

Apoptotic body engulfment by hepatic stellate cells promotes their survival by the JAK/STAT and Akt/NF- κ B-dependent pathways[☆]

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Background/Aims: We have previously shown that phagocytosis of apoptotic bodies (AB) by hepatic stellate cells (HSC) is profibrogenic. As HSC survival is central to the progression of liver fibrosis, our goal was to investigate if phagocytosis induces HSC survival.

Methods: Apoptosis of phagocytosing HSC was studied in the presence of known apoptotic agents. The JAK/STAT- and PI3K/Akt-dependent pathways, NF- κ B activation and expression of the anti-apoptotic proteins Mcl-1 and A1 were evaluated. Apoptosis was assessed after blocking A1 by an siRNA approach.

Results: Phagocytosing HSC were resistant to FasL/cycloheximide or TRAIL-induced apoptosis. Inhibition of the JAK/STAT or PI3K-mediated pathways induced apoptosis of HSC. Phagocytosis induced JAK1/STAT3 phosphorylation, and this was prevented by inhibiting JAK. Translocation of STAT3 to the nucleus was also blocked by JAK inhibition. Mcl-1 expression was upregulated in a JAK-dependent manner. PI3K-dependent phosphorylation of Akt depended on NADPH oxidase activity and superoxide production. NF- κ B activation and subsequent upregulation of A1 was observed, and A1 inhibition induced apoptosis of HSC.

Conclusion: Phagocytosis of AB promotes HSC survival by two pathways, of which the A1 dependent is more significant. This represents a new mechanism by which engulfment of AB contributes to the propagation of liver fibrosis.

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Keywords: Apoptosis; Phagocytosis; NADPH oxidase; Oxidative radicals; Liver fibrogenesis

1. Introduction

Hepatic stellate cells (HSC) play a pivotal role in liver fibrogenesis [1]. Upon induction by fibrogenic stimuli, HSC undergo activation in which they transdifferentiate from a quiescent phenotype to myofibroblasts with production of type I collagen $\alpha 1$ [1]. Survival of HSC is a hallmark of liver fibrosis, and the induction of HSC apoptosis was shown to induce recovery from fibrosis [2–4]. Activated human HSC are resistant to many proapoptotic stimuli, such as serum deprivation, Fas-ligand (FasL), and the main survival signal is the overexpression of Bcl-2 family members [5,6]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) however, was shown to induce apoptosis of activated HSC *via* upregulation of TRAIL-R2/DR5 expression [4]. Accumulating evidence

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Abbreviations: HSC, hepatic stellate cells; TGF- $\beta 1$, transforming growth factor- $\beta 1$; AB, apoptotic bodies; ASMA, α -smooth muscle actin; NADPH oxidase, nicotinamide adenine dinucleotide phosphate reduced oxidase; TAMRA, carboxytetramethyl rhodamine succinimidyl ester.

suggests that reactive oxidative species (ROS) are not only pathological but in many instances they act as second messengers in cell survival pathways [7,8]. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) are activated during oxidative stress [7,9–11]. Upon phosphorylation, STATs translocate to the nucleus and bind DNA [12]. STAT3 phosphorylation is strongly linked to cell survival due to the induction of Bcl-xL [13] and myeloid cell leukemia-1 protein (Mcl-1) [14]. We have recently shown that phagocytosis of apoptotic bodies (AB) by HSC activates nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase, with resulting upregulation of procollagen $\alpha 1(I)$ [15]. Superoxide and hydrogen peroxide (H_2O_2) are known to induce Akt-dependent survival in different systems [16,17]. Because phosphoinositide-3 kinase (PI3K) and Akt are known to regulate nuclear factor κB (NF- κB) activity, it is tempting to speculate that NADPH oxidase would activate NF- κB -dependent survival signals. NF- κB activity is central to the expression of anti-apoptotic proteins, including the Bcl-2 family protein A1 in HSC [4].

Here we report that phagocytosis of AB induces different survival pathways in HSC leading to the propagation of myofibroblasts in the liver. Following the engulfment of AB, an NF- κB -dependent upregulation of the anti apoptotic protein A1 and a STAT3-mediated induction of Mcl-1 are demonstrated.

2. Materials and methods

2.1. Cell culture

Immortalized human HSC line, LX-2 (kindly provided by SL Friedman MD, Mt-Sinai Medical School, NY) and primary rat HSC were used. Rat HSC isolation was performed according to Geerts et al. [18], and the purity was always >95% as assessed by vitamin A fluorescence. Primary HSC were maintained in Medium 199 (Sigma–Aldrich, St. Louis, MO) with 20% FBS. LX-2 cells were cultured in DMEM (Invitrogen) with 5% FBS. Before the experiments the medium was changed to serum-free.

2.2. Generation of apoptotic bodies

To generate carboxytetramethyl rhodamine succinimidyl ester (TAMRA, Invitrogen, Carlsbad, CA) labeled AB, HepG2 cells were incubated with TAMRA (10 μM), then exposed to UV irradiation (100 mJ/cm², 142 s) as described previously [19]. Labeled AB were added to primary HSC or LX-2 cells. Phagocytosis of AB was detected by fluorescence and phase microscopy [15,19].

2.3. Apoptosis experiments

Primary HSC were incubated with AB for 24 h then incubated further in the presence or absence of FasL (5 ng/ml, EMD Chemicals, San Diego, CA), and/or cycloheximide (10 $\mu g/ml$, Sigma–Aldrich) for 18 h, JAK inhibitor, AG490 (50 μM , 1 h, EMD Chemicals), a selective PI3K inhibitor, LY294002 (600 nM, 1 h, EMD Chemicals). For the experiments involving TRAIL, primary HSC were culture-activated for 7 days then exposed to 500 ng/ml recombinant TRAIL (R&D Systems, Minneapolis, MN) overnight in the presence or absence of AB. Active caspase-3 was detected by an *in situ* labeling method using an antibody

to active caspase-3 (FITC/PE detection kit, Cell Technology, Mountain View, CA). Nuclei were labeled with DAPI (Vector Laboratories, Burlingame, CA). Apoptosis was assessed by the characteristic nuclear apoptotic changes (DAPI) and by the presence of active caspase-3. Biochemical assays to assess caspase activity were not done as HSC with engulfed AB would have given false positive signal. Five hundred cells in 3 different fields in each of 3 different experiments were counted.

2.4. Cytochrome *c* reduction assay

Primary HSC were plated and exposed to AB. The supernatant was collected 2 h after exposure to AB. Cytochrome *c* (160 μM) (Sigma–Aldrich, St. Louis, MO) was added in the presence (100 U/ml) or absence of superoxide dismutase (SOD) (Sigma–Aldrich, St. Louis, MO), and incubated for 20 min at 37 °C. The values of superoxide-dependent reduction of cytochrome *c* were obtained by subtracting the absorbance readings at 550 nm of the samples without SOD from those with SOD. The final result was reported as fold changes above the control values [20].

2.5. Western blot analysis

To detect JAK/STAT and Akt phosphorylation, LX-2 cells were incubated in serum free medium for 16 h then exposed to AB with or without inhibitors, AG490 (50 μM), LY294002 (20 μM), or apocynin (100 μM). Cells were then collected at different time points. The protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and 20 μg of protein was separated by SDS–PAGE. The blots were incubated with the appropriate antibodies for 16 h at 4 °C: anti-phospho-JAK1 polyclonal antibody (1:500, EMD-Calbiochem), anti-phospho-STAT3 monoclonal antibody (1:250, Cell Signaling Technology, Danvers, MA), anti-JAK1 and anti-STAT3 polyclonal antibodies (1:300, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho Akt and anti-Akt polyclonal antibodies (1:1000, Cell Signaling Technology), and anti-GAPDH polyclonal antibody (1:3000, Trevigen, Gaithersburg, MD). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the blots were developed by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). To detect Mcl-1 and A1 expression, primary HSC were exposed to AB in the presence or absence of AG490 (50 μM), LY294002 (600 nM), apocynin (100 μM) or CAPE (1 ng/ml). Western blot analysis was performed as above using Mcl-1 antibody (1:1000, Millipore Billerica, MA), A1 polyclonal antibody (1:300, Biovision, Mountain View, CA). The data were normalized to the expression of β -actin (1:1000, Santa Cruz Biotechnology).

2.6. cDNA transfection

pCMV6-XL6-SOCS3 (Origene, Rockville, MD), and the control vector were transfected into LX-2 cells (transfection efficiency 60–70%), using the Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation.

2.7. RNA interference

siRNA to A1 (Ambion Austin, TX), and negative control labeled (Alexa Fluor 488) or unlabeled siRNA (Qiagen, Valencia, CA) were transfected into primary HSC with 30 nM siRNA in RiboJuice™ (Novagen, Madison, WI), according to the manufacturer's instructions. Scrambled siRNA in the same concentration was used as control. The cells were exposed to AB in the presence or absence of FasL and cycloheximide, as above and apoptosis was assessed.

2.8. STAT3 nuclear translocation assay

Primary HSC were incubated with TAMRA-labeled AB in the presence or absence of AG490 (50 μM) for 3 h, then fixed with 1% paraformaldehyde. After washes, the cells were incubated in methanol

at 20° for 2 min then washed, incubated in 10% FBS/PBS for 1 h at 37 °C, and stained with the anti-STAT3 antibody (1:200, Santa Cruz Biotechnology). An FITC-conjugated secondary antibody (1:2000, Invitrogen) was applied, and the nuclei were stained with DAPI. The results were analyzed by fluorescence microscopy.

2.9. Electrophoretic mobility shift assay (EMSA)

LX-2 cells were serum-starved and incubated with AB for 2 h then nuclear extracts were prepared using a cytosolic/nuclear extraction kit (Pierce Biotechnology). The NF- κ B nuclear translocation was determined using Biotin 3' DNA labeling kit and lightshift chemiluminescent EMSA kit (Pierce Biotechnology). The NF- κ B consensus oligonucleotide (5'-AGTTGAGGGGACTTCCAGGC-3') was end-labeled with biotin then the nuclear extract was mixed with the biotin-labeled NF- κ B oligonucleotide. The lysate was subjected to electrophoresis then transferred to a nylon membrane. The membrane was exposed to the chemiluminescent substrate, and the resulting chemiluminescence was detected by autoradiography.

2.10. Statistical analysis

All data represent at least three experiments and expressed as means \pm SD. Differences between groups were compared using one-way analysis of Variance (ANOVA) associated with the Dunnett's test. Statistical significance was assumed when $p < 0.05$.

3. Results

3.1. Primary HSC engulfing apoptotic bodies are protected against apoptotic stimuli

To determine if phagocytosis is protective against known proapoptotic stimuli, primary HSC were exposed to AB in the presence or absence of cycloheximide and Fas ligand or TRAIL. The treatment including cycloheximide, blocking protein synthesis is known to induce apoptosis of primary HSC while FasL alone is less effective [21]. Apoptosis was detected by active caspase-3/DAPI labeling. Cycloheximide and Fas ligand treatment induced apoptosis of HSC, while in phagocytosing HSC this was reduced by 31% compared to cells not exposed to AB (Fig. 1A). As TRAIL induces apoptosis of activated HSC, primary HSC were culture-activated and exposed to TRAIL in the presence or absence of AB. TRAIL induced a significant apoptosis of HSC while phagocytosis of AB decreased this by 46%. These data suggest that phagocytosis of AB has a protective, anti-apoptotic effect on HSC.

3.2. Inhibition of JAK1/STAT3 and PI3K induces apoptosis of phagocytosing HSC

As ROS production has been linked to the activation of the JAK/STAT and PI3K-dependent survival pathways [7,9,22,23], next we studied these pathways in phagocytosing HSC. Primary HSC 2 days after isolation were exposed to TAMRA-labeled AB in the presence or absence of the JAK inhibitor AG490 (50 μ M) or the

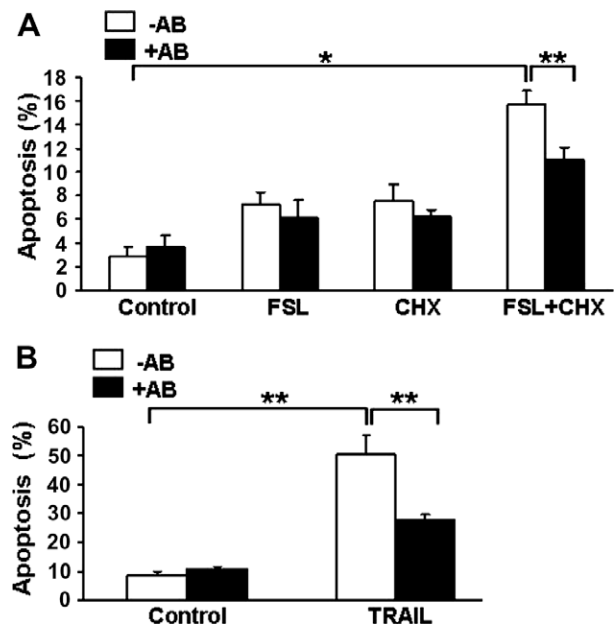


Fig. 1. Phagocytosis of apoptotic bodies induces HSC survival. (A) Exposure to AB did not change the apoptotic rate of primary HSC ($3.7\% \pm 0.9$) compared to control ($2.9\% \pm 0.2$). FasL (FSL) combined with cycloheximide (CHX) induced a $15.8\% (\pm 1.1)$ increase in HSC apoptosis. This was decreased to $11\% (\pm 1.0)$ in phagocytosing HSC $N=5$, $p < 0.001$, $**p < 0.05$. (B) TRAIL-induced apoptosis of primary, culture activated HSC ($50.6\% \pm 6.1$) was significantly decreased in phagocytosing HSC ($27.6\% \pm 1.8$). Apoptosis was based on the detection of active caspase-3/DAPI staining. Data are normalized to control, set as 1. $N = 4$, $**p < 0.05$.

PI3K inhibitor LY294002 (600 nM), or both to assess if these pathways play a role. Immunohistochemistry detecting the active caspase-3 subunit and DAPI staining were performed, and the rate of apoptosis quantified. Biochemical assessment of apoptosis was not done as the lysis of the cells with intracellular AB would have resulted in a false-positive signal. In parallel, propidium iodide (PI) staining was performed as well, to detect cellular necrosis. In both the control group and the AB treated group, the rate of apoptosis was low (Fig. 2A a, b) however, it increased significantly in the AG490 plus AB-treated group (Fig. 2A c). Combining the inhibitors of both pathways, we detected further increase in apoptosis of HSC, as shown by the number of active caspase-3 positive cells with the characteristic nuclear condensation/fragmentation (Fig. 2A d, overlay image). As illustrated in Fig. 2B, AG490 and LY294002 treatment with these conditions induced apoptosis which was slightly decreased by phagocytosis. Combining JAK and PI3K inhibition, the rate of apoptosis rose further ($78.3\% \pm 3.1$), and there was some reduction in phagocytosing cells, however, the apoptotic rate still remained significant. The percentage of PI positive cells was lower than 0.5% among all groups (data not shown). These data indicate that in phagocytosing HSC cell survival mediated by a JAK/STAT and PI3K-dependent pathway.

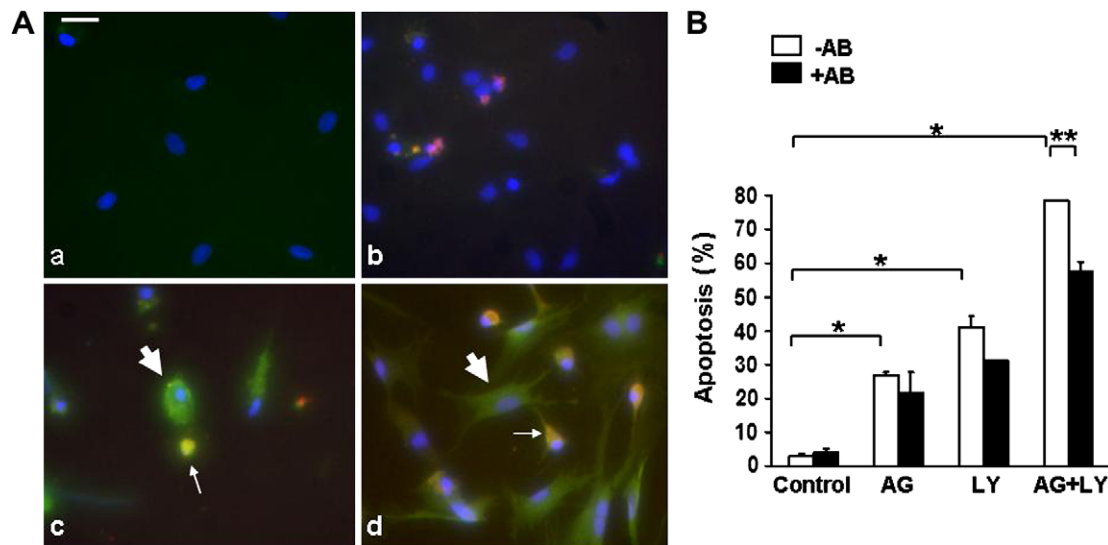


Fig. 2. Blocking the JAK/STAT and/or PI3K pathways induces apoptosis of phagocytosing HSC. (A) DAPI (blue) nuclear staining and immunofluorescence to detect active caspase-3 (green) were performed on rat primary HSC treated for 24 h with TAMRA-labeled AB (red) in the presence or absence of AG490 (AG) and/or PI3K inhibitor LY294002 (LY). DMSO treated cells served as control. (a) control HSC were only DAPI-positive. (b) In HSC exposed to AB, no active caspase-3 signal is detectable. (c) AG490-treated phagocytosing HSC are positive for active caspase-3, indicating higher apoptotic rate. (d) In phagocytosing HSC exposed to both AG490 and LY294002 the apoptosis was further induced. Arrows point to AB where active caspase-3 (green), DAPI (blue), and TAMRA (red) co-localize. Arrowhead: caspase-3 positive HSC. Bar: 10 μ m. (B) AB exposure did not change the apoptotic rate ($4.0\% \pm 0.9$) compared to control ($2.9\% \pm 0.8$). AG490 induced $26.9\% (\pm 1.4)$ of apoptosis and LY294002 induced $40.8\% (\pm 1.0)$ of apoptosis in HSC which was slightly decreased by phagocytosis (not statistically significant). Combined inhibition of both pathways induced $78.3\% (\pm 3.1)$ apoptosis, and this somewhat decreased by phagocytosis of AB, however, the apoptotic rate remained significant ($57.4\% \pm 2.8$). $N = 4$, $*p < 0.001$.

3.3. Engulfment of apoptotic bodies induces activation of JAK1 and STAT3, and STAT3 translocation to the nucleus

Because inhibition of JAK activation after AB engulfment induced apoptosis, we next investigated whether the JAK1/STAT3 pathways are activated in phagocytosing HSC. Western blot analysis showed that phagocytosis of AB induced both JAK1 and STAT3 phosphorylation in a time-dependent manner (Fig. 3A). As chemical inhibition of JAK1 phosphorylation may not be specific, to confirm these data, LX-2 cells were transfected with a SOCS3 expression plasmid prior to exposing them to AB. JAK1 and STAT3 phosphorylation were inhibited in the SOCS3-transfected cells (data not shown). As a positive control for JAK1 and STAT3 phosphorylation, we used leptin, a known cytokine to induce these pathways in HSC [24,25] (Fig. 3B). To assess if the phosphorylated STAT3 translocates to the nucleus, immunohistochemistry on primary, phagocytosing HSC in the presence or absence of the JAK inhibitor, AG490 was performed. STAT3 translocated to the nucleus in AB-exposed cells (Fig. 3C b, c), and this was blocked by AG490 treatment (Fig. 3C d), confirming that engulfment of AB indeed induces STAT3 activation and nuclear translocation. Interestingly, JAK1 and STAT3 phosphorylation could also be partially reduced by inhibiting PI3K activity, suggestive of a crosstalk between the two signaling pathways (Fig. 3D).

3.4. Mcl-1 expression is upregulated in phagocytosing HSC

Since STAT3 is known to be at the center of cell survival pathways [14,26,27], and Mcl-1 expression is regulated by STAT3 at a transcriptional level [28,29], next we tested if phagocytosis of AB upregulates Mcl-1 in HSC. Western blot analysis was performed on cytosolic extracts of primary HSC. After engulfment of AB, Mcl-1 was upregulated, and this was inhibited by prior incubation with AG490. Densitometry data show that phagocytosis of AB induces 1.83-fold upregulation of Mcl-1, while this is inhibited by blocking JAK activity (Fig. 4). IL-6 served as positive control to study induction of Mcl-1.

3.5. Phagocytosis of AB induces PI3K-dependent phosphorylation of Akt

Our previous data showed that inhibition of PI3K with LY294002 induced apoptosis in phagocytosing HSC therefore, survival of HSC may also involve the activation of an Akt-dependent pathway. As PI3K activation following phagocytosis is known to occur in concert with the production of reactive oxidative species (ROS) [16], first we performed a cytochrome *c* assay to demonstrate ROS release (Fig. 5A). Next, HSC were exposed to AB in the presence or absence of LY294002, and AG490, to see if there is a crosstalk between the two signaling

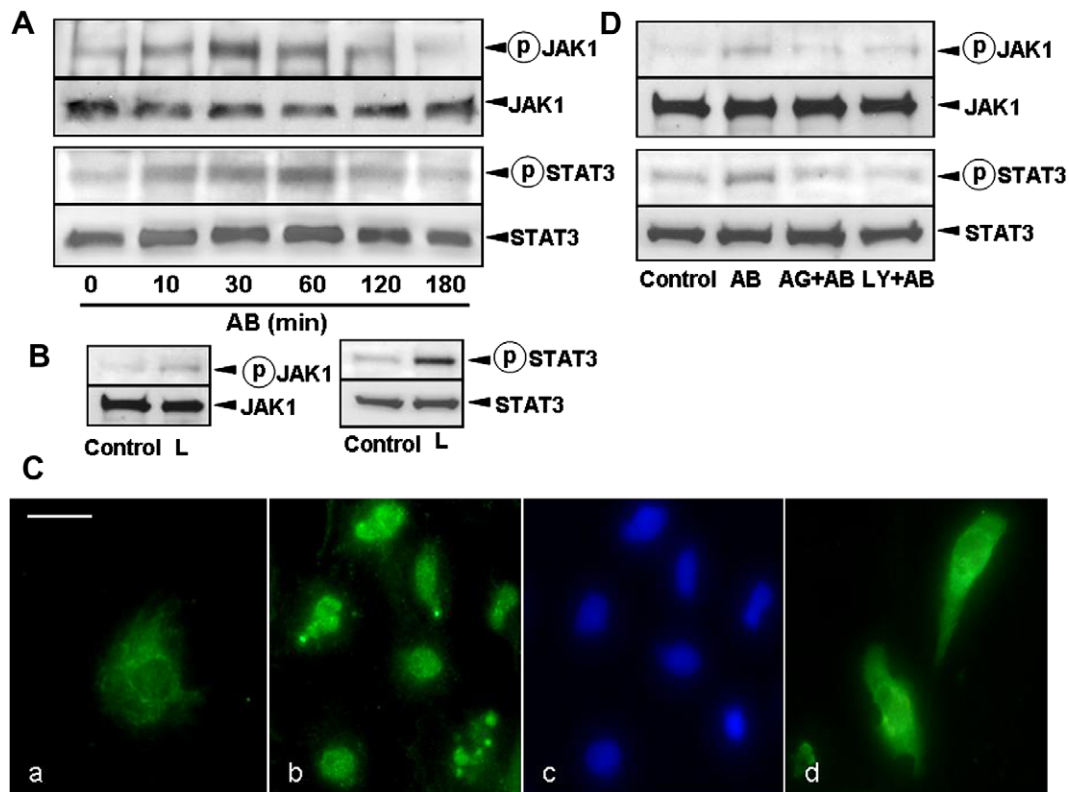


Fig. 3. Phagocytosis of AB induces JAK/STAT phosphorylation and STAT3 nuclear translocation. (A) LX-2 cells were exposed to AB, and the cell lysate was collected for immunoblotting using antibodies to phospho-JAK1, total JAK1, phospho-STAT3, and total STAT3. The assay showed that AB reduced JAK1 and STAT3 phosphorylation in a time-dependent manner. (B) As a positive control, phosphorylation of JAK1 and STAT3 were induced in LX-2 cells by leptin (75 ng/ml, 10 min). (C) Immunocytochemistry using anti-STAT3 antibody was performed on rat primary HSC. (a) In control cells (not exposed to AB), STAT3 is cytoplasmic. (b, c) Incubation with AB induced STAT3 nuclear translocation in HSC, detected by the colocalization of the STAT3 and DAPI signal (d) AG490 treatment in AB-exposed HSC inhibited STAT3 translocation. STAT3: green, DAPI: blue. Bar: 20 μm. (D) JAK1 and STAT3 phosphorylation could also partially be inhibited by inhibiting PI3K activity with preincubation of HSC by LY294002, suggestive of a crosstalk between the two signaling pathways.

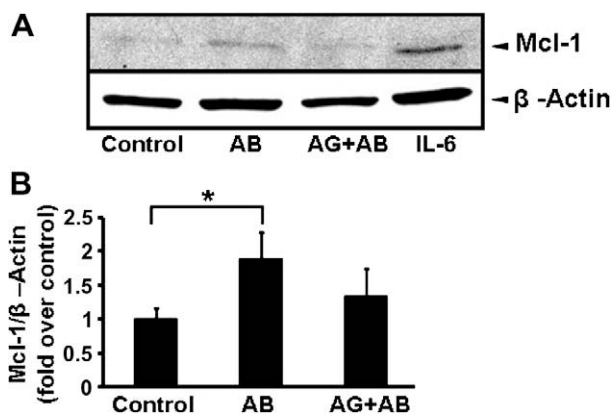


Fig. 4. Phagocytosis of AB induces Mcl-1 expression in HSC. Primary rat HSC were treated with AB for 24 h in the presence or absence of AG490. Western blot analysis was performed on the cytosolic extracts to detect changes in Mcl-1 expression. Mcl-1 expression increased in AB treated cells; this was inhibited by preincubation of cells with the JAK inhibitor, AG490. IL-6 treatment was used as a known inducer of Mcl-1 expression. Densitometry showed that phagocytosis of AB induced a 1.8-fold increase (± 0.3) in expression, normalized to β -actin and presented as fold over control. $N = 4$, $*p < 0.05$.

pathways. Treatment of the cells with the NADPH oxidase inhibitor apocynin was done as well, to test if Akt phosphorylation is dependent on NADPH oxidase activity and ROS production. Engulfment of AB induced phosphorylation of Akt in a time-dependent manner, and this was inhibited by the PI3K inhibitor LY294002 and apocynin (Fig. 5B). Inhibition of JAK had less effect on Akt phosphorylation. Taken together, phagocytosis of AB induces an Akt-dependent survival pathway, which depends on ROS production by NADPH oxidase and PI3K activation.

3.6. Phagocytosis of AB induces NF- κ B activation

NF- κ B is central to regulating apoptosis and cell survival in HSC [30,31], and phagocytosis was shown to activate NF- κ B in macrophages [32]. To study if NF- κ B activation occurs in HSC after phagocytosis, and to explore the signaling pathways leading to its activation; HSC were exposed to AB after preincubation with the NF- κ B inhibitor CAPE, JAK inhibitor AG490, the PI3K inhibitor LY294002, or the NADPH

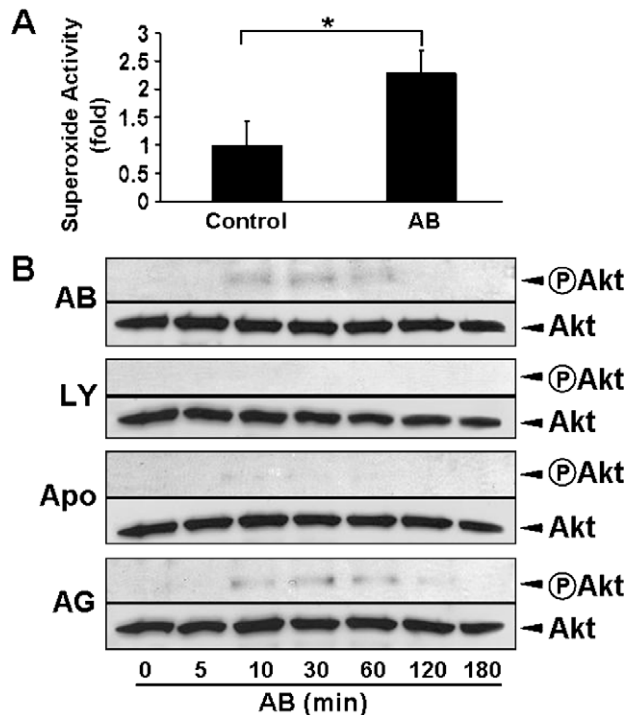


Fig. 5. Phagocytosis of AB induces ROS production and Akt phosphorylation. (A) LX-2 cells were incubated with AB for 6 h and cytochrome *c* assay was used to evaluate ROS production. Phagocytosis of AB induced 2.2 ± 0.4 -fold of superoxide activity compared to control. $N = 4$, $^*p < 0.05$. (B) LX-2 cells were exposed to AB with or without apocynin (NADPH oxidase inhibitor), AG490 or LY294002, and the cell lysates were collected for immunoblotting using antibodies to phospho-Akt and total Akt. AB transiently induced Akt phosphorylation, which was blocked by apocynin and LY294002, respectively, but not AG490.

oxidase inhibitor apocynin. Nuclear extracts were obtained and an EMSA was performed. Phagocytosis of AB strongly induced NF- κ B activation, and this was inhibited by the NF- κ B inhibitor CAPE (Fig. 6). Inhibiting the PI3K also reduced NF- κ B activation significantly, while the reduction by inhibiting the JAK-mediated pathway or the NADPH oxidase was more modest. This is consistent with our previous data where inhibition of the PI3K activation induced apoptosis of phagocytosing HSC. This suggests that NF- κ B activation is an integral part of the phagocytosis-induced HSC survival, and that this depends on PI3K activity.

3.7. Phagocytosis of AB induces upregulation of the anti-apoptotic protein A1 and inhibition of A1 expression induces apoptosis of HSC

One of the main targets of NF- κ B is the Bcl-2 family member A1, and it is known to play a major role in HSC survival [4]. Thus, the next question is whether A1 is upregulated following phagocytosis. We performed Western blot analysis on the cytosols of phagocytosing HSC cultured in the presence or absence of AG 490 and LY294002. We found that A1 indeed was

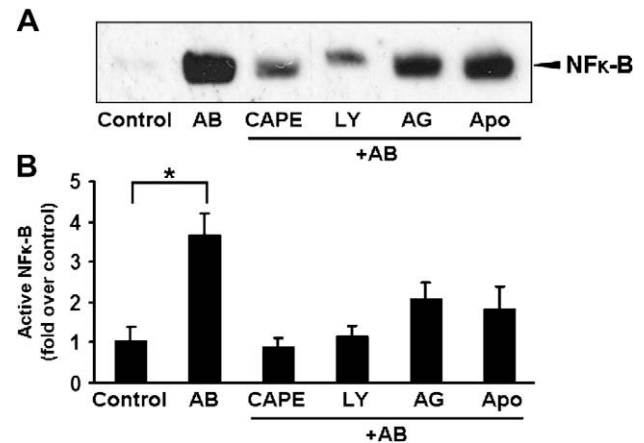


Fig. 6. Phagocytosis of AB induces NF- κ B activation. LX-2 cells were incubated in a serum free medium then exposed to AB with or without prior preincubation with CAPE (1 ng/ml, 14 h), LY294002 (600 nM, 1 h), AG490 (50 μ M, 1 h), or apocynin (100 μ M, 1 h). Engulfment of the AB by LX-2 cells induced a significant activation of NF- κ B (3.65 ± 0.55 -fold), and this was inhibited by CAPE, PI3K inhibition, and to a lesser extent by JAK and NADPH oxidase inhibition. Densitometry shows data from 3 experiments. $^*p < 0.005$.

upregulated following engulfment of AB in a PI3K-dependent manner (Fig. 7A, B). To study if inhibition of A1 induces apoptosis of phagocytosing HSC, the cells were transfected with either scrambled siRNA or siRNA to A1. A1 expression was confirmed by Western blot analysis (Fig. 7C). At baseline (HSC exposed to AB) apoptosis increased following A1 inhibition suggesting that A1 is an important anti apoptotic protein in HSC (Fig. 7D). After treating phagocytosing HSC with FasL and cycloheximide, A1 inhibition caused a significant increase in apoptosis even in phagocytosing cells. These data indicate that A1 expression is one of the major survival pathways induced in HSC following phagocytosis.

4. Discussion

HSC survival during liver injury is an important factor in propagation of liver fibrosis. The exact mechanisms responsible for maintaining an active HSC population however, are not well understood. Our study describes a novel mechanism linking chronic liver injury with resulting hepatocyte apoptosis to phagocytosis of AB and the induction of survival signals in HSC.

The presence of phagocytic cells in an adequate number at the site of tissue injury is essential to maintain homeostasis, first, by the clearance of apoptotic debris and/or pathogens, second, by producing anti-inflammatory cytokines such as TGF- β . Survival of professional macrophages phagocytosing AB was previously demonstrated [32,33], playing a role in the resolution of inflammation. Increased production of TGF- β by phagocytosing HSC was shown by us [15]. As clearance

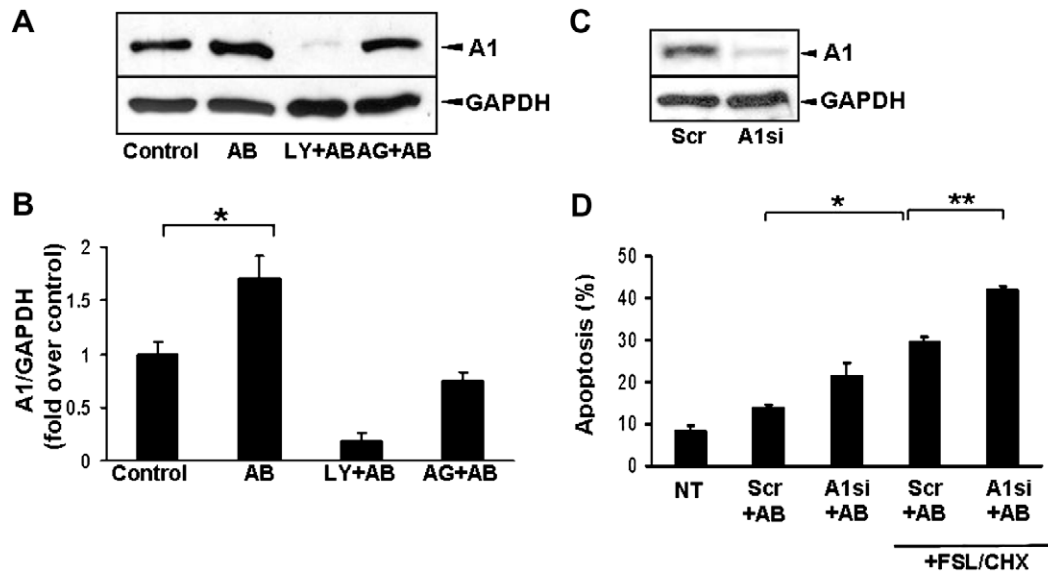


Fig. 7. A1 expression is induced in phagocytosing hepatic stellate cells. (A) LX-2 cells were exposed to AB in the presence or absence of LY294002, or AG490. Western blot analysis was done to study the anti apoptotic protein, A1 expression. (B) The quantitative densitometry analysis shows that phagocytosis of AB upregulated A1 expression (1.7 ± 0.2 -fold), and this was inhibited by blocking the PI3K activity. Inhibiting the JAK pathway, however, had only a modest effect on A1 expression $N = 4$, $^*p < 0.05$. To study the anti-apoptotic effect of A1, primary rat HSC were transfected with A1 siRNA (30 nM) and exposed to AB. (C) Western blot analysis shows the inhibition of A1 expression by the siRNA. (D) Primary HSC were transfected with either scrambled (Scr) or A1 siRNA (A1si), exposed to AB, in the presence or absence of FasL and cycloheximide. Caspase-3/DAPI staining was performed to assess apoptosis. In the Scr+AB group the apoptosis was ($14.1\% \pm 0.8$). This was increased by FasL and CHX ($29.6\% \pm 1.2$). A1 siRNA transfection induced higher apoptosis rate at baseline (only AB-exposed cells) and after FasL/CHX treatment ($42.1\% \pm 0.7$). Mean \pm SED, $N = 4$, $^*p < 0.001$, $^{**}p < 0.005$.

of AB by HSC induces their activation and consequent production of ECM components, it is imperative that we determine the fate of phagocytosing HSC. In our study we have shown that in phagocytosing HSC there are different anti-apoptotic pathways induced, including a JAK1/STAT3-dependent pathway and a NADPH oxidase-dependent PI3K/Akt/NF- κ B induction pathway. ROS in given circumstances have been described as pro-apoptotic [34,35] and also anti-apoptotic [7,8]. The reason for this controversy is that the effects of ROS largely depend on the redox conditions of the cell, concentration and availability of antioxidants, the source, amount and time course of ROS production [36]. ROS can act as second messengers in given circumstances inducing anti-apoptotic pathways in different cell-types [8,37]. The signaling pathways that govern these responses are just beginning to emerge and include the activation of the PI3K/Akt-mediated pathway and NF- κ B [37]. PI3K is an important regulatory element of the NADPH oxidase complex and plays a role in the regulation of superoxide production in neutrophils [38]. Phagocytosis-induced activation of the PI3K leading to Akt phosphorylation was demonstrated in several studies [33,38,39]. Akt is a crucial survival signaling factor in several cell types including HSC [17]. In HSC, leptin protects cells from cycloheximide or TRAIL induced apoptosis *via* phosphorylation of Akt [40,41]. ROS exposure is also known to induce

Akt-dependent survival pathways [22,37,42]. In the studies by Wang et al. oxidative stress-induced activation of the PI3K/Akt pathway was required for cell survival and inhibition of the PI3K induced apoptosis in multiple cell types [43]. Consistent with these, we found that PI3K-dependent Akt phosphorylation follows phagocytosis of AB, and inhibition of this pathway induces apoptosis of HSC. Inhibiting the NADPH oxidase with apocynin reduced phosphorylation of Akt, suggesting that NADPH oxidase activation is preceding Akt phosphorylation. As NF- κ B is a potent pro-survival transcription factor in activated HSC [30,44–46], and its inhibition could cause apoptosis of activated HSC [4], next we tested if phagocytosis of AB induces NF- κ B activation. Phagocytosis induced significant activation of NF- κ B, this was PI3K/Akt-dependent, and partially inhibited by apocynin in HSC, suggesting that NADPH oxidase-derived ROS play a role in its activation. To further elucidate the possible target of NF- κ B, we found that A1, an anti-apoptotic Bcl-2 family member was upregulated upon phagocytosis, and this was inhibited by the PI3K inhibitor. NF- κ B-mediated A1 upregulation was shown to be required for survival of activated macrophages following phagocytosis [47], and also crucial in the survival of activated HSC [4]. To assess if indeed A1 expression is a dominant mechanism of HSC survival following phagocytosis, A1 expression was inhibited by an siRNA approach. This has resulted

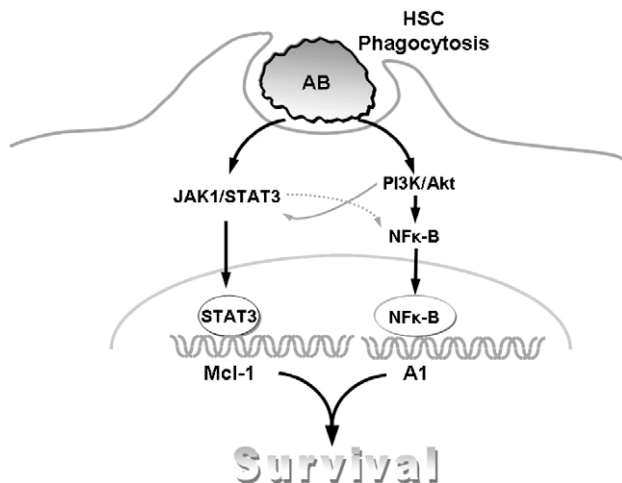


Fig. 8. Phagocytosis of AB by HSC induces different survival pathways. Engulfment of AB induces a JAK1/STAT3 mediated survival pathway leading to an upregulation of Mcl-1. Phagocytosis also induces Akt phosphorylation *via* the PI3K, and this contributes to NF-κB activation. As a result, the A1 anti apoptotic protein is upregulated in phagocytosing HSC, in a PI3K-dependent manner.

in a significant increase in apoptosis of phagocytosing cells suggesting that A1 is a key anti-apoptotic protein (see Fig. 8).

We have also described the activation of a JAK/STAT mediated survival pathway in phagocytosing HSC. STAT3 is considered to be central to multiple anti apoptotic pathways [26,48]. Abrogation of STAT3 activation by an antisense strategy increased tumor cell apoptosis and decreased Bcl-xL expression in head and neck tumors [49], while in a different study, expressing the truncated, inhibitory form of STAT3 induced cell death in melanoma [50]. In our studies we found that following phagocytosis, STAT3 became phosphorylated and translocated to the nucleus, and these events were inhibited by blocking JAK1 activity. One of the targets of STAT3 is Mcl-1 which belongs to the Bcl-2 family of anti-apoptotic proteins, and contributes to the control of mitochondrial integrity, which is critical for maintaining cell viability [51]. We found that Mcl-1 was upregulated following phagocytosis of AB in primary HSC and that this was inhibited by blocking JAK1. This upregulation, albeit less significant as the activation of the NF-κB/A1 axis may also play a role in cell survival following phagocytosis. Other potential targets of STAT3 such as survivin [52,53] may also induce HSC survival. Survivin was shown to be upregulated in different models of liver fibrosis [54]. As to how JAK1 activation occurs following phagocytosis, is not yet elucidated. JAK activation may occur *via* phagocytosis-induced engagement of integrins [55], or alternatively, it could be the result of crosstalk between the PI3K/JAK pathways [56]. Indeed, we found evidence in our studies that inhibition of PI3K may affect JAK1 and STAT3 phosphorylation.

This crosstalk has been demonstrated in other experimental models especially involving ROS-mediated signaling pathways [57].

In summary, these studies have delineated a novel pathway of HSC survival. We have shown that phagocytosis of AB induces different survival signals in HSC, resulting in NF-κB and A1 activation/upregulation, and to a lesser degree Mcl-1 induction. As survival of activated, phagocytosing HSC contributes to the propagation of liver fibrosis, therapeutic strategies, targeting these pathways may be useful in inducing apoptosis of activated HSC, which may result in the reversal of fibrosis.

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