Circulating Neutrophils from Trauma Patients Induce Apoptosis Through Dephosphorylation of Epithelial Cell Caspase-8

Song Hui Jia MD PhD [3]
Jean Parodo BSc [3]
Emmanuel Charbonney MD [2,3]
Jennifer L. Y. Tsang MD [2,3,4]
Sang Yang Jia [3]
Ori D. Rotstein MD MSc1 [1,3]
Andras Kapus MD PhD [1,3]
John C. Marshall MD [1,2,3]

From the Departments of Surgery [1] and Critical Care Medicine [2], and the Keenan Research Centre of the Li Ka Shing Knowledge Institute [3], St. Michael's Hospital, and the Department of Critical Care Medicine, Sunnybrook Health Sciences Centre[4], University of Toronto,

Address Correspondence to:

John C. Marshall MD
St. Michael's Hospital,
4th Floor Bond Wing, Rm. 4-007,
30 Bond Street,
Toronto, Ontario
M5B 1W8

Phone:416-864-5225
Fax: 416-864-5141

Email: marshallj@smh.ca

Abstract

Activated neutrophils can cause oxidant-mediated bystander injury to host cells. This injury has previously been ascribed to the direct effects of oxidants on membrane phospholipids. We show here that oxidants released by neutrophils activated *in vivo* in survivors of multiple trauma or *in vitro* by exposure to bacterial lipopolysaccharide (LPS) can also promote the apoptotic death of epithelial cells, through the SHP-1-mediated dephosphorylation of epithelial cell caspase-8. Neutrophils from a cohort of patients who sustained serious injury induced the apoptotic death of cultured epithelial cells in a manner that was dependent on the activity of the NADPH oxidase and the generation of neutrophil-derived reactive oxygen intermediates. Caspase-8 is constitutively tyrosine phosphorylated in a panel of resting epithelial cells, but undergoes dephosphorylation in response to hydrogen peroxide (H_2O_2), activated neutrophils, or inhibition of Src kinases. Mutation of either of 2 key caspase-8 tyrosine residues, Y397 and Y465 to a non-phosphorylatable phenylalanine accelerates the apoptosis of epithelial cells transfected with caspase-8, while mutation of the same tyrosine residues to the phosphomimetic glutamic acid, or transfection with the Src kinases Lyn or c-Src inhibits H_2O_2-induced apoptosis. Exposure to either H_2O_2 or LPS-stimulated neutrophils, increases the phosphorylation and activity of the phosphatase SHP-1, increases the activity of caspases-8 and -3, and accelerates epithelial cell apoptosis. Together these data reveal a novel mechanism for neutrophil-mediated tissue injury through oxidant-dependent SHP-1-mediated dephosphorylation of caspase-8 resulting in enhanced epithelial cell apoptosis.

Word Count: 234

Acutely ill patients sustaining severe traumatic injuries develop abnormalities of systemic immunologic and metabolic homeostasis that result in impaired physiologic organ function and enhanced susceptibility to infectious complications. Prominent among these abnormalities are systemic neutrophil activation [1] and increased apoptosis of epithelial cells and lymphocytes [2]. The mechanisms underlying the increased cellular apoptosis in acutely ill patients are poorly characterized. Augmented circulating levels of soluble Fas ligand [3, 4] and reduced cellular activity of Bcl2 [5] have been implicated in experimental models. Increased local neutrophil influx is a characteristic finding, and has been hypothesized to play a contributory role [6].

Activated neutrophils are implicated in the pathogenesis of inflammatory tissue injury [1, 7-9]. Tissue damage has been attributed to direct cellular injury by neutrophil proteases such as elastase [10], or to the effects of neutrophil-derived oxidants on lipids in cell membranes, resulting in the necrotic death of the target cell [11]. However extensive necrotic cell death is uncommon in tissues from patients suffering the sequelae of traumatic injury [12], and experimental inflammatory lung injury is characteristically accompanied by evidence of concomitant activation of apoptosis [13, 14]. The potential for neutrophils to induce apoptotic cell death has received little attention, although oxidants such as superoxide anion and hydrogen peroxide – products of the neutrophil NADPH oxidase – are potent stimuli for the induction of apoptosis [15], and *in vitro* exposure of A549 lung epithelial cells to hydrogen peroxide evokes a complex transcriptional response, activating pathways leading to cell cycle arrest, activation of p53, and increased activity of caspase-3 [16].

The catalytic activity of the apical caspase of the extrinsic pathway of apoptosis, caspase-8, is regulated through the phosphorylation of two key tyrosine residues at Y397 and Y465 [17, 18]. Caspase-8 contains an immunoreceptor tyrosine-based inhibition motif (ITIM)-like domain (IxYxxL)[19] at Y310 in the p18 active fragment of the enzyme[20]. Binding of the membrane-associated tyrosine phosphatase SHP-1 to this motif results in SHP-1 mediated dephosphorylation of tyrosine residues at Y397 and Y465, followed by activational cleavage of caspase-8, activation of caspase-3, and the progression of apoptosis [18]. We hypothesized that oxidants from activated neutrophils might regulate the caspase-8 phosphorylation state in epithelial cells, and so alter their survival. Here we show that neutrophils harvested from patients who have sustained multiple trauma can induce the apoptotic death of cultured epithelial cells through the activation of the NADPH oxidase and the generation of reactive oxygen intermediates. Exposure of epithelial cells to either activated neutrophils or hydrogen peroxide (H_2O_2) results in increased activity of the phosphatase SHP-1, which in turn dephosphorylates caspase-8 and activates the extrinsic pathway of apoptosis. These observations reveal a novel mechanism through which neutrophils can induce cellular injury during acute inflammation.

Results

Trauma Neutrophils Trigger the Oxidant-dependent Apoptosis of Cultured Epithelial Cells

We studied eight trauma patients at a mean of 3 days following injury; demographic characteristics are summarized in Table 1. Co-culture of patient neutrophils with subconfluent cultures of A549 or HEK293 epithelial cells resulted in increased epithelial cell apoptosis reflected in increased hypodiploid DNA in permeabilized cells, detected by flow cytometry as the uptake of propidium iodide (**Fig. 1a**). Enhanced apoptosis was accompanied by reduced tyrosine phosphorylation of caspase-8 (**Fig. 1b**) and increased cleavage of pro-caspase-8 to its active p10 and p18 moieties (**Fig. 1c**). To probe the potential role of neutrophil-derived oxidants in this accelerated apoptotic death, we treated trauma patient neutrophils with diphenylene iodonium (DPI, 10 μM), an inhibitor of the NADPH oxidase, catalase (10 μg/ml) to enhance the degradation of H_2O_2, or the antioxidant glutathione (10 μM); each increased caspase-8 tyrosine phosphorylation (**Fig. 1b**), increased caspase-8 cleavage (**Fig. 1c**), and attenuated the pro-apoptotic effects of trauma neutrophils (**Fig. 1d**), suggesting a mechanism involving neutrophil-derived oxidants.

Activated Neutrophils Induce Epithelial Cell Apoptosis

We used co-cultures of a panel of epithelial cells and neutrophils from healthy human donors activated *in vitro* by inflammatory stimuli to study the mechanism of neutrophil-mediated epithelial cell apoptosis. Co-culture of A549 lung epithelial cells with neutrophils alone resulted in modestly increased rates of apoptosis measured as hypodiploid DNA in permeabilized cells (**Fig. 2a**) or as binding of exteriorized phosphatidyl serine (**Fig. 2b**). Activation of neutrophils by LPS (1 μg/mL) resulted in significantly increased rates of A549 cell apoptosis when compared with quiescent

neutrophils alone. LPS alone had no effect on epithelial cell survival. The A549 cell line was derived from a human lung carcinoma [21]. To determine whether PMN could induce the apoptotic death of primary, non-transformed cells, we repeated these studies in a primary human distal airway cell line, BEAS-2B, and in human embryonic kidney (HEK-293) cells. Similar results were obtained (data not shown).

Transfection of wild-type caspase-8 into A549 cells led to increased DNA cleavage (**Fig. 2c**); transfection of A549 cells with a cleavage resistant mutant caspase-8 C377S [22] led to apoptosis rates that did not differ from those of non-transfected cells, or cells transfected with empty vector (**Fig. 2c**), confirming that caspase-8 activation is sufficient to induce epithelial cell apoptosis.

The Caspase-8 Phosphorylation State Regulates Epithelial Cell Survival

Caspase-8a can be phosphorylated on Y397 (corresponding to Y380 in caspase-8b) and Y465; phosphorylation renders caspase-8 resistant to autocatalytic cleavage, and so inhibits the progression of apoptosis that has been initiated through the extrinsic pathway [17, 18]. Caspase-8 is tyrosine phosphorylated in resting A549 cells (**Fig. 3a**). The addition of LPS, 1 μg/ml, to cultures had no effect on the phosphorylation state of caspase-8, whereas TNFα (50 ng/ml) or an agonistic anti-Fas antibody (100 ng/ml) – agents that induce A549 cell apoptosis (data not shown) - resulted in moderate caspase-8 dephosphorylation (**Fig. 3a**). We transfected A549 cells with vectors containing wild-type caspase-8, or mutant caspase-8 in which Y397 or Y465 had been mutated to non-phosphorylatable phenylalanine (Y397F and Y465F) or to the phosphomimetic amino

acid, glutamic acid (Y397E and Y465E) [23]. Overexpression of caspase-8 resulted in increased A549 cell apoptosis (**Fig. 3b**), in association with increased activity of caspase-8 and caspase-3 (**Fig. 3c**). Caspase activity and rates of apoptosis were further increased in cells transfected with the non-phosphorylatable caspase-8 mutants; conversely, transfection with pseudophosphorylated caspase-8 Y397E or Y465E resulted in rates of apoptosis that did not differ from those of empty vector controls (**Fig. 3b**). Increased caspase-8 activity in cells transfected with wild-type caspase-8 or the Y397F mutant was associated with increased caspase-3 cleavage and nuclear translocation (**Fig. 3d**). Thus dephosphorylation of epithelial cell caspase-8 results in increased epithelial cell apoptosis.

Oxidants Induce Epithelial Cell Apoptosis and Caspase-8 Dephosphorylation

Treatment of either A549 cells, BEAS-2B cells or HEK-293 cells with hydrogen peroxide (H_2O_2, 100 μM) resulted in a time-dependent increase in apoptosis (**Fig. 4a**), and in rapid caspase-8 dephosphorylation (**Fig. 4b**) with cleavage of caspases-8 and -3 to their active forms (**Fig. 4c**). H_2O_2 also induced a time-dependent reduction in levels of the endogenous antioxidant, GSH (**Fig. 4d**). Conversely, the addition of GSH (10 mM) to co-cultures of epithelial cells and LPS-activated neutrophils attenuated the increased rates of apoptosis (**Fig. 4e** shows data from co-cultures of PMN and BEAS-2B cells; similar effects were seen using A549 cells and HEK-293 cells, data not shown). Together these experiments show that exogenous oxidants can induce caspase-8 dephosphorylation and epithelial cell apoptosis, and that this process can be inhibited by endogenous antioxidants.

The Caspase-8 Phosphorylation State and Epithelial Cell Survival are Regulated by a Src Kinase

The Src kinase family member, lyn, phosphorylates caspase-8 in inflammatory neutrophils [18], while constitutively active Src phosphorylates caspase-8 in HEK293 cells [17]. Caspase-8 and lyn co-immunoprecipitated in A549 cells; treatment of cultures with either H_2O_2 or the Src kinase inhibitor, PP2 (10 μM), resulted in dissociation of caspase-8 and lyn, and dephosphorylation of caspase-8 (**Fig. 5a**), in association with accelerated epithelial cell apoptosis (**Figs. 4a, 5b**). Caspase-8 also co-immunoprecipitated with c-Src; pretreatment with H_2O_2 disrupted this interaction (**Fig.5c**). Overexpression of either c-Src or lyn attenuated the pro-apoptotic effects of H_2O_2 in A549 cells, an effect that could be reversed by the broad spectrum Src kinase inhibitor, PP2 (**Fig. 5d**). Thus Src family kinases phosphorylate caspase-8 in A549 epithelial cells, and their kinase activity is inhibited by H_2O_2.

SHP-1 is Phosphorylated by H_2O_2, and Dephosphorylates Epithelial Cell Caspase-8

Caspase-8 is dephosphorylated by the Src homology domain 2 containing phosphatase (SHP-1) in neutrophils [18]. SHP-1 activity is enhanced through its phosphorylation on Y536 [24]. SHP-1 was expressed in A549 cells. Exposure to H_2O_2 resulted in tyrosine phosphorylation of SHP-1, reaching maximal levels by 60 minutes (**Fig. 6a**), in association with increased SHP-1 activity (**Fig. 6b**). Overexpression of SHP-1 in A549 cells resulted in increased rates of apoptosis, both in resting cells, and in response to H_2O_2 (**Fig. 6c**). Moreover overexpression of both caspase-8 and SHP-1 resulted in increased cleavage of caspase-8 to its active form (**Fig. 6d**). SHP-1 and

caspase-8 did not associate in resting epithelial cells; however exposure to H_2O_2, a stimulus which induces both SHP-1 phosphorylation (**Fig. 6a**) and A549 cell apoptosis (**Fig. 4a**), resulted in co-immunoprecipitation of caspase-8 and SHP-1 (**Fig. 6e**). When A549 cells were transfected with myc-tagged constructs of SHP-1 or caspase-8, exposure to H_2O_2 resulted in increased SHP-1 phosphorylation, and decreased caspase-8 phosphorylation (**Fig. 6f**). In aggregate, these studies suggest that the caspase-8 phosphorylation state in cultured epithelial cells is regulated by dynamic interactions between Src kinases and the SHP-1 phosphatase, and that hydrogen peroxide, by phosphorylating and activating SHP-1, can direct the net consequences of this interaction towards caspase-8 dephosphorylation, with resultant epithelial cell apoptosis.

Activated Neutrophils Increase SHP-1 Phosphorylation and Activity, While Src Kinases Inhibit Neutrophil-Mediated Cell Death

Finally we sought to determine whether neutrophil-derived oxidants could induce SHP-1 activation and caspase-8 dephosphorylation, and so provide a mechanistic explanation for neutrophil-mediated induction of epithelial cell apoptosis. Consistent with what we had observed using trauma patient neutrophils, co-culture of A549 (**Fig. 7a**) or HEK293 (**Fig. 7b**) cells with LPS-activated neutrophils resulted in enhanced epithelial cell apoptosis. Pretreatment of neutrophils with either DPI (10 μM) or catalase (10 μg/ml) blocked the LPS-induced increase in apoptosis, but had no effect on the increase resulting from exposure to neutrophils alone. Co-culture with LPS-activated neutrophils increased SHP-1 activity (**Fig. 7c**) in A549 cells. Following transfection of A549 cells with c-myc-tagged SHP-1, exposure to LPS-activated neutrophils resulted in

increased SHP-1 phosphorylation (**Fig. 7d**), and when caspase-8-transfected A549 cells were co-cultured with activated neutrophils, caspase-8 was dephosphorylated (**Fig. 7e**). Finally, transfection of A549 cells with plasmids encoding either Lyn or c-Src attenuated the increased rates of apoptotic death resulting from culture with LPS-activated neutrophils (**Fig. 7f**). Together these studies recapitulate changes seen following culture of epithelial cells with trauma patients' neutrophils, and confirm that activated neutrophils induce epithelial cell apoptosis.

Discussion

The maintenance of normal tissue architecture is a dynamic process, regulated by the reciprocal processes of programmed cell death and cellular regeneration. A spectrum of acute stresses that threaten the survival of the whole organism can modulate this balance. At the interface between the multicellular host and its environment, the epithelial cell is particularly vulnerable to injury. Both neutrophils and epithelial cells are constitutively apoptotic. The *in viv*o half-life of the neutrophil is only 6 to 8 hours [25], while that of the epithelial cell ranges from several days for gastrointestinal epithelial cells [26] to upwards of 18 months for lung epithelial cells [27]. Increased epithelial cell apoptosis is a prominent feature of a variety of inflammatory disorders, including ulcerative colitis [28], *C. difficile* colitis [29], pancreatitis [30], experimental influenza [31], renal ischemia/reperfusion injury [32], ventilator-induced lung injury [3], sepsis [12], trauma [33], and toxin-induced liver injury [34] .

Our studies establish a novel mechanism of neutrophil-mediated cytotoxicity during inflammation. Circulating neutrophils freshly isolated from patients who have sustained severe traumatic injury trigger the apoptotic death of a panel of cultured epithelial cells. This mechanism of injury can be recapitulated *ex vivo* using lipopolysaccharide-stimulated neutrophils from healthy volunteers. The pro-apoptotic activity of the neutrophil is dependent on the production of reactive oxygen intermediates – in particular hydrogen peroxide - and occurs through the SHP-1-mediated tyrosine dephosphorylation of epithelial cell caspase-8, a process that, in turn, is inhibited by over-expression of Src family kinases.

Immune cell activation is finely regulated through the interaction of Src family kinases and phosphatases of the Src homology-containing domain (SHP) family with conserved immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibition domains (ITIMs). These domains serve as a docking site for an SH-2 domain-containing phosphatase such as SHP-1, SHP-2, or SHIP [35]. They are present within the intracellular tail of immune cell receptors such as those of B or T cells, and the Fc receptor on monocytes [36] or transmembrane adapter proteins such as DAP12 [37]. The classical ITIM sequence consists of 6 amino acids with a consensus sequence I/V/LxYxxL/V; phosphorylation of the tyrosine residue results in recruitment of an SH2 domain-containing phosphatase and initiates inhibitory signaling [19]. Caspase-8 contains a modified ITIM sequence at Y310 that lacks a hydrophobic amino acid in the Y-2 position. Despite this, however, it is able to recruit SHP-1 [20], and mutation of Y310

to the nonphosphorylatable phenylalanine prevents the increased apoptosis when HL-60 cells are co-transfected with SHP-1 and caspase-8 [18]. Moreover caspase-8 functions as an adapter protein, interacting with death receptors of the CD95 family to transduce pro-apoptotic signals from the environment, and with the p85 regulatory subunit of PI3 kinase to activate PI3 kinase and support cellular activities such as adhesion and motility [38].

The tyrosine phosphatase, SHP-1 is expressed in hematopoietic cells and at lower levels in epithelial cells [39]. It is activated through the phosphorylation of Y536 and Y564 by the Src kinase, lyn [40]. Lyn activity, in turn, is increased following exposure to reactive oxygen species [41]. Hydrogen peroxide at concentrations encountered *in vivo* [42] induced SHP-1 tyrosine phosphorylation, resulting in a physical interaction between SHP-1 and caspase-8; in consequence, caspase-8 was dephosphorylated and its catalytic activity increased. In support of the hypothesis that neutrophil-derived oxidants play a similar role, an inhibitor of the NADPH oxidase (DPI) or antioxidants (GSH or catalase), attenuated the epithelial cell apoptosis induced by co-culture with trauma neutrophils.

Neutrophil-mediated mechanisms of host cell cytotoxicity are numerous, reflecting the prodigious capacity of the neutrophil to respond to local infectious challenge or to tissue injury [43-45]. Oxidants generated through the neutrophil NADPH oxidase cause oxidation of membrane phospholipids and induce necrotic cell death. In the clinical setting of critical illness, however, it is striking that organ dysfunction is disseminated beyond the initiating site of injury or infection, and that the local histologic changes are pathologically bland in nature, with minimal evidence of necrosis [46].

Our studies were undertaken *ex vivo*, using neutrophils from victims of major trauma and a panel of cultured epithelial cells. Animal models are consistent with this hypothesis [6], although it is disarmingly easy to establish efficacy in an animal model, and conversely enormously challenging to translate these observations into effective human therapies [47]. Nonetheless, strategies that either inhibit the capacity of the neutrophil to activate an oxidative burst response, or that enable epithelial cells to resist the consequences of this process, may represent novel interventional approaches that can minimize neutrophil-mediated cellular injury, minimize the adverse consequences of activation of an innate immune response, and ultimately lead to increased patient survival following a broad spectrum of acute inflammatory stimuli.

Materials and Methods

Trauma Patients We recruited patients sustaining multisystem trauma (ISS >16) who were admitted to the Trauma-Neurosurgical Intensive Care Unit of St. Michael's Hospital; patients were studied as early as possible following injury, and serially over the duration of the ICU stay. The protocol was reviewed by the Human Ethics Review Committee of St. Michael's Hospital and written informed consent was obtained from the patient or a surrogate decision-maker.

Cells Circulating neutrophils were obtained from consenting healthy human volunteers by density gradient centrifugation using Ficoll-Hypaque as previously described [48], and resuspended in polypropylene tubes in Dulbecco's Modified Minimum Essential Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. A549 cells (ATCC CCL-185) and Human Embryonic Kidney (HEK)-293 cells (ATCC CRL-1573) were similarly cultured in supplemented DMEM. Human distal airway BEAS-2B cells (ATCC CRL-9609) were cultured in bronchial epithelial cell basal medium (BEBM, Lonza Canada Inc., Shawinigan Que.) supplemented with BEGM SingleQuot (Catalog #CC-4175, Lonza Canada) containing BBE, insulin, hydrocortisone, GA-1000, epinephrine, retinoic acid, transferrin, and triiodothyronine. Cells were cultured in 100x20 mm tissue culture plates, and experiments performed at 80% confluency.

Antibodies and Reagents Antibodies used in these studies were murine monoclonal anti-caspase-8 (1:1000; Calbiochem), agonistic anti-CD95 monoclonal antibody, CH11 (100 ng/ml; Biosource International, Camarillo CA), murine monoclonal anti-phosphotyrosine (1:2000, Upstate Technologies), anti-caspase-3 (1:1000, Santa Cruz Biotechnology),

murine monoclonal anti-lyn (1:1000; Santa Cruz Biotechnology), anti C-Src (1:1000, Santa Cruz Biotechnology;), murine monoclonal anti-SHP-1 (1:1000; Santa Cruz Biotechnology), murine monoclonal anti-β actin (Santa Cruz Biotechnology; 1:4000), and murine monoclonal anti-c-myc (1:1000; Santa Cruz Biotechnology).

LPS from *E. coli* O111:B4 (Sigma-Aldrich, Mississauga, Canada) was used at a concentration of 1 μg/ml. Recombinant TNFα (BioSource International, Camarillo, CA) was used at a concentration of 50 ng/ml. The Src kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; Calbiochem) was used at a concentration of 10 μM; hydrogen peroxide (H_2O_2; Sigma-Aldrich) was used at a concentration of 100 μM.

Neutrophil:Epithelial Cell Co-cultures Neutrophils were added to epithelial cell cultures at a ratio of 5:1 ($2.5X10^6$ PMN to $5X10^5$ epithelial cells), and incubated at 37^oC with or without LPS (1 μg/ml) for a further 18 hours. Plates were then washed three times with supplemented medium to remove PMN, and epithelial cell cultures trypsinized to detach cells from the culture plates.

Construction of Plasmids Total RNA from neutrophils from healthy human volunteers was extracted using TRIzol reagent and 1 μg RNA transcribed to first-strand cDNA using the Superscript II system (Invitrogen); the resultant cDNA was amplified by PCR using the Expand^{TM} High Fidelity PCR System (Roche) and the primer sets shown in Table 1. Amplified fragments were cloned into the pcDNA3.1/Myc-His vector (Invitrogen) according to the manufacturer's instructions. The recombinant plasmids were transfected into DH5a-competent cells (Invitrogen) and colonies identified by restriction enzyme digestion and sequencing.

Construction of Mutant Caspase-8 Plasmids Tyrosine residues in the caspase-8 molecule were mutated using site-directed primers to mutate the tyrosine residue (TAT or TAC) to phenylalanine (TTT or TTC). Pseudophosphorylation mutants of the same sites were created by mutating the relevant tyrosine (Y) residues (TAT or TAC) to glutamic acid (E; GAG) [23]. Finally a catalytically inactive mutant of caspase-8 was created by mutating cysteine (C) 377 to serine (S) [22]; primer sequences are shown in Table 2.

Mutations were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and a caspase-8/myc-his plasmid as a template to perform the mutant strand synthesis reaction. After Dpn I digestion of the amplified product, the mutant DNA was transfected to XL1- blue supercompetent cells, and colonies identified by restriction enzyme digestion and sequencing.

Cell Transfection Cells were transfected with plasmids containing wild-type or mutant caspase-8 as previously described [18]. Briefly, 4 μg plasmid and 10 μl Fugene 6 reagent (Roche) in 200 μl of serum-free medium were added to A549 cell cultures in 10 cm plates at 60% confluency. Cells were cultured for 24 hours, then washed, and cultured for a further 48 hours prior to study.

Assay of Epithelial Cell Apoptosis Epithelial cells were detached from culture plates by trypsinization, washed with PBS, and resuspended in supplemented medium. Cells were permeabilized by the addition of Triton-X100 and incubated for 10 minutes in the dark with propidium iodide (50 μg/ml). The characteristic DNA fragmentation of apoptosis was quantified as the percentage of hypodiploid DNA in permeabilized cells by flow cytometry using a BD FACS CANTO cytofluorometer with BD FACS DIVA software [49];

a minimum of 10,000 events was recorded. Alternatively, early events during the expression of apoptosis were assessed by detecting exteriorized phosphatidyl serine by flow cytometry as the binding of Annexin V as described [48].

Western Blot and Co-immunoprecipitation Studies Cells were lysed in lysis buffer (10mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM PMSF, 1mM Na_3VO_4, 10μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose (Amersham Pharmacia Biotech), and probed with the appropriate primary antibody. Bands were detected with an HRP-conjugated second antibody at a dilution of 1:4000 using the ECL western blotting detection system (Amersham Pharmacia Biotech). Blots were stripped and reprobed with an anti-actin antibody to confirm equal loading.

For immunoprecipitation studies, cell lysates were centrifuged at 12000 rpm for 10 min. Supernatants were pre-cleared with protein G sepharose beads (Amersham Biosciences) for one hour, then re-centrifuged to remove the beads. Protein concentration in the lysates was measured using the BCA protein assay (Pierce), then 5 μl of anti-caspase-8 (Calbiochem), or 15 μl of anti-SHP1 (Santa Cruz), or 5 μl of anti-lyn (Santa Cruz) was added to 500 μl of supernatant, and incubated for 1 hour at 4^o C before adding protein G beads (20 μl) and incubating for an additional 1 hour or overnight. Suspensions were centrifuged and beads washed three times in PBS, then boiled in Laemmli buffer for 5 minutes prior to SDS-PAGE and western analysis. Western analysis of the immunoblots used anti-phosphotyrosine (clone 4G10) 1:1000 (Upstate), or other antibodies as noted.

Measurement of GSH Levels: GSH levels were assayed using a DTNB (5,5'-dithiobis[2-nitrobenzoic acid])-based assay as previously described [50]. A549 cells (5 X 10^6/ml) were sonicated for 30 seconds on ice in 300 µL of 5% 5-sulfosalicylic acid. Following centrifugation for 10 minutes at 10,000G, nonprotein sulfhydryls in the supernatant were quantified as the reduction of DTNB by its conversion to TNB (5-thiol-2 nitrobenzoic acid), measured at 412 nm using a spectrophotometer. GSH levels were expressed as nmol/100 µl/µg protein by comparison to a standard curve.

Assay of Activity of Caspases-3 and -8: Caspase activity was determined using a caspase assay kit (BioSource International). Cell lysates sere incubated overnight with 25 µl of synthetic substrate that is preferentially cleaved by caspase-3 (Ac-DEVD-pNA) or caspase-8 (Ac-IETD-pNA). Release of the colorimetric substrate was measured at 405 nm in 96 well plates using a colorimetric plate reader (Titertek Instruments, Hunstville, AL), and expressed as absorbance at 405 nm per mg protein.

SHP-1 Activity Assay: The release of inorganic phosphate from phosphopeptides was measured using the malachite green assay (Upstate). Briefly, 10x10 ^{6} A549 cells were lysed for 10 minutes on ice in lysis buffer containing 1 mM sodium orthovanadate and protease inhibitors. Suspensions were centrifuged for 15 minutes at 12000g, protein content of the resulting supernatant was determined, and 500 µl supernatant was incubated with 10 µl anti-SHP-1 antibody (Santa Cruz) pre-bound to Protein A sepharose (SantaCruz) for three hours at 4^{o}C. Immunoprecipitates were washed six time in wash buffer (10 mM Tris pH 7.4), then incubated with tyrosine phosphopeptide substrate (RRLIEDAEpYAARG) in 10 mM Tris pH 7.4 for 30 minutes. The reaction was stopped with 100 µl malachite green solution. Absorbance was measured at 620 nm using a

LabSystems Multiskan plate reader and phosphate release in pmol phosphate/minute/μg was determined by comparing absorbance to standard curve.

Immunofluorescence Microscopy: A549 cells were grown on coverslips in 75 mm plates, and transfected with caspase-8/pcDNA3.1/myc plasmids. After 24 hours, cells were washed with PBS, then fixed on coverslips for 30 minutes with 4% paraformaldehyde. Coverslips were then incubated with 100 mM glycine in PBS, washed 4 times, and permeabilized with 0.1% Triton-X-100 in PBS supplemented with 1% albumin. After blocking with 5% albumin in PBS, slides were incubated with primary antibodies (rabbit anti-active caspase-3, 1:1000 and mouse anti-c-myc, 1:1000) for 60 minutes, washed 7 times, and incubated for 60 minutes with secondary antibody (anti-mouse cy3-conjugated and anti-rabbit FITC-conjugated). Slides were washed 7 times, and immunofluorescence detected using an Olympus IX 81microscope (Melville, N.Y.) coupled to an Evolution QEi monochrome camera (Media Cybernetics) using QED InVivo Imaging software and ImagePro Plus 3DS 5.1 softwar (Media Cybernetics).

Statistics: Data are presented as means ± standard deviation. Intergroup comparisons were performed using one-way ANOVA followed by Dunnett's t test, with the α level for statistical significance set at $p<0.05$. Studies were repeated a minimum of 3 times.

Table 1. Demographic Characteristics of the Study Population

Baseline Characteristics	
Age (SD)	37 ± 15 years
Males (%)	8 (100)
Injury Severity Score (SD)	32 ± 8
Units packed red blood cells in first 24 hours (median; range)	3 (0 – 31)
Time to sampling (hours; SD)	73 ± 45
Outcomes	
Days mechanical ventilation (SD)	11 ± 7
ICU days (SD)	13 ± 6
Survival (%)	8 (100)

Table 2. Primers for Plasmid Construction

Caspase-8 ***Upstream primer*** (containing a Hind III site and a Kozak sequence):

5'-GCAAGCTTGCCACCATGGACTTCAGCAGAAATCT-3'

Downstream primer (containing an Xba I site):

5'-GCTCTAGAATCAGAAGGGAAGACAAGT-3'

SHP-1 ***Upstream primer*** (containing a Hind III site and a Kozak sequence):

5'-GCAAGCTTGCCACCATGGTGAGGTGGTTTCACCGA-3'

Downstream primer (containing an Xba I site):

5'-GCTCTAGACTTCCTCTTGAGGGAACCCT-3'

Lyn ***Upstream primer*** (containing a BamH I site and a Kozak sequence):

5'-GCGGATCCGCCACCATGGGATGTATAAAATCAAAA-3'

Downstream primer (containing an Xba I site):

5'-GCTCTAGAAGGCTGCTGCTGGTATTGCCCT-3'

Caspase-8 Y→F

Tyr 397 ***Upstream primer*** 5'-GGAGCAACCCTTTTTAGAAATGG-3'

Downstream primer 5'-CCATTTCTAAAAAGGGTTGCTCC-3'

Tyr 465 ***Upstream primer*** 5'-GAAGTGAACTTTGAAGTAAGC-3'

Downstream primer 5'-GCTTACTTCAAAGTTCACTTC-3'

Caspase-8 Y→E

Tyr 397 ***Upstream primer*** 5’-GGAGCAACCCGAGTTAGAAATGG-3’

Downstream primer 5’-CCATTTCTAACTCGGGTTGCTCC-3’

Tyr 465 ***Upstream primer*** 5’-GAAGTGAACGAGGAAGTAAGC-3’

Downstream primer 5’-GCTTACTTCCTCGTTCACTTC-3’

Caspase-8 Y377S

Upstream primer 5’-ATTCAGGCTAGTCAGGGGG-3’

Downstream primer 5’-CCCCCTGACTAGCCTGAAT-3’

Legends for Figures

Figure 1. Trauma Neutrophils Induce Caspase-8 Dephosphorylation and the Apoptotic Death of Epithelial Cells

(a) PMN (2.5 X 10^6) harvested from healthy laboratory controls or from patients who had sustained multiple traumatic injuries were cultured for 18 hours with 0.5 X 10^6 A549 cells or HEK293 cells. Control PMN induced a modest degree of epithelial cell apoptosis; trauma PMN induced significantly greater degrees of epithelial cell apoptosis; Results are means ± SD, N=8; *$p<0.05$ vs no PMN, ** $p<0.001$ vs no PMN and <0.05 vs control PMN. **(b)** A549 cells were cultured alone, or with 2.5 X10^6 trauma PMN, with or without a pharmacologic inhibitor of the NADPH oxidase (DPI, 10 μM), catalase (10 μg/ml), or glutathione (10 μM). A549 cell lysates were immunoprecipitated with anti-caspase-8, then precipitates probed with anti-caspase-8 (upper panel) or anti-phosphotyrosine (lower panel). Trauma neutrophils induced caspase-8 dephosphorylation, an effect that was partially blocked by DPI, catalase, or GSH. **(c)** A549 cell lysates prepared as in 1b were analyzed by Western blot analysis using an antibody to the p18 active form of caspase-8 (upper panel); blots were stripped and reprobed with antibody to β-actin (lower panel) to confirm equal loading. The graph demonstrates the fold change in expression of active caspase-8 from control levels, as measured by densitometry; N=3, *$p<0.05$. **(d)** A549 cells and HEK293 cells were cultured with (black bars) or without (clear bars) trauma PMN. Addition of DPI (hatched bars), catalase (light gray bars), or GSH (dark gray bars) significantly attenuated

the pro-apoptotic effects of trauma PMN. Results are means ± SD, N=8; *p<0.01 vs trauma PMN alone.

Figure 2. LPS-Stimulated Neutrophils Induce Epithelial Cell Apoptosis

(a). Freshly isolated neutrophils alone induced a significant increase in rates of epithelial cell apoptosis. While the addition of LPS (1 μg/ml) to A549 cultures was without effect, exposure to LPS-stimulated neutrophils resulted in a further significant increase in rates of epithelial cell apoptosis (Mean ± SD, N=6; *p<0.01 vs control cells, **p<0.01 vs neutrophils alone). **(b).** Similar results were observed when early changes of epithelial cell apoptosis were evaluated by flow cytometry as the binding of Annexin V (Mean ± SD, N=3; *p<0.05 vs control cells, **p<0.05 vs neutrophils alone. **(c).** To confirm that caspase-8 cleavage was necessary for epithelial cell apoptosis, A549 cells were transfected with either wild-type caspase-8, or the cleavage-resistant mutant caspase-8 C377S. Rates of apoptosis were increased following transfection with caspase-8, but not following transfection with the C377S mutant or empty plasmid (N=6; *p<0.01 vs C377S or empty plasmid).

Figure 3. The Caspase-8 Tyrosine Phosphorylation State Regulates Epithelial Cell Survival

(a). Confluent cultures of A549 cells were incubated with LPS (1 μg/ml), TNFα (50 ng/ml), or the agonistic anti-CD95 MAb, CH11 (100 ng/ml). Lysates were immunoprecipitated with anti-caspase-8, and immunoprecipitates probed with an antiphosphotyrosine antibody. Exposure to TNFα or CH11 resulted in tyrosine

dephosphorylation of caspase-8. **(b).** Rates of apoptosis, quantified by propidium iodide uptake in permeabilized cells, were increased in A549 cells transfected with wild-type myc-caspase-8 construct (dark gray bar), and further increased when transfected with either a Y397F or Y465F mutant caspase-8 construct (black bars); in contrast, cells transfected with the non-phosphorylatable Y397E or Y465E constructs (light gray bars) manifested rates of apoptosis that did not differ from those of empty vector controls (N=6; *$p<0.01$ vs non-transfected controls or phosphorylation-resistant mutants; **$p<0.01$ vs wild-type caspase-8 transfectants; ***$p<0.01$ vs non-phosphorylatable mutants, p=NS vs empty vector controls). **(c).** A549 cells transfected with wild-type caspase-8/c-myc (dark gray bars) showed increased activity of caspases-8, and -3; caspase activity was further increased when cells were transfected with the non-phosphorylatable Y397F constructs (black bars; N=5; *$p<0.05$ vs cells transfected with empty vector, **$p<0.05$ vs cells transfected with wild-type caspase-8). **(d).** Transfected A549 cells were stained with FITC-labeled anti-c-myc (green) or ethidium bromide-labeled antibody to the active fragment of caspase-3 (red); nuclei were stained with the nuclear dye, DAPI (blue). Cells were evaluated by immunofluorescence microscopy 24 hours following transfection. Cells transfected with caspase-8 showed enhanced caspase-3 cleavage, reflected in enhanced expression of active caspase-3; cells transfected with the Y397F non-phosphorylatable mutant showed further activation of caspase-3, and increased caspase-3 nuclear translocation.

Figure 4. Hydrogen Peroxide Induces Epithelial Cell Apoptosis

(a). Culture of A549 cells, BEAS-2B cells, or HEK-293 cells with exogenous hydrogen peroxide (H_2O_2, 100μM) led to progressively increased rates of *in vitro* apoptosis, (N=6 for A549 cells, N=3 for BEAS-2B and HEK-293 cells; * $p<0.05$). **(b).** Exogenous H_2O_2 also induced rapid dephosphorylation of caspase-8. Caspase-8 was immunoprecipitated from cell lysates (upper blot) then precipitates were probed with anti-phosphotyrosine antibody (lower blot) **(c).** Whereas minimal spontaneous activational cleavage of caspases-8 (55 kDa) and -3 (34 kDa) was evident following 24 hours of *in vitro* culture in control A549 cells, the addition of H_2O_2 resulted in a time-dependent increase in the cleavage of both to their active moieties (18 and 20 kDa respectively). **(d).** H_2O_2 also produced a dose- and time-dependent decrease in the levels of the endogenous intracellular antioxidant, glutathione (GSH; N=4; *$p<0.05$ versus time 0). **(e).** Addition of glutathione (GSH, 10 mM) to the culture medium significantly attenuated the capacity of neutrophils to induce BEAS-2B cell apoptosis (N=6; ** $p<0.01$ vs cells cultured without GSH).

Figure 5. Src Kinase Activity Promotes Epithelial Cell Survival

(a). Caspase-8 and the Src kinase, lyn, co-immunoprecipitated, albeit weakly, in resting A549 cells. Addition of H_2O_2 (100 μM) or the Src kinase inhibitor, PP2 (10 μM) resulted in disruption of these interactions. Cell lysates were immunoprecipitated with antibodies to caspase-8 (left) or lyn (right), then precipitates were probed by Western blot analysis using anti-caspase-8 (upper panel) or anti-lyn (lower panel). Dotted arrows denote the immunoglobulin heavy chain. **(b).** Addition of PP2 (10 μM) to cell cultures resulted in a time-dependent increase in rates of apoptosis, evaluated as uptake of propidium iodide in

permeabilized cells (N=3 for each). **(c).** c-Src co-immunoprecipitated with caspase-8 in A549 lung epithelial cells; exposure to H_2O_2 (lanes 2 and 4) blocked this interaction. **(d).** A549 cells were transfected with a plasmid containing c-Src, lyn or an empty vector (pcDNA3.1) and cultured with either H_2O_2 or PP2. Overexpression of either c-Src or lyn inhibited the pro-apoptotic effects of H_2O_2, but not those resulting from Src kinase inhibition (N=6; *$p<0.01$ compared with non-transfected cells or cells transfected with vector only).

Figure 6. The Tyrosine Phosphatase, SHP-1, Dephosphorylates Epithelial Cell Caspase-8

(a). Exposure of A549 cells to H_2O_2 (100 μM) resulted in a time-dependent increase in the tyrosine phosphorylation of SHP-1. A549 cell lysates were immunoprecipitated with anti-SHP-1, then precipitates probed by Western analysis with antiphosphotyrosine. **(b).** Exposure of A549 cells to H_2O_2 resulted in increased SHP-1 activity as measured by the malachite green assay (N=3; * $p<0.05$). **(c).** A549 cells were transfected with SHP-1 or empty vector (pcDNA3.1); rates of apoptosis were quantified as uptake of propidium iodide 3 days following transfection. While exposure to H_2O_2 increased rates of epithelial cell apoptosis, overexpression of SHP-1 increased apoptosis in both resting A549 cells, and A549 cells exposed to H_2O_2 (N=6; *$p<0.01$ vs cells not exposed to H_2O_2, ** $p<0.01$ vs cells not transfected with SHP-1) **(d).** A549 cells were co-transfected with caspase-8 and SHP-1. Lysates were immunoprecipitated with anti-c-myc, then probed with antibodies to total or active caspase-8. Cleavage of caspase-8 to its active form was increased by co-transfection. **(e).** A549 cell lysates were immunoprecipitated with anti-

caspase-8 (left) or anti-SHP-1 (right), and precipitates probed by Western analysis with anti-SHP-1 (upper panel) or anti-caspase-8 (lower panel). Exposure to H_2O_2 increased the physical interactions between SHP-1 and caspase-8. **(f).** A549 cells were transfected with myc-tagged constructs encoding SHP-1 (left) or caspase-8 (right), then immuno-precipitated with anti-c-myc, and immunoblotted with either anti-c-myc (upper panel) or anti-phosphotyrosine (lower panel). Exposure of transfected cells to H_2O_2 (100 μM) increased the tyrosine phosphorylation of transfected SHP-1, and concomitantly, decreased the phosphorylation of caspase-8.

Figure 7. LPS-activated Neutrophils Induce SHP-1 Phosphorylation and Activity, and Increase Caspase-8 Dephosphorylation

(a,b). Neutrophils freshly isolated from healthy volunteers were cultured with either medium, DPI (10 μM), or catalase (10 μg/ml), with or without LPS (1 μg/ml), then added to subconfluent cultures of A549 epithelial cells (**Fig. 7a**) or HEK293 cells (**Fig. 7b**). Pretreatment with either DPI or catalase attenuated the increase in epithelial cell apoptosis resulting from exposure to LPS-activated neutrophils (N=5 for each; *$p<0.05$ compared with LPS-treated neutrophils alone). **(c).** SHP-1 phosphatase activity measured using the malachite green assay was increased when A549 cells were cultured with resting neutrophils, and further increased when A549 cells were cultured with LPS-activated neutrophils (N=3; *$p<0.05$ vs A549 cells alone, **$p<0.05$ vs resting PMN). **(d).** A549 cells were transfected with myc-tagged SHP-1, and lysates immunoprecipitated with anti-myc, then probed with anti-phosphotyrosine. Exposure to LPS-stimulated neutrophils resulted in enhanced tyrosine phosphorylation of SHP-1. **(e).** A549 cells

were transfected with myc-tagged caspase-8, immunoprecipitated with anti-myc, and probed with either anti-myc (upper panel) or antiphosphotyrosine (lower panel). Co-culture with LPS-activated neutrophils resulted in reduced tyrosine phosphorylation of the transfected caspase-8 construct. **(f).** A549 cells were transfected with myc-tagged constructs encoding the Src family kinase members Lyn (gray bars) or c-Src (black bars), then incubated with neutrophils and/or LPS. Over-expression of Src family kinases provided protection against the apoptosis-inducing activity of LPS-activated neutrophils. N=4; * $p<0.05$.

Author Contributions

S-H.J. designed and performed the experiments and analyzed the data; J.P performed experiments; E.C. recruited trauma patients and performed experiments; S.J. performed experiments; J.T. performed experiments and assisted in preparation of the manuscript; O.D.R. assisted with interpretation of data; A.K. assisted with the design of experiments and interpretation of data; J.C.M. designed the experiments, analyzed the data and wrote the manuscript.

Supported by grants from the Canadian Institutes of Health Research #MOP-62908 and MOP-129493.

Reference List

1. Brown,K.A. *et al.* Neutrophils in development of multiple organ failure in sepsis. *Lancet* **368**, 157-169 (2006).

2. Hotchkiss,R.S. & Nicholson,D.W. Apoptosis and caspases regulate death and inflammation in sepsis. *Nat. Rev. Immunol.* **6**, 813-822 (2006).

3. Imai,Y. *et al.* Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome. *JAMA* **289**, 2104-2112 (2003).

4. Wesche-Soldato,D.E. *et al.* In vivo delivery of Caspase 8 or Fas siRNA improves the survival of septic mice. *Blood* **106**, 2295-2301 (2005).

5. Coopersmith,C.M. *et al.* Inhibition of intestinal epithelial apoptosis and survival in a murine model of pneumonia-induced sepsis. *JAMA* **287**, 1716-1721 (2008).

6. Perl,M., Lomas-Neira,J., Chung,C.S., & Ayala,A. Epithelial cell apoptosis and neutrophil recruitment in acute lung injury-a unifying hypothesis? What we have learned from small interfering RNAs. *Mol. Med.* **14**, 465-475 (2008).

7. Smith,J.A. Neutrophils, host defense, and inflammation: A double-edged sword. *J. Leuk. Biol.* **56**, 672-686 (1994).

8. Weiss,S.J. Tissue destruction by neutrophils. *N. Engl. J. Med.* **320**, 365-376 (1989).

9. Nathan,C. & Ding,A. Nonresolving inflammation. *Cell* **140**, 871-882 (2010).

10. Smedly,L.A. *et al.* Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *J. Clin. Invest.* **77**, 1233-1243 (1986).

11. Ramaiah,S.K. & Jaeschke,H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol. Pathol.* **35**, 757-766 (2007).

12. Hotchkiss,R.S. *et al.* Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit Care Med* **27**, 1230-1251 (1999).

13. Mantell,L.L. *et al.* Unscheduled apoptosis during acute inflammatory lung injury. *Cell Death Differ.* **4**, 600-607 (1997).

14. Kawasaki,M. *et al.* Protection from lethal apoptosis in lipopolysaccharide-induced acute lung injury in mice by a caspase inhibitor. *Am. J. Pathol.* **157**, 597-603 (2000).

15. Mayadas,T.N. & Cullere,X. Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol.* **26**, 388-395 (2005).

16. Dandrea,T. *et al.* The transcriptosomal response of human A549 lung cells to a hydrogen peroxide-generating system: relationship to DNA damage, cell cycle arrest, and caspase activation. *Free Radic. Biol. Med.* **36**, 881-896 (2004).

17. Cursi,S. *et al.* Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J.* **25**, 1895-1905 (2006).

18. Jia,S.H., Parodo,J., Kapus,A., Rotstein,O.D., & Marshall,J.C. Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. *J. Biol. Chem.* **283**, 5402-5413 (2008).

19. Daëron,M., Jaeger,S., Du Pasquier,L., & Vivier,E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. *Immunol. Rev.* **224**, 11-43 (2008).

20. Daigle,I., Yousefi,S., Colonna,M., Green,D.R., & Simon,H.-U. Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils. *Nature Med.* **8**, 61-67 (2002).

21. Lieber,M., Smith,B., Szakal,A., Nelson-Rees,W., & Todaro,G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* **17**, 62-70 (1976).

22. Hasegawa *et al.* ASC-mediated NF-kappaB activation leading to interleukin-8 production requires caspase-8 and is inhibited by CLARP. *J. Biol. Chem.* **280**, 15122-15130 (2005).

23. Necula,M. & Kuret,J. Site-specific pseudophosphorylation modulates the rate of tau filament dissociation. *FEBS Lett.* **579**, 1453-1457 (2005).

24. Zhang,Z., Shen,K., Lu,W., & Cole,P.A. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *J. Biol. Chem.* **278**, 4668-4674 (2003).

25. Savill,J.S., Wyllie,A.H., Henson,J.E., Henson,P.M., & Haslett,C. Macrophage phagocytosis of aging neutrophils in inflammation. *J. Clin. Invest.* **83**, 865-875 (1989).

26. Komai,M. & Kimura,S. Effects of restricted diet and intestinal flora on the life span of small intestine epithelial cells in mice. *J. Nutr. Sci. Vitaminol. (Tokyo)* **25**, 87-94 (1979).

27. Rawlins,E.L. & Hogan,B.L. Ciliated epithelial cell lifespan in the mouse trachea and lung. *Am. J. Physiol Lung Cell Mol. Physiol* **295**, L231-L234 (2008).

28. Gitter,A.H., Wullstein,F., Fromm,M., & Schulzke,J.D. Epithelial barrier defects in ulcerative colitis: characterization and quantification by electrophysiological imaging. *Gastroenterology* **121**, 1320-1328 (2001).

29. Mahida,Y.R., Makh,S., Hyde,S., Gray,T., & Borrriello,S.P. Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: Induction of interleukin 8 production and apoptosis after cell detachment. *Gut* **38**, 337-347 (1996).

30. Takeyama,Y. Significance of apoptotic cell death in systemic complications with severe acute pancreatitis. *J. Gastroenterol.* **40**, 1-10 (2005).

31. Herold,S. *et al.* Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J. Exp. Med.* **205**, 3065-3077 (2008).

32. Wu,H. *et al.* TLR4 activation mediates kidney ischemia/reperfusion injury. *J. Clin. Invest.* **117**, 2847-2859 (2007).

33. Hotchkiss,R.S. *et al.* Rapid onset of intestinal epithelial and lymphocyte apoptotic cell death in patients with trauma and shock. *Crit. Care Med.* **28**, 3207-3217 (2000).

34. Ku,N.O., Toivola,D.M., Strnad,P., & Omary,M.B. Cytoskeletal keratin glycosylation protects epithelial tissue from injury. *Nat. Cell Biol.* **12**, 876-885 (2010).

35. Blank,U., Launay,P., Benhamou,M., & Monteiro,R.C. Inhibitory ITAMs as novel regulators of immunity. *Immunol. Rev.* **232**, 59-71 (2009).

36. Abram,C.L. & Lowell,C.A. The expanding role for ITAM-based signaling pathways in immune cells. *Sci. STKE.* **2007**, re2 (2007).

37. Ivashkiv,L.B. Cross-regulation of signaling by ITAM-associated receptors. *Nat. Immunol.* **10**, 340-347 (2009).

38. Zhao,Y., Sui,X., & Ren,H. From procaspase-8 to caspase-8: revisiting structural functions of caspase-8. *J. Cell Physiol* **225**, 316-320 (2010).

39. Lorenz,U. SHP-1 and SHP-2 in T cells: two phosphatases functioning at many levels. *Immunol. Rev.* **228**, 342-359 (2009).

40. Xiao,W., Ando,T., Wang,H.Y., Kawakami,Y., & Kawakami,T. Lyn- and PLC-beta3-dependent regulation of SHP-1 phosphorylation controls Stat5 activity and myelomonocytic leukemia-like disease. *Blood* **116**, 6003-6013 (2010).

41. Yan,S.R. & Berton,G. Regulation of Src family tyrosine kinase activities in adherent human neutrophils. Evidence that reactive oxygen intermediates produced by adherent neutrophils increase the activity of the p58c-fgr and p53/56lyn tyrosine kinases. *J. Biol. Chem.* **271**, 23464-23471 (1996).

42. Van de Bittner,G.C., Dubikovskaya,E.A., Bertozzi,C.R., & Chang,C.J. In vivo imaging of hydrogen peroxide production in a murine tumor model with a chemoselective bioluminescent reporter. *Proc. Natl. Acad. Sci. U. S. A* **107**, 21316-21321 (2010).

43. Segel,G.B., Halterman,M.W., & Lichtman,M.A. The paradox of the neutrophil's role in tissue injury. *J. Leukoc. Biol.* **89**, 359-372 (2011).

44. Borregaard,N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657-670 (2010).

45. Mantovani,A., Cassatella,M.A., Costantini,C., & Jaillon,S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat. Rev. Immunol.* **11**, 519-531 (2011).

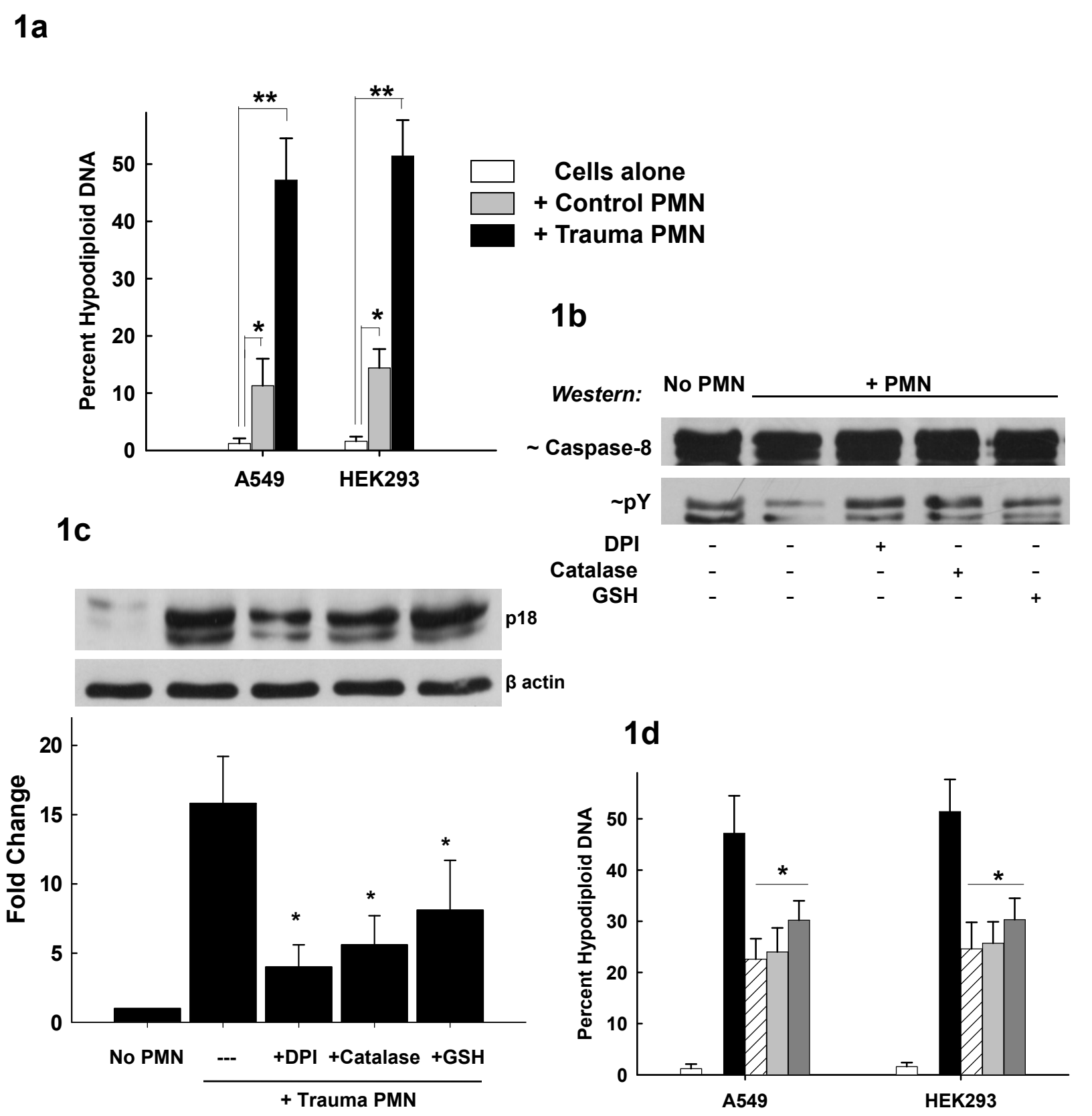
46. Hotchkiss,R.S. & Karl,I.E. The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* **348**, 238-250 (2003).

47. Marshall,J.C. Such stuff as dreams are made on: Mediator-targeted therapy in sepsis. *Nature Rev. Drug Disc.* **2**, 391-405 (2003).

48. Jia,S.H. *et al.* Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. *J. Clin. Invest.* **113**, 1318-1327 (2004).

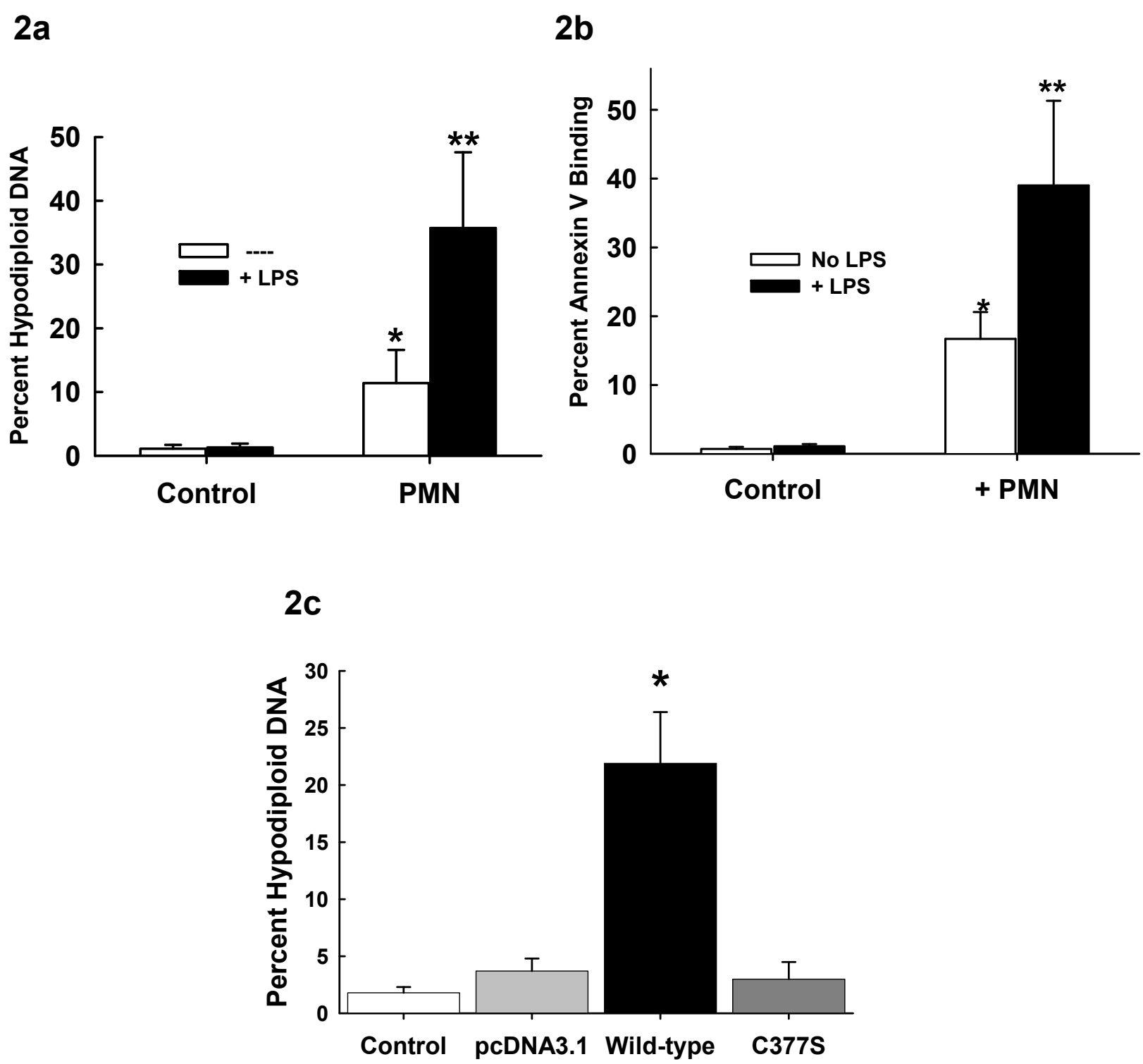
49. Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F., & Riccardi,C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271-276 (1991).

50. Watson,R.W.G., Rotstein,O.D., Jimenez,M., Parodo,J., & Marshall,J.C. Augmented intracellular glutathione inhibits Fas-triggered apoptosis of activated human neutrophils. *Blood* **89**, 4175-4181 (1997).


1a
Percent Hypodiploid DNA
0
10
20
30
40
50
**
**
*
*
A549
HEK293
Cells alone
+ Control PMN
+ Trauma PMN
1b
Western:
No PMN
+ PMN
~ Caspase-8
~pY
DPI - - + - -
Catalase - - - + -
GSH - - - - +
1c
p18
β actin
Fold Change
0
5
10
15
20
*
*
*
No PMN

+DPI
+Catalase
+GSH
+ Trauma PMN
1d
Percent Hypodiploid DNA
0
10
20
30
40
50
*
*
A549
HEK293

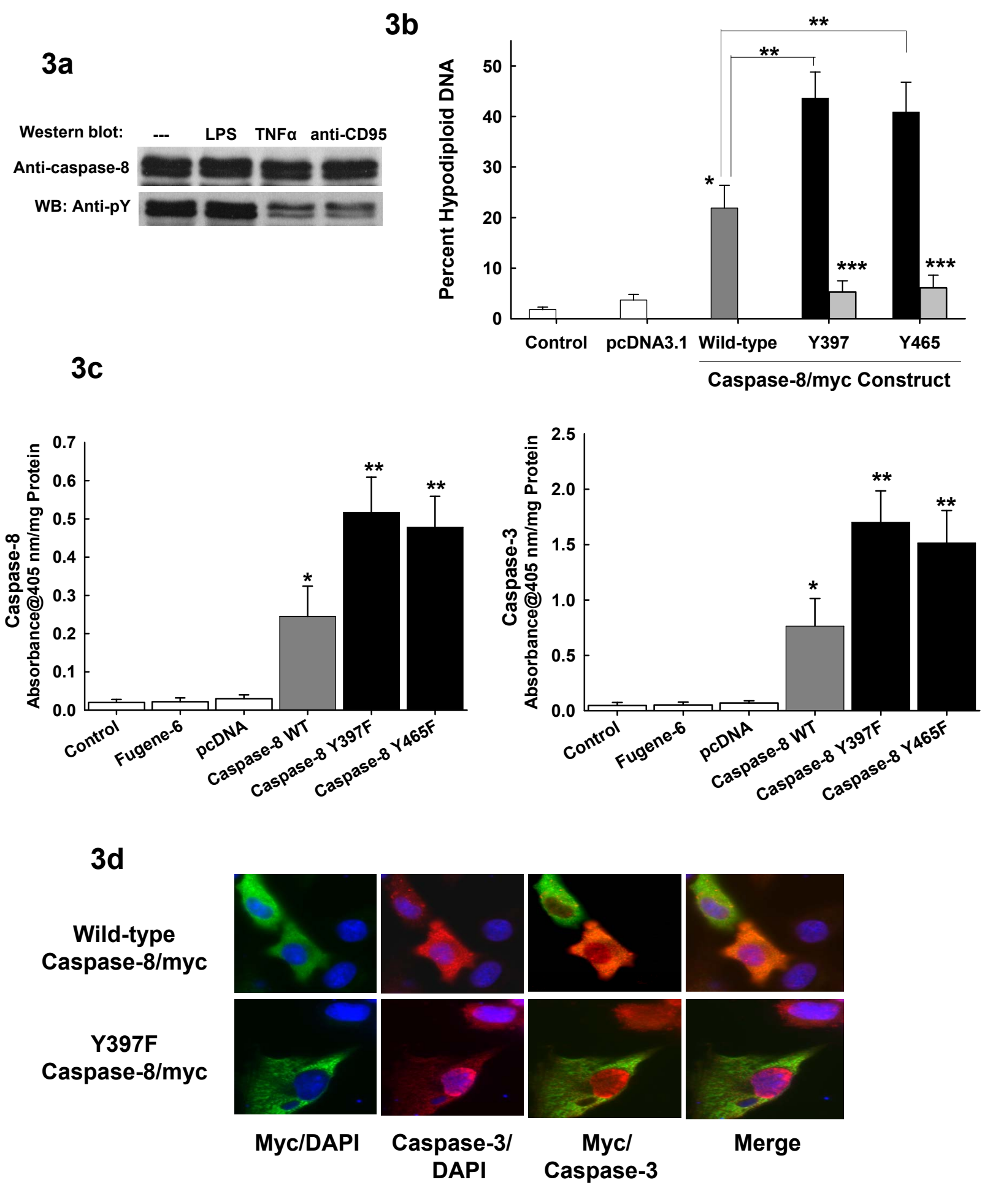
Figure 1


2a
Percent Hypodiploid DNA
50
40
30
20
10
0

+ LPS
*
**
Control
PMN
2b
Percent Annexin V Binding
50
40
30
20
10
0
No LPS
+ LPS
*
**
Control
+ PMN

2c
Percent Hypodiploid DNA
30
25
20
15
10
5
0
*
Control
pcDNA3.1
Wild-type
C377S

Figure 2.


3a
Western blot:

LPS
TNFα
anti-CD95
Anti-caspase-8
WB: Anti-pY
3b
Percent Hypodiploid DNA
Control
pcDNA3.1
Wild-type
Y397
Y465
Caspase-8/myc Construct
3c
Caspase-8
Absorbance@405 nm/mg Protein
Caspase-3
Absorbance@405 nm/mg Protein
Control
Fugene-6
pcDNA
Caspase-8 WT
Caspase-8 Y397F
Caspase-8 Y465F
3d
Wild-type
Caspase-8/myc
Y397F
Caspase-8/myc
Myc/DAPI
Caspase-3/
DAPI
Myc/
Caspase-3
Merge

Figure 3

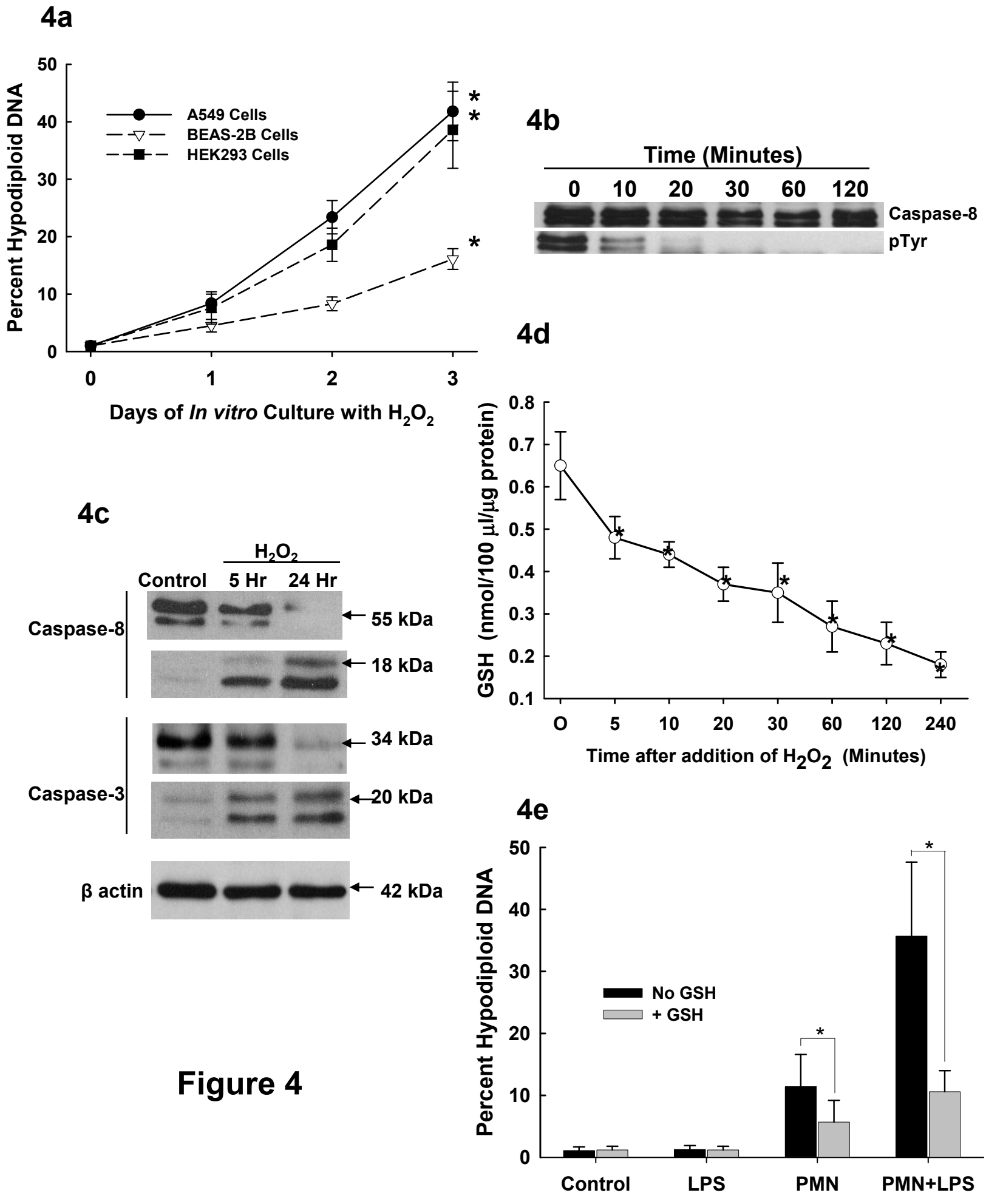
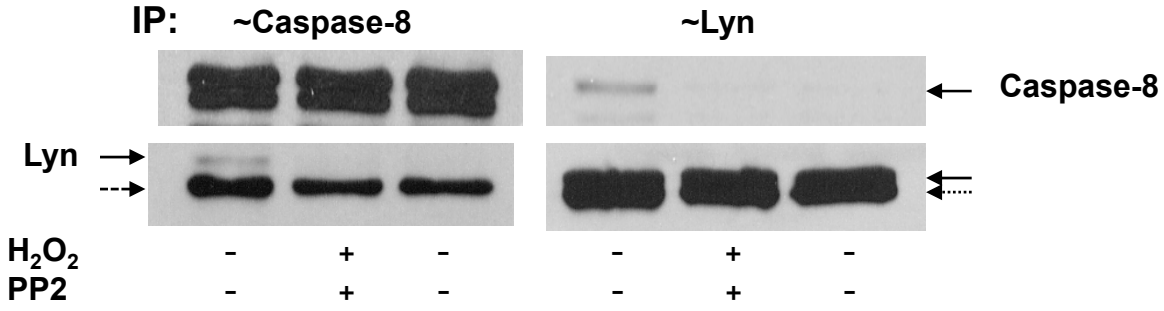
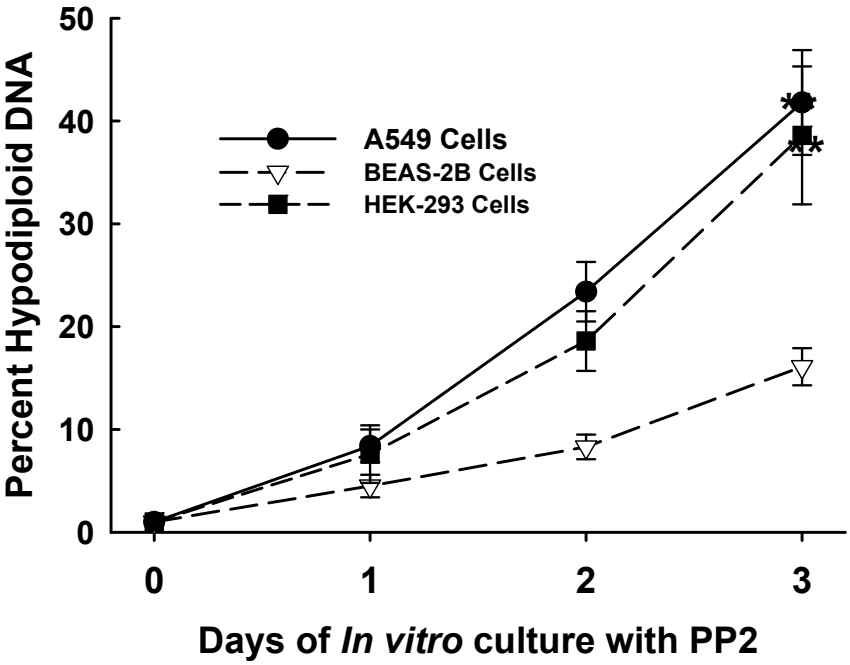

4a
Percent Hypodiploid DNA
A549 Cells
BEAS-2B Cells
HEK293 Cells
Days of In vitro Culture with H2O2
4b
Time (Minutes)
0 10 20 30 60 120
Caspase-8
pTyr
4c
H2O2
Control 5 Hr 24 Hr
Caspase-8
55 kDa
18 kDa
Caspase-3
34 kDa
20 kDa
β actin
42 kDa
4d
GSH (nmol/100 µl/µg protein)
Time after addition of H2O2 (Minutes)
4e
Percent Hypodiploid DNA
No GSH
+ GSH
Control
LPS
PMN
PMN+LPS

Figure 4

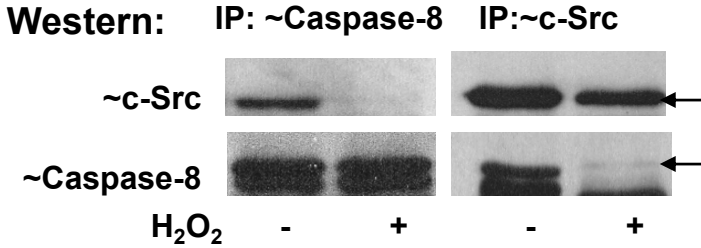
Figure 5



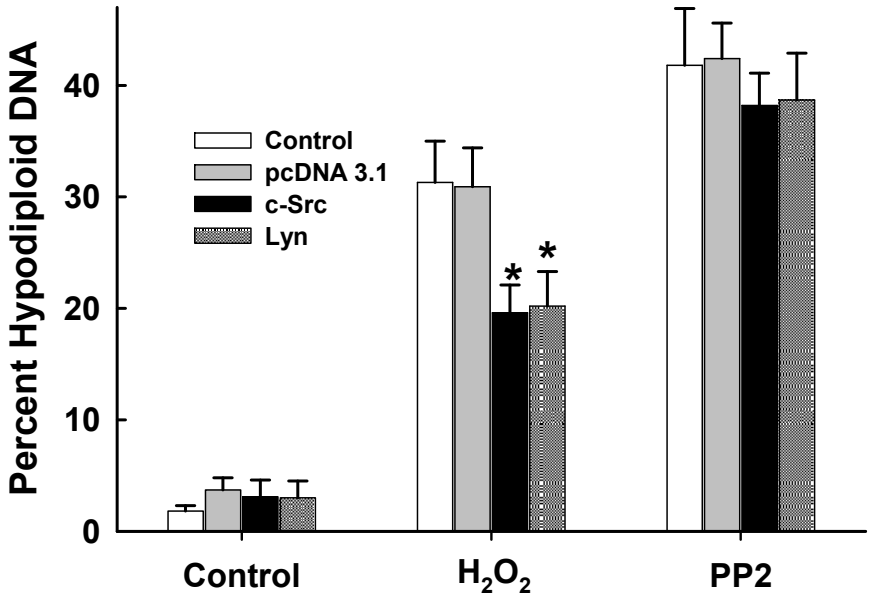
5b

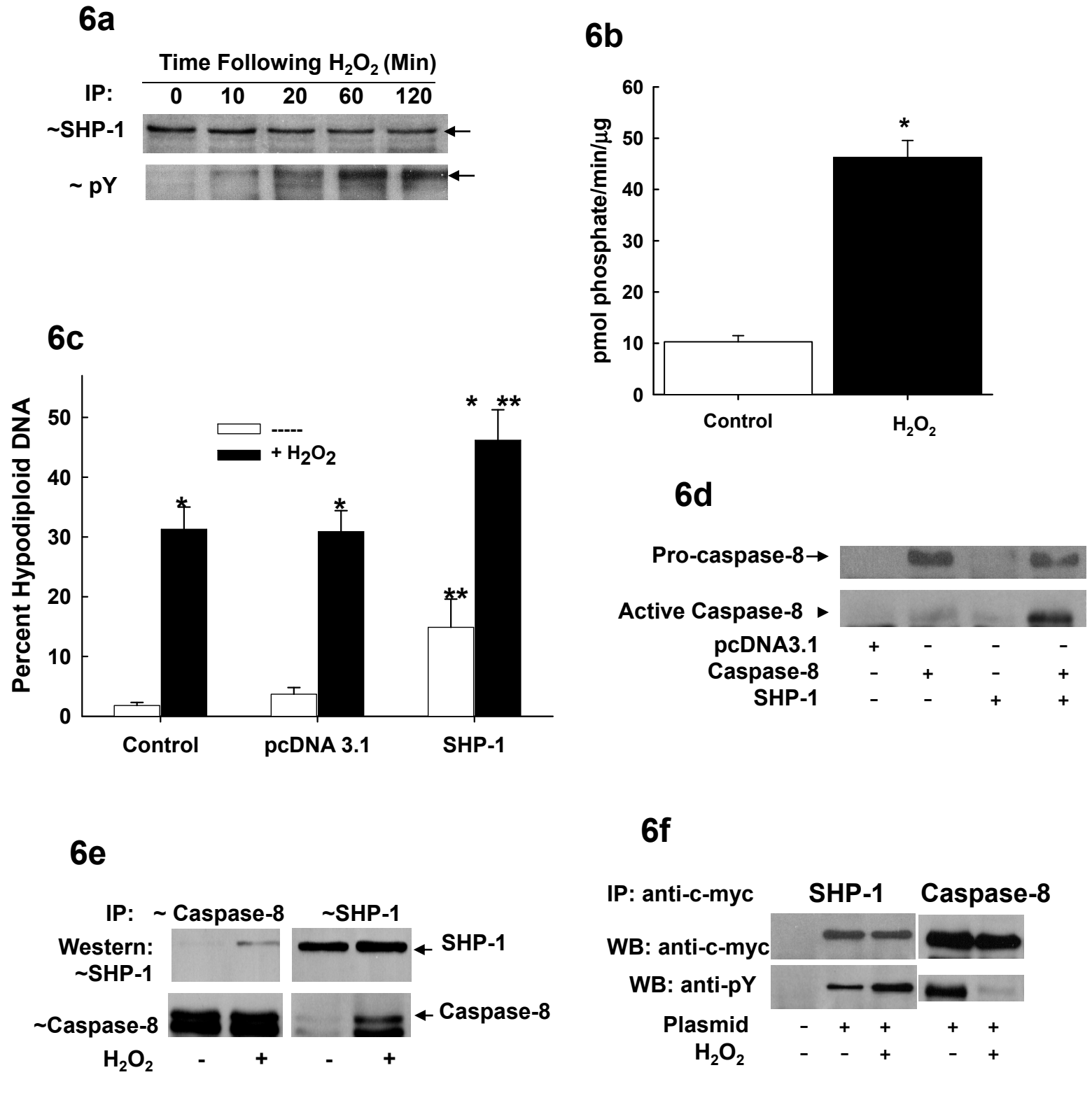


5c



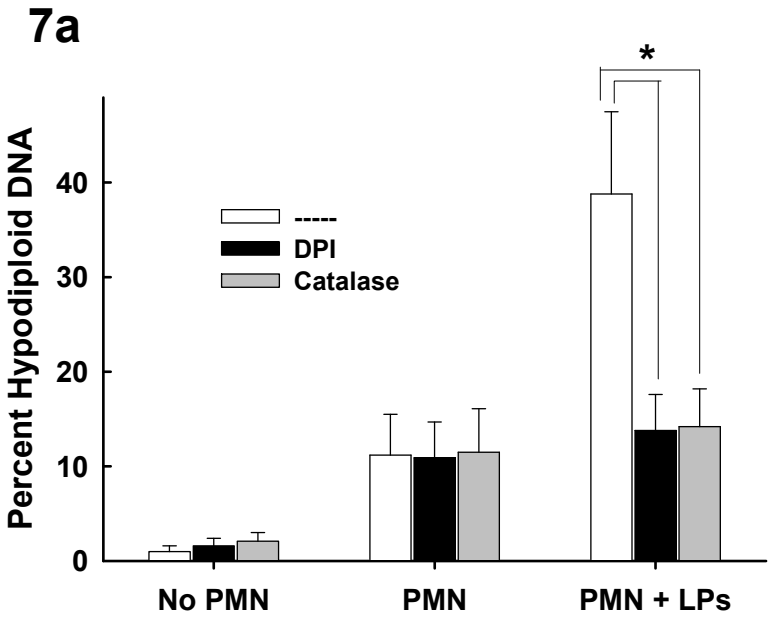
5d



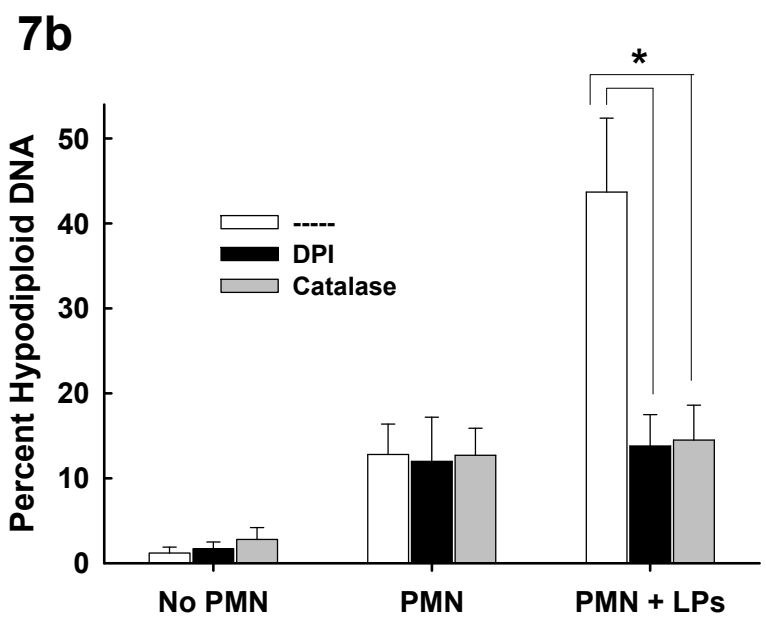

6a
Time Following H_2O_2 (Min)
IP:
0
10
20
60
120
~SHP-1
~ pY
6b
pmol phosphate/min/µg
60
50
40
30
20
10
0
*
Control
H_2O_2
6c
Percent Hypodiploid DNA
50
40
30
20
10
0

+ H_2O_2
*
*
**
**
Control
pcDNA 3.1
SHP-1
6d
Pro-caspase-8
Active Caspase-8
pcDNA3.1 + - - -
Caspase-8 - + - +
SHP-1 - - + +
6e
IP: ~ Caspase-8
~SHP-1
Western: ~SHP-1
SHP-1
~Caspase-8
Caspase-8
H_2O_2 - + - +
6f
IP: anti-c-myc
SHP-1
Caspase-8
WB: anti-c-myc
WB: anti-pY
Plasmid - + + + +
H_2O_2 - - + - +

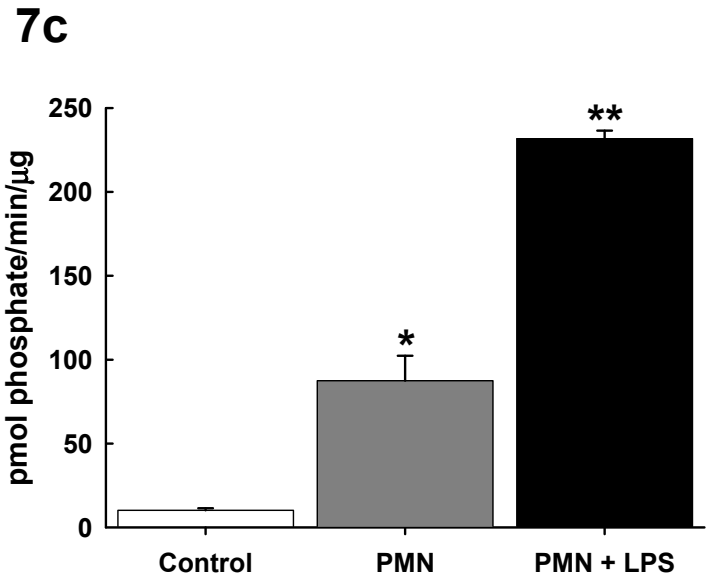
Figure 6

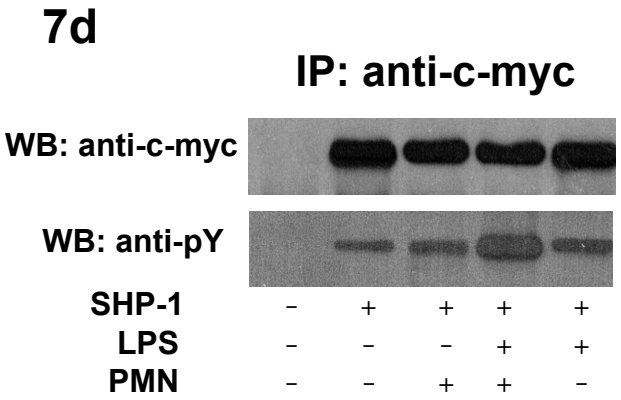

7a
Percent Hypodiploid DNA
40
30
20
10
0
*

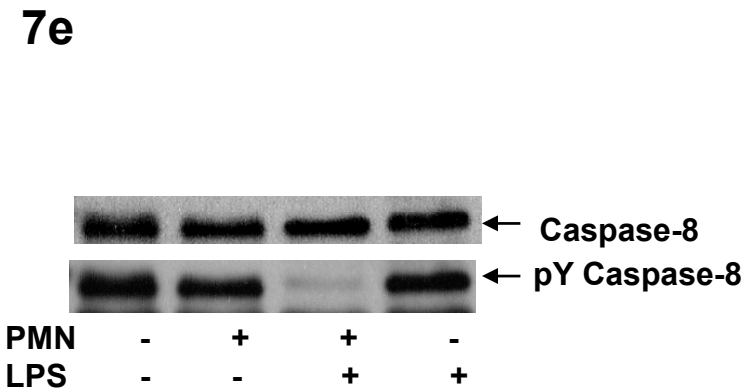
DPI
Catalase
No PMN
PMN
PMN + LPs


7b
Percent Hypodiploid DNA
50
40
30
20
10
0
*

DPI
Catalase
No PMN
PMN
PMN + LPs


7c
pmol phosphate/min/µg
250
200
150
100
50
0
**
*
Control
PMN
PMN + LPS


7d
IP: anti-c-myc
WB: anti-c-myc
WB: anti-pY
SHP-1 - + + + +
LPS - - - + +
PMN - - + + -


7e
Caspase-8
pY Caspase-8
PMN - + + -
LPS - - + +

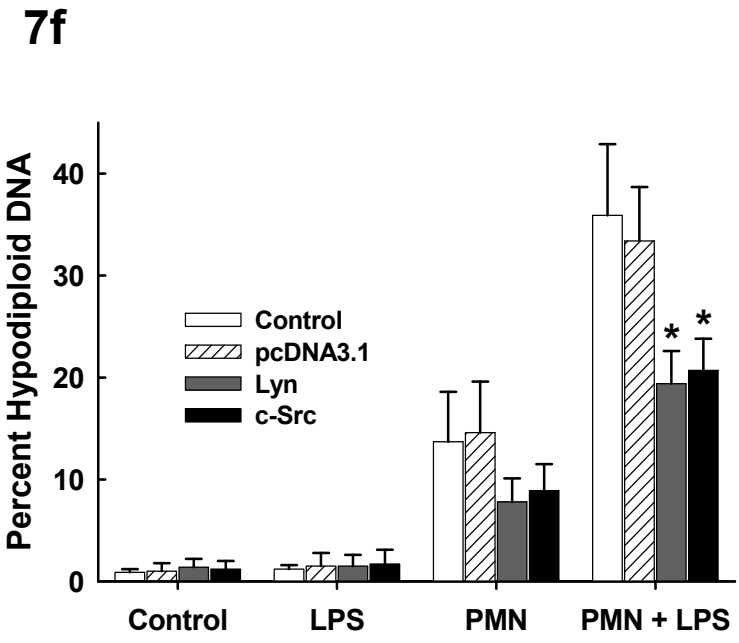

7f
Percent Hypodiploid DNA
40
30
20
10
0
Control
pcDNA3.1
Lyn
c-Src
*
*
Control
LPS
PMN
PMN + LPS

Figure 7.