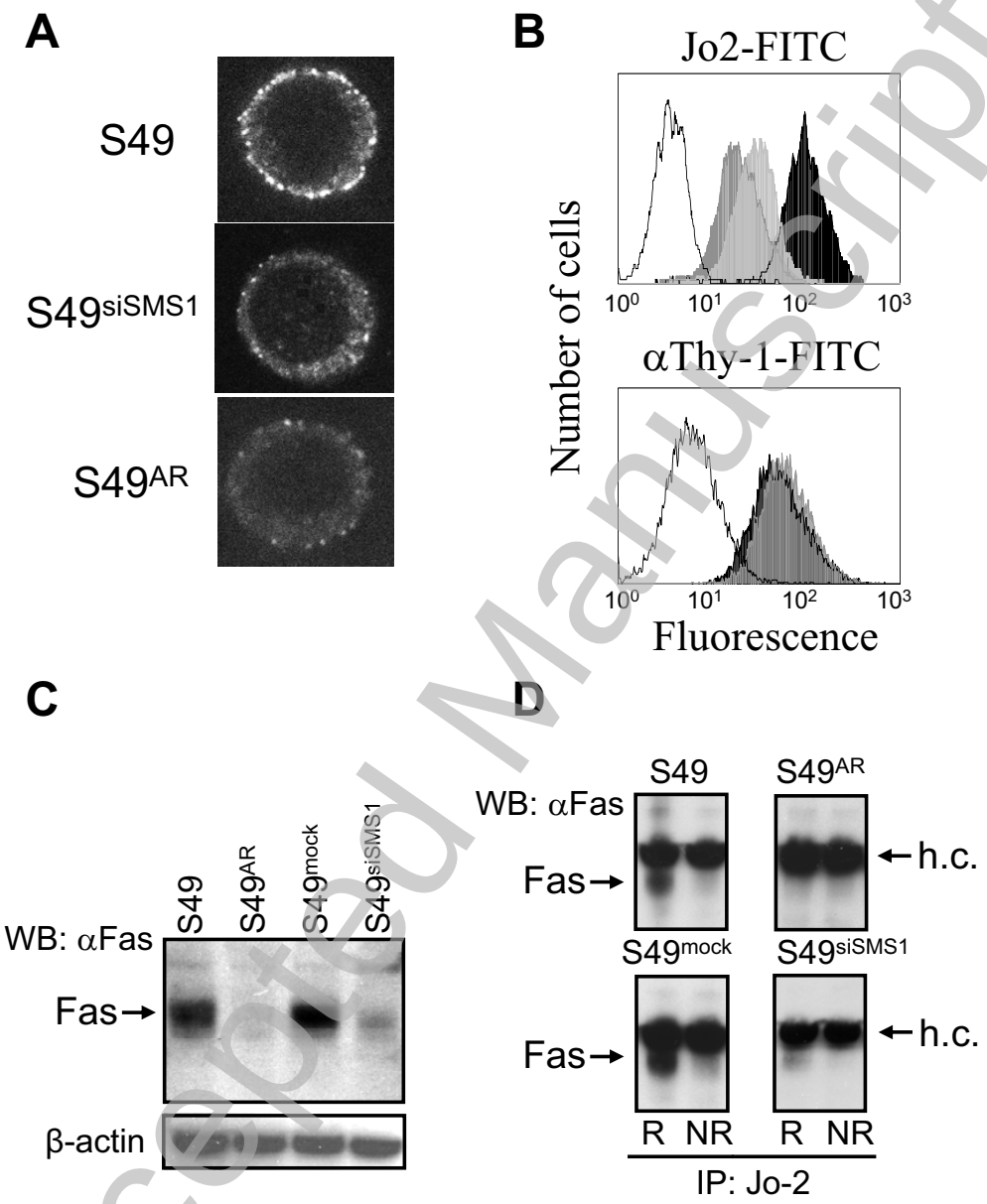

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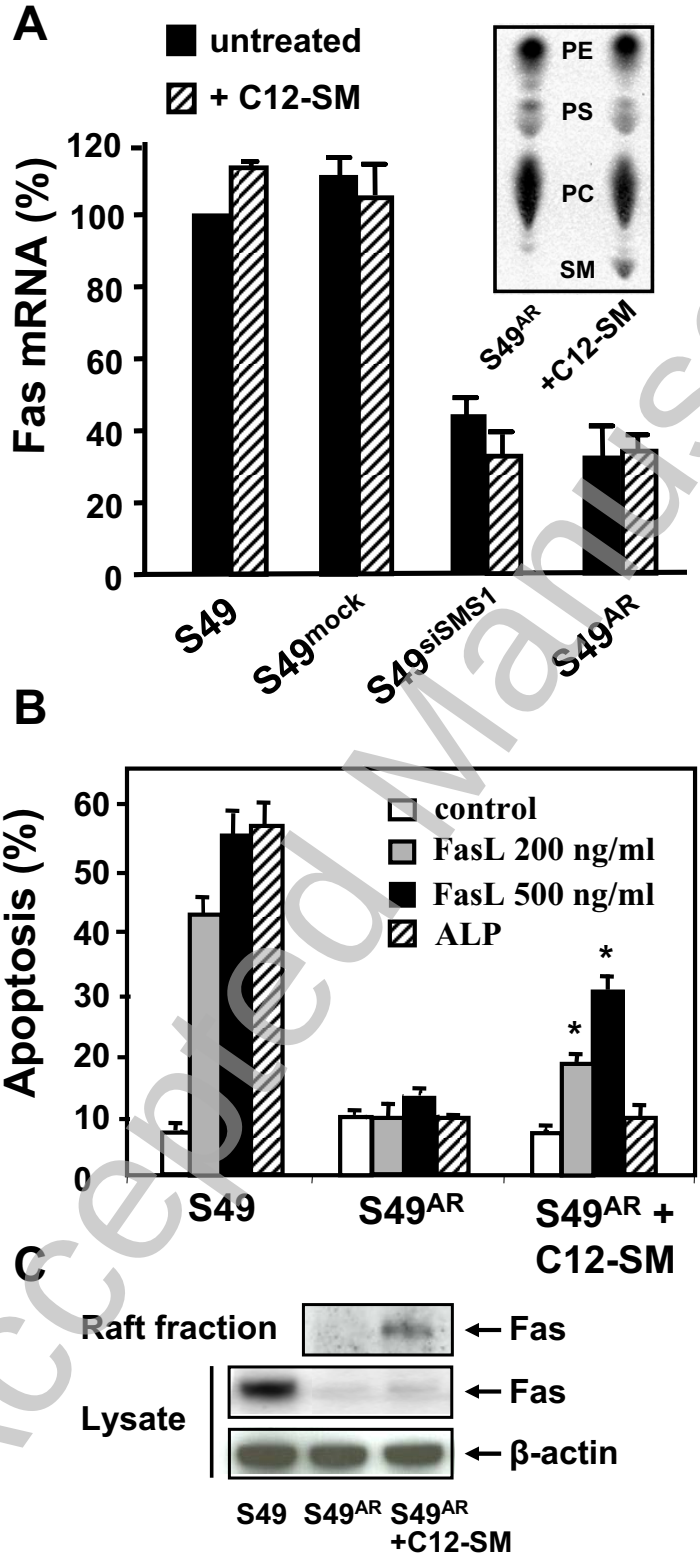

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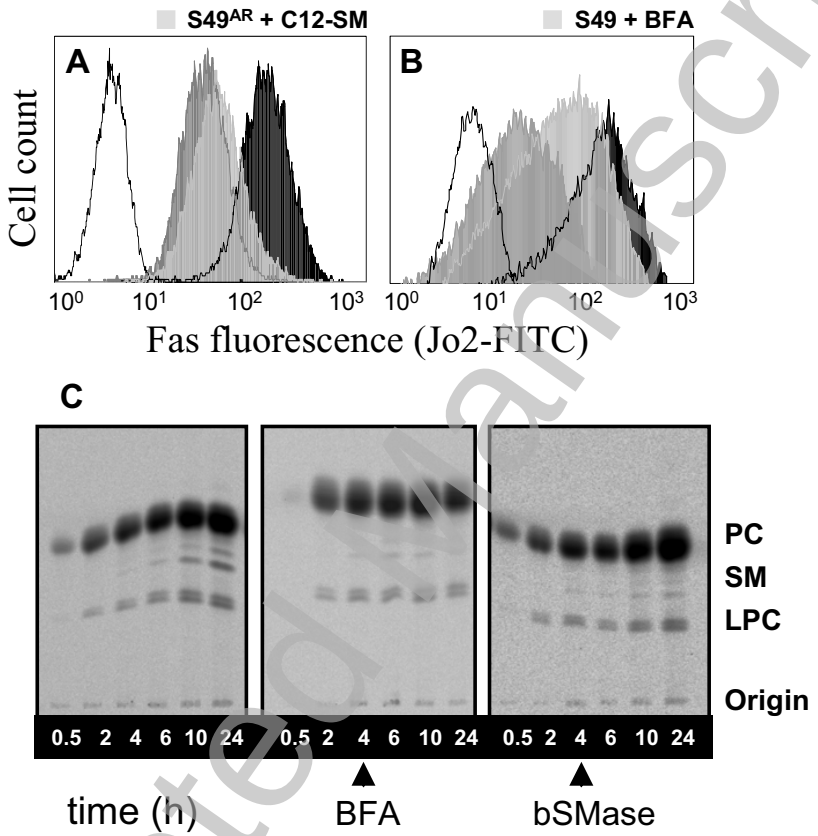

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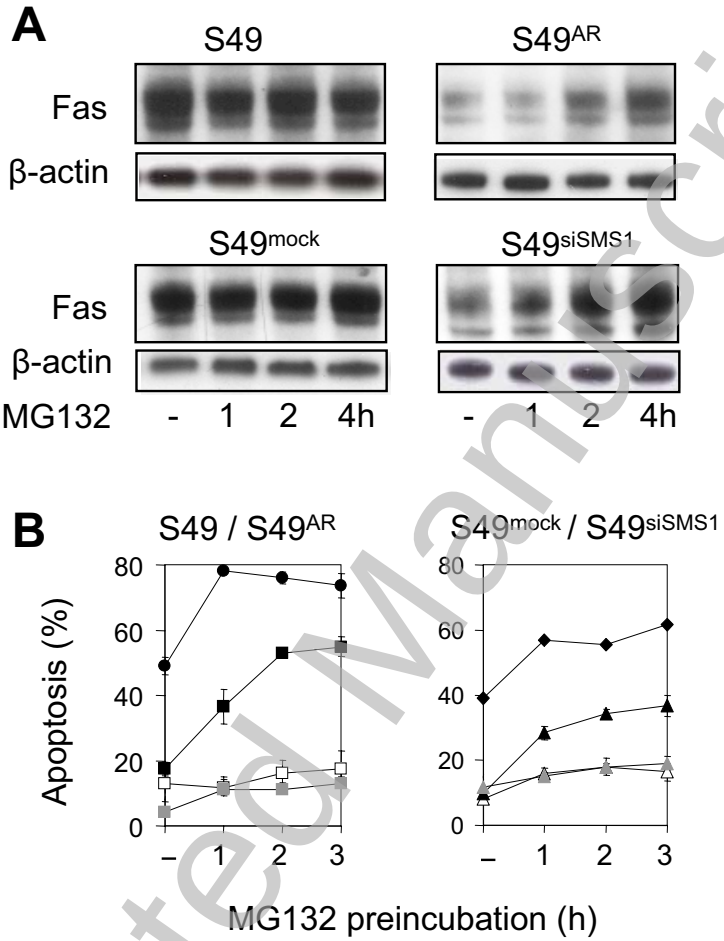

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