Dynamic Regulation of Neutrophil Survival through Tyrosine Phosphorylation or Dephosphorylation of Caspase-8*

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Song Hui Jia¹, Jean Parodo¹,⁵, Andras Kapus¹,⁵, Ori D. Rotstein¹,⁵, and John C. Marshall¹,⁵,‡⁴,§

From the ¹Department of Surgery and the ⁵Interdepartmental Division of Critical Care Medicine, University of Toronto, Toronto, Ontario and the ⁴Sepsis Research Laboratory, Li Ka Shing Knowledge Institute, St. Michael’s Hospital, Toronto, Ontario M5B 1W8, Canada

Efficient expression of innate immunity is critically dependent upon the capacity of the neutrophil to be activated rapidly in the face of an acute threat and to involute once that threat has been eliminated. Here we report a novel mechanism regulating neutrophil survival dynamically through the tyrosine phosphorylation or dephosphorylation of caspase-8. Caspase-8 is tyrosine-phosphorylated in freshly isolated neutrophils but spontaneously dephosphorylates in culture, in association with the progression of constitutive apoptosis. Phosphorylation of caspase-8 on Tyr-310 facilitates its interaction with the Src-homology domain 2 containing tyrosine phosphatase-1 (SHP-1) and enables SHP-1 to dephosphorylate caspase-8, permitting apoptosis to proceed. The non-receptor tyrosine kinase, Lyn, can phosphorylate caspase-8 on Tyr-397 and Tyr-465, rendering it resistant to activation and cleavage and inhibiting apoptosis. Exposure to lipopolysaccharide reduces SHP-1 activity and binding to caspase-8, caspase-8 activity, and rates of spontaneous apoptosis. SHP-1 activity is reduced and Lyn increased in neutrophils from patients with sepsis, in association with profoundly delayed apoptosis; inhibition of Lyn can partially reverse this delay. Thus the phosphorylation and dephosphorylation of caspase-8, mediated by Lyn and SHP-1, respectively, represents a novel, dynamic post-translational mechanism for the regulation of neutrophil apoptosis whose dysregulation contributes to persistent neutrophil survival in sepsis.

Sepsis, a life-threatening disorder that arises through a dysregulated systemic host inflammatory response to infection, is the most common cause of morbidity and mortality for critically ill patients admitted to an intensive care unit. With an annual incidence of 300 cases per 100,000 population and a fatality rate of 30% over the first month, it rivals acute myocardial infarction and stroke as a modifiable cause of mortality in the developed world (1, 2). Sepsis is effected through changes in the expression of literally thousands of genes (3), resulting in systemic alterations in metabolic and immunologic homeostasis, and in fundamental changes in the kinetics of cell survival. Accelerated apoptosis results in the depletion of lymphocytes and certain epithelial cells, whereas inhibition of a constitutive apoptotic program leads to the prolonged survival of activated polymorphonuclear neutrophils (4, 5).

Neutrophils, the cardinal cellular effectors of the immediate innate host response to injury, have been implicated in the pathogenesis of a variety of acute and chronic inflammatory disorders. Neutrophils damage host tissues, because their effector mechanisms, both oxidant-mediated and non-oxidant-mediated, are non-specific, with the result that bystander tissue injury is an invariable concomitant of neutrophil activation (6). The extent of this tissue injury is limited by the termination of a neutrophil-mediated inflammatory response through the programmed cell death or apoptosis of the neutrophil (7). In health, the in vivo life span of the neutrophil is 6–8 h (8): ~10¹¹ neutrophils are released each day from bone marrow stores, whereas an equal number die an apoptotic death (9). Their removal not only eliminates a potentially activated cell population from the host, but also triggers transcriptional programs in the phagocytosing cell that are anti-inflammatory and reparative in nature (10). Thus neutrophil apoptosis is fundamental to the maintenance of normal immunologic homeostasis.

Neutrophil apoptosis is inhibited in vitro by a wide variety of inflammatory stimuli of both host and microbial origin that are present in the inflammatory microenvironment (11, 12), and in a number of inflammatory diseases, including arthritis (13), inflammatory bowel disease (14), and sepsis (5, 15). Prolonged survival is dependent on the transcription of anti-apoptotic survival factors such as granulocyte macrophage-colony stimulating factor, interleukin-1β, and Mcl-1 (16, 17). We now show that neutrophil apoptosis can also be regulated rapidly, dynamically, and post-transcriptionally through the tyrosine phosphorylation and dephosphorylation of caspase-8, the apoptotic membrane-associated enzyme of the extrinsic pathway of caspase-mediated apoptosis. Caspase-8 activation is inhibited by its tyrosine phosphorylation at Tyr-397 and Tyr-465, a process that is effected by the tyrosine kinase, Lyn, whereas the tyrosine phosphatase, SHP-1,² binding to a phosphorylated tyrosine residue at Tyr-310, dephosphorylates caspase-8 and permits its cleavage and activation. These observations reveal a novel mechanism for the dynamic regulation of neutrophil survival.

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1 To whom correspondence should be addressed: St. Michael’s Hospital, 4th Floor Bond Wing, Rm. 4-007, 30 Bond St., Toronto M5B 1W8, Ontario. Tel.: 416-864-5225; Fax: 416-864-5141; E-mail: marshallj@smh.toronto.on.ca.

2 The abbreviations used are: SHP-1, Src-homology domain 2 containing tyrosine phosphatase-1; CHO, Chinese hamster ovary; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PTTP, α-bromo-4-hydroxyacetophenone; LPS, lipopolysaccharide.
vival during inflammation through the tyrosine phosphorylation and dephosphorylation of caspase-8.

**EXPERIMENTAL PROCEDURES**

**Study Subjects**—We studied healthy laboratory volunteers and critically ill patients hospitalized in the Medical Surgical Intensive Care Unit of the Toronto General Hospital who met clinical criteria for severe sepsis (18) and had a sepsis score (19) of 3 or higher. Informed consent was obtained in each case from the volunteer or a family member; the study protocol was reviewed and approved by the Human Research Ethics Board of the University Health Network.

**Neutrophil Isolation and Culture**—We obtained up to 20 ml of whole blood into heparinized tubes by venipuncture from healthy volunteers or through an indwelling arterial line from septic patients. We isolated neutrophils by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient as previously described (5); cell populations were consistently >95% neutrophils, and viability as assessed by trypan blue exclusion routinely exceeded 95%. Polymorphonuclear neutrophils were resuspended in polypropylene tubes at a concentration of 1 x 10⁶ cells/ml in supplemented Dulbecco’s modified Eagle’s medium (Invitrogen).

**Cell Lines and Reagents**—CHO cells were obtained from ATCC (number CCL-61) and cultured in α-minimal essential medium with 10% fetal bovine serum and 1% penicillin/streptomycin solution. HL-60 cells were also obtained from ATCC (number CCL-240), and cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin. In some studies, HL-60 cells were induced to undergo differentiation to a neutrophil-like phenotype through the addition of 1 μM all-trans-retinoic acid to the culture medium (20).

Antibodies (dilutions and suppliers) used for these studies were murine monoclonal anti-caspase-8 (1:000, Calbiochem), murine monoclonal anti-β-actin (1:4000, Sigma), murine monoclonal anti-phosphotyrosine (1:2000, Upstate), murine monoclonal anti-phosphoserine (1:20, Calbiochem), murine monoclonal anti-c-Myc (1:1000, Santa Cruz Biotechnology), murine monoclonal anti-SHP-1 (1:1000, Santa Cruz Biotechnology), murine monoclonal anti-Lyn (1:1000, Santa Cruz Biotechnology), anti-mouse IgG horseradish peroxidase-conjugated (1:4000, Amersham Biosciences), and anti-rabbit IgG horseradish peroxidase-conjugated (1:4000, Amersham Biosciences). PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), a selective inhibitor of Src family kinases (21), and protein-tyrosine phosphatase inhibitor 1 (PTPI, α-bromo-4-hydroxyacetophenone, 10 μM) (22), were purchased from Calbiochem.

**Quantification of Apoptosis**—We measured rates of apoptosis by flow cytometry, quantifying the uptake of propidium iodide in Triton X-100 permeabilized cells as previously described (5, 23). Briefly, Triton X-100-permeabilized neutrophils or HL-60 cells were incubated with propidium iodide (50 μg/ml) and analyzed using a Coulter Epics XL-MCL cytofluorometer (Hialeah, FL). A minimum of 5000 events was collected and analyzed.

CHO cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (24). CHO cells were plated in 6-well plates at 50% confluency prior to transfection. After 48 h of culture, cells were washed twice with phosphate-buffered saline, and 2 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (500 ng/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline) was added. Following 1-h incubation at 37 °C, 2 ml of acidic isopropanol (0.04 M HCl in absolute isopropanol) was added, and the cells were suspended by vigorous pipetting. The contents of the wells were then transferred to Eppendorf tubes and centrifuged at 12,000 rpm for 5 min. Color development was read at 570 nm using a Milton Roy Spectronic 1001 Plus spectrophotometer.

**Western Blot and Immunoprecipitation Studies**—We lysed 2 x 10⁶ polymorphonuclear neutrophils in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotonin). Cell lysates were run on a 10% SDS-PAGE gel, transferred to nitrocellulose (Amer sham Biosciences), and probed with the appropriate primary antibody. Bands were detected with an horseradish peroxidase-conjugated second antibody at a dilution of 1:4000 using the ECL Western blotting detection system (Amer sham Biosciences). Blots were stripped and reprobed with a monoclonal antibody to β-actin at a 1:4000 dilution, to confirm equal loading of the gels.

For immunoprecipitation studies, cell lysates were centrifuged at 12,000 rpm for 10 min. Supernatants were pre-cleared with protein G-Sepharose beads (Amersham Biosciences) for 1 h, and 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 Biotechnology), or 5 μl of anti-Lyn (Santa Cruz Biotechnology) was added to 500 μl of supernatant, and incubated for 1 h at 4 °C before adding Protein G beads (20 μl) and incubating for an additional 1 h or overnight. Suspensions were centrifuged, and beads washed three times in phosphate-buffered saline, then boiled in Laemmli buffer for 5 min prior to SDS-PAGE and Western analysis. Western analysis of the immunoblots used anti-phosphotyrosine (clone 4G10) 1:2000 (Upstate), anti-phosphoserine (16B4, mouse) 1:20 (Calbiochem), or other antibodies as noted.

**Caspase-8 Activity Assay**—We measured caspase-8 activity using either a fluorometric substrate (Ac-Ile-Glu-Asp-amino-4-methylcoumarin, Upstate) or a colorimetric substrate (IETD-pNA, BioVision). For the former, 10 x 10^6 cells were lysed in chilled lysis buffer according to the manufacturer’s protocol. After measuring protein concentration, 50 μl of the cell lysate supernatant was incubated with 7 μl of the caspase-8 fluorometric substrate in a 96-well plate. Fluorescence was measured using a fluorometer at an excitation wavelength of 380 nm and an emission wavelength at 460 nm, and caspase-8 activity was expressed as picomoles/second/mg of protein. For assay of caspase-8 using a colorimetric substrate, cell lysates were incubated with 25 μl of a specific substrate for caspase-8 in a 96-well plate. Plates were incubated overnight, and color development was measured using a colorimetric plate reader (LabSystems Multiskan, Ascent Software) at 405 nm; caspase-8 activity was expressed as absorbance at 405 nm/mg of protein.
Caspase-8 Phosphorylation Regulates Neutrophil Apoptosis

**Caspase-8 Activity** (A) and **Percent Apoptosis** (B) in neutrophils from healthy volunteers were constitutively apoptotic, measured as the uptake of propidium iodide in Triton X-100-permeabilized cells after 21 h of *in vitro* culture. LPS (1 µg/ml) inhibited neutrophil apoptosis; rates were similarly inhibited in circulating neutrophils harvested from critically ill patients with sepsis (n = 4–6/group; *, p < 0.05 versus controls). B. Inhibition of caspase-8 using the reversible tetrapeptide inhibitor, IETD-CHO, resulted in a dose-dependent delay in constitutive neutrophil apoptosis, whereas transfection of HL-60 cells with wild-type, but not catalytically inactive C377S caspase-8 resulted in increased rates of spontaneous apoptosis (results are means ± S.D.; *, p < 0.05, n = 3). Caspase-8 catalytic activity measured as the cleavage of the tetrapeptide IETD-AMC increased over time in cultures of quiescent neutrophils, but decreased in neutrophils that had been exposed to LPS (n = 4, p < 0.05). Similarly, spontaneous cleavage of pro-caspase-8, generating the active p20 moiety, was apparent by Western blot during 2 h of *in vitro* culture (D); LPS prevented caspase-8 cleavage.

**SHP-1 Activity Assay**—The release of inorganic phosphate from phosphopeptides was measured using the malachite green assay (Upstate). Briefly, 10 × 10⁶ polymorphonuclear neutrophils were lysed for 10 min on ice in lysis buffer containing 1 mM sodium orthovanadate and protease inhibitors. Suspensions were centrifuged for 15 min at 12,000 × *g*, protein content of the resulting supernatant was determined, and 500-µl supernatant was incubated with 10 µl of anti-SHP-1 antibody (Santa Cruz Biotechnology) prebound to Protein A-Sepharose (Santa Cruz Biotechnology) for 3 h at 4 °C. Immunoprecipitates were washed six times (Invitrogen) according to the manufacturer’s instructions. The recombinant plasmids were transfected into DH5α-vector competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and sequencing.

**Construction of Mutant Caspase-8 Plasmids**—Candidate tyrosine residues in the caspase-8 molecule that were predicted to be potential phosphorylation sites (NetPhos 2.0 Server) to phenylalanine (TTT or TTC): Tyr-310 in wash buffer (10 mM Tris, pH 7.4), then incubated with tyrosine phosphopeptide substrate (RRLIEDA-EpYAARG) in 10 mM Tris, pH 7.4, for 30 min. The reaction was stopped with 100 µl of malachite green solution. Absorbance was measured at 620 nm using a LabSystems Multiskan plate reader, and phosphate release in picomoles of phosphate/min/µg was determined by comparing absorbance to standard curve.

**Construction of Plasmids**—Total RNA from neutrophils from healthy human volunteers was extracted using TRIzol reagent, and 1 µg of RNA transcribed to first-strand cDNA using the Superscript II system (Invitrogen); the resultant cDNA was amplified by PCR using the Expand™ High Fidelity PCR System (Roche Applied Science) and the following primer sets: caspase-8 upstream primer (containing a HindIII site and a Kozak sequence), 5′-GCAAGCTTGGCC-ACCATGGACTTCAAGCAGAA-ATCT-3′; caspase-8 downstream primer (containing an XbaI site), 5′-GCTCTAGAATCAGAAGGGAAGACAAGT-3′; SHP-1 upstream primer (containing a HindIII site and a Kozak sequence), 5′-GCAAGCTTGGCCACCATGGTGAGGTGGTTTC-3′; SHP-1 downstream primer (containing an XbaI site), 5′-GCTCTAGAATCCCTCTGGAGGGAACCCCT-3′; Lyn upstream primer (containing a BamHI site and a Kozak sequence), 5′-GGCGATCCGCCACCATGGATGTA-6.3 AATTCAAAA-3′; and Lyn downstream primer (containing an XbaI site), 5′-GCTCTAGAAGGCTGCTGTGTGGTTTC-3′.

Amplified fragments were cloned into the pcDNA3.1/Myc-His vector (Invitrogen) according to the manufacturer’s instructions. The recombinant plasmids were transfected into DH5α-competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and sequencing.

**Construction of Mutant Caspase-8 Plasmids**—Candidate tyrosine residues in the caspase-8 molecule that were predicted to be potential phosphorylation sites (NetPhos 2.0 Server, Technical University of Denmark) were mutated using the following site-directed primers to mutate the tyrosine residue (TAT or TAC) to phenylalanine (TTT or TTC): Tyr-310...
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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 DpnI digestion of the amplified product, the mutant DNA was transfected into XL1-blue supercompetent cells, and colonies identified by restriction enzyme digestion and sequencing.

Cell Transfection—The above plasmids (4 µg) were transfected into CHO cell (2 × 10^5 cells/plate) or HL-60 cells (1 × 10^6 cells/ml) using FuGENE 6 (10 µl) reagent (Roche Applied Science). Cultures were maintained for 1 day, then washed and re-cultured for an additional 24 h. Transfection efficiency of HL-60 cells, as judged by immunofluorescence microscopy at 24 h, was ~20%.

Statistical Analysis—Results are reported as the means ± S.D. of n experiments, unless otherwise noted. Comparison of means was by Student’s t test, or analysis of variance with post hoc testing by Student-Newman-Keuls test when more than two groups were analyzed. The α level for statistical significance was set at p < 0.05.

RESULTS

Constitutive Neutrophil Apoptosis and Its Inhibition by LPS Are Caspase-8-dependent—Consistent with our previous observations (5), we found that neutrophils from healthy volunteers are constitutively apoptotic, as quantified by the uptake of propidium iodide by permeabilized cells. More than 50% of quiescent neutrophils were apoptotic following 24 h of *in vitro* culture; co-culture with lipopolysaccharide (1 µg/ml) significantly inhibited this process. Even in the absence of exogenous stimulation, neutrophils harvested from critically ill septic patients with sepsis manifested a further degree of inhibition of *in vitro* apoptosis (Fig. 1A).

The mechanisms of constitutive neutrophil apoptosis are not well defined, however spontaneous activation of both the extrinsic (27) and intrinsic (28) pathways has been described. When we treated quiescent neutrophils with a cell-permeant tetrapeptide inhibitor of caspase-8 catalytic activity, IETD-CHO (29), we observed dose-dependent inhibition of apoptosis, implicating caspase-8 in the initiation of constitutive neutrophil apoptosis (Fig. 1B). A necessary role for active caspase-8 in constitutive myeloid cell apoptosis was further suggested by studies showing that transfection with wild-type, but not cata-

A

| SPREDSESQTLKTVYQMSKPRGCYLIN |
| NHNFRAKREVKPIHSIRDRNGTHDLAGAL |
| TTFEEHFEIKHPHDCVTEQVYEFKLYQLM |
| DSHSMDTFICLISQHGKIGYIYDGQXAPI |
| YELTSQFTGLKCPMLAGPKVFFIQACGD |
| NYQGIPVETD |

B

| FIGURE 2. Caspase-8 is tyrosine-phosphorylated in neutrophils. A, the amino acid sequence of the p18 and p10 fragments of caspase-8, demonstrating predicted tyrosine phosphorylation sites at Tyr-310 (which incorporates the canonical SHP-1 binding motif, YXXL), at Tyr-397, and at Tyr-465. B, treatment of neutrophils with LPS (1 µg/ml) resulted in significant and sustained tyrosine phosphorylation and lesser degrees of serine phosphorylation. Lysates were immunoprecipitated with anti-caspase-8 and probed with anti-phosphotyrosine antibody. Phosphotyrosine was detectable immediately following cell isolation, however rapid spontaneous dephosphorylation of caspase-8 occurred, and by 2 h, phospho-caspase-8 was essentially undetectable (C). In contrast, exposure to LPS (1 µg/ml) resulted in sustained, and even increased tyrosine phosphorylation of caspase-8 (D).

(upstream: 5’-GAGCAATTTTGGATTTTG-3’; downstream: 5’-GGAATCGAAAGATTTGTC-3’); Tyr-397 (upstream: 5’-GAGCAACCCCTTTTAGAATGG-3’; downstream: 5’-CCATTTTCAAAAAGGGTTGTTC-3’); and Tyr-465 (upstream: 5’-GAAGTTGAACTTTAGAATGC-3’; downstream: 5’-GCTTACTTCAAGTTCAC-3’).

Pseudophosphorylation mutants of the same sites were created by mutating the relevant tyrosine (Y) residues (TAT or TAC) to glutamic acid (E; GAG) (25), using the following primers: Y310E (upstream: 5’-GAGCAATTTTGGATTTTG-3’; downstream: 5’-GGAATCGAAAGATTTGTC-3’); Y397E (upstream: 5’-GAGCAACCCCTTTTAGAATGG-3’; downstream: 5’-CCATTTTCAAAAAGGGTTGTTC-3’); and Y465E (upstream: 5’-GAAGTTGAACTTTAGAATGC-3’; downstream: 5’-GCTTACTTCAAGTTCAC-3’).

Finally a catalytically inactive mutant of caspase-8 was created by mutating cysteine 377 to serine (26), using the following primers: C377S (upstream: 5’-ATTACGGCTAGTCAGGGG-3’; downstream: 5’-CCCCCTGACTAGCTGAAT-3’).
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FIGURE 3. Phosphorylation of caspase-8 on tyrosine residues Tyr-397 and Tyr-465 inhibits caspase-8 activation and reduces apoptosis. CHO cells were transfected with a caspase-8a/c-Myc construct (2 μg with 5 μl of Fugene-6, lane 3), or caspase-8a/c-Myc construct in which the specific tyrosine residue was mutated to phenylalanine (Y310F, Y397F, and Y465F, 4 μg with 10 μl of Fugene-6, lanes 4–8). Lane 1 represents untransfected CHO cells, and lane 2, CHO cells transfected with pcDNA3.1/c-Myc plasmid alone (4 μg with 10 μl of Fugene-6). CHO cell lysates were immunoprecipitated with anti-c-Myc and then Western blotted with anti-caspase-8 or anti-phosphotyrosine. Y397F and Y465F transfectants showed reduced tyrosine phosphorylation (Fig. 3A) and increased caspase-8 cleavage (Fig. 3B). C, promyelocytic HL-60 cells were transfected with caspase-8 wild-type, or Y310F, Tyr-397, or Tyr-465 mutants and cultured for the indicated number of days. Transfection with wild-type caspase-8 or the Y310F mutant (*) resulted in increased rates of spontaneous apoptosis over time compared with untransfected cells or cells transfected with the empty vector. Transfection with either the Y397F or Y465F mutant caspase-8 (†) resulted in a further increase in spontaneous apoptosis (p = 0.05, analysis of variance). D, after 6 days of culture, HL-60 cells transfected with either Y397F or Y465F showed modestly increased rates of apoptosis, whereas cells transfected with the pseudophosphorylation mutants Y397E and Y465E had significantly lower rates of apoptosis than cells transfected with wild-type caspase-8 (n = 5, p < 0.05 versus cells transfected with caspase-8 wild type).

lytically inactive caspase-8 C377S (26), induced the spontaneous apoptosis of HL-60 cells (Fig. 1B). We further found that caspase-8 is spontaneously activated in circulating neutrophils isolated from healthy volunteers, as reflected in both increased caspase-8 catalytic activity (Fig. 1C), and increased cleavage of pro-caspase-8 (Fig. 1D) detectable as early as 30 min during in vitro culture. Caspase-8 activation could be blocked, and basal caspase-8 activity further reduced, by exposure to LPS (Fig. 1, C and D).

Caspase-8 Is Tyrosine-Phosphorylated in Resting, and LPS-stimulated Neutrophils—At least eight isoforms of caspase-8 have been identified (30). Caspase-8/a, a 55.7-kDa protein comprising 496 amino acids, and caspase-8/b, a 53.7-kDa protein of 479 amino acids, are the most widely distributed and catalytically active; other isoforms such as c-FLIP (31) and caspase-8L (32) serve as inhibitors of caspase-8 activation. Following engagement and trimerization of a death receptor of the Fas/TNFr family, the adaptor protein, FADD, is recruited to the intracellular tail of the receptor complex. This interaction, in turn, results in recruitment of pro-caspase-8 that binds FADD through homotypic interactions with the death effector domain in the N terminus of pro-caspase-8 (33, 34). Dimerization of pro-caspase-8 leads to its activation, and cleavage to yield a larger p18, and a smaller p10 fragment; active caspase-8 consists of a tetramer composed of two p18 and two p10 subunits (35).

Protein phosphorylation represents a rapid, dynamic, and reversible post-translational modification that can modulate intracellular protein function by modifying protein-protein interactions (36). The activity of caspase-9 is known to be regulated by serine phosphorylation (37, 38), and the catalytic activity of the p18 fragment of caspase-8 has been shown to be similarly regulated (39). It has been recently reported that caspase-8/b activity can be also regulated by tyrosine phosphorylation on Tyr-380, located in the 10-amino acid linker region between p18 and p10 (40).

We identified potential phosphorylation sites in the caspase-8a molecule using NetPhos 2.0 (Technical University of Denmark). Three particularly strong candidate sites (score ≥0.900) were identified: Tyr-310, which is located in the p18...
moiety and incorporates the canonical SHP-1 binding motif, YXXL (41), Tyr-397, corresponding to Tyr-380 in caspase-8b, and Tyr-465 in the p10 moiety (42) (Fig. 2A). Co-immunoprecipitation studies using freshly isolated resting neutrophils confirmed serine phosphorylation of pro-caspase-8, but showed much stronger evidence of tyrosine phosphorylation (Fig. 2B).

Whereas spontaneous dephosphorylation of caspase-8 occurred in resting polymorphonuclear neutrophils (Fig. 2C), LPS exposure resulted in sustained, and even increased levels of phosphotyrosine accumulation (Fig. 2D), that persisted for at least 20 h (data not shown).

Caspase-8 Activity Is Regulated by Tyrosine Phosphorylation at Tyr-397 and Tyr-465—To evaluate the role of phosphorylation of these tyrosine residues on cell survival, we transfected CHO cells and promyelocytic HL-60 cells with wild-type caspase-8, or mutant caspase-8 in which the tyrosine residue was mutated to non-phosphorylatable phenylalanine, or to glutamic acid to create a pseudo-phosphorylated amino acid residue (25). CHO cells transfected with caspase-8 Y397F and Y465F, but not Y310F, showed decreased phosphotyrosine accumulation (Fig. 3A) and increased levels of active caspase-8 (Fig. 3B), as well as increased caspase-8 activity and increased cell death (data not shown). Similarly, HL-60 cells transfected with mutant caspase-8 Y397F or Y465F showed increased rates of apoptosis over time (Fig. 3C), whereas rates were reduced in cells transfected with the pseudophosphorylation mutants Y397E and Y465E (Fig. 3D). The activities of both caspase-8 and the downstream effector caspase, caspase-3 (Fig. 3E), were increased in HL-60 cells transfected with the Y397F and Y465F mutants. Together these studies reveal that the pro-apoptotic activity of caspase-8 is further amplified when the phosphorylation of tyrosine residues Tyr-397 and Tyr-465 is prevented and establish a role for tyrosine phosphorylation in the tonic inhibition of caspase-8 activation.

The Tyrosine Phosphatase, SHP-1, Dephosphorylates Caspase-8—Having shown increased caspase-8 activity following dephosphorylation of tyrosine residues 397 and 465, we sought to identify a phosphatase that might mediate this effect. SHP-1 has

FIGURE 4. Interactions between caspase-8 and SHP-1 promote neutrophil apoptosis. A, lysates of neutrophils treated with LPS or SHP-1 inhibitor were immunoprecipitated with anti-caspase-8 (left) or anti-SHP-1 (right), and the immunoprecipitates reprobed with antibodies to caspase-8 (top), SHP-1 (middle), or phosphotyrosine (bottom). Treatment with LPS disrupted interactions between caspase-8 and SHP-1; LPS or SHP-1 inhibition promoted increased phosphorylation of caspase-8. B, neutrophil SHP-1 activity as measured by the malachite green assay was significantly reduced by LPS or by exposure to the SHP-1 inhibitor, PTP1 (n = 4, *p < 0.05). C, neutrophils from healthy volunteers were cultured with or without α-bromo-4-hydroxyacetophenone, 10 μM, an inhibitor of SHP-1; rates of apoptosis were evaluated as uptake of propidium iodide in permeabilized cells (n = 5, *p < 0.01). D, incubation of neutrophils with either LPS or the SHP-1 inhibitor also reduced the activity of caspases-8 (top) and 3 (bottom) in cell lysates (n = 3–5/group, *p < 0.05).
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Figure 5. Phosphorylation of caspase-8 at Tyr-310 promotes caspase-8 dephosphorylation and activation by SHP-1. A, CHO cells were transfected with SHP-1 and c-Myc-tagged wild-type caspase-8, or c-Myc-tagged caspase-8 in which Tyr-310 had been mutated to phenylalanine. Lysates were immunoprecipitated with either anti-caspase-8 (top panel) or anti-SHP-1 (bottom panel), then immunoblotted with anti-c-Myc to detect the transfected caspase-8. Whereas wild-type caspase-8 co-immunoprecipitated with SHP-1, the Y310F mutant did not. CHO cells were transfected with plasmids for caspase-8, SHP-1, or both. Caspase-8 was tyrosine-phosphorylated, and survival, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay, decreased, in cells transfected with the caspase-8 plasmid alone. Co-transfection resulted in dephosphorylation of caspase-8, and further decreased CHO cell survival; transfection with the empty pcDNA3.1 vector or exposure to Fugene-6 alone was without effect (*, p < 0.05 versus control cells; †, p < 0.05 compared with caspase-8 alone; ††, p < 0.05 compared with caspase-8 alone; n = 5).

been shown to bind to death receptors of the CD95 family in neutrophils and to block the anti-apoptotic effects of growth factors such as granulocyte macrophage-colony stimulating factor (43). Moreover, neutrophils from moth-eaten mice lacking functional SHP-1 show multiple abnormalities reflecting increased cellular activation (44). Thus we hypothesized that SHP-1 can interact directly with, and dephosphorylate caspase-8.

Co-immunoprecipitation studies demonstrated that SHP-1 physically associates with caspase-8 and that exposure to LPS disrupts this association (Fig. 4A). Exposure of neutrophils to either LPS or PTPI (10 μM) reduced SHP-1 activity (Fig. 4B). Treatment of neutrophils with PTPI markedly inhibited apoptosis (Fig. 4C), and reduced activity of caspases-8 and -3 (Fig. 4D), in association with sustained tyrosine phosphorylation of caspase-8 that persisted for at least 20 h (data not shown).

To further characterize interactions between caspase-8 and SHP-1, we transfected CHO cells and HL-60 cells with constructs encoding Myc-tagged caspase-8a, Myc-tagged SHP-1, or both. SHP-1 and caspase-8 co-immunoprecipitated in CHO cells transfected with both constructs; mutation of Tyr-310 in the SHP-1 recognition motif blocked this interaction (Fig. 5A). Caspase-8 was tyrosine-phosphorylated in cells transfected with a caspase-8/c-Myc construct alone; co-transfection with SHP-1 resulted in dephosphorylation of caspase-8, and increased rates of cell death (Fig. 5B). Endogenous SHP-1 expression increased over time in retinoic acid-matured HL-60 cells, and SHP-1 co-immunoprecipitated with caspase-8 (data not shown). Transfection of resting HL-60 cells with either caspase-8 or SHP-1 resulted in increased apoptosis; co-transfection further increased rates of spontaneous apoptosis (Fig. 5C). When HL-60 cells were co-transfected with SHP-1 and the Y310F mutant plasmid, in which the tyrosine residue in the SHP-1 binding motif had been mutated to phenylalanine, rates of apoptosis were similar to those of cells transfected with caspase-8 alone (Fig. 5C), consistent with the interpretation that SHP-1 binding to caspase-8 is necessary for its pro-apoptotic activity. Similar results were observed when HL-60 cells were matured with retinoic acid for 3 days prior to transfection (data not shown). Together these data suggest that SHP-1 initiates apoptosis through its ability to bind and dephosphorylate caspase-8.

The Src Kinase, Lyn, Phosphorylates Caspase-8—Non-receptor tyrosine kinases of the Src family, in particular Lyn, have been implicated in the inhibition of neutrophil apoptosis (43,
of both caspase-8 (pro-form 55 kDa) and caspase-3 (pro-form 34 kDa) to their active forms (18 and 20 kDa, respectively) as determined by Western blot analysis of lysates of control and LPS-treated cells, and abrogated the anti-apoptotic effects of exposure to LPS (alone, NS).

We hypothesized that these activities arise through the capacity of Lyn to phosphorylate caspase-8. Caspase-8 and Lyn co-immunoprecipitated in LPS-stimulated neutrophils; preincubation of cells with the Src kinase inhibitor PP2 (10 μM), for 1 h prior to exposure to LPS prevented this interaction, and blocked caspase-8 tyrosine phosphorylation (Fig. 6A). Moreover, PP2 pre-treatment increased the activities of caspase-8 (Fig. 6B), increased the cleavage of caspases-8 and -3 to their active forms, and increased rates of apoptosis in LPS-treated cells (Fig. 6C).

When CHO cells were transfected with Myc-tagged constructs expressing caspase-8, Lyn, or both, co-expression of Lyn increased the tyrosine phosphorylation of caspase-8, and abrogated the pro-apoptotic effects observed with caspase-8 transfection alone (Fig. 6D). Rates of apoptosis were similarly reduced in HL-60 cells co-transfected with caspase-8 and Lyn, when compared with caspase-8 alone; the anti-apoptotic effects
of Lyn were absent in HL-60 cells co-transfected with the Y397F and Y465F caspase-8 mutants, implicating these tyrosine residues as targets of Lyn-induced phosphorylation (Fig. 6E). Together these data show that the Src kinase, Lyn, can phosphorylate caspase-8, reducing its activity and inhibiting apoptosis.

Neutrophils from Patients with Sepsis Show Sustained Phosphorylation of Caspase-8—Circulating neutrophils from critically ill patients with sepsis manifest profound delays in apoptosis, in association with biochemical features of cellular activation (5, 15). We hypothesized that sustained caspase-8 phosphorylation contributes to the persistence of activated neutrophils in critically ill septic patients. Caspase-8 activity was significantly reduced in neutrophils harvested from patients with sepsis (Fig. 7A), as were protein levels and bioactivity of SHP-1 (Fig. 7B). In contrast, septic patient neutrophils showed increased expression of Lyn (Fig. 7C). Septic neutrophils further showed significant and sustained tyrosine phosphorylation of caspase-8; PP2 treatment in vitro resulted in dephosphorylation of caspase-8, and significantly increased rates of apoptosis (E) (n = 4 – 6 per group, *, p < 0.01 versus controls, †, p < 0.05 versus septic neutrophils without PP2).

DISCUSSION
The ability to mount an effective response to an external threat is crucially dependent upon the capacity of immune effector cells to become rapidly activated in the presence of...
danger, but to restore a state of quiescence once that danger has passed. For the neutrophil, the first cell population recruited to a site of inflammatory challenge, these twin imperatives are accomplished primarily through the dynamic regulation of cell survival by apoptosis (7). The shortest lived cells in the human body, neutrophils are constitutively apoptotic, with a normal \textit{in vivo} lifespan that is measured in hours (8). Multiple stimuli present in the microenvironment of inflammation, including bacterial factors such as LPS, lipoteichoic acid, mannann, and short chain fatty acids from anerobic bacteria, and host-derived inflammatory mediators such as interleukin-1, tumor necrosis factor, interleukin-6, granulocyte colony stimulating factor, and leukotriene B4, can activate anti-apoptotic signaling pathways leading to the transcription of inhibitor of apoptosis proteins, and facilitating prolonged cell survival (47). Conversely, the phagocytosis of bacteria (48, 49) or fungi (50) triggers neutrophil apoptosis and promotes the resolution of inflammation.

Delayed neutrophil apoptosis requires activation of anti-apoptotic signaling pathways (51) and the transcription of anti-apoptotic survival genes (5, 16). We hypothesized that the progression of apoptosis might also be inhibited more rapidly through post-translational modifications of key apoptotic enzymes and focused our attention on phosphorylation of caspase-8, because serine phosphorylation of caspase-9 has previously been shown to regulate the activity of the latter (37). Our observations reveal a novel model for the early regulation of apoptosis in response to external stimuli through the phosphorylation and dephosphorylation of caspase-8 (Fig. 8). Caspase-8 is tyrosine-phosphorylated in freshly isolated human neutrophils, but undergoes rapid dephosphorylation in culture resulting in caspase-8 activation and progression of the apoptotic cascade. On the basis of \textit{in silico} prediction of probable tyrosine phosphorylation sites in the caspase-8a molecule, confirmed by mutational studies in which each of these is mutated to the structurally similar, but non-phosphorylatable amino acid, phenylalanine, we identified three tyrosine residues as key to this regulatory process.

Tyr-310 in the pro-domain of the caspase-8 molecule occurs in the context of the tetrapeptide motif, YXXL, known to bind the tyrosine phosphatase, SHP-1 (41, 43). Using co-immunoprecipitation studies (Fig. 4A), we confirmed that caspase-8 can bind SHP-1. Mutation of Tyr-310 to phenylalanine disrupted the physical interaction of caspase-8 and SHP-1 (Fig. 5A) and abrogated the pro-apoptotic consequences of SHP-1 transfection (Fig. 5C). Moreover, whereas caspase-8 was tyrosine-phosphorylated when transfected into CHO cells, co-transfection of caspase-8 and SHP-1 resulted in the dephosphorylation of caspase-8. A pro-apoptotic role for caspase-8-SHP-1 interactions was supported by the observations that inhibition of SHP-1 resulted in profound suppression of constitutive neutrophil apoptosis (Fig. 4C) and that normal neutrophils exposed to LPS, or neutrophils harvested from patients with sepsis, showed reduced SHP-1 activity (Fig. 7B).

Mutational studies using both CHO cells and promyelocytic HL-60 cells revealed that the phosphorylation of tyrosine residues at both Tyr-397 and Tyr-465 regulates the activity of caspase-8 and -3 (Figs. 3E and cell survival (Fig. 3, C and D). Tyr-397, corresponding to Tyr-380 in caspase-8b (40), lies in the linker region between the catalytically active p18 and p10 fragments of caspase-8, whereas Tyr-465 is in the p10 fragment (Figs. 2A and 8). Several lines of investigation suggest that a Src kinase, likely Lyn, phosphorylates these regulatory tyrosine residues. Caspase-8 and Lyn were co-immunoprecipitated in neutrophils (Fig. 6A). Inhibition of Src kinases with the broad spectrum inhibitor, PP2, prevented this interaction, blocked caspase-8 phosphorylation, and increased caspase-8 cleavage, caspase-8 activity, and rates of apoptosis in LPS-treated neutrophils (Fig. 6, A–C). Co-transfection of caspase-8 and Lyn into CHO cells or HL-60 cells resulted in increased tyrosine phosphorylation of caspase-8 (Fig. 6D), and increased rates of cell survival (Fig. 6, D and E). Importantly this pro-survival effect was not seen in HL-60 cells transfected with mutant Y397F or Y465F caspase-8 (Fig. 6E), providing further evidence of a functional role for these two residues.

Lyn activation has been associated with resistance to apoptosis in myeloid (52) and B cell malignancies (53) and in delayed apoptosis of human neutrophils exposed to granulocyte macrophage-colony stimulating factor (45). Lyn expression was increased in neutrophils harvested from critically ill septic patients (Fig. 7C), and \textit{in vitro} exposure of septic neutrophils to PP2 resulted in tyrosine dephosphorylation of caspase-8 and partially abrogated the sepsis-induced
delay of apoptosis (Fig. 7E). At the same time, the expression and activity of SHP-1 was reduced in septic neutrophils (Fig. 7B). Thus delayed neutrophil apoptosis during life-threatening systemic inflammation arises in part, though not exclusively, through an acquired imbalance in the regulation of caspase-8 phosphorylation, with increased expression of the anti-apoptotic kinase, Lyn, and reduced expression of the pro-apoptotic phosphatase, SHP-1. That other anti-apoptotic mechanisms are involved is suggested by the fact that inhibition of Lyn only partially reverses the apoptotic delay.

Inflammatory stimuli are known to induce the transcription of a variety of anti-apoptotic genes, including members of the inhibitor of apoptosis protein family and anti-apoptotic members of the Bcl-2 family (54, 55); whether they are also increased in sepsis is unknown.

Neutrophil activation has been implicated in the inflammatory injury of a variety of disease processes prominent among which are such common and disabling disorders as arthritis (13), chronic obstructive pulmonary disease (56), stroke (57), and sepsis. Identification of the phosphorylation of caspase-8 as a mechanism regulating neutrophil activity by modulating the lifespan of the neutrophil provides another candidate target for the development of anti-inflammatory therapies for a broad repertoire of human diseases.

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