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# Phosphatidylserine exposure in Fas type I cells is mitochondria-dependent

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**Abstract Previous studies have demonstrated that Fas-triggered activation of effector caspases and subsequent nuclear apoptosis either is mitochondria-independent (type I cells) or relies on mitochondrial amplification of the initial stimulus (type II cells). We show herein that Bcl-2 overexpression in a prototypic type I cell line (SKW6.4) promotes mitochondrial generation of ATP and blocks Fas-triggered plasma membrane externalization of phosphatidylserine (PS). Moreover, overexpression of Bcl-2 attenuates macrophage engulfment of Fas-triggered cells. Fas-mediated DNA fragmentation, on the other hand, remains unaffected in SKW6.4-*bcl-2* cells. These studies thus demonstrate that PS externalization and clearance of cell corpses are mitochondria-dependent events, and show that these events can be dissociated from other features of the apoptotic program, in Fas type I cells.**



*Key words:* Adenosine triphosphate; Apoptosis; Bcl-2; Mitochondrion; Phagocytosis; Phosphatidylserine

## 1. Introduction

The rapid engulfment of apoptotic cells by neighboring macrophages serves to preclude the release of noxious cellular constituents and the tissue scarring that would otherwise ensue [1]. Previous studies have shown that the externalization of phosphatidylserine (PS) [2] and its concomitant oxidation [3] is critical for macrophage recognition and uptake of effete cells. While the apoptotic redistribution of PS has been demonstrated to require extracellular $Ca^{2+}$ [4] and the activation of caspases [5–7], the downstream signaling events have remained poorly understood. We have recently provided evidence that the decline of intracellular ATP and inhibition of the ATP-dependent aminophospholipid translocase may govern the egress of PS during Fas-triggered apoptosis [8]. To further elucidate the mechanism underlying PS externalization, we focused in the present study on Fas-triggered apoptosis in the prototypic type I cell line, SKW6.4. Type I cells are thought to be mitochondria-independent insofar as Bcl-2 fails to abrogate nuclear apoptotic changes upon Fas ligation [9]. By contrast, cell death and the activation of caspases in type II cells were shown to be critically dependent on mitochondrial signaling. Caspase-8-mediated cleavage of cytosolic Bid serves as a link between death receptor activation at the plasma membrane and mitochondrial events; upon cleavage, truncated Bid translocates to mitochondria and initiates the cytochrome *c*-dependent activation of caspases downstream of these organelles [10,11]. The fact that hepatocytes derived from Bid-deficient mice are Fas-resistant and the observation that these animals are protected from anti-Fas antibody-induced hepatic injury [12] suggest that the type II phenotype can be recapitulated in vivo.

In the present study, we demonstrate that overexpression of Bcl-2 blocks PS exposure in Fas type I cells. Moreover, the externalization of PS was found to be dissociated from other indices of apoptosis, including the activation of caspase-3-like enzymes and the induction of nuclear apoptotic changes. Our data also show that Bcl-2 overexpression attenuates clearance of Fas-triggered type I cells by two classes of macrophages, thus suggesting that execution of cell death and clearance of cell corpses are independently regulated processes.

## 2. Materials and methods

### 2.1. Reagents and cell culture

Anti-Fas monoclonal antibodies (clone CH-11) were purchased from Medical and Biological Laboratories (Nagoya, Japan). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone zVAD-fmk and DEVD-AMC were obtained from Enzyme Systems Products (Dublin, CA, USA) and Peptide Institute (Osaka, Japan), respectively. Oligomycin was from Sigma (St. Louis, MO, USA). The human B cell line SKW6.4 (European Collection of Cell Cultures, Salisbury, UK) was cultured in the high glucose version of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 100 μ/ml penicillin and 100 mg/ml streptomycin (Life Technologies), in a humidified atmosphere of 5% $CO_2$ at 37°C. The SKW6.4-*bcl-2* and SKW6.4-*neo* cell lines, stably transfected with retroviral vectors co-expressing the human *bcl-2* cDNA and a neomycin resistance gene, and a control *neo* construct, respectively, were kindly provided by Drs. Andreas Strasser and Lorraine O'Reilly (Melbourne, Australia) and were cultured as previously described [13]. The human monocytic cell line THP-1 (European Collection of Cell Cultures) was maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM Na-pyruvate, and 50 μM 2-mercapto-

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*Abbreviations:* $\Delta\psi_m$, mitochondrial transmembrane potential; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PS, phosphatidylserine; TAMRA, 5(6)-carboxytetramethyl-rhodamine *N*-hydroxysuccimide ester; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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ethanol. To induce differentiation into macrophage-like cells, $5\times10^5$ cells were stimulated with phorbol 12-myristate 13-acetate (Sigma) at 150 nM for 3 days. The murine macrophage cell line J774A.1 (European Collection of Cell Cultures) was cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate.

### 2.2. Western blot

Cell lysates were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 130 V and electroblotted to nitrocellulose membranes for 2 h at 100 V. Membranes were blocked for 1 h with 5% non-fat milk in phosphate-buffered saline (PBS) at room temperature and subsequently probed overnight with monoclonal antibodies directed against Bcl-2 (Dako, Glostrup, Denmark) or polyclonal antibodies directed against glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Trevigen, Gaithersburg, MD, USA). After washing, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) and bound antibody was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Densitometric analysis was performed using a Fuji LAS 1000 Plus densitometer (Fuji, Stockholm, Sweden).

### 2.3. Mitochondrial transmembrane potential

Loss of mitochondrial transmembrane potential ($\Delta\psi_m$) was detected using the cationic fluorescent probe tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Leiden, The Netherlands). Briefly, $0.5\times10^6$ cells were incubated with 25 nM TMRE at 37°C for 20 min. Cells were then pelleted, washed once in PBS and resuspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing 25 nM TMRE. Flow cytometric analysis was performed on a FACScan (Becton Dickinson, San Jose, CA, USA) using the CellQuest software (Becton Dickinson).

### 2.4. ATP determination

The concentration of intracellular ATP was determined using the luciferase-based ATP Bioluminescence Assay Kit CLS II (Boehringer Mannheim, Mannheim, Germany) as previously described [8]. Data are presented as nM ATP based on an ATP standard curve.

### 2.5. PS exposure

PS exposure was determined by flow cytometric detection of annexin V using the protocol outlined in the annexin V-FITC apoptosis detection kit (Pharmingen, BD Biosciences, San Diego, CA, USA). Cells were washed and resuspended in HEPES buffer containing annexin V-FITC and propidium iodide (100 μg/ml) prior to analysis on a FACScan flow cytometer (Becton Dickinson). Ten thousand events were collected and analyzed using the CellQuest software. Low-fluorescence debris and necrotic (propidium iodide-positive) cells were gated out prior to analysis.

### 2.6. Caspase activity

The measurement of DEVD-AMC cleavage, indicative of caspase-3-like enzyme activity, was performed in a fluorometric assay as previously described [14]. Cleavage of the fluorogenic substrate was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence was measured every 70 s during a 30 min period, and fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC.

### 2.7. DNA fragmentation

DNA fragmentation was assessed by conventional agarose gel electrophoresis and flow cytometry. For visualization of DNA laddering, $2\times10^6$ cells were washed once in PBS and resuspended in sample buffer containing RNase (10 mg/ml) for 20 min at room temperature. Cell lysates were then loaded on a 1.8% agarose gel with a digestion gel (0.8% ultrapure agarose, 2% sodium dodecyl sulfate, 0.6 mg/ml proteinase K) at the upper end. The gel was run at 20 V for 14 h and then at 90 V for 2.5 h. After ethidium bromide staining, DNA was visualized under 305 nm UV illumination. For flow cytometric analysis, $1\times10^6$ cells were harvested, washed once in PBS and resuspended in staining solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate in PBS. Cells were then analyzed on a FACScan (Becton Dickinson) using the CellQuest software. Data are presented as the percentage of cells displaying hypodiploid DNA content.

### 2.8. Phagocytosis assays

Phagocytosis was evaluated as described elsewhere [3]. Briefly, target cells were labeled using 50 μM of the fluorescent dye 5(6)-carboxytetramethyl-rhodamine *N*-hydroxy-succimide ester (TAMRA) (Sigma) in serum-free medium for 15 min at 37°C and then cultured in the absence or presence of the apoptotic stimulus. Target cells were then washed twice in serum-free medium and resuspended in fresh medium, and layered onto THP-1 or J774A.1 macrophages at a ratio of 10:1. Cells were co-cultured for 2 h at 37°C and non-engulfed cells were then removed by seven or eight rounds of washing with PBS; the remaining, adherent cells were fixed in 2% paraformaldehyde. Phagocytosis was evaluated by counting macrophages in visible light and thereafter counting macrophage-engulfed cells under UV illumination using a fluorescence microscope at 400× magnification. At least 300 macrophages per experimental condition were scored, and data are presented as the percentage of macrophages positive for uptake of target cells.

### 2.9. Statistics

Data are expressed as mean ± S.D. Samples were analyzed by Student's *t*-test (single comparisons). Differences between means were considered significant when $P<0.05$.

## 3. Results and discussion

### 3.1. Bcl-2 protects against mitochondrial perturbation and blocks PS exposure in Fas-triggered type I cells, yet fails to prevent nuclear apoptotic changes

To address the importance of mitochondria for the externalization of PS, we utilized SKW6.4 (type I) cells stably transfected with Bcl-2. Western blot confirmed a four-fold elevation of Bcl-2 expression in the *bcl-2*-transfected cells as compared to vector-transfected controls (Fig. 1A). Moreover, Fas-induced dissipation of $\Delta\psi_m$ was abrogated upon overex-

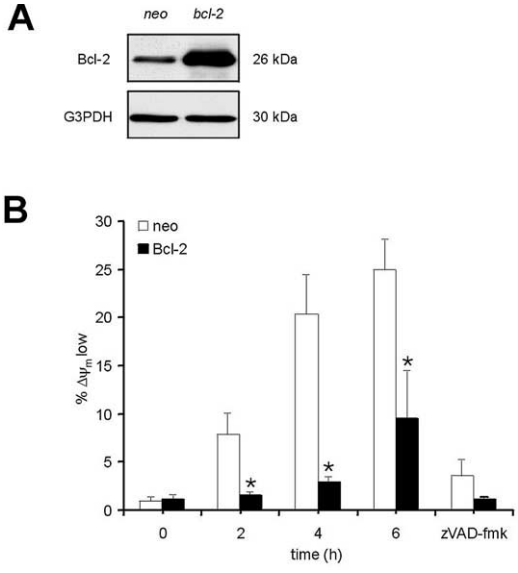


Fig. 1. Bcl-2 overexpression prevents Fas-mediated mitochondrial perturbation in SKW6.4 (type I) cells. A: Total cell lysates of SKW6.4-neo and SKW6.4-*bcl-2* cells were analyzed by Western blot using a Bcl-2-specific antibody. G3PDH was monitored to control for equal loading of protein. B: Cells treated with agonistic anti-Fas antibodies (250 ng/ml) in the presence or absence of the pan-caspase inhibitor zVAD-fmk (10 μM) were assessed for loss of $\Delta\psi_m$. Data shown are expressed as mean ± S.D. ($n=3$). *$P<0.01$ (*neo* vs. *bcl-2*).

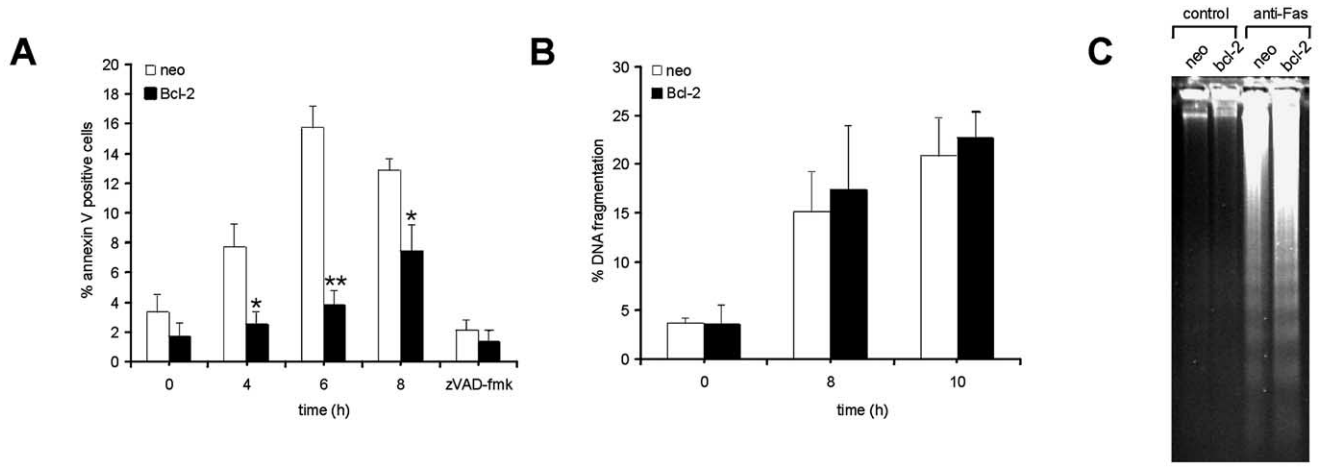


Fig. 2. Bcl-2 overexpression blocks Fas-triggered PS exposure, but fails to prevent nuclear apoptotic changes. SKW6.4-*neo* and SKW6.4-*bcl-2* cells were treated as detailed in the legend to Fig. 1, and assessed for (A) PS exposure, (B) DNA fragmentation, as evidenced by the degree of hypodiploid DNA content, and (C) DNA laddering. Data shown in A and B are expressed as mean ± S.D. ($n = 3$–4). *$P < 0.01$, **$P < 0.001$ (*neo* vs. *bcl-2*). No significant differences in DNA fragmentation were detected between *neo* and *bcl-2* cells.

pression of Bcl-2, thus confirming that the Bcl-2 protein was, indeed, functional (Fig. 1B). As expected, the pan-caspase inhibitor zVAD-fmk also blocked the drop in $\Delta\psi_m$. Hence, for clarification, it should be noted that mitochondrial alterations, as evidenced by the decline in $\Delta\psi_m$, occur in both type I and type II cells; however, such changes are of no consequence for the induction of nuclear apoptosis in type I cells (discussed below). We then studied the externalization of PS following Fas stimulation. As seen in Fig. 2A, SKW6.4-*bcl-2* cells exhibited significantly less PS exposure when compared to their control-transfected counterparts. For comparison, zVAD-fmk was found to block PS externalization completely in both cell lines. Downstream activation of caspase-3-like enzymes was, on the other hand, unaffected in Bcl-2-overexpressing cells (data not shown). Fas-triggered DNA fragmentation was also unaffected by the overexpression of Bcl-2 (Fig. 2B,C). The latter data further support the contention [9] that nuclear apoptotic events transpire independently of mitochondrial alterations in type I cells. Importantly, while Bcl-2 blocks nuclear apoptotic changes [9] and PS externalization [15] in Fas type II cells, our data show, for the first time, that Fas-induced PS exposure and DNA fragmentation can be dissociated in type I cells. A lack of nuclear involvement in the redistribution of PS has also been observed in other models of apoptosis [16–18]. Taken together, it appears that PS externalization, both in type I and type II cells, is a Bcl-2-inhibitable, mitochondria-dependent event.

### 3.2. *Bcl-2 overexpression promotes mitochondrial ATP production and prevents the decline in ATP upon apoptosis induction*

We previously identified an Epstein–Barr virus-transformed B cell line (Raji) that undergoes apoptosis upon Fas ligation, yet is defective for PS exposure [14]. We have also shown that Raji cells retain normal levels of intracellular ATP upon Fas stimulation; however, when ATP levels are suppressed, Fas triggering results in apoptotic PS exposure in these cells [8]. In line with these observations, basal levels of ATP in SKW6.4-*bcl-2* cells were significantly higher than in *neo* cells, and the Fas-triggered dissipation of ATP was effectively blocked by Bcl-2 (Fig. 3A). To confirm that this effect of Bcl-2 was due to the maintenance of mitochondrial function (as opposed to a promotion of extramitochondrial ATP production), SKW6.4-*bcl-2* and *neo* cells were incubated with oligomycin, an inhib-

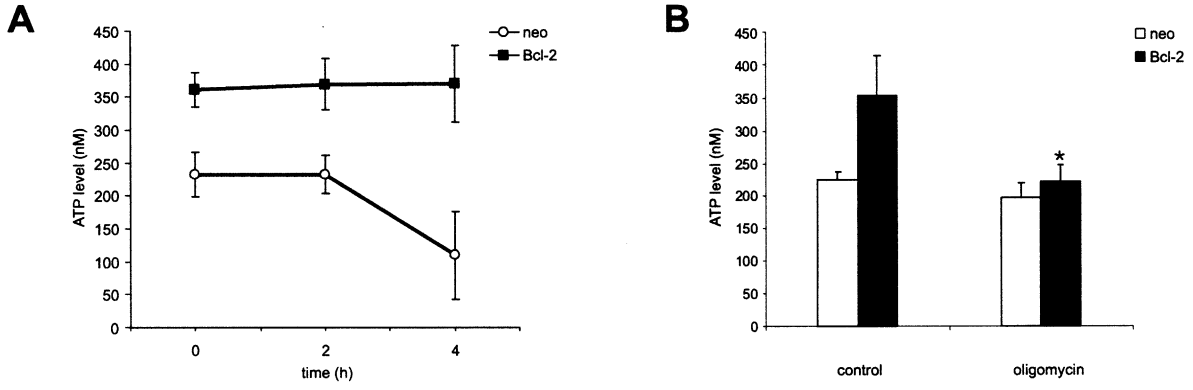


Fig. 3. Overexpression of Bcl-2 promotes mitochondrial ATP production. A: Intracellular ATP levels were determined in SKW6.4-*neo* and SKW6.4-*bcl-2* cells upon treatment with agonistic anti-Fas antibodies (250 ng/ml) for the indicated timepoints. Data are shown as mean ± S.D. ($n = 4$). B: SKW6.4-*neo* and SKW6.4-*bcl-2* cells were incubated for 30 min in the presence or absence of oligomycin (5 μg/ml) and ATP levels were determined. Data shown are expressed as mean ± S.D. ($n = 4$). *$P < 0.05$ (oligomycin vs. medium alone).

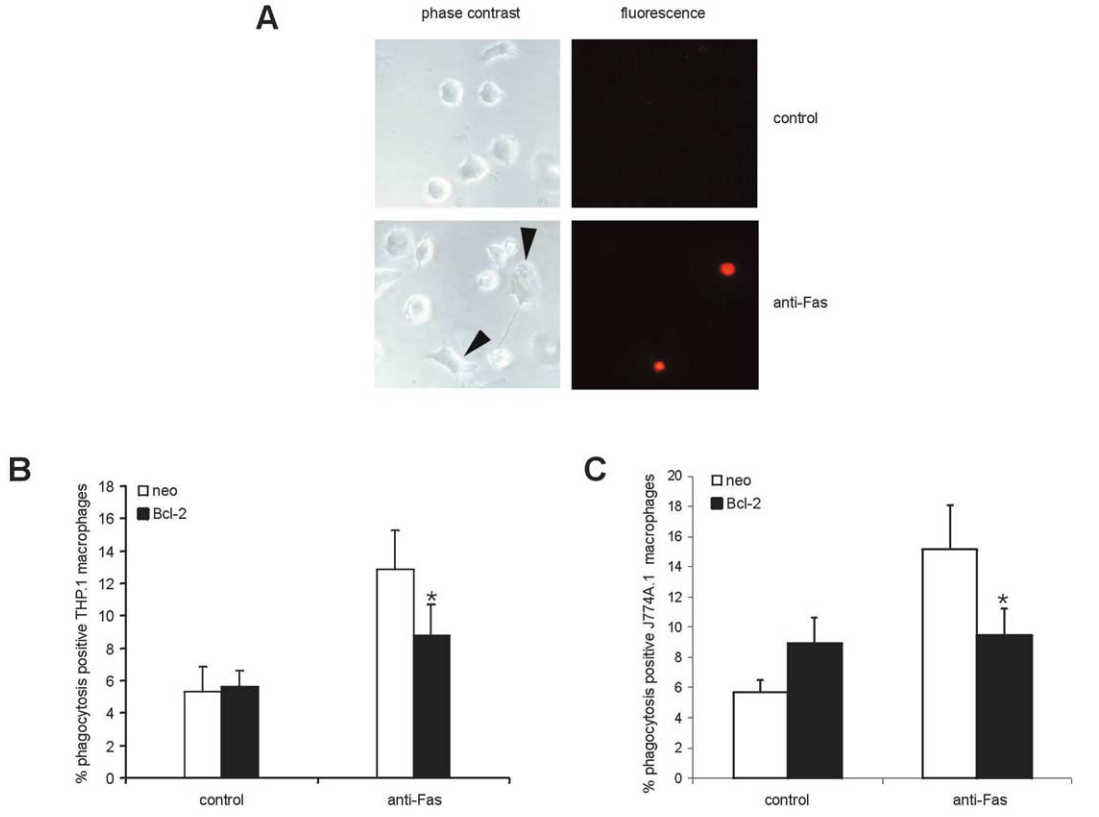


Fig. 4. Bcl-2 overexpression attenuates macrophage clearance of Fas-triggered type I cells. A: Visualization of THP-1 macrophages upon co-cultivation with apoptotic (Fas stimulation, 5 h) versus untreated SKW6.4-*neo* cells. Arrows indicate macrophages that have ingested TAMRA-labeled target cells. Original magnification 400×. B: Quantification of macrophage engulfment of SKW6.4-*neo* and SKW6.4-*bcl-2* cells. The percentages of THP-1 macrophages positive for uptake of TAMRA-labeled target cells are depicted. C: J774A.1 macrophage engulfment of target cells treated as above. Data shown in B and C are mean ± S.D. ($n = 4$). *$P < 0.05$ (*neo* vs. *bcl-2*).

itor of the mitochondrial $F_1F_0$-ATPase. Under these conditions, intracellular ATP in the Bcl-2-overexpressing cell line dropped to a level comparable to that seen in the vector-transfected control (Fig. 3B). These data indicate that, in addition to its established anti-apoptotic properties, Bcl-2 might also have a more general role in the preservation of mitochondrial function. Indeed, recent studies suggest that Bcl-2 can improve oxidative phosphorylation through modulation of mitochondrial adenine nucleotide translocation [19,20]. Furthermore, the current observations, in conjunction with our previous findings in Raji cells [8], underscore the link between the decline in intracellular ATP during apoptosis and the externalization of PS, at least in the Fas model.

### 3.3. *Bcl-2 overexpression attenuates macrophage clearance of Fas-triggered type I cells: implications for the therapeutic manipulation of apoptosis*

We have previously shown that Fas-triggered cells that fail to expose PS remain unengulfed when co-cultured with macrophages, while PS-positive cells are readily removed, thus serving to underscore the importance of PS exposure for phagocytic clearance of Fas-triggered cells [3]. Since overexpression of Bcl-2 inhibited PS externalization in SKW6.4 cells but not other features of apoptosis, we wished to assess whether phagocytic recognition of these cells also was impaired. For this purpose, Fas-triggered SKW6.4-*bcl-2* cells and their vector-transfected counterparts were co-cultured with THP-1 macrophages, and the degree of phagocytosis was determined. As seen in Fig. 4A,B, Bcl-2 overexpression significantly attenuated macrophage engulfment of SKW6.4 cells. Similar data were obtained upon co-cultivation of cells with the murine macrophage cell line J774A.1 (Fig. 4C). Therefore, while nuclear apoptotic alterations in Fas type I cells are mitochondria-independent ([9]; the present study), the externalization of PS and subsequent recognition of cells by macrophages appears to be governed by mitochondrial events. Hence, although Fas-triggered SKW6.4-*bcl-2* cells are committed to death and sustain DNA fragmentation, a classical manifestation of apoptosis, they remain functionally 'un-dead' since they fail to undergo macrophage clearance. The implications of these findings for in vivo conditions are readily apparent: if cells that are triggered to undergo apoptosis escape phagocytic clearance then the 'meaning' of cell death is precluded, as such cells are likely to undergo secondary necrosis, an event that will ultimately lead to inflammation and tissue scarring.

## 4. Concluding remarks

The current data indicate that the cellular manifestations of Fas-triggered apoptosis are governed by subprograms or parallel processes, some of which rely on mitochondrial events and others which are independent thereof. Specifically, while SKW6.4 cells can be classified as type I based on the mitochondria-independent, nuclear manifestations of apoptosis, these cells are nevertheless mitochondria-dependent from the

point of view of PS externalization and macrophage engulfment. Hence, under certain circumstances, the execution phase of apoptosis can be divorced from the resolution phase. An increased understanding of the molecular mechanisms underlying *both* stages of cell death may aid in the design of novel therapeutic strategies in diseases characterized by excessive or unscheduled cell loss by apoptosis.

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