

# Phosphatidylinositol 3-Kinase Controls Antineutrophil Cytoplasmic Antibodies—Induced Respiratory Burst in Human Neutrophils

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**Abstract.** Antineutrophil cytoplasmic antibodies (ANCA) activate human polymorphonuclear neutrophils (PMN) primed with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) *in vitro*. Phosphatidylinositol 3-kinase (PI3-K) and the protein-serine/threonine kinase Akt have been implicated in the control of the phagocyte respiratory burst. The hypothesis that PI3-K controls the ANCA-induced respiratory burst was tested. TNF- $\alpha$ -primed PMN were stimulated with a monoclonal antibody to myeloperoxidase (MPO) and with PR3- and MPO-ANCA, respectively. Akt activation was assessed with phospho-specific antibodies. Superoxide release was measured with ferricytochrome. ANCA antigen translocation was assessed by fluorescence-activated cell sorter. The effect of TNF- $\alpha$  and MPO-ANCA on Akt signaling was studied with immunoprecipitation and glutathione S-transferase pull-down assays. Western blotting revealed rapid transient Akt phosphorylation during TNF- $\alpha$  priming and a second phosphorylation after ANCA. PI3-K inhibition by LY294002 blocked both Akt phos-

phorylation and superoxide generation. A total of  $20 \pm 3$  nmol  $O_2^-/0.75 \times 10^6$  PMN/45 min was released after stimulation with PR3-ANCA. LY294002 (5  $\mu$ M) decreased this amount to  $0.3 \pm 2.6$  nmol ( $n = 10$ ,  $P < 0.05$ ); the MPO-ANCA values were  $23 \pm 3$  versus  $1.6 \pm 3.6$  ( $n = 10$ ,  $P < 0.05$ ). p38 MAPK inhibition with 10  $\mu$ M SB202190 that also decreased ANCA-induced superoxide generation prevented S473 phosphorylation of Akt in response to TNF- $\alpha$  and to ANCA. However, SB202190 but not LY294002 abrogated TNF- $\alpha$ -mediated ANCA antigen surface translocation, demonstrating that superoxide generation and ANCA antigen translocation proceed by separate mechanisms. Akt, PAK1, and Rac1 existed as cytosolic complex in resting PMN. TNF- $\alpha$  stimulation increased association of PAK1 with Akt. An MPO monoclonal antibody did not alter the Akt signaling complex further. The data demonstrate the importance of PI3-K for the ANCA-induced PMN oxidant production.

Antineutrophil cytoplasmic antibodies (ANCA) are detected in patients with microscopic polyangiitis, Wegener's granulomatosis, Churg-Strauss syndrome, and pauci-immune necrotizing crescentic glomerulonephritis (1–3). ANCA may play an important role in the pathogenesis of glomerulonephritis and vasculitis. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) primes neutrophils for an ANCA-induced activation, resulting in release of toxic granule proteins and cytokines, upregulation of adhesion molecules, and respiratory burst (4–8). Ultimately, ANCA-activated neutrophils damage endothelial cells *in vitro* (9–11). However, signaling events that control ANCA-induced neutrophil activation are not completely understood. We reported previously that p38 mitogen-activated protein kinase (MAPK)

and extracellular signal-regulated kinase (ERK) are important pathways that mediate ANCA-induced respiratory burst, acting particularly during TNF- $\alpha$  priming (12). Phosphatidylinositol 3-kinase (PI3-Kinase) is another kinase that controls cellular functions. PI3-kinase generates phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>) and phosphatidylinositol-3,4-diphosphate (PIP<sub>2</sub>). Both products are important for the recruitment of the serine/threonine kinase Akt to the plasma membrane, where it can be phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) at T308, and by PDK2 at S473. In a previous study, we reported that p38 MAPK-dependent MAPK-activated protein kinase (MK-2) can function as PDK2 and cause phosphorylation of Akt at S473 in neutrophils stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) and PIP<sub>3</sub> (13). A critical role of PI3-kinase in septic granulocyte responses and the respiratory burst has been demonstrated (14). Recently, Ben-Smith *et al.* (15) demonstrated that ANCA can activate the p101/110 $\gamma$  PI3-K isoform, whereas conventional Fc $\gamma$ R ligation activates the p85/p110 isoform. In the present study, we further investigated the role of PI3-Kinase and Akt in the ANCA-induced respiratory burst in human neutrophils. We show that ANCA activates Akt via both the p38 MAPK and PI3-K

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pathways. Also, TNF- $\alpha$ , an agent essential to priming polymorphonuclear neutrophils (PMN) for subsequent ANCA-induced activation, stimulates Akt by both PI3-K and p38 MAPK-dependent mechanisms. Characterization of the Akt signaling module showed that Akt, PAK1, and Rac1 exist in complex in resting PMN cytosol and that TNF- $\alpha$  stimulation caused increased association of PAK1 with Akt.

Both the PI3-K and p38 MAPK exert control over the ANCA-induced respiratory burst. p38 MAPK activation plays a key role in TNF- $\alpha$ -induced translocation of ANCA, whereas PI3-K is essential to triggering the ANCA-induced respiratory burst. As PI3-K can be an upstream activator of p38 MAPK, activation of PI3-K seems to play a key role in the activation of human neutrophils by TNF- $\alpha$  and ANCA. Pharmacologic inhibition of these kinases may attenuate or block ANCA-induced vascular and glomerular inflammation.

## Materials and Methods

### Materials

Plasmagel was obtained from Zeptomatrix Corporation (Buffalo, NY), and Ficoll-Hypaque was obtained from Sigma (Deisenhofen, FRG). Trypan blue, phosphate-buffered saline (PBS), and Hanks' balanced salt solution (HBSS) were obtained from Biochrom (Berlin, FRG), and recombinant TNF- $\alpha$  was obtained from R&D Systems (Wiesbaden, FRG). Bovine erythrocyte superoxide dismutase (SOD; 2,500–7,000 U/mg protein), ferricytochrome C, cytochalasin B, FMLP, and phorbol myristate acetate (PMA) were from Sigma. The monoclonal antibody (mAb) to PR3 was from CLB (CLB 12.8, Amsterdam, Netherlands), and the mAb to myeloperoxidase (MPO) was from Dako (MPO-7, Hamburg, FRG). Dihydro-rhodamine-1,2,3 (DHR) was from Molecular Probes (Eugene, OR). The specific polyclonal rabbit antibody to phospho-S473 Akt was from New England Biolabs (Wiesbaden, FRG); the HRP-labeled secondary donkey antibody was from Amersham Pharmacia biotech (Freiburg, FRG); and LY294002, SB202190, and PD98059 were purchased from Calbiochem (Bad Soden, FRG). Ninety-six-well microtiter plates were from TPP-Company (Munich, FRG). Endotoxin-free reagents and plastic disposables were used in all experiments.

### Isolation of Human PMN and Culture Conditions

PMN from healthy human donors were isolated from heparinized whole blood by red blood cell sedimentation with plasma gel, followed by Ficoll-Hypaque density gradient centrifugation. Erythrocytes were lysed by incubation with hypotonic saline for 15 s. PMN were spun down ( $200\times g$ , 7 min) and reconstituted in HBSS with calcium and magnesium (HBSS<sup>++</sup>). Ten microliters of neutrophil suspension was incubated with 40  $\mu$ l of trypan blue for 5 min at room temperature. Cells were counted in duplicate using a hemocytometer and considered viable if able to exclude trypan blue. The cell viability was detected in every cell preparation and found to be >99%. The percentage of PMN in the suspension was >95% by a Wright-Giemsa staining and by light microscopy.

### Preparation of Immunoglobulins

Human immunoglobulin G (IgG) was prepared from patients with biopsy-proven Wegener's granulomatosis (two PR3-ANCA) and microscopic polyangiitis (two MPO-ANCA) as well as from two healthy control subjects as described recently (6). Plasma samples were obtained from freshly drawn blood and kept at  $-20^{\circ}\text{C}$ . Plasma was

filtered through a 0.2- $\mu$ m syringe filter (Gelman Sciences, Ann Arbor, MI) and applied to a protein G affinity column (Pharmacia, Uppsala, Sweden). Bound Ig were eluted with 0.1 M glycine-HCl buffer (pH 2.75; elution buffer). After the antibodies emerged, the pH was immediately adjusted to pH 7.0 using 1 M Tris-HCl (pH 9.0). A mouse monoclonal to MPO (MPO-7, IgG1 $\kappa$ ) and an isotype-matched control (IgG1 $\kappa$ ) were purchased from Dako (Hamburg, FRG). Before use, IgG preparations were centrifuged at  $10,000\times g$  for 5 min to remove aggregates.

### Measurement of Superoxide by the Ferricytochrome C Assay

Superoxide was measured using the assay of SOD-inhibitable reduction of ferricytochrome C as described by Pick and Mizel (16). Briefly, freshly isolated PMN or PMN cultured for the indicated time periods were pretreated with 5  $\mu$ g/ml cytochalasin B for 15 min at  $4^{\circ}\text{C}$ . Cells ( $0.75\times 10^6$ ) were primed with 2 ng/ml TNF- $\alpha$  for 15 min at  $37^{\circ}\text{C}$  before anti-MPO mAb or human ANCA preparations were added. No priming was performed when cells were stimulated with PMA or FMLP. The final concentrations were 2.5  $\mu$ g/ml for the mAb to MPO, 100  $\mu$ g/ml for purified IgG preparations, 25 ng/ml of PMA, and  $10^{-7}$  M of FMLP. All experiments were set up in duplicate. The samples were incubated in 96-well plates at  $37^{\circ}\text{C}$  for up to 60 min, and the absorption of samples with and without 300 U/ml SOD was scanned repetitively at 550 nm using a Microplate Autoreader. The final ferricytochrome C concentration was 50  $\mu$ M, and the final cell concentration was  $3.75\times 10^6$ /ml. No activating effect was seen when human and mouse control antibodies were used or when cells were incubated with 2 ng/ml TNF- $\alpha$ . The baseline activity of TNF- $\alpha$ -treated PMN was determined in every experiment and was factored for each condition.

### Measurement of Respiratory Burst by DHR Oxidation to Rhodamin

The generation of reactive oxygen radicals was additionally assessed using DHR as described previously (12). In brief, neutrophils ( $1\times 10^7$ /ml HBSS) were loaded with DHR (1  $\mu$ M) for 10 min at  $37^{\circ}\text{C}$ . After 15 min of priming with 2 ng/ml TNF- $\alpha$ , cells were divided and  $5\times 10^5$  cells were incubated with the stimuli in a total assay volume of 100  $\mu$ l. Preincubation with LY294002 or buffer control was done for 30 min on ice before the priming. After 45 min, the reactions were stopped by adding 900  $\mu$ l of ice-cold PBS/1% bovine serum albumin. Samples were analyzed using a FACScan (Becton Dickinson, Heidelberg, FRG). Data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined. For each condition, the mean fluorescence intensity (MFI, representing the amount of generated reactive oxygen radicals) is reported.

### Western Blot Analysis for Phosphorylated Akt

PMN were incubated at a concentration of  $2\times 10^6$ /ml in the presence of buffer control or with the indicated stimuli. Because cytochalasin B can affect signal transduction, no pretreatment with this compound was performed. Samples were harvested and cell lysates were prepared by resuspending cells in 20  $\mu$ l of ice-cold lysing solution (20 mM Tris-HCl [pH 8.0] containing 138 mM NaCl, 1% Triton X-100, 1% NP-40, 20 mM NaF, 2 mM ethylenediaminetetraacetic acid, 10% glycerol, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 0.1 mM quercetin, 5 mM iodoacetamide). Samples were stored for 5 min on ice and centrifuged at  $13,000\times g$  for 5 min at  $4^{\circ}\text{C}$ .

Supernatant was recovered, and the protein concentration was estimated by bicinchoninic acid (BCA) protein assay (Pierce, Munich, FRG). Samples were incubated for 5 min at 95°C in loading buffer (250 mM Tris-HCl [pH 6.8] with 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.01% bromophenol blue), and 50 µg of protein per lane was loaded on 10% SDS-polyacrylamide gel, electrophoresed, and blotted onto polyvinylidene difluoride membrane by semi-dry technique. The membrane was blocked in 5% skim milk/0.05% Tween/PBS overnight at 4°C. Phosphorylation was detected using specific antibody to phospho-S473 Akt (1,000 dilution) and a horseradish peroxidase-labeled secondary antibody (1:1,000). Blot was developed by incubation in a chemiluminescence substrate (ECL, Amersham, Pharmacia, Freiburg, Germany) and exposed to an x-ray film.

### Assessment of ANCA-Antigen Expression by Flow Cytometry

Flow cytometry was used to evaluate the effect of LY294002 and SB202190 on PR3 and MPO expression on neutrophils. Immunostaining was performed as described previously (12). Cells not pretreated with cytochalasin B were preincubated with 10 µM of the inhibitor or the same dilution of DMSO for 30 min on ice, followed by treatment with 2 ng/ml TNF-α or buffer control for 15 min at 37°C. Cells were pelleted at 200× *g* for 7 min at 4°C and resuspended in 1 ml of ice-cold PBS. After washing in HBSS without Ca<sup>2+</sup>/Mg<sup>2+</sup>, cells were incubated with dilutions of mAb to PR3, MPO, or an isotype control followed by a secondary FITC-conjugated F(ab)<sub>2</sub>-fragment of goat anti-mouse IgG. Flow cytometry was performed on the same day using a FACScan, and 10,000 events per sample were collected.

### Glutathione S-Transferase Pull-Down Assay

Neutrophils (2 × 10<sup>7</sup>) were prewarmed at 37°C for 5 min before stimulation with or without TNF-α (2 ng/ml) or the mAb to MPO (5 µg/ml). The reactions were stopped by centrifugation followed immediately by the addition of 200 µl of immunoprecipitation lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (vol/vol) Triton X-100, 0.5% (vol/vol) Nonidet P-40, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 20 mM sodium orthovanadate, 10 µM *p*-nitrophenol phosphate, 20 mM NaF, 5 mM phenylmethylsulfonyl fluoride, 21 µg/ml aprotinin, and 5 µg/ml leupeptin. After centrifugation at 15,000× *g* for 15 min at 4°C, cleared lysates were incubated with 5 µl of anti-Akt antiserum overnight with continuous rotation at 4°C. Protein A-Sepharose beads (15 µl) were then added, and samples were rotated for an additional 2 h at 4°C. Beads were washed once by centrifugation in Krebs buffer and then resuspended in 50 µl of 2× Laemmli buffer and boiled for 3 min. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membrane, and blocked with 5% milk/TTBS for 1 h. Blots were probed with anti-PAK1 (1:1,000), anti-Hsp27 (1:1,000), anti-MK-2 (1:2,000), or anti-Akt (1:1,000) antiserum in 5% bovine serum albumin/TTBS (wt/vol) and peroxidase-conjugated secondary antibody in 5% milk/TTBS (wt/vol). Products were visualized by chemiluminescence.

### Statistical Analyses

Results are given as mean ± SEM. Comparisons between two groups were done using paired Wilcoxon rank tests. Comparisons between multiple groups were done using Kruskal-Wallis tests. Specific differences between multiple groups were then determined by use of a Bonferroni *post hoc* test on the ranked values.

## Results

### Activation of Akt during TNF-α Incubation with Subsequent ANCA Stimulation

To investigate the effect of both TNF-α-mediated priming and ANCA stimulation on Akt activation, we performed Western blot analysis. Akt S473 phosphorylation was measured during incubation with the priming concentration of 2 ng/ml TNF-α (*n* = 5; Figure 1) and up to 60 min after incubation with the mAb to MPO after a 15-min TNF-α exposure (*n* = 4; Figure 2). Figure 1 indicates that TNF-α induced a rapid but transient increase in Akt phosphorylation. Densitometric analysis shows that the peak phosphorylation occurred at 10 min, decreasing thereafter (Figure 1B). When PMN were primed for 15 min and subsequently stimulated with the mAb to MPO, we observed a second phosphorylation event that did not occur in the presence of an isotype control (Figure 2). Here, the peak was observed at 15 min and phosphorylation decreased afterward. Corresponding optical density (OD) measures for the time course study using the mAb to MPO are given in Figure 2B (*n* = 4). The difference between the mAb to MPO and the isotype control was significant at 15 and 30 min. On the basis

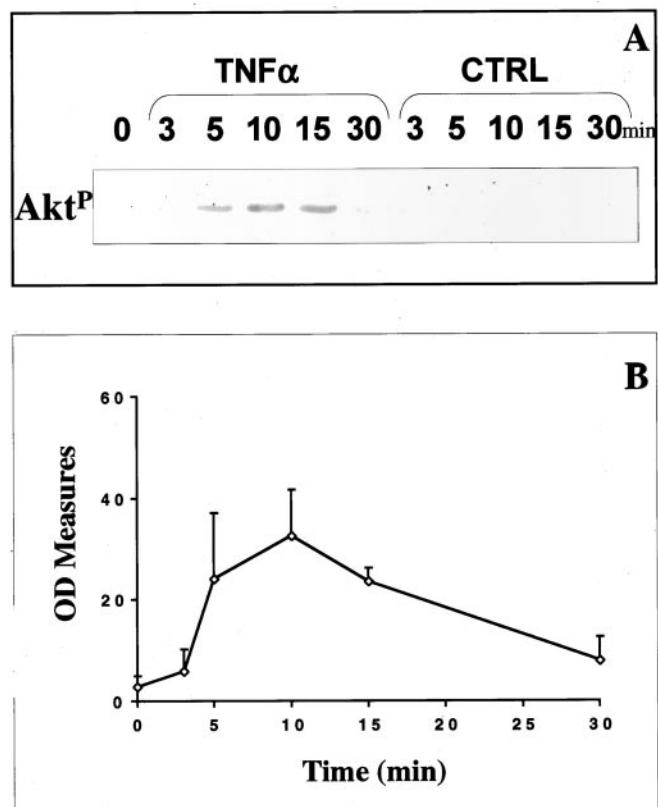
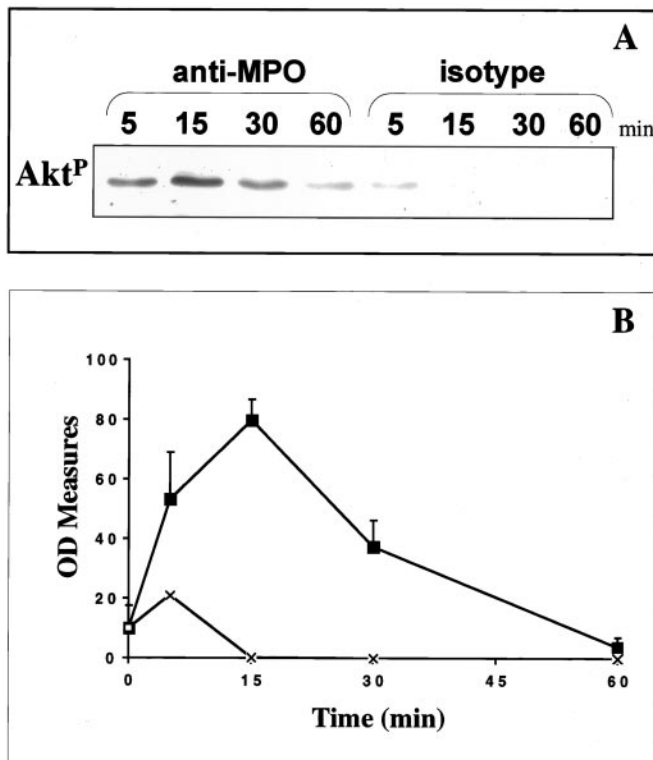


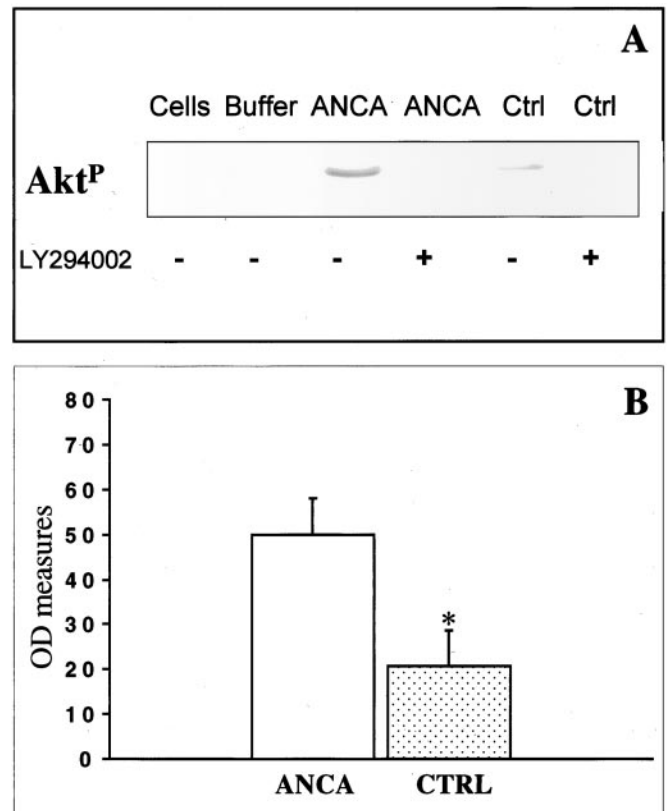
Figure 1. Phosphorylation of Akt in response to tumor necrosis factor α (TNF-α) priming was studied by immunoblotting. Cell aliquots were obtained after 0, 3, 5, 10, 15, and 30 min of TNF-α priming with 2 ng/ml and buffer control (CTRL), respectively. At each time point, S473-phosphorylated Akt was determined by immunoblotting, and a representative example of five independent experiments is shown (A). The corresponding densitometric analysis is shown in B (*n* = 5). These data show that TNF-α priming results in transient Akt phosphorylation.





**Figure 2.** Phosphorylation of Akt in response to a monoclonal antibody (mAb) to human myeloperoxidase (MPO) was investigated by immunoblotting. Cells were primed for 15 min with 2 ng/ml TNF- $\alpha$  and subsequently incubated with 2.5  $\mu$ g/ml mAb to MPO and the same concentration of an isotype control, respectively. Samples were harvested after 5, 15, 30, and 60 min. At each time point, S473-phosphorylated Akt was determined by immunoblotting, and a representative example of four independent experiments, using neutrophils from different donors, is shown (A). The corresponding densitometric analysis is shown in B ( $n = 4$ ). The difference between the mAb to MPO and the isotype control was  $P < 0.01$  at 15 min and  $P < 0.05$  at 30 min. These data show that stimulation with an mAb to MPO but not with a control antibody results in Akt phosphorylation.

of these results, we selected the 15-min time point for additional experiments. A total of eight experiments were done, and the OD measures for the stimulation with 2.5  $\mu$ g/ml mAb to MPO was  $84 \pm 4$  and for an equal amount of monoclonal control antibody  $7 \pm 2$  ( $P < 0.01$ ). Also, an mAb to PR3 (15  $\mu$ g/ml) resulted in increased Akt phosphorylation, compared with an equal amount of the isotype control (OD measures  $28 \pm 5$  to  $15 \pm 6$ ,  $n = 3$  independent experiments). This reaction was weaker when compared with the mAb to MPO. We next tested the effect of human PR3- and MPO-ANCA preparations on Akt phosphorylation (Figure 3). These results indicate significantly increased Akt phosphorylation in response to ANCA compared with IgG preparations from healthy control subjects. The corresponding OD measures are depicted in Figure 3B. To confirm that Akt phosphorylation was mediated by PI3-kinase activity, we preincubated cells in two independent experiments with LY294002. Figure 3A shows that pharmacologic inhibition of PI3-kinase with LY294002 abrogated Akt phosphorylation.



**Figure 3.** The effect of human antineutrophil cytoplasmic antibodies (ANCA) preparations on S473 phosphorylation of Akt was studied by immunoblotting. Cells were primed for 15 min with 2 ng/ml TNF- $\alpha$  and subsequently incubated with buffer control (buffer), 100  $\mu$ g/ml human ANCA (ANCA), and the same amount of control immunoglobulin G preparations from healthy donors (Ctrl), respectively. Samples were harvested after another 15 min, and S473-phosphorylated Akt was determined by immunoblotting. In two independent experiments, cells were preincubated with 10  $\mu$ M LY294002, and a representative example is shown (A). The corresponding densitometric analysis is shown in B. For these studies, seven independent experiments were performed, each using different neutrophil donors. We tested two different human PR3-ANCA (4 $\times$  patient 1, and 3 $\times$  patient 2), two different human MPO-ANCA (3 $\times$  patient 1, and 3 $\times$  patient 2), and two different human controls (3 $\times$  donor 1, and 4 $\times$  donor 2) in parallel. The ANCA results are pooled in the white bar, and the human controls are pooled in the dotted bar (\* $P < 0.05$ ).

#### Activation of Akt Depends on Activity of PI3-Kinase and p38 MAPK but Not on ERK

To determine the role of MAPK in Akt activation, we compared the effect of LY294002, SB203680, and PD98059 on phosphorylation of Akt. Neutrophils ( $2 \times 10^6$ ) were preincubated with the inhibitors for 30 min before treatment with 2 ng/ml TNF- $\alpha$  (Figure 4A) or the mAb to human MPO (Figure 4B), respectively. S473 phosphorylation of Akt was assessed after 15 min of incubation with either stimulus. These data indicate that blocking PI3-K and p38 MAPK but not ERK abrogates Akt phosphorylation, indicating that p38 MAPK regulates Akt activation in human neutrophils that respond to TNF- $\alpha$  and to mAb to MPO.

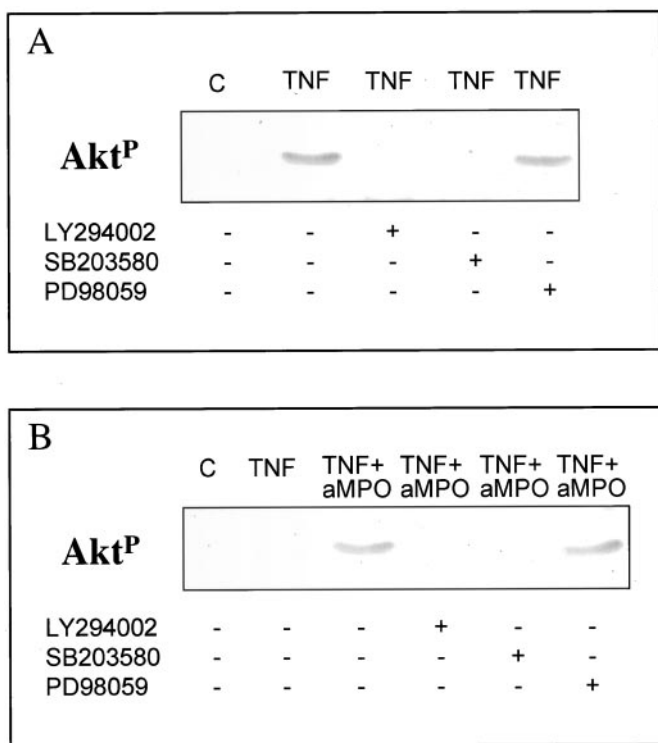


Figure 4. The effect of 10  $\mu$ M LY294002, 10  $\mu$ M SB203680, and 25  $\mu$ M PD98059 on phosphorylation of Akt was studied by immunoblotting. Neutrophils ( $2 \times 10^6$ ) were preincubated with the inhibitors for 30 min on ice. Samples were stimulated with 2 ng/ml TNF- $\alpha$  (A) or the mAb to human MPO (B) for 15 min, respectively ( $n = 2$  independent experiments). These data indicate that inhibition of phosphatidylinositol 3-kinase (PI3-K) with LY294002 and inhibition of p38 mitogen-activated protein kinase (MAPK) with SB202190 but not inhibition of extracellular signal-regulated kinase (ERK) with PD98059 abrogates Akt phosphorylation.

*Effect of PI3-K Inhibition on ANCA-Induced Respiratory Burst*

We showed previously that both p38 MAPK and ERK control ANCA-induced respiratory burst (12). Here, we investigated whether activation of the PI3-K/Akt pathway was of functional significance for ANCA-induced PMN activation. We established a dose-response curve for the effect of the PI3-K irreversible inhibitor wortmannin on the ANCA-induced superoxide generation. PMN were preincubated with increasing concentrations of wortmannin (1 to 50 nM) before the priming with TNF- $\alpha$  and the subsequent stimulation with the mAb to human MPO. Using the assay of SOD-inhibitable reduction of ferricytochrome C, our results demonstrate that inhibition of PI3-K with wortmannin decreased superoxide production over the entire assay period of 60 min in a dose-dependent manner. This effect occurred already at small concentrations of 2.5 mM. Respiratory burst was completely abrogated at 25 mM. For clarity, although tested in a continuous superoxide assay, the data are given for the representative 45-min time point of activation ( $n = 3$ ; Figure 5). Another set of experiments was performed using the highly specific PI3-K blocker LY294002 that inhibits PI3-k by competing with ATP

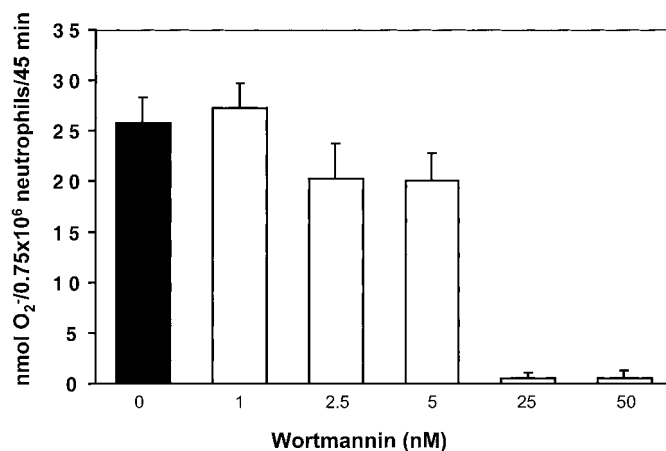


Figure 5. Using wortmannin, the effect of PI3-K inhibition on superoxide release of TNF- $\alpha$ -primed neutrophils was determined in experiments using the mAb to MPO ( $n = 3$  independent experiments). Superoxide release was measured using a continuous assay of superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C (■, controls). Cells were preincubated with increasing concentrations of wortmannin (1 to 50 nM), and the representative 45-min time point is depicted. These experiments indicate a dose-dependent inhibition of respiratory burst by wortmannin.

for its substrate binding site. Figure 6 shows that preincubation with LY294002 significantly abrogated superoxide generation in response to the mAb to MPO ( $n = 4$ ). Almost complete inhibition occurred already at a concentration as low as 2.5  $\mu$ M. We next investigated the effect of LY294002 on the

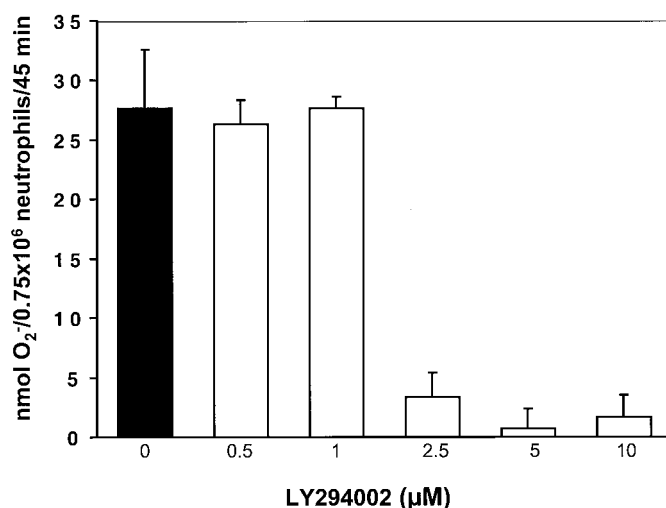
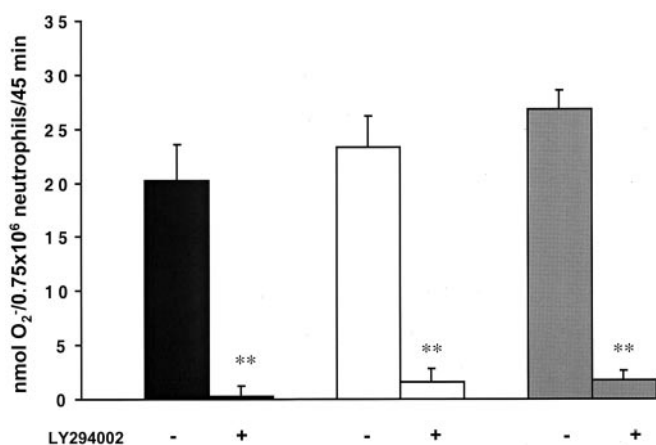


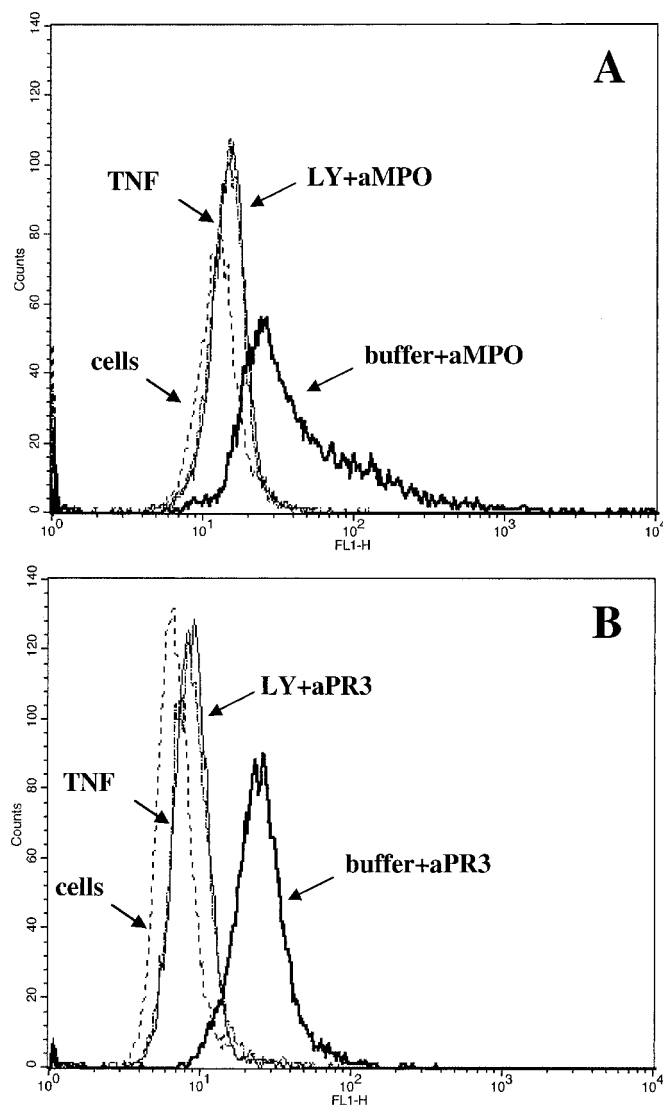
Figure 6. The effect of the specific PI3-K inhibitor LY294002 on superoxide release of TNF- $\alpha$ -primed neutrophils was determined in experiments using the mAb to MPO ( $n = 4$  independent experiments). Superoxide release was measured using a continuous assay of SOD-inhibitable reduction of ferricytochrome C (■, controls). Cells were preincubated with increasing concentrations of LY294002 (0.5 to 10  $\mu$ M) and the representative 45 minutes time point is depicted. These experiments indicate a dose-dependent inhibition of respiratory burst by LY294002.

respiratory burst in response to human ANCA (Figure 7). PR3-ANCA preparations from two different patients and MPO-ANCA preparations from two different patients were tested. A total of  $20.2 \pm 3.4$  nmol  $O_2^-/0.75 \times 10^6$  PMN/45 min were released after stimulation with human PR3-ANCA, and  $5 \mu\text{M}$  LY294002 decreased this amount to  $0.3 \pm 2.6$  nmol ( $n = 10$ ;  $P < 0.05$ ); these numbers were  $23.3 \pm 2.9$  versus  $1.6 \pm 3.6$  for MPO-ANCA ( $n = 10$ ;  $P < 0.05$ ). No effect of LY294002 was seen when  $25 \text{ ng/ml}$  PMA was used to activate PMN ( $52.3 \pm 2.2$  nmol  $O_2^-/0.75 \times 10^6$  PMN/45 for stimulation with PMA in the absence and  $53.0 \pm 3.8$  nmol in the presence of  $5 \mu\text{M}$  LY 294002;  $n = 3$ ).

We used the dihydrorhodamine oxidation test as a second independent assay to assess the effect of PI3-K inhibition on ANCA-induced respiratory burst in a cytochalasin B-free system. The MFI, representing the amount of generated reactive oxygen radicals, was  $10 \pm 2$  in untreated cells and  $14 \pm 2$  in cells primed with  $2 \text{ ng/ml}$  TNF- $\alpha$ . The MFI value increased to  $147 \pm 14$  in TNF- $\alpha$ -primed neutrophils activated with the mAb to MPO and was decreased by preincubation with LY294002 to  $13 \pm 2$ . Stimulation of TNF- $\alpha$ -primed neutrophils with an mAb to PR3 resulted in an MFI of  $28 \pm 1$ . This number was decreased to  $13 \pm 2$  by  $5 \mu\text{M}$  LY294002 ( $n = 3$ ). As observed with Akt phosphorylation, the response to the mAb to PR3 was weaker than to the mAb to MPO. A typical experiment is shown in Figure 8. These results confirm the assay of SOD-inhibitable reduction of ferricytochrome C, showing that PI3-K inhibition decreased the respiratory burst in response to ANCA.



**Figure 7.** The inhibitory effect of LY294002 on superoxide release was studied in experiments using human PR3-ANCA ( $n = 10$ ) and human MPO-ANCA ( $n = 10$ ) in addition to the murine mAb to MPO ( $n = 12$ ). ■, PR3-ANCA; □, MPO-ANCA; ▤, mAb to MPO. In each of the 10 independent experiments using human ANCA, preparations from two different PR3-ANCA patients and two different MPO-ANCA patients were tested. Superoxide release was measured using a continuous assay of SOD-inhibitable reduction of ferricytochrome C. These results demonstrate that specific inhibition of PI3-K with  $5 \mu\text{M}$  LY294002 significantly abrogated superoxide generation (\*\* $P < 0.01$ ).



**Figure 8.** With the use of the dihydrorhodamine oxidation test, the effect of LY294002 on the respiratory burst of TNF- $\alpha$ -primed neutrophils activated with  $5 \mu\text{g/ml}$  mAb to MPO (A) or  $15 \mu\text{g/ml}$  mAb to PR3 (B) was assessed in parallel. Each diagram shows the results from unstimulated cells (cells), TNF- $\alpha$ -primed cells (TNF), and TNF- $\alpha$ -primed cells treated with the mAb to MPO (aMPO) or PR3 (aPR3) after preincubation with buffer, or  $5 \mu\text{M}$  LY294002 (LY), respectively. The increase in fluorescence intensity represents the oxidation of the nonfluorescent dihydrorhodamine to the green fluorescent rhodamin reflecting the amount of generated reactive oxygen radicals. A typical of three independent experiments is shown. Inhibition of PI3-K abrogated the respiratory burst in TNF- $\alpha$ -primed neutrophils challenged with the mAb to MPO and PR3, respectively.

### Effect of PI3-K Blockade on Translocation of ANCA Antigens

We found previously that p38 MAPK but not ERK controls the translocation of ANCA antigens from the granules to the cell surface (12). We show in the present study that p38 MAPK and PI3-K participate in Akt phosphorylation and therefore performed flow cytometry to assess the role of PI3-K pathway in the surface expression of PR3 and MPO during TNF- $\alpha$

priming. PMN were preincubated with LY294002 or buffer control for 30 min before the incubation with 2 ng/ml TNF- $\alpha$ . Figure 9 shows that TNF- $\alpha$  increased the amount of PR3 and MPO on the cell membrane and that LY294002 did not inhibit this process. Parallel experiments confirm our previous data demonstrating that preincubation with SB202190 decreased TNF- $\alpha$ -induced translocation of PR3 (MFI from  $270 \pm 30$  to  $134 \pm 37$ ) and MPO ( $26 \pm 6$  to  $9 \pm 1$ ). These experiments indicate that translocation of ANCA antigens is not controlled by PI3-K-dependent activation of Akt. They are consistent with the finding that ANCA translocation requires p38 MAPK activation.

#### Effect of TNF- $\alpha$ Priming and ANCA Stimulation on the Composition of the Akt-Signaling Module

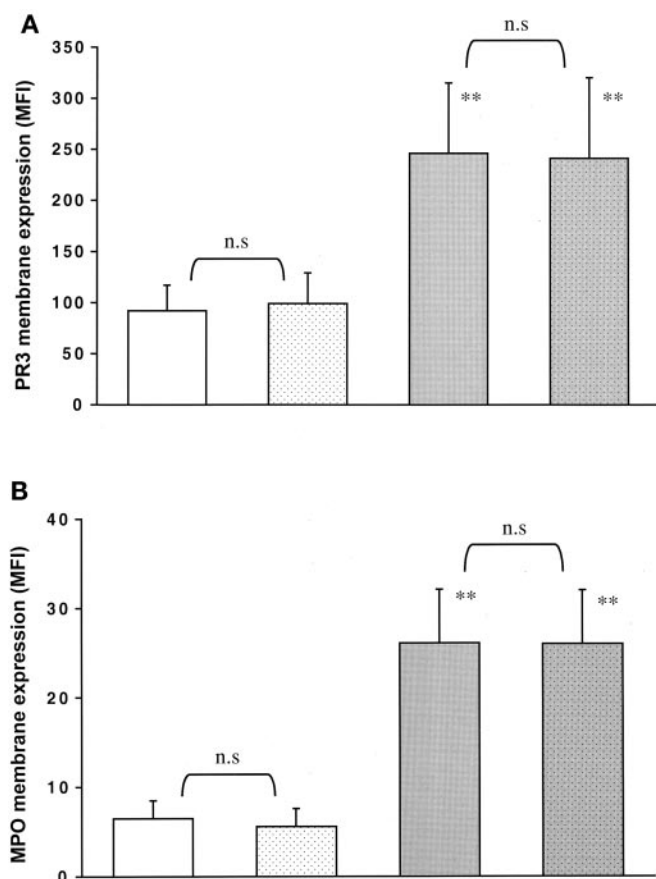
We demonstrated previously that Akt exists in a signaling complex with p38 MAPK, MK-2, and HSP27 (13). The com-

position of this complex may change in response to stimuli. Therefore, changes in response to TNF- $\alpha$  and ANCA in the association of Akt with other kinases important to PMN activation was investigated using a GST pull-down assay. GST-fused Akt was expressed in *Escherichia coli* and immobilized on glutathione-Sepharose beads. PMN were stimulated with the priming concentration of 2 ng/ml TNF- $\alpha$  or buffer control, respectively. After 15 min, cells were lysed and incubated with the GST Sepharose beads. Because cytoskeleton reorganization is essential in the PMN respiratory burst, we examined the presence in the Akt module of proteins that help regulate the cytoskeleton. The proteins attached to the beads were separated by SDS-PAGE and immunoblotted for HSP27, PAK1, and Rac1 as described in the Methods section. Figure 10 shows that Akt and PAK1 exist in a complex in resting PMN. TNF- $\alpha$  stimulation (2 ng/ml for 15 min) caused a 60% increase in the amount of PAK associated with Akt. Rac1 and HSP27 expression was unchanged, and consecutive stimulation with either an mAb to MPO or a control mAb caused no additional change in the Akt signaling complex (data not shown).

#### Discussion

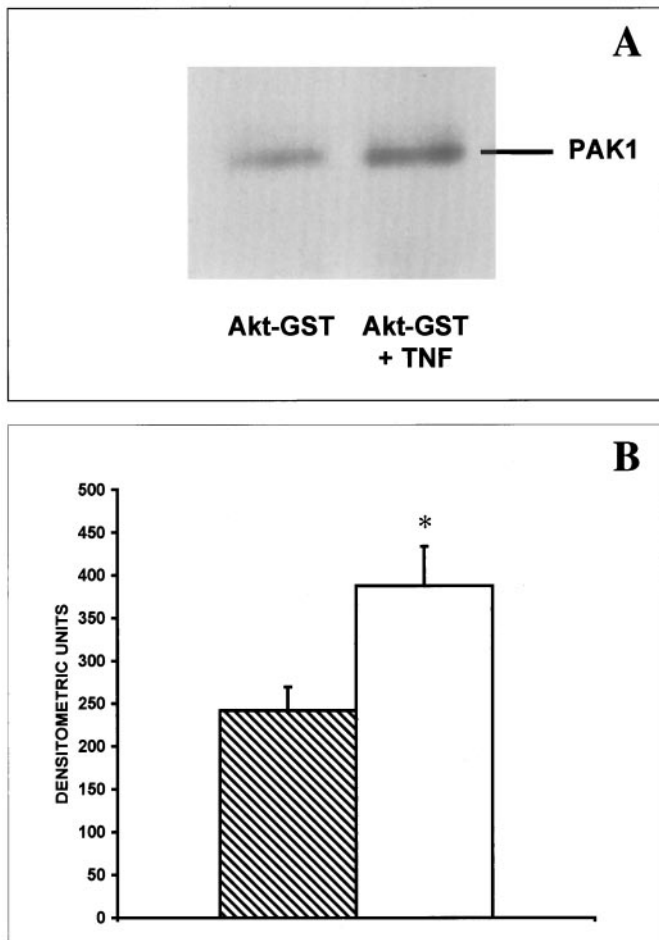
ANCA are detected in patients with small-vessel vasculitis and necrotizing crescentic glomerulonephritis. Several lines of evidence suggest that ANCA-activated neutrophils and monocytes contribute to the inflammatory process, causing necrotizing vascular and glomerular inflammation. *In vitro* incubation of TNF- $\alpha$ -primed neutrophils with ANCA results in activation by mechanisms that are incompletely understood. We tested the hypothesis that ANCA activate human neutrophils via PI3-K-dependent activation of the Akt pathway. Akt can be activated by either products of PI3-K or the p38 MAPK substrate MK-2. Our data indicate that both priming with low concentrations of TNF- $\alpha$  and subsequent ANCA stimulation activate the serine/threonine kinase Akt. Interestingly, we show that Akt activation during TNF- $\alpha$  priming and ANCA antigen translocation occurs via a p38 MAPK-dependent pathway. Using the pharmacologic inhibitor LY294002, we demonstrated that blocking PI3-K activation prevents ANCA-induced Akt phosphorylation and superoxide generation. This effect was independent of ANCA antigen translocation from the cytoplasm to the cell surface shown previously to depend on p38 MAPK activity. Characterization of the Akt signaling module showed that Akt, PAK1, HSP27, and Rac1 exist in complex in resting PMN cytosol and that TNF- $\alpha$  stimulation caused increased association of PAK1 with Akt.

Several stimuli can activate the PI3-K and Akt pathways in neutrophils, including lysophosphatidylcholine (17), arachidonic acid (18), activation of  $\beta$ 2-integrins (19), Fc $\gamma$ -receptors, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-8, lipopolysaccharide (LPS), and FMLP (20–23). Here, we demonstrate that the proinflammatory cytokine TNF- $\alpha$  stimulates Akt, even when used in small priming concentrations. This activation was transient, peaking at 10 min. Subsequent incubation of TNF- $\alpha$ -primed neutrophils with ANCA induced a second strong Akt phosphorylation. This effect did not occur when control antibodies were used, sug-



**Figure 9.** The effect of the PI3-K inhibitor LY294002 on translocation of ANCA antigens during TNF- $\alpha$  priming was assessed by flow cytometry using primary mAb to PR3 and MPO. Unstimulated neutrophils (open bars) and TNF- $\alpha$ -stimulated cells (gray bars) in the absence or presence (dotted bars) of 10  $\mu$ M LY294002 were assayed simultaneously ( $n = 6$  independent experiments). The membrane expression of PR3 (A) and MPO (B) is depicted as fluorescence intensity. These data show that TNF- $\alpha$  increased membrane expression of PR3 and MPO (\*\* $P < 0.01$ ) and that PI3-K inhibition did not affect TNF- $\alpha$ -mediated membrane translocation of ANCA antigens (NS).





**Figure 10.** The effect of TNF- $\alpha$  on the composition of the Akt signaling module was studied using a glutathione S-transferase pull-down assay. Polymorphonuclear neutrophils (PMN) were stimulated with the priming concentration of 2 ng/ml TNF- $\alpha$  or buffer control, respectively. After 15 min, cells were lysed and incubated with the GST-fused Akt that was immobilized on Sepharose beads. The proteins attached to the beads were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted for the small GTP-binding protein (PAK1). Panel A shows typical of six immunoblotting experiments for PAK1 using different neutrophil donors, indicating that Akt and PAK1 exist in a complex in resting PMN and that TNF- $\alpha$  caused an increase in the amount of PAK associated with Akt. The densitometric analysis is shown in B (\* $P < 0.05$ ).

gesting that specific interaction of ANCA with the expressed antigens was responsible for Akt phosphorylation. Akt is a serine/threonine kinase that becomes fully activated after phosphorylation at both 308Thr and 473Ser. Phosphatidylinositol-dependent kinase 1 phosphorylates Akt at the 308Thr site. For FMLP, we showed previously that phosphorylation at 473Ser can be achieved by a p38 MAPK-dependent mechanism, where the p38 MAPK substrate MK-2 can act as phosphatidylinositol-dependent kinase 2 phosphorylating Akt at the 473Ser site (13). In this study, we used 10  $\mu$ M SB202190 to inhibit the p38-MAPK pathway by competing with ATP for the ATP binding site on p38-MAPK and observed a decreased Akt

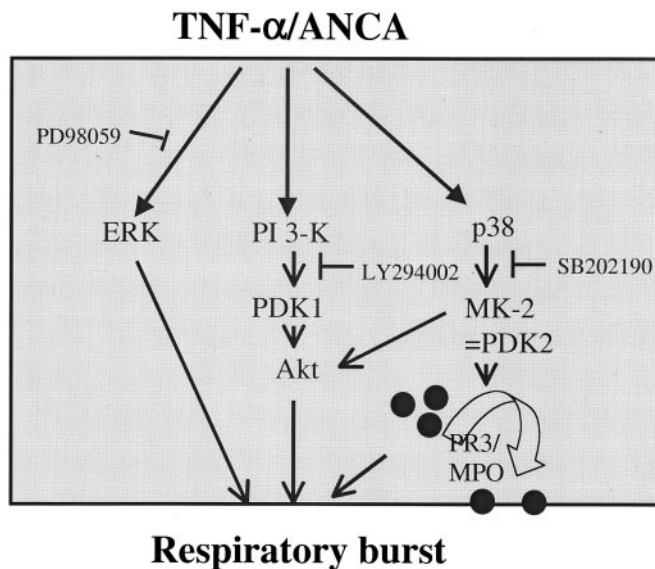
phosphorylation in response to either TNF- $\alpha$  or ANCA. Thus, we extend our previous observation and show that TNF- $\alpha$ - and ANCA-mediated 473Ser phosphorylation of Akt is dependent on p38 MAPK activity.

The PMN respiratory burst and the translocation of ANCA antigens require changes in the cytoskeleton (24–26). We have shown previously that Akt, p38 MAPK, MK-2, and HSP27 form a signaling complex in human PMN and that cellular activation alters the composition of that complex. We reasoned that the association of Akt with cytoskeleton regulatory proteins might be a point of convergence of signaling pathways involved in the response to TNF- $\alpha$  and ANCA. As HSP27 can function as an actin regulatory protein, we examined whether activation by TNF- $\alpha$  or the mAb to MPO would result in the association of Akt with HSP27 or other cytoskeletal regulatory proteins. Using Akt pull-down assays, we determined that Akt is associated with the actin regulatory proteins Rac1 and PAK1 and confirmed its association with HSP27. Exposure to TNF- $\alpha$  caused an increased association of PAK1 with Akt. No additional effect was observed in response to the mAb to MPO. Akt has been shown recently to control chemotaxis in *Dictyostelium* cells by direct phosphorylation of PAKa, a structural homologue of mammalian PAK1 (27). Akt has also been shown to stimulate PAK phosphorylation in mammalian cells (28). Our data demonstrate for the first time that Akt and PAK form a signaling complex in PMN and that this complex alters its composition in response to priming concentrations of TNF- $\alpha$ . Thus, these data suggest the hypothesis that PAK1 may serve as a downstream effector of Akt during TNF- $\alpha$  priming by altering cytoskeletal composition, enabling subsequent stimulation of a respiratory burst in response to ANCA.

The functional importance of the PI3-K pathway in neutrophils has been demonstrated for migration (29) and by us and others for apoptosis (19,22,23,30,31). Recently Hii and colleagues (18,32) demonstrated that arachidonic acid and FMLP stimulate the respiratory burst via the PI3-K pathway. Ben-Smith *et al.* (15) described very recently a functional role for the p101/p110 $\gamma$  PI3-K isoform for an ANCA-induced neutrophil activation. Our present study confirms a central role of PI3-K in ANCA-induced superoxide generation in human neutrophils.

ANCA stimulate the neutrophil respiratory burst only after priming with subactivating concentrations of inflammatory cytokines, such as TNF- $\alpha$ . We and others demonstrated that TNF- $\alpha$  priming leads to translocation of ANCA antigens from intracytoplasmic granules to the outer cell membrane, resulting in increased ANCA binding to their target antigens. Using the specific inhibitor SB202190, we showed previously that this translocation depends on p38 MAPK activity (12). The present study revealed that p38 MAPK participates in the activation of Akt in response to TNF- $\alpha$  because SB202190 inhibited Akt phosphorylation. Therefore, we addressed the question of whether PI3-K or p38 MAPK controls the ANCA antigen translocation. Our data clearly indicate that pharmacologic inhibition of PI3-K by LY294002 did not block TNF- $\alpha$ -induced increase of ANCA antigen membrane expression. As PI3-K is an upstream activator of p38 MAPK, it is unlikely that





**Figure 11.** Proposed scheme of PI3-K- and MAPK-mediated respiratory burst stimulation in TNF- $\alpha$ -primed PMN activated by ANCA. PI3-K, ERK, and p38 MAPK are activated during TNF- $\alpha$  priming. P38 MAPK is central for increasing membrane expression of ANCA antigens, enabling subsequent PMN activation by ANCA. PI3-K and P38 MAPK cooperate in Akt activation. P38 MAPK activates MAPK-activated protein kinase that in turn serves as PKD2 to phosphorylate Akt. ANCA additionally stimulate PI3-K/Akt, resulting in full-blown PMN activation. In this illustration, results from this study, our previous studies (12,13), and the work by Ben-Smith *et al.* (15) are incorporated.

TNF- $\alpha$  modulates ANCA translocation by the Akt pathway even through p38 MAPK activation. Alternatively, p38 MAPK might alter translocation through MK-2-dependent activation of Hsp27 or other substrates that lead to cytoskeletal rearrangement. A proposed model of PI3-K- and MAPK-mediated respiratory burst stimulation in TNF- $\alpha$ -primed PMN activated by ANCA is depicted in Figure 11.

In summary, our data are consistent with the finding that TNF- $\alpha$ -induced ANCA antigen translocation occurs by p38 MAPK but not Akt-dependent mechanisms. However, the ANCA-induced respiratory burst requires PI3-K activation and possibly proceeds by an Akt-dependent pathway. Pharmacologic inhibition of PI3-K or p38 MAPK may be useful in limiting neutrophil inflammation in patients with ANCA vasculitis.

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See related editorial, “ANCA Are Pathogenic—Oh Yes They Are,” on pages 1977–1979.