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Aspirin and salicylate suppress polymorphonuclear apoptosis delay mediated by pro-inflammatory stimuli

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Running title: Salicylates decrease PMN survival

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Nonstandard abbreviations: ASA: acetyl salicylic acid; COX: cyclooxygenase; G-CSF: granulocyte-colony stimulating factor; GM-CSF: granulocyte monocyte-colony stimulating factor; IL: Interleukin; LPS: lipopolysaccharide; LXA4: 15-epi-lipoxin A4; NaSal: sodium salicylate; NFκB: Nuclear Factor kappa B; PMN: polymorphonuclear leukocyte; TNFα: tumor necrosis factor alpha.

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Abstract

During inflammation, polymorphonuclear leukocytes (PMN) apoptosis can be delayed by different pro-inflammatory mediators. Classically, it has been accepted that the widely used anti-inflammatory drug acetyl salicylic acid (ASA) exerts its action through inhibition of cyclooxygenases and subsequent prostaglandin synthesis. We hypothesized that another anti-inflammatory action of ASA could be the shortening of PMN survival. We found that at therapeutic concentrations (1-3 mM) ASA and its metabolite salicylate (NaSal), but not Indomethacin or Ibuprofen, counteracted the prolonged PMN survival mediated by lipopolysaccharide (LPS) through inhibition of NFκB activation. Both salicylates also inhibited IL-1α or acidic conditions antiapoptotic activity. Higher concentrations of both drugs had a direct apoptotic effect. Salicylates were not effective when PMN apoptosis delay was induced by GM-CSF, a NFκB-independent cytokine. Promotion of PMN survival by the combination of IL-1α and LPS was also reversed by salicylates, but higher concentrations were required. ASA concentrations that did not trigger PMN death, increase the Zymosan- or TNFα-mediated proapoptotic effect. The LPS- and IL-1α but not GM-CSF-mediated antiapoptotic effect was markedly reduced in PMN from donors who had ingested ASA. Using a thioglycolate-induced peritonitis model, we showed that in ASA or NaSal-treated mice there was not only a decrease in the number of cells recruited but also an increase in the percentage of apoptotic PMN as well as an enhancement of phagocytosis compared with controls. Our findings demonstrate that acceleration of PMN apoptosis by turning off the NFκB-mediated survival signals elicited by pro-inflammatory stimuli is another anti-inflammatory action of ASA and NaSal.

Introduction

Polymorphonuclear leukocytes (PMN) are short-lived phagocytic leukocytes that are rapidly recruited from the bloodstream to the site of tissue damage or infection. PMN dominate the early response against invading microbes. During inflammation, and to optimize PMN bactericidal function, a range of inflammatory mediators including interleukin-1 alpha (IL-1α), granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte-CSF (GM-CSF), lipopolysaccharide (LPS) or even acidic conditions function to prolong PMN lifespan (Trevani et al., 1999; Akgul et al., 2001). After killing the phagocytosed microbe, PMN die by apoptosis. Both *in vitro* and *in vivo* evidence suggest that PMN apoptosis and PMN clearance by macrophages are the major mechanisms for promoting resolution of inflammation (Savill and Fadok, 2000). In fact, dysregulation of apoptosis will lead to persistence of cell exudates at inflammatory sites and has been implicated in the pathogenesis of chronic inflammatory diseases including glomerulonephritis and systemic lupus erythematosus (Taylor et al., 2000).

Acetyl salicylic acid (ASA), also named aspirin, is a widely used non-steroidal anti-inflammatory drug. Although suppression of pro-inflammatory prostaglandins synthesis by inhibition of cyclooxygenases (COX) is the major effect of ASA and its metabolite, salicylate (Mitchell et al., 1993; Xu et al., 1999), other mechanisms, including the synthesis of 15-epi-lipoxin A4 (LXA4) and the release of adenosine (Cronstein et al., 1999) or nitric oxide (Paul-Clark et al., 2004) also appear to be involved in the anti-inflammatory action of ASA.

Nuclear factor kappa B (NFκB) is a critical regulator of the innate early pathogen response, playing an important role in promoting inflammation, in the control of cell proliferation and survival of many cell types. NFκB normally exists as an inactive cytoplasmic complex whose predominant form is a heterodimer composed of p50 and

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p65 subunits tightly bound to inhibitory proteins of the IκB family (Baldwin, 1996). NFκB is activated by numerous diverse agents including cytokines, viral infection, UV radiation and free radicals. Upon activation, the inhibitors are phosphorylated and rapidly degraded allowing NFκB to translocate into the nucleus to activate target genes (Birbach et al., 2002). The *in vitro* observation that ASA and sodium salicylate (NaSal) inhibit activation of NFκB transcription factor has suggested that these non-steroidal anti-inflammatory drugs may also act at the transcriptional level (Kopp and Ghosh, 1994). In PMN, activation of NFκB controls the expression of many anti-apoptotic genes including members of the Bcl-2 family (Moulding et al., 2001). We therefore hypothesize that salicylates also exert their anti-inflammatory action by interfering with PMN survival.

Methods

Isolation of human PMN. PMN were isolated from peripheral blood drawn from healthy donors who had provided informed written consent subsequent to approval from institutional ethic committees. PMN were isolated by Ficoll Hypaque (δ=1077) gradient centrifugation (Ficoll; Pharmacia, Uppsala, Sweden) (Hypaque; Winthrop Products, Buenos Aires, Argentina) and Dextran sedimentation, as previously described (Trevani et al., 1999). Cell suspensions contained >96% neutrophils, as determined by May Grunwald-Giemsa-stained cytopreps, and the levels of monocyte contamination were always <0.2%, as evaluated by CD14 staining and flow cytometry. The cells were suspended in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Invitrogen) at a concentration of 2.5 x 10^6/ml.

Cell cultures. PMN suspensions (100 μl aliquots) were placed in 96-well flat-bottom microplates in a humidified incubator at 37 ºC and 5% CO_2. After a 30 min incubation without (controls) or with non-steroid antiinflammatory drugs (ASA, NaSal, Indomethacin, Ibuprofen, all from Sigma, Saint Louis, MO), cells were stimulated with LPS, IL-1α, GM-CSF (Sigma) or the pH was adjusted to 6.5 by addition of isotonic HCl. All drugs were freshly prepared for each experiment and dissolved in RPMI with exception of Indomethacin that was dissolved in ethanol. The final ethanol concentrations (0.1% v/v) did not induce PMN cytotoxic effects.

Quantitation of PMN apoptosis and viability by fluorescence microscopy. Cells were analyzed for changes in morphology and viability by labeling cells with a mixture of the fluorescent DNA-binding dyes: acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to

differentiate between viable and nonviable cells (Pozner et al., 2005). Necrotic cells never exceeded 1%.

Quantitation of PMN apoptosis by propidium iodide staining and flow cytometry. Hypodiploid cells were detected by flow cytometry as previously described (Pozner et al., 2005). PMN were suspended in 1 ml of ice-cold 70% ethanol for 30 min, washed three times, and then resuspended in a solution containing RNAse (1 mg/ml) (Sigma) and propidium iodide (2 µg/ml) (Sigma). After 15 min, cells were analyzed by flow cytometry. The red fluorescence of individual nuclei was measured using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Annexin-V binding assay. Phosphatidylserine expression was detected by Annexin-V binding and PI staining using a commercial kit (Sigma). Briefly, 20 h after different treatments, PMNs ($1.5x10^5$) were washed and incubated for 30 min with Annexin-V-FITC at 37ºC. Cells were then resuspended in 400 µl of binding buffer containing PI and immediately analyzed by flow cytometry.

Degradation of IκBα by Western blot analysis. Whole-cell lysates were prepared using a 3% sodium dodecyl sulphate (SDS) lysis buffer (Boehringer Mannheim, Mannheim, Germany). Lysates were boiled for 5 min and protein was quantified using the bicinchoniancidic protein assay (Pierce, Rockford, IL). Samples were then frozen at -80ºC until use. Equal amounts of proteins (40 µg per sample) were separated by electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were then electro-transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell, Keene, NH). After blocking, membranes were incubated overnight at 4ºC with a rabbit

polyclonal anti-IκBα antibody (BD Pharmigen, Franklin Lake, NJ) followed by a goat anti-rabbit-horseradish peroxidase secondary antibody (Cell Signaling technologies Inc, Beverly, MA). Protein bands were visualized by enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech, England).

Animals and drug dosing. Male BALB/c mice (18-22 g) between 8-10 wk of age were housed and bred in the animal facility, Hematological Research Institute under the Argentine Home Office regulations for the care and use of animals. ASA (200 mg/kg), NaSal (200 mg/kg) or vehicle was dosed orally 24 and 1 h before initiation of peritonitis or blood collection.

Isolation of murine PMN. Blood samples were obtained by puncture of the retro-orbital plexus. PMN were isolated by Ficoll-Hypaque (δ=1090) gradient centrifugation followed by Dextran sedimentation and then resuspended in RPMI-1640 ($2.5x10^6$/ml) supplemented with 10% fetal calf serum (Gomez et al., 2005). Monocyte contamination determined by May-Grunwald Giemsa-stained blood smears was always < 10%.

Animal model of inflammation. Mice were injected intraperitoneally with thioglycolate (3%) in sterile PBS as previously described (Potter et al., 2003). Exudates were collected 4 h after stimulation by lavaging the cavity with 5 ml of PBS/EDTA (5 mM) and then samples were kept on ice. Lavage fluids were stained with Turk's solution (0.01% crystal violet in 3% acetic acid) and cell counts were performed by light microscopy using a Neubauer hemocytometer. Phagocytosis was scored on May-Grunwald stained cytospins. Between 300 and 400 macrophages were counted and the phagocytosis was expressed as the percentage of macrophages that contained >50% of

an apoptotic body within its body (Fadok et al., 1992). PMN apoptosis was quantified by flow cytometry, co-staining the cells with Annexin-V-FITC (Sigma) and biotinylated anti-Gr-1 antibody (which specifically recognizes PMN) followed by Streptavidin-PE (BD PharMingen).

Statistical analysis. Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test to determine significant differences between groups. P values < than 0.05 were considered statistically significant.

Results

Aspirin and NaSal inhibit delay of PMN apoptosis induced by several pro-inflammatory stimuli.

In the first experiments, we analyzed the effect of ASA and its metabolite, NaSal on PMN life span. When apoptosis was revealed by fluorescence microscopy using the fluorescent DNA-binding dyes acridine orange and ethidium bromide, we found that both drugs alone significantly triggered PMN programmed cellular death only at the highest concentration employed (necrosis was always <1%.) (Figure 1 A). Similar results were obtained when apoptosis was analyzed by Annexin-V-FITC/PI staining (Figure 1 B). In agreement with previous reports (Colotta et al., 1992), we observed that stimulation of PMN with LPS (0.5 μg/ml) promotes cell survival. However, pre-incubation with ASA or NaSal significantly suppressed the LPS-mediated increase in PMN survival in a concentration dependent manner (Figure 2 A and B).

Because, in addition to LPS, cytokines and the acidic conditions of the inflammatory milieu can also modulate PMN death rate (Colotta et al., 1992; Trevani et al., 1999), we also studied the effect of salicylates on the prolonged PMN survival mediated by three different stimuli: IL-1α, GM-CSF and acidic conditions. Table 1 summarizes the results obtained by detection of hypodiploid nuclei in permeabilized cells stained with PI and flow cytometry. Either ASA or NaSal at low concentration (1mM) almost completely prevented the IL-1α (10 ng/ml) or pH 6.5 antiapoptotic effect. In contrast, the cellular death protection mediated by GM-CSF (10 ng/ml) was not changed by PMN incubation with ASA or NaSal. Although at higher concentrations of both drugs the GM-CSF cytoprotection was slightly reduced, it was still significantly different compared to salicylates alone (Figure 3).

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When the delay in PMN apoptosis was induced by the simultaneous addition of LPS (0.5 μg/ml) and IL-1α (10 ng/ml), ASA and NaSal (3 mM) were also effective, (Control:45±7; LPS+IL-1α:10±4; ASA+LPS+IL-1α:29±7*; NaSal+LPS+IL-1α:38±6* % of apoptotic cells, n=4, *$p<0.05$ vs LPS + IL-1α).

ASA and NaSal synergized with pro-apoptotic stimuli

During the course of inflammation, macrophages, lymphocytes and/or mast cells produce TNFα (Vassalli, 1992; Tracey and Cerami, 1993). It has been shown that while prolonged incubations (>12 h) of human neutrophils with TNFα can cause a decrease in apoptosis, it can also induce programmed cell death in a sub-population of cells at earlier times of incubation (<8 h) (Murray et al., 1997). Thus, we examined the effect of ASA on apoptosis induced by a 3 h exposure to TNFα and other proapoptotic stimulus such as Zymosan. Although at these early times ASA was unable to induce PMN apoptosis *per se*, it did enhance programmed cell death mediated by TNFα (10 ng/ml) or Zymosan (150 μg/ml) (Figure 4). Similar results were obtained with NaSal (data not shown).

Salicylates prolong PMN survival by a cyclooxygenase independent pathway

Whereas ASA inhibits COX-1 and -2 activities by acetylating the enzyme (Mitchell et al., 1993), salicylic acid interferes with COX-2 transcription (Xu et al., 1999). Although the involvement of COX-2 in the LPS-mediated delay of PMN apoptosis has not been described, it is known that LPS triggers COX-2 transcription in several cell types including neutrophils (Maloney et al., 1998). Thus, in the next experiments we analyzed whether other NSAIDs not structurally related with salicylates, but acting through COX inhibition, were able to accelerate PMN death. We found that in contrast to salicylates,

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preincubation of PMN with Indomethacin (100 μM) or Ibuprofen (100 μM) did not modify the antiapoptotic activity exerted by LPS (0.5 μg/ml) (Control: 55±11; LPS: 27±4 and Indomethacin+LPS: 24±6; Ibuprofen+LPS: 27±5 % of apoptotic cells, n=5) suggesting that salicylates effect was independent of COX .

It has been previously described that inhibition of NFκB activation might be another mechanism by which ASA or NaSal exerts its anti-inflammatory action (Kopp and Ghosh, 1994). The involvement of NFκB pathway was evaluated directly by Western blot analysis of its inhibitor, IκBα. As shown in Figure 5, while LPS (0.5 μg/ml) degraded intracellular IκBα, pretreatment of PMN with ASA or NaSal markedly inhibited this effect. In contrast, Indomethacin or Ibuprofen had no effect.

LPS and IL-1α, but not GM-CSF, antiapoptotic effect is inhibited by ASA ingestion

To take these experiments further, we examined the antiapoptotic effect of LPS (0.5 μg/ml), IL-1α (10 ng/ml) or GM-CSF (10 ng/ml) on PMN obtained from individuals who had taken 2.0 g of ASA the day before sampling. Remarkably, while GM-CSF effect was not modify, the LPS- or IL-1α-mediated delay of PMN death was significantly reduced in PMN from ASA-treated donors (Figure 6).

ASA and NaSal promote resolution of inflammation by accelerating PMN apoptosis.

In the next experiments we examined the effect of both salicylates in the removal of PMN using the thioglycolate-elicited peritonitis experimental model. In ASA-or NaSal-treated-animals we observed a dramatic reduction in the total cell number recruited 4 h after thioglycolate-injection (Figure 7 A). Since nuclear morphological changes alone

did not allow the accurate identification of the cell type that was undergoing apoptosis, we determined the presence of apoptotic PMN by co-staining the cells with Annexin-V-FITC and biotinylated anti-Gr-1 antibody (which specifically recognizes PMN) followed by Streptavidin-PE (Fadok et al., 1992). Figure 7 B demonstrates that in ASA or NaSal-treated animals there was a significant increase in the percentage of PMN showing Annexin-V positive cells. This was consistent with a 2.5 fold increase in the percentage of phagocytosis (Figure 7 C and Fig. 8). This data strongly suggest that both ASA and NaSal accelerate resolution of inflammation by shortening PMN life span and promoting engulfment of PMNs by resident macrophages. Finally, we analyzed whether the apoptosis of PMN from ASA- or NaSal-treated mice was also increased *ex-vivo* after LPS- or IL-1α-stimulation. As observed in human donors, the apoptosis delay mediated by both compounds was significantly reduced in PMN from ASA or NaSal-treated mice (Control: 39±2, LPS: 22±3***, IL-1α: 24±4***, ASA: 39±4, LPS + ASA: 34±4$^{\#}$, IL-1α + ASA: 34±3$^{\#}$, NaSal: 40±3, LPS + NaSal: 36±5$^{\#}$, IL-1α + NaSal: 37±2$^{\#}$, n=8, ***$p<0.001$ vs Control and $^{\#}p<0.05$ vs LPS or IL-1α alone).

Discussion

Our observations revealed that both *in vitro and ex-vivo,* ASA and NaSal abrogated the survival signals elicited in human PMN by several different pro-inflammatory stimuli. Considering that the observed salicylate effect was obtained using similar concentrations to those attained in plasma during anti-inflammatory therapy (1-3 mM), (Weissmann, 1991) our data strongly suggest that interference with PMN survival represents an additional mechanism by which both salicylates exert its anti-inflammatory action. Of note, higher ASA or NaSal concentrations showed a direct pro-apoptotic effect. Although these are not common therapeutic doses, as salicylates accumulate at the mildly acidic environment (Brooks and Day, 1991; Weissmann, 1991) it cannot be rule out that such salicylates levels might be reached at sites of inflammation. Interestingly, we demonstrated that not only cytokines or LPS-mediated prolongation of PMN lifespan was abrogated by ASA or salicylate but also the prevention mediated by extracellular acidosis, a condition frequently associated not only with a variety of inflammatory conditions (Edlow and Sheldon, 1971) but also with tumor microenvironments (Tannock and Rotin, 1989).

Although COX activity or transcription is inhibited by ASA and NaSal respectively (Vane, 1971; Xu et al., 1999), this pathway appeared not to be involved in acceleration of PMN death since Indomethacin or Ibuprofen, structurally unrelated inhibitors of COX, were unable to modify the antiapoptotic activity exerted by LPS. The observation that LPS-mediated degradation of the NFκB inhibitor, IκBα, was completely suppressed by pretreatment with ASA or NaSal, revealed that blockade of NFκB activation is at least one of the signaling pathways mediating the salicylate-induced shortening of PMN half-life. Moreover, our data showing that Indomethacin or Ibuprofen did not block LPS-mediated degradation of IκBα and that prolongation of PMN survival mediated by

GM-CSF, a cytokine that exerts its effect independently of NFκB translocation (McDonald et al., 1997), was not modified by salicylates, further supports the involvement of this transcription factor in ASA or NaSal proapoptotic activity. It is noteworthy that under conditions where apoptotic nuclei were not yet observed (3 hours post ASA or salicylate treatment) both drugs were capable to potentiate the pro-apoptotic effect of TNFα or Zymosan. These results are in agreement with previous reports showing that NFκB inhibitors induced PMN apoptosis or augmented the early pro-apoptotic effects of TNFα (Ward et al., 1999).

It was previously demonstrated that in rat primary neuronal cultures and hippocampal slices, activation of NFκB also accounted for the protective action of salicylates against neurotoxicity elicited by glutamate (Grilli et al., 1996). Therefore, it is quite possible that, depending on whether the stimulus for NFκB activation triggers anti-apoptotic proteins or pro-apoptotic proteins salicylates could selectively promote cell survival or accelerate programmed cell death respectively.

During the preparation of this manuscript Derouet M *et al* (Derouet et al., 2006), reported that NaSal greatly enhanced neutrophils apoptosis by modification in the rate of Mcl-1 turnover through modulation of the activities of members of the MAPK family. Thus, it appears that several pathways might be involved in NaSal-mediated regulation of PMN survival. More experiments are required to determine whether MAPK transduction pathway is also regulated by ASA.

Recent data obtained from *in vivo* inflammation models indicate that the reduction of leukocyte accumulation within the inflammed areas is another relevant mechanism by which salicylates exert their anti-inflammatory action. Whereas in the air-pouch model both ASA and salicylate-mediated inhibition of PMN influx was associated with the release of adenosine (Cronstein et al., 1999), in thioglycolate-induced peritonitis ASA-

mediated induction of nitric oxide release potently inhibits leukocyte/endothelium interaction preventing leukocyte migration (Paul-Clark et al., 2004). This latter effect, which was not shared by NaSal, was related to the acetylation of the active site of inducible COX and generation of a family of lipid mediators called the epi-lipoxins, mainly LXA4. Using the same model, we not only found that both ASA and NaSal treated-animals showed a marked decrease in leukocyte influx, but we also demonstrate that removal of cells from the inflammed peritoneal cavity was associated with an increased percentage of apoptotic PMN that correlated with an enhanced macrophage phagocytosis. Although we did not directly address the mediator involved in these *in vivo* salicylates phenomena, our data and previous findings showing that ASA inhibits the production of several inflammatory mediators through the inhibition of NFκB activation (Pernerstorfer et al., 1999; Tegeder et al., 2001; Yoo et al., 2001), suggest that the reduced accumulation of peritoneal exudates cells and the augmentation of PMN apoptosis in ASA- or NaSal-treated mice could be related to NFκB inhibition resulting in both, a reduced transduction of PMN antiapoptotic signals and suppression of pro-inflammatory substances responsible for the delay in PMN survival. In the same line of evidence, it was demonstrated that administration of a membrane-permeable IκBα repressor resulted in a diminished infiltration of cells together with an increased apoptosis of leukocytes at sites of inflammation (Blackwell et al., 2004).

Finally, we examined the effect of ASA intake in human *ex-vivo* experiments and we found that the antiapoptotic activity of LPS or IL-1α, but not of GM-CSF, was significantly reduced in PMN from normal individuals who had ingested ASA. The fact that the increased PMN apoptosis was only observed with those agonists acting through NFκB, strength the involvement of this signaling pathway in the ASA effect. It is well known that cyclooxygenase inhibition by aspirin is dose-dependent, rapid and

irreversible and the length of its effect depends on cell capability to synthesize new protein (Higgs GA, Br. Med. Bull. 1983). In contrast, the kinetics of NFkB inhibition by aspirin is less known. Considering that it was demonstrated that ASA binding to IKK beta kinase, although not covalent, is very slowly reversible (Yin MJ, Nature 1998), it is possible to speculate that a similar *de novo* protein synthesis-dependent mechanism also regulates the extent of NFkB inhibition mediated by aspirin. In this regard it would be conceivable that while a single dose of aspirin would transiently block NFkB pathway, a regular ingestion (e.g., treatment for chronic inflammatory diseases) would result in a permanent suppression. However, more studies are required to determine the precise pharmacokinetics of NFkB inhibition by salicylates.

In conclusion, our results provide another mechanism by which salicylates reduce leukocyte accumulation and promote resolution of inflammation: turning off the PMN NFκB-mediated survival signals elicited by pro-inflammatory stimuli, acceleration of PMN apoptosis and enhancement of PMN removal from the inflammatory area.

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References

Akgul C, Moulding DA and Edwards SW (2001) Molecular control of neutrophil apoptosis. *FEBS Lett* **487**:318-322.

Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**:649-683.

Birbach A, Gold P, Binder BR, Hofer E, de Martin R and Schmid JA (2002) Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. *J Biol Chem* **277**:10842-10851.

Blackwell NM, Sembi P, Newson JS, Lawrence T, Gilroy DW and Kabouridis PS (2004) Reduced infiltration and increased apoptosis of leukocytes at sites of inflammation by systemic administration of a membrane-permeable IkappaBalpha repressor. *Arthritis Rheum* **50**:2675-2684.

Brooks PM and Day RO (1991) Nonsteroidal antiinflammatory drugs--differences and similarities. *N Engl J Med* **324**:1716-1725.

Colotta F, Re F, Polentarutti N, Sozzani S and Mantovani A (1992) Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**:2012-2020.

Cronstein BN, Montesinos MC and Weissmann G (1999) Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NFkappaB. *Proc Natl Acad Sci U S A* **96**:6377-6381.

Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ and Edwards SW (2006) Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. *J Immunol* **176**:957-965.

Edlow DW and Sheldon WH (1971) The pH of inflammatory exudates. *Proc Soc Exp Biol Med* **137**:1328-1332.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL and Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148**:2207-2216.

Gomez SA, Fernandez GC, Camerano G, Dran G, Rosa FA, Barrionuevo P, Isturiz MA and Palermo MS (2005) Endogenous glucocorticoids modulate neutrophil function in a murine model of haemolytic uraemic syndrome. *Clin Exp Immunol* **139**:65-73.

Grilli M, Pizzi M, Memo M and Spano P (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* **274**:1383-1385.

Kopp E and Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**:956-959.

Maloney CG, Kutchera WA, Albertine KH, McIntyre TM, Prescott SM and Zimmerman GA (1998) Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *J Immunol* **160**:1402-1410.

McDonald PP, Bald A and Cassatella MA (1997) Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* **89**:3421-3433.

Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ and Vane JR (1993) Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci U S A* **90**:11693-11697.

Moulding DA, Akgul C, Derouet M, White MR and Edwards SW (2001) BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol* **70**:783-792.

Murray J, Barbara JA, Dunkley SA, Lopez AF, Van Ostade X, Condliffe AM, Dransfield I, Haslett C and Chilvers ER (1997) Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* **90**:2772-2783.

Paul-Clark MJ, Van Cao T, Moradi-Bidhendi N, Cooper D and Gilroy DW (2004) 15-epi-lipoxin A4-mediated induction of nitric oxide explains how aspirin inhibits acute inflammation. *J Exp Med* **200**:69-78.

Pernerstorfer T, Stohlawetz P, Hollenstein U, Dzirlo L, Eichler HG, Kapiotis S, Jilma B and Speiser W (1999) Endotoxin-induced activation of the coagulation cascade in humans: effect of acetylsalicylic acid and acetaminophen. *Arterioscler Thromb Vasc Biol* **19**:2517-2523.

Potter PK, Cortes-Hernandez J, Quartier P, Botto M and Walport MJ (2003) Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells. *J Immunol* **170**:3223-3232.

Pozner RG, Negrotto S, D'Atri LP, Kotler ML, Lazzari MA, Gomez RM and Schattner M (2005) Prostacyclin prevents nitric oxide-induced megakaryocyte apoptosis. *Br J Pharmacol* **145**:283-292.

Savill J and Fadok V (2000) Corpse clearance defines the meaning of cell death. *Nature* **407**:784-788.

Tannock IF and Rotin D (1989) Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* **49**:4373-4384.

Taylor PR, Carugati A, Fadok VA, Cook HT, Andrews M, Carroll MC, Savill JS, Henson PM, Botto M and Walport MJ (2000) A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J Exp Med* **192**:359-366.

Tegeder I, Pfeilschifter J and Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* **15**:2057-2072.

Tracey KJ and Cerami A (1993) Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* **9**:317-343.

Trevani AS, Andonegui G, Giordano M, Lopez DH, Gamberale R, Minucci F and Geffner JR (1999) Extracellular acidification induces human neutrophil activation. *J Immunol* **162**:4849-4857.

Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* **231**:232-235.

Vassalli P (1992) The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* **10**:411-452.

Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C and Rossi AG (1999) NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem* **274**:4309-4318.

Weissmann G (1991) Aspirin. *Sci Am* **264**:84-90.

Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M and Wu KK (1999) Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci U S A* **96**:5292-5297.

Yoo CG, Lee S, Lee CT, Kim YW, Han SK and Shim YS (2001) Effect of acetylsalicylic acid on endogenous I kappa B kinase activity in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **280**:L3-9.

Footnotes

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Legends for Figures

Figure 1. PMN were treated with or without ASA or NaSal. Apoptosis was evaluated 20 h later by fluorescence microscopy (mean±SEM, n=10) (A) or Annexin-V-FITC/PI staining (B). One representative experiment from 4 independent experiments performed in duplicate is shown in B. ***p<0.001 vs control.

Figure 2. PMN with or without ASA or NaSal were treated or not with LPS (0.5 µg/ml). Apoptosis was evaluated 20 h later by fluorescence microscopy (mean±SEM, n=10) (A) or propidium iodide staining and flow cytometry (B). One representative experiment from 5 independent experiments performed in duplicate is shown in B. ***p<0.001 vs ASA or NaSal 0 mM (open column), $^{\#\#}$p<0.01 vs ASA or NaSal 0 mM (closed column).

Figure 3. PMN with or without ASA or NaSal were treated or not with GM-CSF (10 ng/ml). Apoptosis (mean±SEM, n=4) was evaluated 20 h later by propidium iodide staining and flow cytometry. ***p<0.001 vs ASA or NaSal 0 mM (open column), $^{\#}$p<0.05 vs ASA or NaSal 0 mM (closed column), $^{\Psi}$p<0.05 or $^{\Psi\Psi}$p<0.01 vs the same concentration of ASA or NaSal without GM-CSF.

Figure 4. PMN were incubated without or with ASA before Zymosan (150 µg/ml) or TNFα (10 ng/ml) addition. After 3 h, apoptotic nuclei (mean±SEM, n=4) were detected by fluorescence microscopy. *p<0.05 vs control, $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 vs TNFα or Zymosan alone.

Figure 5. PMN incubated for 15 min without or with ASA (3mM), NaSal (3mM), Indomethacin (Ind, 100 μM) or Ibuprofen (Ibu, 100 μM) were stimulated with LPS (0.5 μg/ml) for 40 min. IκBα levels were determined by Western blot analysis (mean ± SEM, n=5). *$p<0.05$ vs control, $^{\#}p<0.05$ vs LPS alone.

Figure 6. PMN obtained from donors who had (white bars) or had not (black bars) ingested ASA (2 g) were stimulated with LPS (0.5 μg/ml), IL-1α (10 ng/ml) or GM-CSF (10 ng/ml). Apoptosis was quantified 20 h later by fluorescence microscopy (mean ± SEM, n=9). *$p<0.05$ and ***$p<0.001$ vs control (closed column), $^{\#}p<0.05$ vs LPS or IL-1α (closed column).

Figure 7. Total cell number (A), percentage of apoptotic PMN determined by two-color flow cytometry analysis (B) and percentage of phagocytosis (C) are shown for peritoneal lavage recovered from mice after thioglycolate-induced inflammation. Horizontal bars denote means (n=5). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs control.

Figure 8. Representative photomicrographs of May Grunwald-Giemsa stained cytospins of peritoneal lavage cells from BALB/c mice. Cells shown were recovered from mice treated orally with vehicle (A), ASA (B) or NaSal (C) after thioglycolate-induced inflammation. The presence of engulfed PMN within the cytoplasm of macrophages is identified by arrows. Original magnification 400X.

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Table 1. Effect of salicylates on the prolonged PMN survival mediated by several pro-inflammatory mediators.

	Apoptosis (%)		
	Control	ASA (1 mM)	NaSal (1mM)
None	40 ± 3	41 ± 4	45 ± 3
pH 6.5	28 ± 3[a]	43 ± 3[b]	44 ± 3[b]
IL-1α (10 ng/ml)	20 ± 5[a]	34 ± 6[b]	36 ± 5[b]
GM-CSF (10 ng/ml)	19 ± 5[a]	21 ± 10	20 ± 5

[a]$p<0.05$ vs None/Control, [b]$p<0.05$ vs pH 6.5 or IL-1α without ASA or NaSal/Control (n=10).

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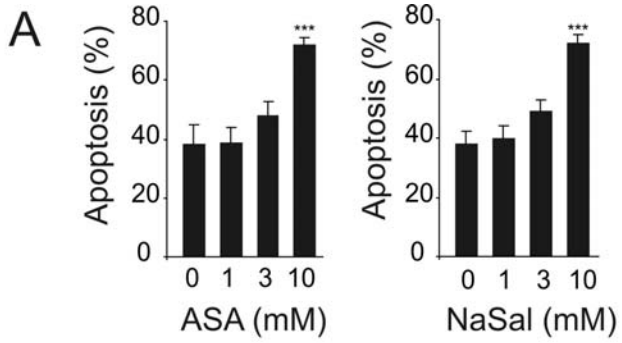

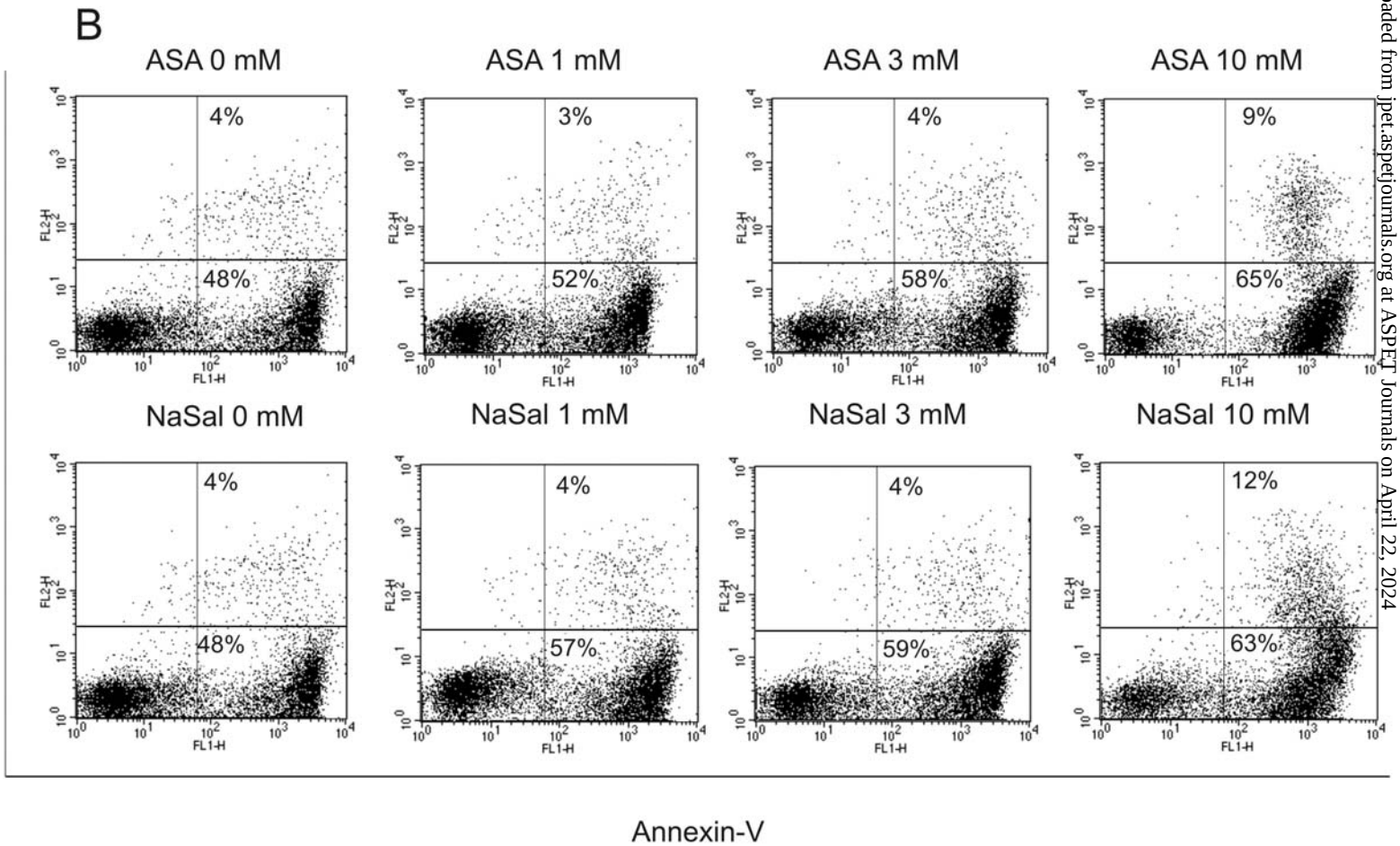

Figure 1

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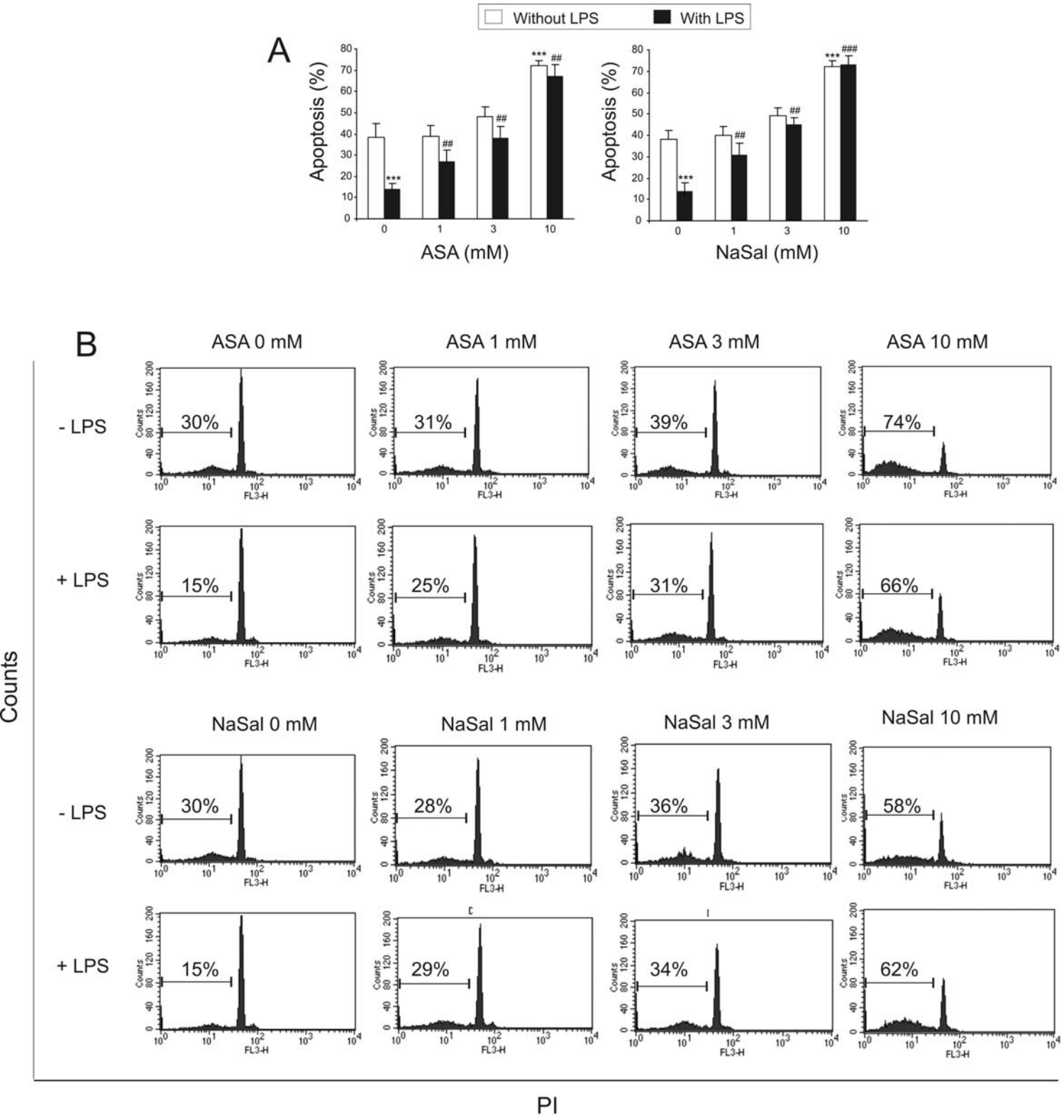

Figure 2

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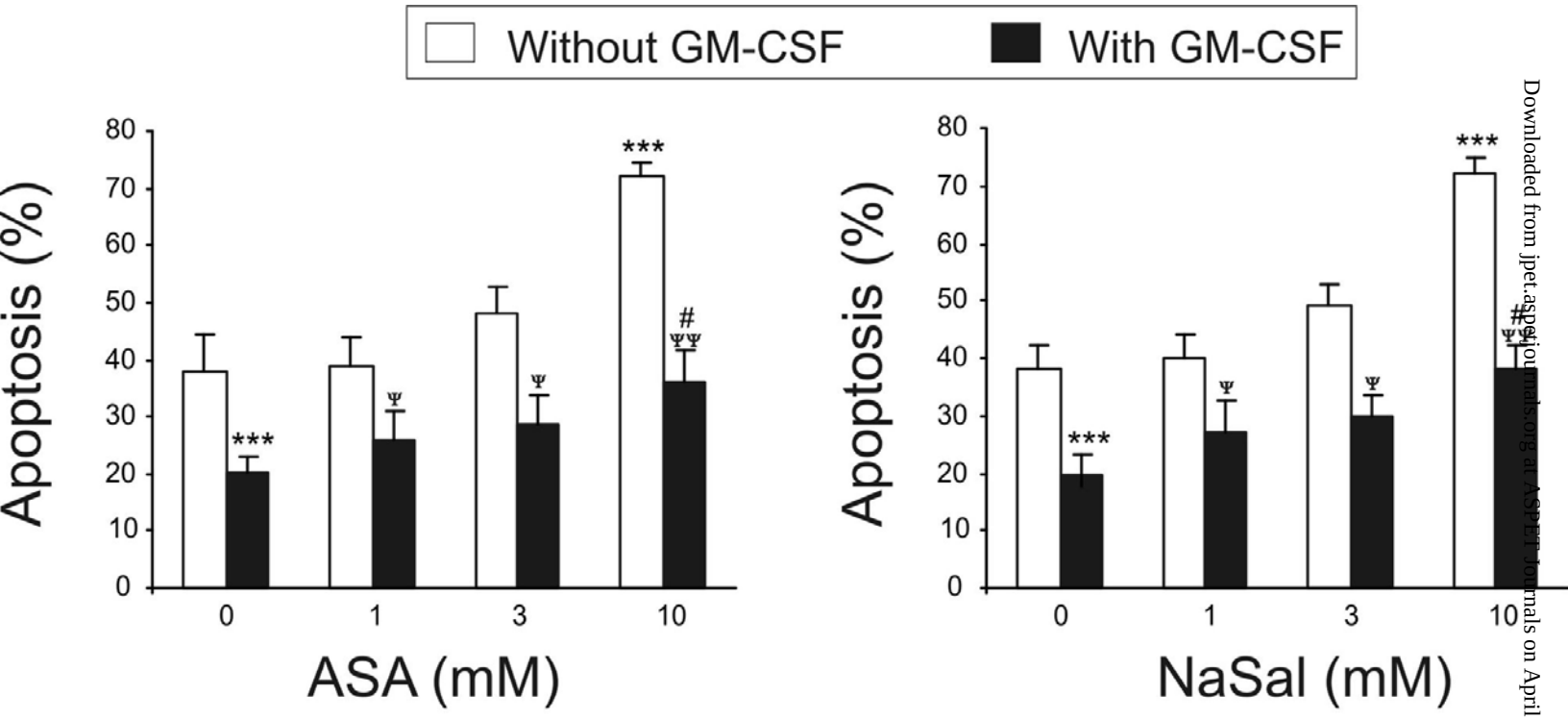

Figure 3

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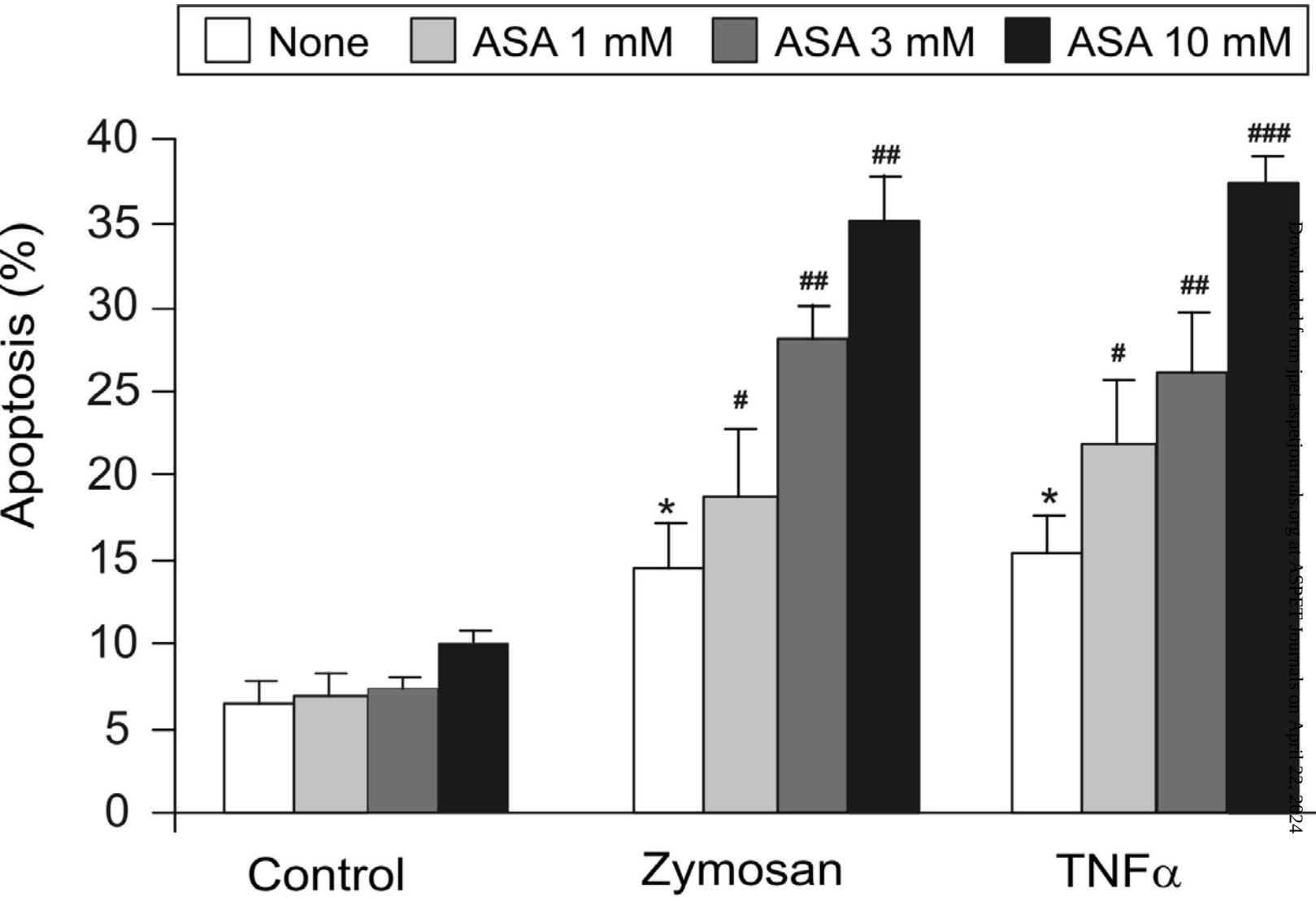

Figure 4

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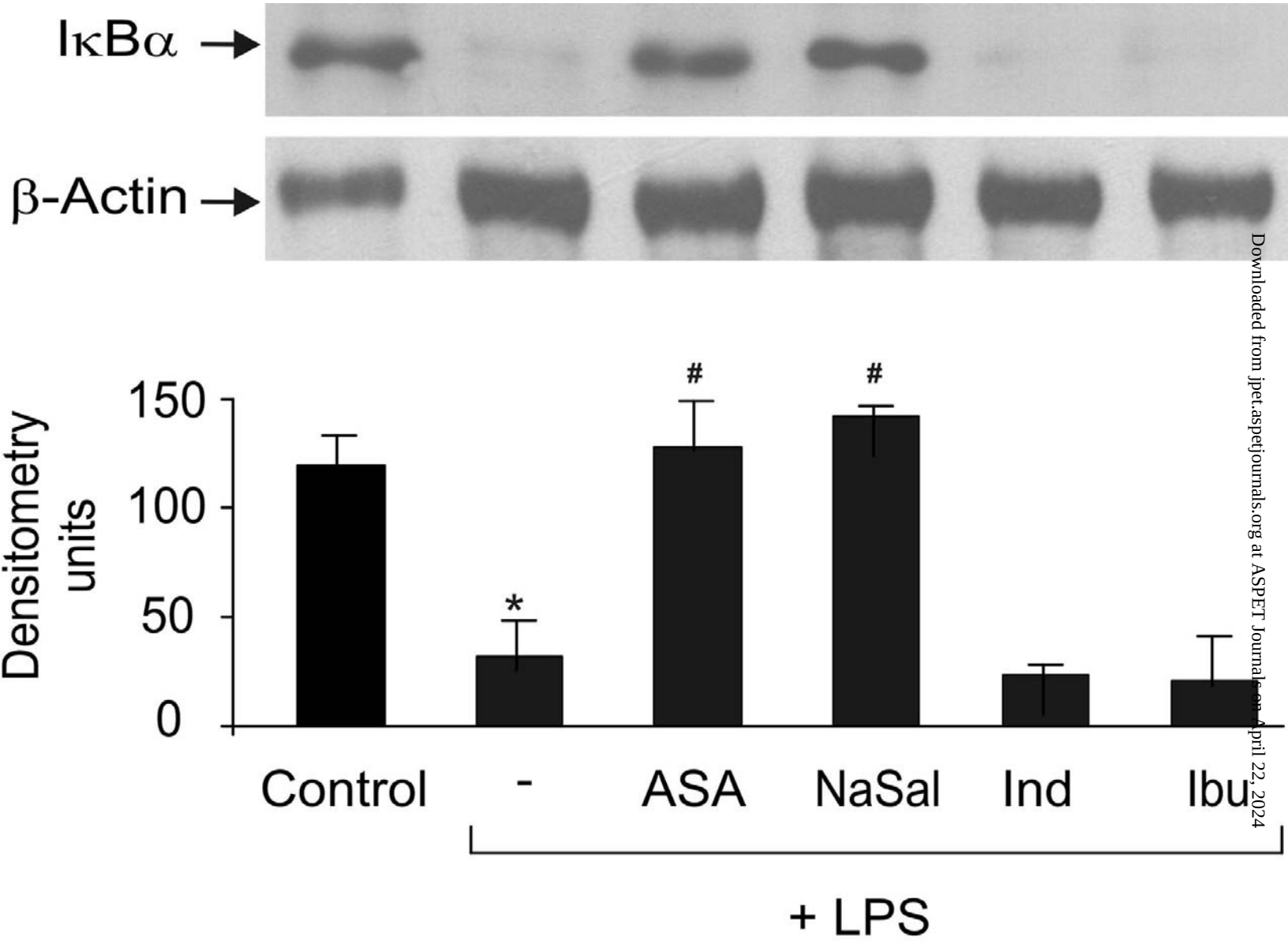

Figure 5

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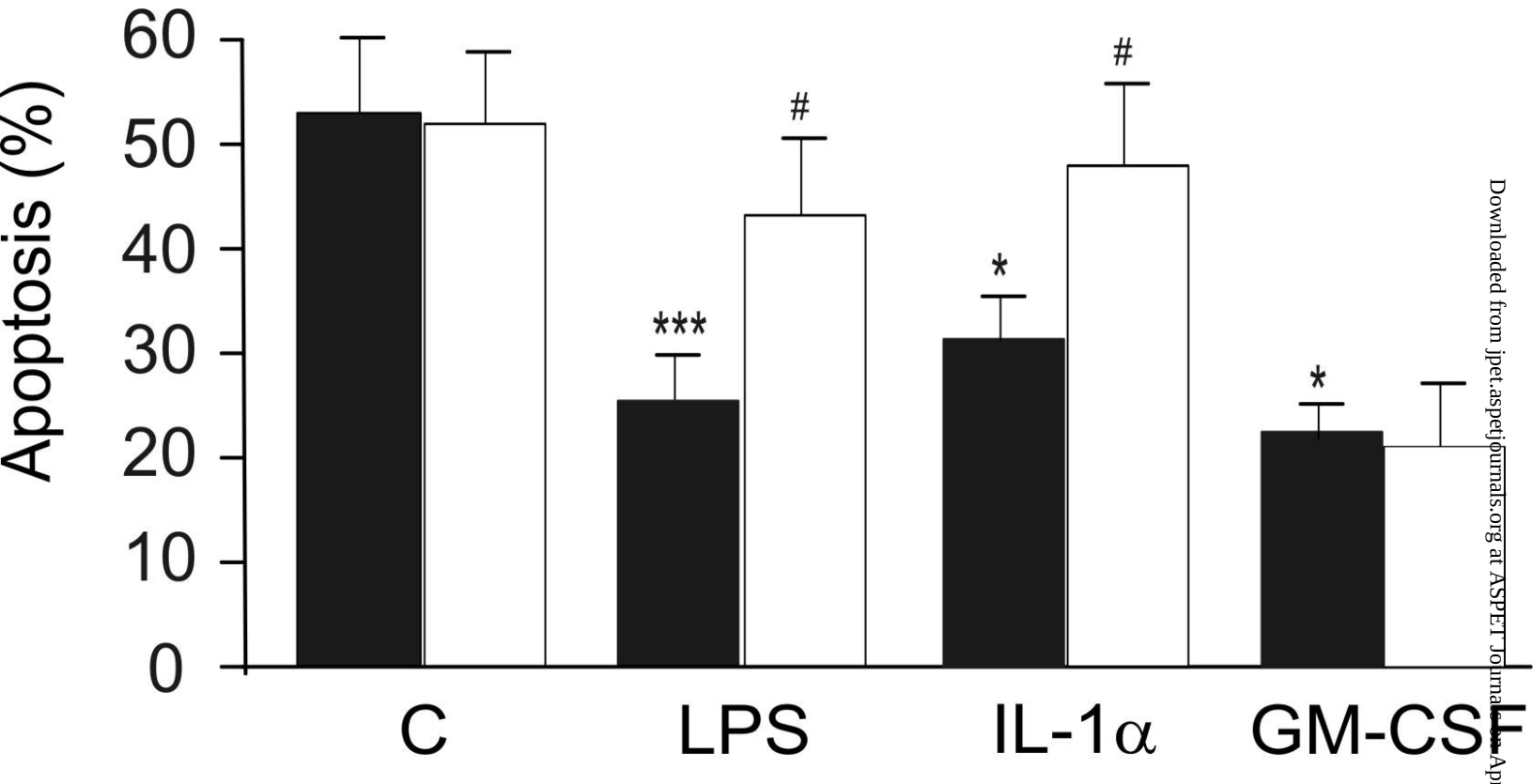

Figure 6

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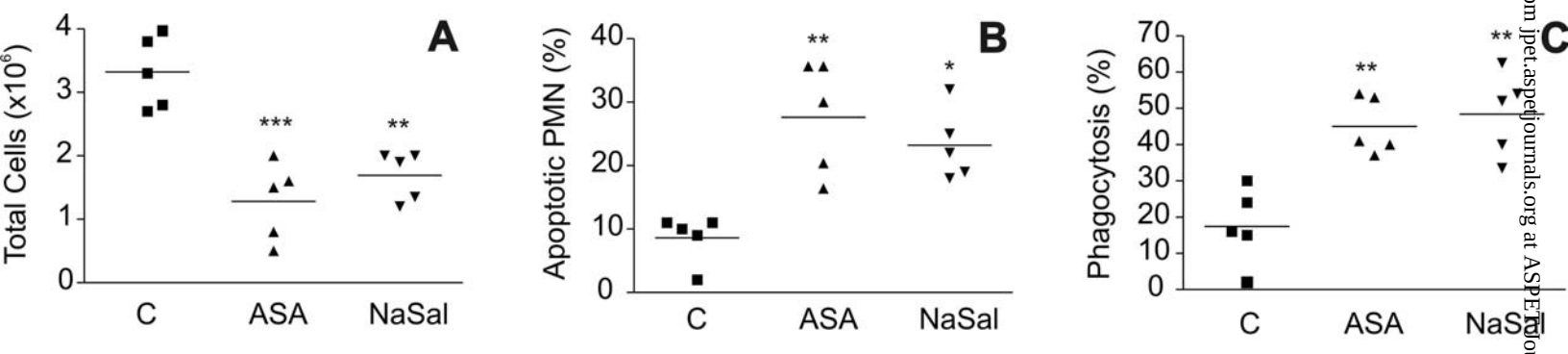

Figure 7

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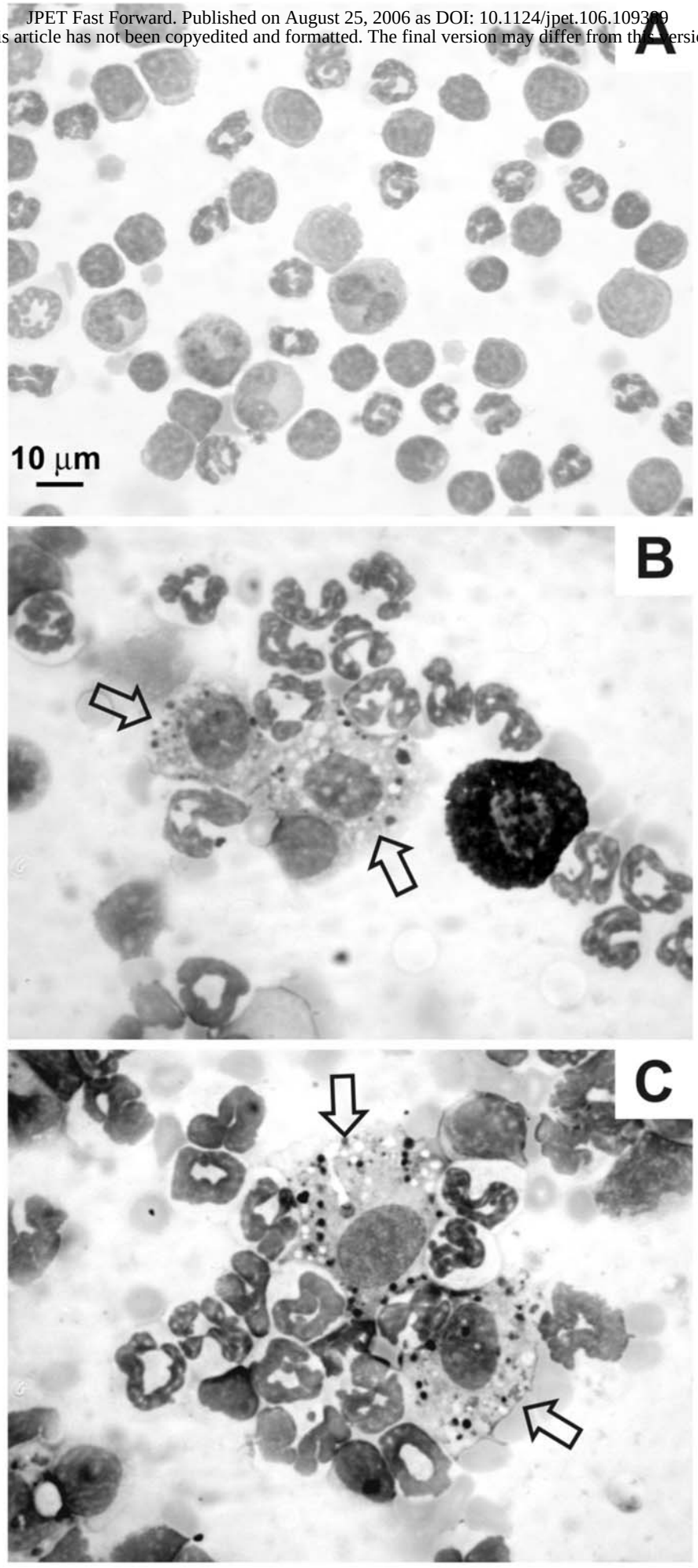

Figure 8