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Cardiovascular, Pulmonary and Renal Pathology

The Involvement of the Apoptosis-Modulating Proteins ERK 1/2, Bcl-x_L and Bax in the Resolution of Acute Inflammation *in Vivo*

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Inflammatory cell recruitment, activation, and apoptosis are highly regulated processes involving several checkpoints controlling the resolution of inflammation. We investigated the role of the mitogen-activated protein kinase (MAPK) signaling pathway (ie, ERK1/2) and apoptosis-regulating Bcl-2 family members (ie, Bcl-x_L and Bax) in the resolution of a rat carrageenan-induced pleurisy model. The specific ERK1/2 inhibitor PD98059 enhanced the resolution of inflammation, whereas the MEK1/2 inhibitor U0126 had no effect and the flavonoid apigenin, a nonspecific inhibitor of ERK1/2 and COX-2, augmented inflammation. Specifically, PD98059 significantly decreased the total number of macrophages and neutrophils in the pleural cavity, mainly by increasing the rate of neutrophil apoptosis, as measured by Annexin V labeling and morphological analysis. Conversely, a specific inhibitor of proapoptotic Bax (V5) increased inflammation, indicating that by preventing apoptosis *in vivo*, resolution of inflammation is delayed. This was associated with a decrease in neutrophil apoptosis and an increase in macrophage and neutrophil numbers perpetuating the inflammatory response. In conclusion, this study shows that ERK1/2, Bax, and Bcl-x_L play important functional roles in the resolution phase of the acute inflammatory response *in vivo* by influencing apoptosis. Importantly, these data may provide novel therapeutic targets for the treatment of inflammatory diseases. *(Am J Pathol 2006, 168:33–41; DOI: 10.2353/ajpath.2006.050058)*

The inflammatory response is a highly regulated process in which the balance between cell survival and apoptosis is orchestrated to ultimately drive and resolve inflammation. In addition, inflammation is controlled by a series of checkpoints, in which an array of endogenous mediators act to either elicit and/or to suppress the inflammatory response, depending on chronology and circumstance.[1] Classically, the onset of acute inflammation is characterized by the recruitment of neutrophils and eosinophils from the bone marrow to the local site of inflammation, regulated by several chemokines and cell adhesion molecules.[2] Here, they are activated by various cytokines [for example, tumor necrosis factor-α, interleukin (IL)-1β, and IL-8], growth factors (GM-CSF), and several other endogenous mediators (for example, PAF and LTB_4). Resultant degranulation of cytotoxic granules and generation of toxic oxygen radicals contribute to surrounding tissue damage. At the peak of the inflammatory response, with regard to leukocyte recruitment, there is an influx of monocytes that differentiate into macrophages *in situ*. It is thought that these professional phagocytes clear apoptotic cells and contribute to resolution by generation of anti-inflammatory cytokines, such as transforming growth factor (TGF)-β_1 and IL-10. On the contrary, phagocytosis of necrotic cells leads to the generation of proinflammatory signals that fuels persistent inflammation and tissue damage.[3] It is therefore thought that the promotion of apoptosis of inflammatory cells, hence efficient clearance by phagocytes, would be a beneficial therapeutic strat-

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egy for inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease.

Recently, we have shown the cellular profile of apoptosis in an acute pleurisy model of inflammation *in vivo*.[4] One of the key events that initiate the resolution of inflammation (24 to 36 hours) is the induction of neutrophil apoptosis, which allows for safe clearance by phagocytes, such as macrophages. During resolution the majority of macrophages migrate to the local lymph node,[5] however we have shown that some macrophages undergo apoptosis *in situ* at 72 hours, when inflammation has almost fully resolved. The signaling molecules and mechanisms that govern inflammatory cell survival and apoptosis have not yet been fully elucidated and more importantly, whether modulating these molecules can prevent or promote the resolution of the inflammatory response in an *in vivo* scenario has not been evaluated. There are numerous signal transduction molecules that play a role in determining the fate of the cell, which are triggered by extracellular signals before committing to caspase-dependent or -independent cell death.[6] The Bcl-2 and mitogen-activated protein kinase (MAPK) pathways have been well characterized in terms of cell apoptosis and cell survival regulation *in vitro*, however their effects on the resolution of inflammation *in vivo* is still unclear. Bax and Bcl-x_L, proapoptotic and anti-apoptotic Bcl-2 family members, respectively, play key roles in the regulation of apoptosis.[7] For example, it has been shown that eosinophils isolated from children with acute asthma had an increased expression of Bcl-2, which was inversely correlated with the expiratory flow rate.[8] In addition, Bax expression is attenuated and Bcl-x_L expression is increased in T cells isolated from the lamina propria from patients with Crohn's disease.[9] This shows that inflammatory cell survival, by means of prosurvival and anti-apoptotic signaling mechanisms are important in the pathogenesis of inflammatory diseases. It has yet to be determined if modifying proteins from the Bcl-2 family can modulate inflammation per se.

The three main cascades by which MAPK homologues are released via MAPK kinase (MEK 1-7) are the extracellular signal-regulated kinase (ERK) (p42, p44, MAPK), c-Jun amino-terminal kinases (JNKs), and p38 MAPK pathways.[10] The endogenous homologues of MAPK all play roles in cell differentiation, apoptosis, stress responses, and inflammation.[11] In inflammatory diseases, such as asthma and rheumatoid arthritis, MAPK expression is increased.[12] ERK1/2 can be activated for a relatively prolonged period of time under certain inflammatory conditions, in which it has been shown that macrophage inhibitory factor (MIF) induces ERK1/2 via $cPLA_2$-induced arachidonic acid generation for a period of 24 hours in culture, which may partially account for the mechanism of ERK-induced cellular proliferation.[13] In addition, macrophage ingestion of apoptotic cells liberate mediators[3] that can influence inflammatory cell survival via MAPK pathways.

Studies have also shown that inhibitors of p38 MAPK, such as RWJ-67657, SB203580, and SB242235, are effective anti-inflammatory agents in suppressing cytokine release in human clinical trials *ex vivo*[14] and also in the rat adjuvant-induced arthritis and in murine collagen-induced arthritis models *in vivo.*[15,16] Inhibitors of ERK 1/2, such as PD98059, have also been shown to inhibit eosinophilic proinflammatory cytokine release, differentiation, and degranulation *in vitro.*[17] Interestingly, flavonoids, such as apigenin, also possess anti-inflammatory properties *in vitro* through antioxidant mechanisms;[18] inhibition of cell adhesion molecule expression, such as ICAM-1,[19,20] and by attenuating the release of inflammatory mediators such as IL-1β-induced PGE_2 via the inhibition of MAPK.[21] *In vivo* studies have shown that flavonoids attenuate the onset of acute inflammation in murine models of carrageenan-induced paw edema and peritonitis, which is attributable to PGE_2 and LTB_4 inhibition.[22] Although flavonoids, such as apigenin, are effective at preventing the onset of inflammation, it is unclear whether flavonoids exert anti-inflammatory effects once inflammation is established and whether they can promote the resolution of inflammation. We therefore hypothesize that inhibiting prosurvival factors, such as ERK 1/2 and Bcl-x_L, or inducing proapoptotic proteins, such as Bax, may lead to the safe clearance of inflammatory cells and aid the resolution of acute inflammation. In this study, we examined the expression profile of apoptosis-modulating proteins during the inflammatory response and investigated whether inhibition of these specific proteins can result in the advancement or delay of the resolution of inflammation.

Materials and Methods

All materials were purchased from Sigma-Aldrich Ltd. (Poole, UK) unless otherwise stated. Bax-inhibiting peptide (V5), PD98059, and U0126 were purchased from Calbiochem (Nottingham, UK). Viscarin (λ-carrageenan) was a kind gift from Marine Colloids Inc. (Philadelphia, PA). Antibodies to Bax, Bcl-x_L, and pERK 1/2 were purchased from Autogen Bioclear (Wiltshire, UK).

Carrageenan-Induced Pleurisy

Male Lewis rats (250 to 300 g) (Tuck and Sons, Battlebridge, UK) were housed in the University of Edinburgh animal facilities in accordance with local guidelines. Animals were fed on a normal diet with tap water *ad libitum* and were housed in a 12-hour light/dark cycle, at 20 to 21°C and with 50% humidity levels. The first described model of carrageenan-induced inflammation was by Winter and co-workers[23] in the paw edema model. Since then, the carrageenan-induced pleurisy model has been further developed and widely used as an acute resolving model of inflammation.[24] Briefly, the animals were anesthetized with halothane, a small incision was made to expose the musculature and 0.15 ml of 1% viscarin (λ-carrageenan) was injected intrapleurally with a blunted 21-gauge needle. Animals were killed by a rising concentration of CO_2 at various time points after carrageenan administration, from the onset (6 hours), the peak (24 hours), and during the resolution of inflammation (36, 48, and 72 hours). Pleural cavities were washed with 1 ml

of 3.15% (w/v) sodium citrate in physiological saline. Total inflammatory cell pellets were harvested for analysis by Western blotting.

In Vivo *Treatment with 2′-Amino-3′-Methoxyflavone (PD98059), 1,4-Diamino-2,3-Dicyano-1,4-Bis(O-Aminophenylmercapto) Butadiene (U0126), or Bax-Inhibiting Peptide V5*

Rats were treated with 0.15 ml of saline control, 0.15 ml of 0.1% or 0.8% dimethyl sulfoxide vehicle control, or with 300 μmol/L PD98059, 300 μmol/L U0126, or 200 μmol/L Bax-inhibiting peptide (V5) intrapleurally 36 hours after injection with 1% carrageenan. Animals were culled by a rising concentration of CO_2 48 hours after injection with carrageenan (12 hours after drug treatment). Pleural cavities were washed with 1 ml of 3.15% (w/v) sodium citrate in physiological saline. Edema formation was measured by weighing the total pleural exudates, and total cell counts were measured with a Coulter Counter (model DN; Coulter Electronics, Luton, UK). An aliquot of 1×10^6 inflammatory cells were washed in PBS three times and labeled with fluorescein isothiocyanate-conjugated Annexin V according to the manufacturer's instructions. Total cell apoptosis was measured by Annexin V labeling according to the manufacturer's instructions and analyzed by the BD FacsCalibur (Becton-Dickinson, Mountain View, CA). Lymphocyte, polymorphonuclear leukocyte (PMNs), and monocyte/macrophage total numbers were counted by light microscopy from hematoxylin and eosin (H&E)-stained cytospin preparations and confirmed by flow cytometry as described.[4] Total neutrophil apoptosis was also measured by morphological analysis by light microscopy from H&E-stained cytospin preparations of pleural inflammatory cells with data presented as the percentage of apoptotic neutrophils from the total number of neutrophils present.

Western Blot Analysis

Inflammatory cell pellets were lysed in the presence of protease inhibitory buffer containing 1 μmol/L phenylmethyl sulfonyl fluoride, 1.5 μmol/L pepstatin A, and 0.2 μmol/L leupeptin. Protein concentrations were determined by the Bradford assay, and equal protein loading was confirmed by β-actin blotting (data not shown). Samples were boiled for 5 minutes with equal volumes of Laemmli gel loading buffer (5 mmol/L Tris, 10% sodium dodecyl sulfate, 10% glycerol, 10% β-mercaptoethanol, and 2 mg/ml bromophenol blue). Total protein equivalents of 10 μg for each sample were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose paper (Amersham, UK) using the Bio-Rad minigel system (Bio-Rad, Hemel Hempstead, UK). Nonspecific antibody binding was blocked with 5% dried milk protein, and blots were incubated with either polyclonal goat or rabbit antibodies against pERK 1/2 (1:1000 dilution), Bax (1:500 dilution), or Bcl-x_L (1:1000 dilution).

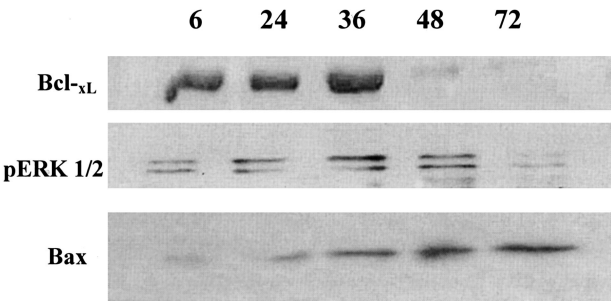

Figure 1. Expression of the prosurvival proteins, pERK 1/2 and Bcl-x_L, and the proapoptotic signaling protein, Bax, during the onset and resolving phase of inflammation. Male Lewis rats were injected intrapleurally with 1% carrageenan and inflammatory cells were harvested at various time points during the onset (6 to 24 hours) and resolving phase of acute inflammation (36 to 72 hours). Equal protein equivalent was determined by Bradford assay and 10 μg was loaded for each sample. Protein expression of pERK1/2, Bcl-x_L, and Bax were measured by electrophoresis and Western blotting. Data are representative of three separate experiments.

Statistics

All experiments were performed with six to eight rats per group, with experiments repeated to verify the original findings. Statistical analysis was performed by a one-way analysis of variance with Bonferroni multiple comparison *post hoc* test with a 95% confidence interval or by a Student's *t*-test as appropriate. Data are expressed as mean ± SEM.

Results

Expression Profiles of Signaling Molecules Involved in Apoptosis

Inflammatory cells from the onset of inflammation in a rat carrageenan-induced pleurisy model (6 to 36 hours after carrageenan) expressed the prosurvival molecules pERK1/2 and Bcl-x_L with very low levels of Bax expression found (Figure 1). Conversely during the resolution of inflammation (48 hours after carrageenan), Bax protein expression was increased with negligible levels of pERK1/2 and Bcl-x_L expression.

Modulation of Proapoptotic or Prosurvival Proteins Can Enhance or Prevent the Resolution of Inflammation in Vivo

Functional studies were conducted to assess whether inhibition of the prosurvival ERK1/2 could alter the resolution of inflammation in the carrageenan-induced pleurisy model. Once inflammation was established in the pleural cavity 36 hours after carrageenan, the flavonoid apigenin, a nonspecific ERK1/2 inhibitor, was given locally by intrapleural injection. Although apigenin did not affect edema, it was found to exacerbate inflammation with an increased total amount of pleural cell numbers of $112.6 \times 10^6 \pm 6.4$ cells compared with vehicle-treated control of $71.7 \times 10^6 \pm 7.3$ cells ($^*P < 0.05$; Figure 2).

The specific ERK1/2 inhibitor PD98059 augmented the resolution of inflammation by reducing cell numbers in

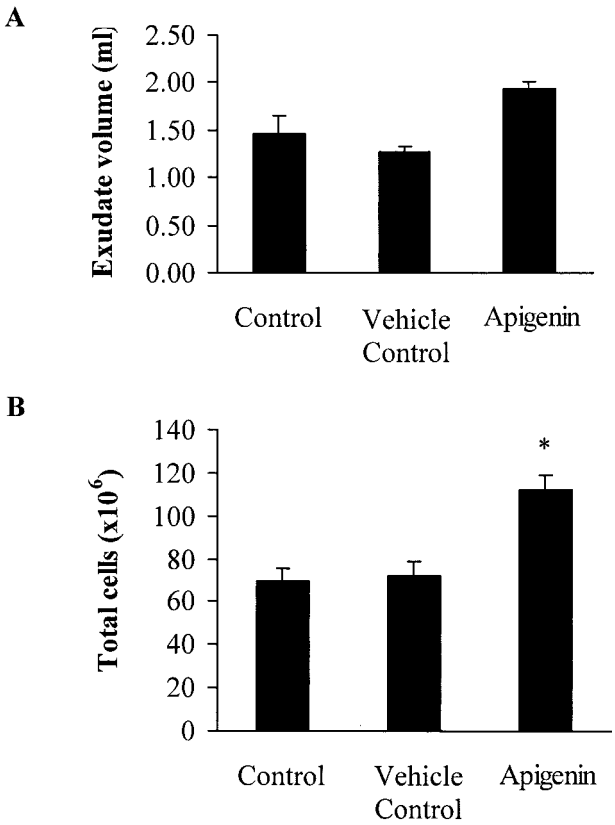

Figure 2. Apigenin, a nonspecific inhibitor of ERK1/2 and COX-2, inhibited the resolution of inflammation. Male Lewis rats were injected intrapleurally with 1% carrageenan and after 36 hours were treated intrapleurally with saline, vehicle-treated control, or 30 μmol/L apigenin. Twelve hours later edema formation (**A**) and total inflammatory cell numbers (**B**) were measured (48 hours after the onset of the carrageenan-induced pleurisy). Data are the mean of six animals per group ± SEM, $^{*}P < 0.05$.

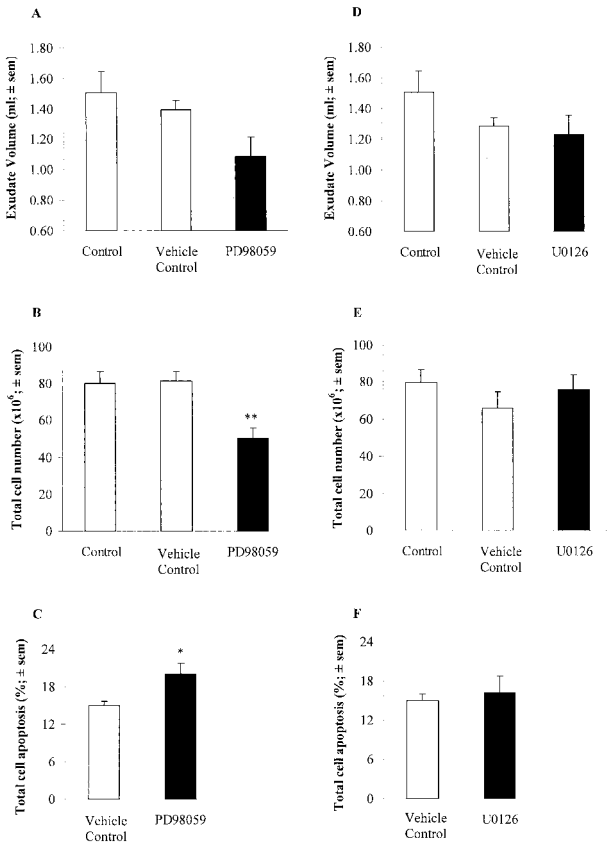

Figure 3. The specific ERK 1/2 inhibitor, PD98059, enhanced the resolution of inflammation, whereas the MEK inhibitor U0126 had no effect. Male Lewis rats were injected intrapleurally with 1% carrageenan and after 36 hours were treated intrapleurally with saline, vehicle-treated control, 300 μmol/L PD98059, or 300 μmol/L U0126. Twelve hours later edema formation (**A** and **D**) and total inflammatory cell numbers (**B** and **E**) were measured (48 hours after the onset of the carrageenan-induced pleurisy). Levels of apoptosis (**C** and **F**) were measured by Annexin V binding and analyzed by flow cytometry. Data are the mean of eight animals per group ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$.

the pleural cavity to $50 \times 10^6 \pm 5.2 \times 10^6$ cells ($n = 8$; Figure 3B) compared with vehicle control of $81 \times 10^6 \pm 5.6 \times 10^6$ cells ($^{**}P < 0.01$) when given 36 hours after carrageenan. PD98059 pretreatment halved the total cell number of PMNs ($^{*}P < 0.05$) and also monocytes/macrophages ($^{***}P < 0.001$) compared with vehicle-treated control (Figure 5A). Edema formation decreased with PD98059 treatment by 30% when compared with saline control; however, PD98059 treatment was not significantly different from vehicle-treated control (Figure 3A). Inflammatory cell apoptosis, measured by Annexin V binding and analyzed by flow cytometry, was significantly enhanced with PD98059 resulting in a 25% increase in binding when compared with vehicle control ($n = 8$, $^{*}P < 0.05$; Figure 3C). Specifically, there was as increase of apoptotic neutrophils with PD98059 pretreatment by 50 ± 6% ($n = 6$, $^{**}P < 0.01$; Figure 5B), which was approximately double the amount of constitutive apoptosis found in the saline and vehicle-treated control groups. Neutrophil apoptosis was further illustrated with H&E-stained inflammatory cells (Figure 5C). In contrast, the MEK1/2 inhibitor U0126 had no effect on the exudate volume, total cell number, or cell apoptosis levels (Figure 3, D–F).

We have shown that by enhancing apoptosis, via inhibition of ERK1/2, there is a significant attenuation of inflammation. On the contrary, it was also important to assess whether direct inhibition of apoptosis can alter the resolution of inflammation. A specific inhibitor of Bax prevented the safe resolution of inflammation with an increase in numbers of inflammatory cells to $121.9 \times 10^6 \pm 16.7 \times 10^6$ cells ($n = 8$; Figure 4) compared with saline alone to $78.3 \times 10^6 \pm 9.5 \times 10^6$ cells ($n = 8$) when administered locally at the peak of inflammation. Specifically, the total amount of PMNs and monocytes/macrophages increased by 42% and 33%, respectively, compared with saline control ($^{*}P < 0.05$, $^{***}P < 0.001$; Figure 5A). There was also a significant decrease (35%, $^{*}P < 0.05$, $n = 8$) in Annexin V binding compared with control, which further supports the hypothesis that the augmented inflammation was due to the prevention of apoptosis, which would normally occur during the resolution phase of inflammation. In particular, Bax inhibition caused a decrease in the number of apoptotic neutrophils, in which only 7.2% of neutrophils were apoptotic within the total number of neutrophils present (Figure 5, B and C; $^{*}P < 0.05$). Interestingly, although TGF-β_1 was prevalent during resolution (36 hours after carrageenan),

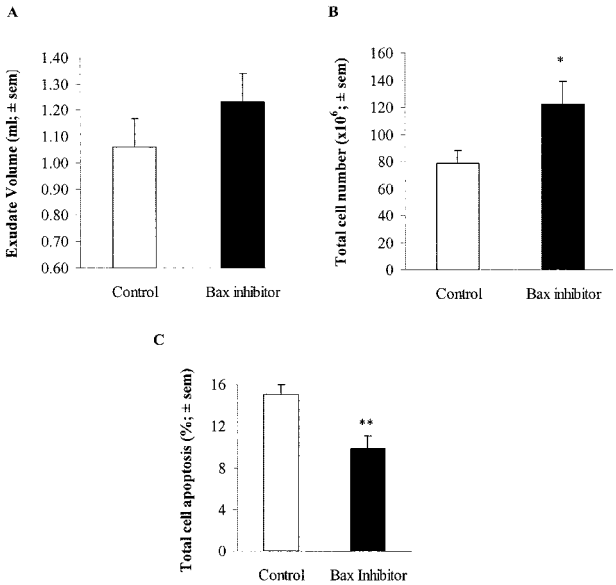

Figure 4. The Bax-inhibiting peptide V5 prevented the safe resolution of inflammation. Male Lewis rats were injected intrapleurally with 1% carrageenan and after 36 hours were treated intrapleurally with saline or 200 μmol/L Bax-inhibiting peptide V5. Twelve hours later edema formation (**A**) and total inflammatory cell numbers (**B**) were measured (48 hours after the onset of the carrageenan-induced pleurisy). Levels of apoptosis (**C**) were measured by Annexin V binding and analyzed by flow cytometry. Data are the mean of 10 animals per group ± SEM, $^{*}P < 0.05$, $^{**}P = 0.01$.

there was no significant difference in TGF-β_1 or IL-10 levels in any of the treatment groups (data not shown).

Discussion

The balance between prosurvival and proapoptotic signals is a highly regulated process involving many checkpoints that regulate cell survival and death.[1,25] Several proinflammatory mediators, such as fMLP,[26] tumor necrosis factor-α,[27] GM-CSF,[28] PAF,[29] LTB_4,[30] influence apoptosis of inflammatory cells,[31] and trigger MAPK pathways. This study has shown that during the initiation of acute inflammation, expression of prosurvival molecules, such as ERK1/2 and Bcl-x_L, are increased. Furthermore, we also show that the expression of these survival factors is down-regulated during the resolution phase. Conversely, the proapoptotic signaling molecule Bax is up-regulated when the inflammation is resolving with low expression levels found during the onset of inflammation. The functional role of cell signaling proteins, such as Bax, MEK1/2, and ERK1/2, in inflammation *in vivo* was previously undetermined.

This study demonstrates for the first time that apoptosis-modulating pharmacological inhibitors can promote or delay the resolution of acute inflammation. Importantly, the specific ERK1/2 inhibitor, PD98059, accelerates resolution of inflammation even after inflammation was established. It is difficult to predict in patients when inflammation will occur, even in chronic inflammatory diseases, and therefore, treatment during flare-ups is more beneficial than a prolonged prophylactic drug treatment regime. Furthermore, this is an advantageous therapeutic strategy that will allow neutrophil infiltration to combat invading pathogens but will limit persistent inflammation and excessive tissue damage. Premature neutrophil apoptosis during infection is likely to be detrimental to host defense and lead to bacterial infection and sepsis. This is a known complication in cystic fibrosis patients in which the *Pseudomonas aeruginosa*-derived exotoxin pyocyanin induces neutrophil apoptosis, which allows inflammatory cells and bacteria to persist contributing to pulmonary damage and patient mortality.[32]

The effects of endogenous or exogenous mediators depend on the kinetics of the synthesis or release or the timing of the administration of the inflammatory process. Studies have shown that in the carrageenan-induced paw edema model, apigenin had an anti-inflammatory effect when given at the onset of inflammation.[33] This effect would be consistent with the suppression of COX-2 inhibiting inflammation when given prophylactically. In the present study, the nonselective ERK1/2 inhibitor apigenin exacerbated inflammation, which in part is likely due to an inhibition of COX-2 because it has been shown that selective COX-2 inhibition by NS-398 and traditional NSAIDs, when given during the peak of inflammation, causes a prolonged inflammation.[34] This is likely due to inhibition of the anti-inflammatory cyclopentenone prostaglandins, 15deoxyδ,[12,14] PGJ_2, and PGD_2, which directly promote the apoptosis of neutrophils and macrophages *in situ.*[4] Further evidence of the anti-inflammatory role of COX-2 was also shown by Wallace and co-workers[35] in which inflammation failed to resolve in COX-2 knockout mice in a model of carrageenan-induced paw inflammation.

Here, we show that pretreatment with the specific ERK1/2 inhibitor PD98059 enhanced the resolution of acute inflammation, with attenuated total cell numbers of monocytes/macrophages and PMNs in the pleural cavity. This correlated with an increase in the number of free apoptotic neutrophils found after pretreatment with PD98059. The increased amount of free apoptotic cells was probably not due to a defective phagocytosis mechanism because previous studies have shown that PD98059 does not affect phagocytosis in many different *in vitro* assays.[36–38] In the present study, we also qualitatively show that the macrophage can still ingest apoptotic cells, as indicated in Figure 5C. The ability to visualize the free apoptotic neutrophils may be a result of a pronounced increase in apoptosis levels and a reduced number of the professional phagocytes (macrophages) in the pleural cavity. The persistence of free apoptotic cells has recently been suggested to be anti-inflammatory dependent on the environment that the macrophage is exposed to.[39,40] Interestingly, autologous administration of apoptotic cells has been clinically proven to help prevent heart, lung, and renal allograft transplant rejection.[41–44] This is thought to occur as a result of tolerogenic changes of immature dendritic cells after the engulfment of the apoptotic cells, which then stimulates inhibitory T-regulatory cells that suppress immune rejection.[45] This clearly shows that the administration of apoptotic cells and their safe engulfment in hu-

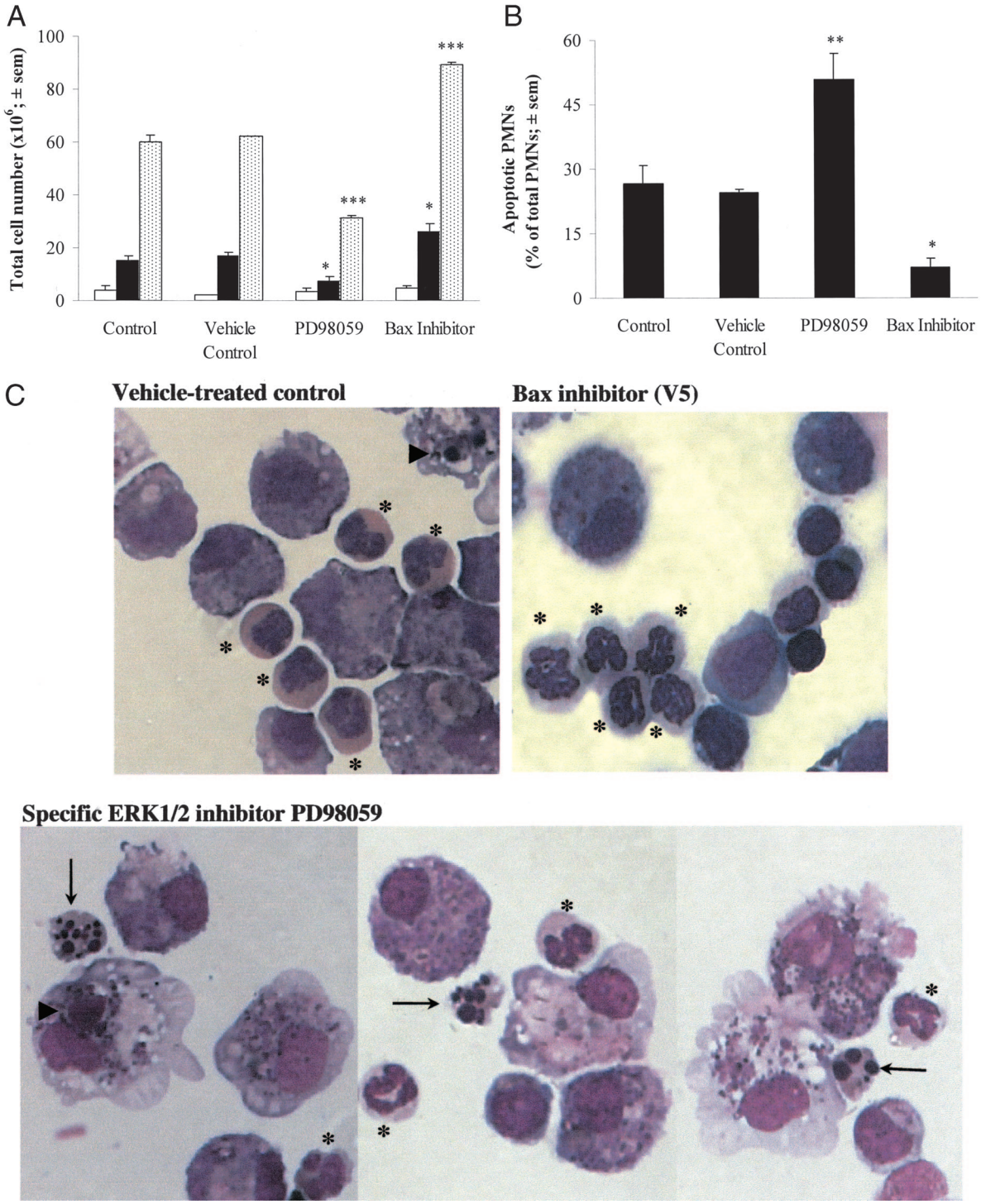

Figure 5. The specific ERK1/2 inhibitor PD98059 augments neutrophil apoptosis, whereas the Bax-inhibiting peptide promoted neutrophil survival. Male Lewis rats were injected intrapleurally with 1% carrageenan and after 36 hours were treated intrapleurally with saline, vehicle-treated control, 300 μmol/L PD98059, or 200 μmol/L Bax-inhibiting peptide V5. **A:** Twelve hours later, the total number of lymphocytes (**open bars**), neutrophils (**filled bars**), and monocytes/macrophages (**dotted bars**) was counted by flow cytometric analysis and differential cell counts by light microscopy. **B:** Neutrophil apoptosis was measured by morphological analysis by light microscopy after H&E staining with data presented as the percentage of apoptotic neutrophils from the total number of neutrophils present. Examples of this in a typical field are illustrated showing the distinct nuclei of apoptotic neutrophils (**arrows**), phagocytosed apoptotic neutrophils (**arrowhead**), and viable neutrophils (**asterisk**) after vehicle, PD98059, or Bax inhibitor (V5) pretreatment. Original magnifications, ×60.

mans has a role in the adaptive and the innate immune system by stimulating tolerance and promoting the resolution of inflammation. In this study, there is an overall diminished inflammatory response with PD98059 treatment in which increased apoptosis of neutrophils leads to an enhanced resolution of inflammation.

The accelerated resolution of inflammation was a specific effect for PD98059 because the MEK1/2 inhibitor

U0126 had no anti-inflammatory effect in terms of inflammatory cell numbers and apoptosis levels *in vivo*. U0126 has been shown to be specific for MEK1/2 and is noncompetitive with ERK and ATP.[46] This indicates that MEK1/2 may be disassociated from the ERK1/2 signaling pathway, which may provide a novel pathway for cell survival in inflammation. Alternatively U0126 could be ineffective in this model. Recently however, administration of U0126 at the onset of inflammation in a murine model of ovalbumin-induced asthma has been shown to be effective in reducing eosinophilia, proinflammatory cytokine release, and airway hyperresponsiveness.[47] In an *in vitro* study, there was a suppression of the release of eotaxin, RANTES, and GM-CSF from human airway smooth muscle cells with the MEK 1/2 inhibitor U0126; however, no effect was observed with a range of concentrations of the ERK1/2 inhibitor PD98059.[48] The authors argue that PD98059 has poor efficacy, which is different from the findings proposed here, in which this compound augmented the resolution of inflammation. The local concentrations of the PD98059 and U0126 compounds were consistent with EC_{50} values shown in other *in vitro* studies.[21]

The Bax-inhibiting peptide V5 is a cell-permeable pentapeptide that blocks the ku70 binding domain and prevents Bax conformational change and mitochondrial translocation.[49] This peptide has been shown to inhibit staurosporine-, UV-, and anti-cancer drug-induced apoptosis *in vitro.*[50] In the present study, inhibition of Bax during the resolving phase of acute inflammation exacerbated the inflammatory response *in vivo*. Specifically, total pleural cell numbers of monocytes/macrophages and PMNs were increased, with a corresponding decrease in the amount of neutrophil apoptosis, after pretreatment with the specific Bax inhibitor. Other studies by Maiuri and co-workers[51] show that induction of Bax via inhibition of nuclear factor-κB in a carrageenan sponge model of chronic inflammation is associated with the apoptosis of neutrophils.

A decrease in total cell apoptosis, which could be attributed mainly to neutrophil apoptosis, was observed with the Bax peptide inhibitor. In contrast, an increase in neutrophil apoptosis contributed to an enhanced resolution with the ERK1/2 inhibitor PD98059. The percent total apoptosis of pleural cells in control and drug-treated animals were relatively small. This likely reflects the difficulty in visualizing and measuring apoptosis due to efficient clearance by phagocytosis. There is also a very limited window of opportunity to visualize free apoptotic cells *in vivo*, in which the estimated duration of detectable apoptosis due to rapid recognition and efficient engulfment by phagocytes is only 1 to 2 hours.[52,53] However, even the levels of apoptosis that we have observed may have significant biological effects because studies have shown that small changes in apoptosis levels can drastically affect the overall total cell number. For example, in the rat liver an increase of 2% apoptosis by histological examination results in an overall 25% reduction in cell number.[54] Furthermore, our previous work shows that an approximate 3% induction of apoptosis contributes to an

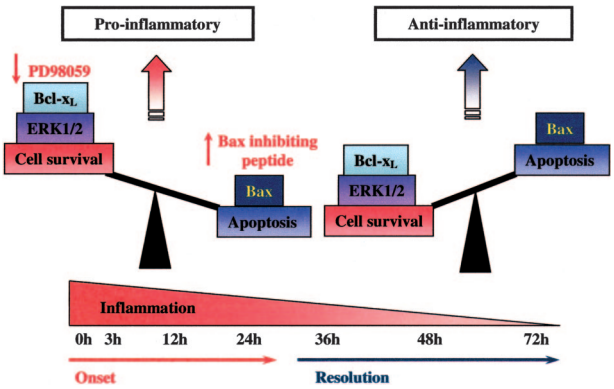

Figure 6. A schematic diagram showing the balance between prosurvival and proapoptotic signaling molecules from the onset to the resolution of acute inflammation. This figure shows that prosurvival proteins such as ERK1/2 and Bcl-x_L predominate during the onset of inflammation, which when inhibited promotes apoptosis and aids resolution. The expression of proapoptotic molecules such as Bax are relatively low at this time point. Conversely, during resolution of inflammation, the balance switches toward the expression of proapoptotic signaling molecules, Bax, which ultimately promotes cell death and safe clearance by phagocytosis.

80% reduction in the number of neutrophils in the carrageenan-induced pleurisy model of inflammation *in vivo*.[4]

Results of previous studies suggest that phagocytosis of apoptotic cells results in release of anti-inflammatory cytokines, such as TGF-β_1[3] and IL-10.[55] Furthermore, the released TGF-β_1 is capable of inhibiting the release of proinflammatory cytokines modulated via p38 MAPK whereby the inhibitor, SB 203580, prevented TGF-β_1-mediated inhibition of lipopolysaccharide-induced tumor necrosis factor-α and MIP-2 release.[56] In this study, however, there was no significant difference in TGF-β_1 or IL-10 levels found using the Bax inhibitor-, PD98059-, or the U0126-treated groups (data not shown). However, silent phagocytosis of early apoptotic cells can occur without affecting cytokine levels.[57] The levels of proinflammatory cytokines, such as tumor necrosis factor-α and IL-1β, are insignificant during the resolution of inflammation; therefore, we were unable to establish whether inducing or preventing apoptosis *in vivo* affects these cytokines in particular.

Although we have clearly shown in this study that manipulation of pathways that influence apoptosis (namely ERK1/2 and Bax) can accelerate the resolution of an acute model of inflammation, the question remains whether our observations could be extended to a more chronic inflammatory scenario. Many of the pathways that we have investigated here can affect cell recruitment and activation, in addition to influencing cell survival and apoptosis. Furthermore, it is now becoming clear that MAPK signaling pathways and proteins involved in the regulation of apoptosis are dysregulated in a number of pathological inflammatory conditions, such as asthma.[12] The challenge now is to investigate whether such manipulation of these pathways influences the resolution phase of inflammation in animal models of chronic inflammation or, more importantly, whether chronic inflammatory diseases in humans can be beneficially influenced. The clinical trials currently underway with compounds that directly influence apoptosis (eg, the caspase inhibitors[58])

and compounds that have an indirect effect on cell survival (eg, JNK, ERK, and p38 kinase inhibitors[59]) will identify whether manipulation of apoptosis would be clinically beneficial for chronic inflammatory diseases.

Overall this study shows that direct modulation of apoptosis through interfering with the signaling of important molecules (ERK 1/2, Bcl-x_L, Bax) can exacerbate or attenuate cell infiltrate and edema formation. Thus evidence from *in vitro* data and from the evidence presented here suggest that promoting apoptosis and safe clearance of effete cells may be a valuable new therapeutic target for inflammatory diseases.[60] There is also a greater need to elucidate effective mechanisms of inducing apoptosis of inflammatory cells *in vivo* and whether this affects the outcome of the disease process once inflammation is established. This study emphasizes that there is an intricate balance of survival and apoptotic signaling pathways that mediate the kinetics of the acute inflammatory response, as illustrated in Figure 6. The enhanced resolution of inflammation by the inhibition of the prosurvival mediators, such as ERK 1/2 with PD98059 may prove to be an important advancement for the development of new treatments of inflammatory diseases.

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