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# C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway

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**C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway.**

*Background.* Studies have shown that survival factors including cytokines and growth factors delay apoptosis of human neutrophils via induction of the phosphatidylinositol-3 kinase (PI 3-K)/Akt pathway. In the present study, we explored whether complement fragment C5a has a modulatory effect on neutrophil apoptosis through this signaling pathway.

*Methods.* Human neutrophils were isolated and treated with C5a for up to 24 hours, with or without wortmannin, a PI 3-K inhibitor, and staurosporine, a caspase-9 activator. Apoptosis was quantified by flow cytometry, using propidium iodide nuclear staining, and confirmed by the detection of DNA fragmentation on gel electrophoresis. PI 3-K downstream signaling events were evaluated by measuring the expression of cytosolic total and phosphorylated Akt and Bad proteins by Western blot analyses, and caspase-9 activity.

*Results.* C5a inhibited neutrophil apoptosis in a dose- and time-dependent manner. The anti-apoptotic effects of C5a were markedly abrogated in the presence of wortmannin. Brief stimulation of neutrophils with C5a induced phosphorylation of Akt and Bad proteins through a PI 3-K–dependent pathway. Caspase-9 activity was minimal in C5a-treated cells, but markedly increased following PI 3-K inhibition by wortmannin. Finally, C5a reduced caspase-9 activity in staurosporine-treated cells.

*Conclusions.* This study demonstrates that C5a inhibits neutrophil apoptosis via a PI 3-K signaling pathway. This effect may be an important mechanism that improves cell survival and function in the inflammatory milieu.

Neutrophils are short-lived leukocytes that are committed to undergo apoptosis under normal conditions [1, 2]. During inflammatory responses, neutrophil transmigration into tissues is associated with increased cellular activation and a significant delay in apoptosis [3]. This regulation of neutrophil survival is influenced by a variety of inflammatory mediators, such as bacterial lipopolysaccharide (LPS) [4, 5], cytokines [4–6], hematopoietic growth factors [7], complement fragments [4, 5], and extracellular matrix proteins [8].

Neutrophil survival requires the active inhibition of apoptosis, which is accomplished either by inhibiting caspases or by preventing their activation. Signaling pathways involving phosphatidylinositol-3 kinase (PI 3-K) have been implicated in several cellular responses including protection from apoptosis [9]. This pathway leads to the activation of Akt, a cytosolic serine/threonine kinase that acts downstream of PI 3-K [10, 11]. The function of Akt is largely undefined, but it acts as an effector of PI 3-K to prevent cells from undergoing apoptosis [10, 11]. Cell permeable inhibitors of PI 3-K such as wortmannin have proved to be critically important not only as more efficient tools to examine the Akt pathway, but also as a fundamental evidence for the function of PI 3-K in a specific receptor-mediated signaling pathway [9].

Activation of the complement cascade via either the classical or alternative pathway results in the generation of several complement fragments, especially C5a, which is a potent immuno-modulatory molecule [12]. C5a has been implicated in the pathogenesis of glomerulonephritis [13] and neutrophil-mediated ischemic re-perfusion injury [14]. It also contributes to the increased adhesiveness of neutrophils that takes place during hemodialysis with cellulose-based membranes, which results in transient margination and pulmonary leukostasis [15, 16]. In this study, we tested the hypothesis that complement factor C5a down-regulates neutrophil apoptosis, using primarily the PI 3-K/Akt signaling pathway.

## METHODS

### Neutrophil isolation

Heparinized whole blood (10 IU/mL) was obtained from healthy volunteers ($N = 11$; age $42 \pm 2$ years).

**Key words:** polymorphonuclear cells, neutrophils, cell death, inflammation, complement, kinase, PI 3-K, Akt, Bad, caspase-9, wortmannin, staurosporine.

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The Human Investigation Review Committee approved collection of blood samples and all participants gave informed consent.

Neutrophils ($20 \times 10^6$/mL) were harvested by Percoll gradient (63/69%) followed by hypotonic lysis of erythrocytes as previously described [17]. Cell purity (>95%) and viability (>99%) were assessed by the Trypan blue exclusion method. Cells were re-suspended in RPMI-1640 supplemented with 10 mmol/L L-glutamine, 24 mmol/L $NaHCO_3$, (Mallinckrodt, Paris, KY, USA), 10 mmol/L HEPES (Sigma Chemical Co., St. Louis, MO, USA), 100 U/mL streptomycin (Irvine Scientific, Santa Ana, CA, USA) and 10% fetal calf serum (FCS) for culture conditions.

### Cell culture

To examine the effect of C5a on neutrophil apoptosis, cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$, with rising concentrations of complement component C5a (0.5 to 2 μg/mL; Sigma), and with or without wortmannin (100 nmol/L; Sigma), a selective inhibitor of PI 3-kinase. After 6 and 24 hours of incubation, cell aliquots were processed for quantification of apoptosis as described below.

### Quantitative detection of apoptosis by flow cytometry

Apoptosis was quantified by flow cytometry using propidium iodide (PI) nuclear staining, as previously described [18]. In brief, cell aliquots were centrifuged and pellets were fixed in 2 mL of ice-cold 70% ethanol for a minimum of two hours. Cells were then washed in phosphate-buffered saline (PBS), re-suspended in 0.5 mg/mL of RNAse (Type XII-A; Sigma), and incubated for 15 minutes at room temperature. Propidium iodide (50 μg/mL) was then added and cell suspensions were kept on ice in the dark for 15 to 30 minutes until analyzed by flow cytometry. Flow cytometric analysis was carried out at a flow rate of 1000 events/second by using a dual laser flow cytometer (FACscan; Becton Dickinson, San Jose, CA, USA). A total of 10,000 events were counted. Cell debris and clumps were excluded from the analysis by gating single cells in the forward and side light scatters. Propidium iodide was excited using the 488-nm ultraviolet line of the Argon laser. For each experiment, neutrophils were immediately fixed after isolation, and analyzed first, to determine the gates delineating the hypodiploid cell population with low PI uptake. Neutrophils with low PI uptake were considered apoptotic. Acquired data were analyzed with Mac-based software (FlowJo; Tree Star, Inc., San Carlos, CA, USA). The proportion of apoptotic neutrophils was defined as the number of apoptotic cells divided by the total number of cells × 100.

### Qualitative detection of apoptosis by DNA electrophoresis

DNA fragmentation was analyzed by agarose gel electrophoresis as previously prescribed [19]. In brief, neutrophils ($10 \times 10^6$/mL) were washed and lysed overnight at 37°C, in extraction buffer containing 50 mmol/L Tris, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS) and 250 μg/mL proteinase K. DNA was precipitated with twice the volume of ethanol and one-tenth the volume of 3 mol/L sodium acetate, and re-suspended in Tris-EDTA (10 mmol/L Tris and 1 mmol/L EDTA at pH 8.0) containing 250 μg/mL of RNAse. Purified DNA was electrophoresed in 1.2% agarose gel containing ethidium bromide. DNA was visualized under ultraviolet illumination and photographed using Polaroid film.

### Western blot analyses for Akt and Bad proteins

To examine PI 3-K downstream signaling events, the expression of cytosolic total and phosphorylated Akt and Bad proteins was measured by Western blot analyses. In brief, neutrophils ($10 \times 10^6$ cells) were incubated with rising concentrations of C5a (0 to 2 μg/mL) for five minutes in the presence or absence of wortmannin (100 nmol/L) at 37°C. Following this brief incubation period, cells were centrifuged at $450 \times g$ for five minutes. To the cell pellets, cell lysis buffer [10 mmol/L Tris-HCl at pH 7.6, 140 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF)] was added, and the suspension was incubated for 30 minutes at 4°C. Protein concentration was determined using the Bradford reagent (Sigma). The cell lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter. The membranes were blocked using non-specific interactions with PBS-T (PBS at pH 7.4 with 0.1% Tween 20) containing 5% blocking agent for one hour. Immunoblotting was performed using a rabbit anti-human Akt polyclonal antibody (Cell Signaling, Beverly, MA, USA) or a mouse anti-human Phospho-Akt (within the C-terminus at Ser473) monoclonal antibody (Cell Signaling). After a 12-hour incubation at 4°C, the membranes were washed with PBS-T and respectively incubated with secondary anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase for one hour at room temperature. After three washes with PBS-T, immunoreactive bands were visualized by using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions.

Similar experiments were performed to examine the expression of cytosolic total and phosphorylated BAD proteins, using rabbit anti-human Bad or anti-human Phospho-Bad (at Ser136) polyclonal antibodies (Cell Signaling).

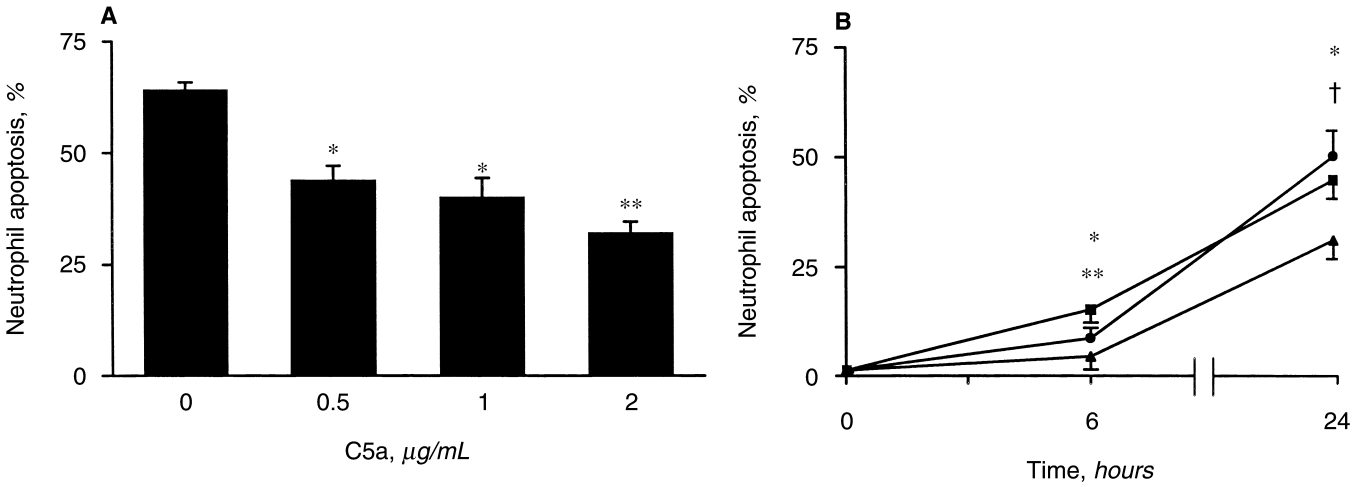


**Fig. 1. Dose- and time-dependent effect of C5a on apoptosis of human neutrophils.** (*A*) Dose-dependent effect. Cells were treated with C5a (0 to 2 μg/mL) for 24 hours, and processed for quantification of apoptosis by flow cytometry. **P* = 0.01 vs. control conditions; ***P* = 0.005 vs. control conditions. Data are means ± SEM of 10 experiments. (*B*) Time-dependent effect. Cells were control (●) or treated with C5a alone (2 μg/mL; ▲) or C5a plus wortmannin (100 nmol/L; ■) for 6 and 24 hours, and processed for quantification of apoptosis by flow cytometry. **P* = 0.02 C5a vs. control conditions; ***P* = 0.02 C5a + wortmannin vs. C5a conditions; †P = 0.04 C5a + wortmannin vs. C5a conditions. Data are means ± SEM of 7 experiments.

Densitometric analyses of the images were performed using the Scion Image β3b acquisition and analysis software (Scion Corp., Frederick, MD, USA). Optical density (OD) calibration was used for all analyses using a generic mapping of brightness to OD. This calibration was used for comparison of lane bands in the same digital image.

### Measurement of caspase-9 activity

To examine the effect of C5a on caspase-9 activity, we used a commercially available colorimetric assay (R & D Systems, Minneapolis, MN, USA). In brief, neutrophils ($2 \times 10^6$ cells) were incubated alone, or with wortmannin (100 nmol/L) or C5a (2 μg/mL) in the presence or absence of wortmannin. In addition, cells were incubated with staurosporine (0 to 20 μmol/L), a known stimulator of caspase-9 activity. The latter experiments were performed with or without a 30-minute pre-exposure to C5a (2 μg/mL). Following a four-hour incubation at 37°C, cells were pelleted at 250 × *g* for 10 minutes. The supernatant was gently removed and discarded while the cell pellet was lysed with a commercially provided lysis buffer. The cell lysate was incubated on ice for ten minutes and then centrifuged at 10,000 × *g* for one minute. Caspase-9 activity was measured in a 96-well flat bottom microplate. Cell lysate reaction buffer containing 5 μmol/L dithiothreitol (DTT; 50 μL) and LEHD-p-nitroaniline (LEHD-pNA; 5 μL), a caspase-9 colorimetric substrate, were added to the microplate and incubated for one hour at 37°C. The plate was read at 405-nm single wavelength, using a microplate reader (MRX-500; Dynatech Laboratories Inc., Chantilly, VA, USA). Background control values were subtracted from the experimental results. Results are expressed as OD and fold change in caspase-9 activity.

### Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences version 10.0 (SPSS, Chicago, IL, USA). Comparisons between groups were made by ranked non-parametric Kruskall Wallis analysis of variance (ANOVA) or Friedman test, and two-tailed Mann Whitney test (unpaired and paired) for continuous variables. Results are expressed as means ± standard error of the mean (SEM). Differences were considered statistically significant at *P* values of less than 0.05.

## RESULTS

### Time- and dose-dependent effect of C5a on neutrophil apoptosis

The dose-dependent effect of C5a on neutrophil apoptosis is shown in Figure 1A ($N = 10$). Compared with unstimulated conditions (64.4 ± 2.0%), neutrophils treated with 0.5 μg/mL (43.8 ± 3.3%), 1 μg/mL (39.9 ± 4.5%) and 2 μg/mL (31.5 ± 2.9%) of C5a had marked delay in apoptosis ($P \leq 0.01$ vs. unstimulated conditions). This effect was confirmed by gel electrophoresis. Indeed, as shown in Figure 2, compared with unstimulated conditions, inter-nucleosomal DNA fragmentation was almost undetectable at 24 hours in cells treated with rising concentrations of C5a.

Time-dependent effect of C5a on neutrophil apoptosis is shown in Figure 1B ($N = 7$). Compared with unstimulated conditions, neutrophils treated with C5a (2 μg/mL) displayed marked delay in cellular apoptosis at 6 (8.6 ±

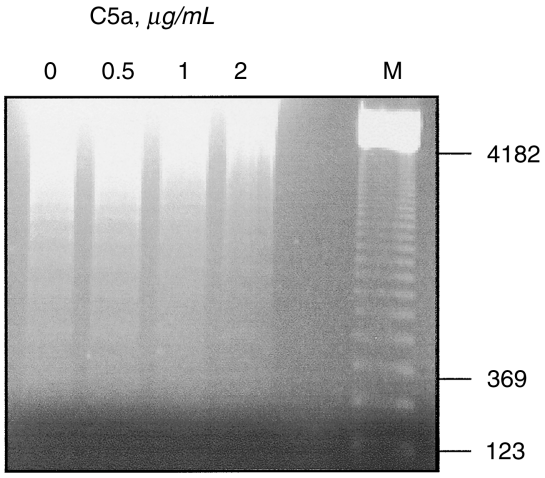


**Fig. 2. Gel electrophoresis of intact and fragmented DNA isolated from C5a-treated human neutrophils.** Cells were treated with C5a (0 to 2 μg/mL) for 24 hours, and processed for quantification of apoptosis by gel electrophoresis. Lane M, 123 bp ladder.

2.4% vs. 4.4 ± 1.1%; $P = 0.018$) and 24 hours (50.3 ± 5.9% vs. 31.1 ± 6.2%; $P = 0.018$).

We next explored whether the anti-apoptotic effects of C5a were mediated in part by the PI 3-K-signaling pathway. As shown in Figure 1B, compared with unstimulated conditions, the anti-apoptotic effects of C5a were markedly reduced in the presence of wortmannin (a specific PI 3-K inhibitor) at 6 (15.1 ± 2.9% vs. 4.4 ± 1.1%; $P = 0.018$) and 24 hours (44.9 ± 4.3% vs. 31.1 ± 6.2%; $P = 0.043$).

### Time- and dose-dependent effect of C5a on Akt phosphorylation

C5a-mediated downstream signaling events leading to delayed apoptosis are unknown. As shown above, since wortmannin provided indirect evidence for the involvement of PI 3-K/Akt in a specific receptor-mediated signaling pathway, we next explored whether C5a directly induces Akt phosphorylation in human neutrophils. Time-dependent effect of C5a on Akt phosphorylation was rapid, and the peak effect was observed after three to five minutes of cell exposure to C5a (data not shown).

Figure 3 shows a representative immunoblot and pooled data from four experiments examining Akt phosphorylation of C5a-treated neutrophils in the presence or absence of wortmannin. Western blot analyses revealed that human neutrophils constitutively express Akt protein (data not shown). Akt phosphorylation was minimal at time 0, and markedly increased at five minutes in the presence of C5a (Fig. 3A), which was abrogated by wortmannin ($P = 0.03$ by Friedman test; Fig. 3B). Indeed, compared with unstimulated conditions, 0.5, 1 and 2 μg/mL of C5a resulted in a respective 24%, 108% and 130% ($P = 0.07$) increase in Akt phosphorylation, which was reduced by 43% in the presence of wortmannin ($P = 0.07$).

### Dose-dependent effect of C5a on Bad phosphorylation

Figure 4 shows a representative immunoblot and pooled data from five experiments examining Bad phosphorylation of C5a-treated neutrophils in the presence or absence of wortmannin. Western blot analyses revealed that human neutrophils constitutively express Bad protein (data not shown). Bad phosphorylation was minimal at time 0, but markedly increased at five minutes in the presence of C5a (Fig. 4A), and was reduced in the presence of wortmannin ($P = 0.02$ by Friedman test; Fig. 4B). Indeed, compared with unstimulated conditions, 0.5, 1 and 2 μg/mL of C5a resulted in a respective 56% ($P = 0.08$), 71% ($P = 0.04$) and 147% ($P = 0.04$) increase in Bad phosphorylation, which was reduced by 34% in the presence of wortmannin ($P = 0.04$).

### Effect of C5a on caspase-9 activity

Figure 5 shows pooled OD data from six experiments examining caspase-9 activity of untreated, wortmannin- and C5a-treated neutrophils in the presence or absence of wortmannin. Caspase-9 activity was undetectable at time 0, and detectable at four hours in untreated and C5a-treated cells. Compared with untreated cells, caspase-9 activity decreased by only 8% in the presence of C5a. However, compared with C5a-treated cells, the addition of C5a and wortmannin together resulted in a 229% increase in caspase-9 activity ($P = 0.03$). Of note, caspase-9 activity also increased by 95% in wortmannin-treated cells compared with untreated cells.

In exploring whether caspase-9 activation by staurosporine, a potent caspase-9 activator, could be inhibited by pre-exposure of cells to C5a, a dose-dependent effect of staurosporine on caspase-9 activity was observed. Indeed, 5, 10 and 20 μmol/L of staurosporine resulted in a 0.7 ± 0.2-fold, 0.6 ± 0.1-fold, and 1.7 ± 0.8-fold increase in caspase-9 activity, respectively. However, compared with the 20 μmol/L dose of staurosporine, pre-exposure of cells to C5a resulted in a 2.7 ± 0.5-fold decrease in caspase-9 activity ($P = 0.028$; $N = 6$).

## DISCUSSION

Under normal conditions, cells are constantly negotiating a balance of factors and signals that determine whether survival or apoptosis will be the chosen course of action. Elements controlling this balance include cellular and genomic integrity, availability of growth and survival factors, and death signals generated during immune responses. Aberrant inputs into survival/apoptosis control figure prominently in human disease ranging from degenerative disorders to cancer.

In humans, little is known about the function and

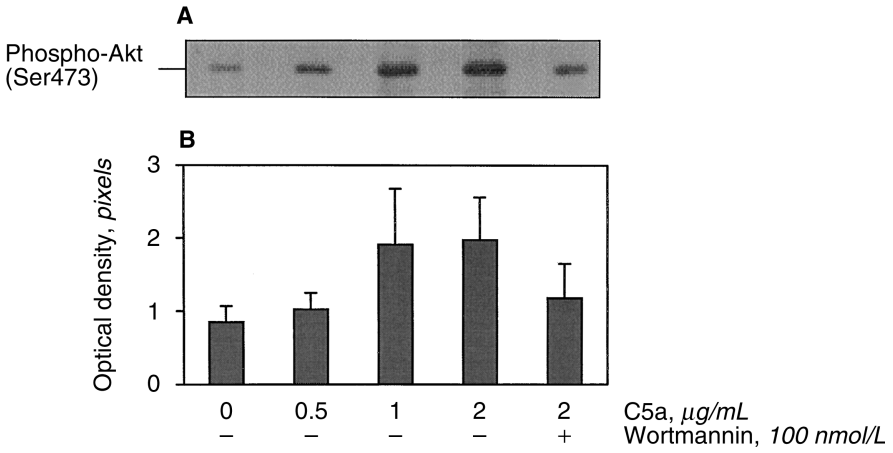


**Fig. 3. Dose-effect of C5a on phosphorylation of Akt protein in human neutrophils.** Cells were treated with C5a (0 to 2 μg/mL) ± wortmannin (100 nmol/L) for 5 minutes, and cell lysates were immunoblotted with either anti-Akt or anti-phosphorylated (Ser473) Akt antibody. *(A)* The upper panel is a representative immunoblot of Akt phosphorylation. *(B)* The lower panel represents the pixel values of Akt phosphorylation that were pooled from four separate experiments, yielding similar results ($P = 0.03$ by Friedman test).

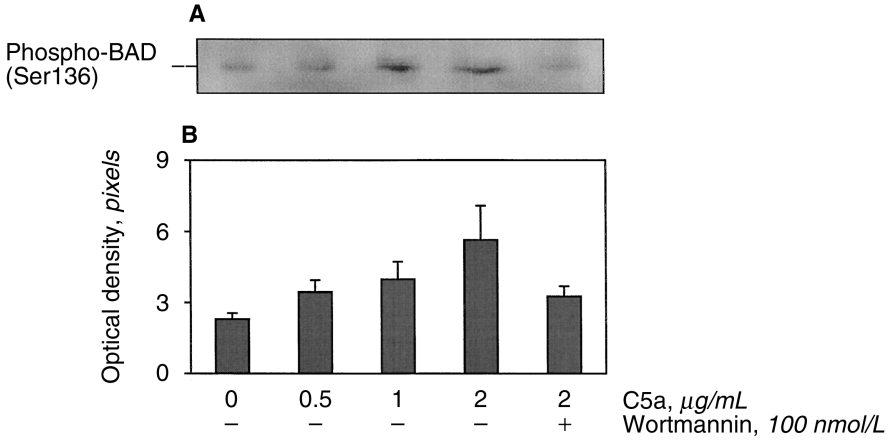


**Fig. 4. Dose-effect of C5a on phosphorylation of BAD protein in human neutrophils.** Cells were treated with C5a (0 to 2 μg/mL) ± wortmannin (100 nmol/L) for five minutes, and cell lysates were immunoblotted with either anti-BAD or anti-phosphorylated (Ser136) BAD antibody. *(A)* A representative immunoblot of BAD phosphorylation. *(B)* Pixel values of BAD phosphorylation that were pooled from five separate experiments, yielding similar results ($P = 0.02$ by Friedman test).

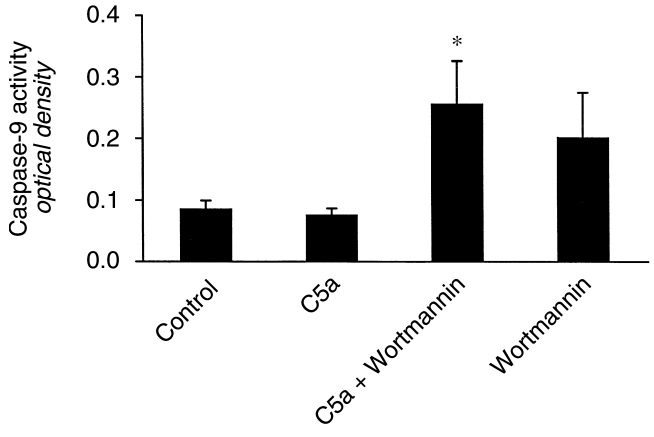


**Fig. 5. Effect of C5a on caspase-9 activity.** Neutrophils were incubated alone (control), with wortmannin (Wt) (100 nmol/L) or C5a (2 μg/mL) ± Wt. *$P = 0.03$ vs. C5a alone. Data are means ± SEM of 6 experiments.

survival of neutrophils in acutely inflamed tissues. According to experimental data, the process of leukocyte transmigration into tissues is associated with increased cellular activation and a significant delay of spontaneous apoptosis [3]. Once in situ, the regulation of neutrophil survival is critical to the propagation and resolution of inflammation. Dysregulation of this process has been implicated in the pathogenesis of prolonged or inappropriate inflammation in severe infection, autoimmune disorders, and possibly atherosclerosis [20, 21]. In chronic inflammatory diseases, persistent accumulation and activation of neutrophils is associated with tissue injury leading to organ dysfunction [22, 23]. At sites of inflammation, the fate of neutrophils is influenced by a variety of mediators, including microbial products such as LPS, cytokines, and hematopoietic growth factors [4–8]. These mediators have been shown to delay neutrophil apoptosis by inducting the PI 3-K/Akt signaling transduction pathway [7].

Signaling pathways involving PI 3-K protect cells from undergoing apoptosis. Akt is a major hub for the input and output of kinase signaling pathways, and plays a critical role in survival signaling. Indeed, this protein kinase is activated downstream from PI 3-K, and once phosphorylated, Akt inhibits the pro-apoptotic Bcl-2 family member Bad and directly inhibits caspase-9 [10, 11].

Indeed, Bad phosphorylation results in its dissociation from the anti-apoptotic Bcl-2 family member $Bcl_{xL}$, which in turn prevents caspase-9 activation [24, 25].

In the present study, we explored whether complement fragment C5a modulates neutrophil apoptosis using a PI 3-K–dependent pathway. The results indicate that C5a inhibits neutrophil apoptosis in a dose- and time-dependent manner. In addition, neutrophil stimulation with C5a induced rapid phosphorylation of Akt and Bad proteins. Further, both the anti-apoptotic effects of C5a and Akt/Bad phosphorylation were diminished in the presence of wortmannin, a specific PI 3-K inhibitor. Caspase-9 activity minimally decreased in C5a-treated cells, but markedly increased when PI 3-K was inhibited by wortmannin. Finally, C5a reduced caspase-9 activity in staurosporine-treated cells.

C5a is a complement fragment that has multifunctional properties [12]. It plays an important role in the pathogenesis of complement-mediated glomerulonephritides [13]. In addition, C5a is a potent chemo-attractant for neutrophils. Indeed, it induces the up-regulation of adhesion molecules on the cell-surface of neutrophils, which promotes transmigration into inflammatory tissues [12, 14, 16]. The systemic effects of C5a following complement activation by bio-artificial surfaces such as during hemodialysis also are well documented [15]. In addition, elevated complement factor D (an essential enzyme of the alternative pathway of complement) concentration has been observed in renal failure, which also may have pathophysiological consequences on C5a generation [26].

Complement-mediated downstream signaling events leading to delayed apoptosis are unknown. Our data indicate that the PI 3-K/Akt signaling pathway mediates the survival-promoting effect of C5a. These findings are in agreement with other studies, which have shown that hematopoietic growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin also can delay programmed cell death of neutrophils and vascular smooth muscle cells, respectively, by activating the PI 3-K pathway [7, 27].

There are several limitations in our study that are worthy of mention. Inhibition of PI 3-K in human neutrophils had a partial effect on C5a-mediated cell survival, suggesting for the role of additional survival signaling pathway that are independent of PI 3-K/Akt. The p44/42 mitogen-activated protein kinase (MAPK), also known as the extracellular signal-regulated kinase or ERK pathway, can be activated by growth factors/mitogens, and has been linked to prolonged cellular survival [7, 28, 29]. It remains to be determined whether C5a employs this pathway to mediate neutrophil survival.

The C5a concentrations used in our experiments were significantly higher than that encountered in the plasma of patients undergoing hemodialysis or during sepsis (less than 0.1 μg/mL) [30, 31]. However, these concentrations are still clinically relevant, as tissue levels of inflammatory mediators are higher than systemic levels. Indeed, among patients suffering from pneumonia, C5a levels in broncho-alveolar lavage fluids can range between 1 and 10 μg/mL [32].

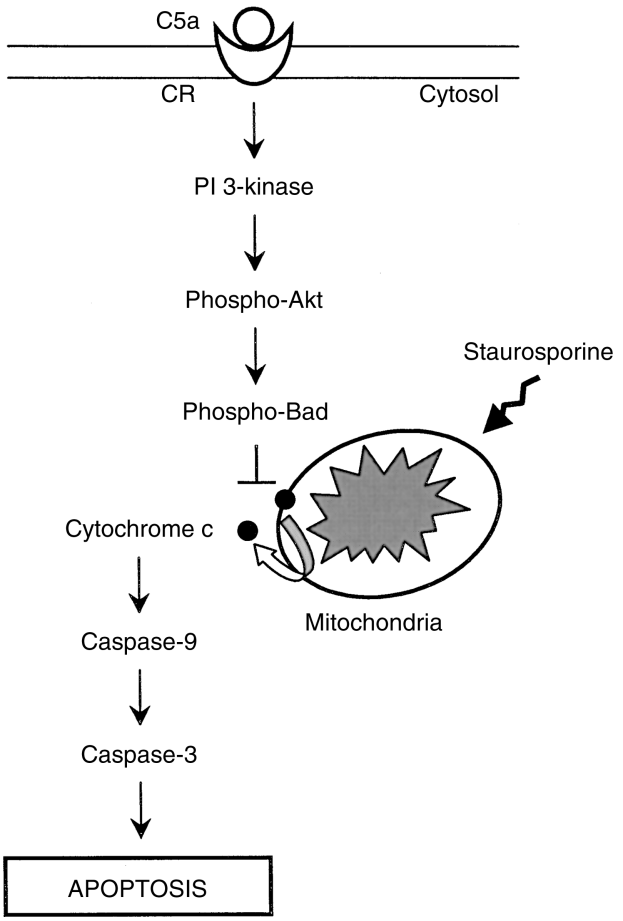


**Fig. 6. Proposed mechanisms for C5a-mediated delayed apoptosis in human neutrophils.** C5a binds to complement receptor (CR), which activates phosphatidyl inositol 3-kinase, leading to Akt phosphorylation. This in turn phosphorylates Bad protein, which may prevent mitochondrial leakage of cytochrome c. The caspase cascade is consequently inhibited, leading to delayed cellular apoptosis (↓ = activation; ⊥ = inhibition).

We failed to observe a marked reduction in caspase-9 activity of C5a-treated neutrophils compared with untreated cells, whereas PI 3-K inhibition with wortmannin, irrespective of C5a, enhanced caspase-9 activity. Previous studies have shown that Akt activation can independently phosphorylate pro-caspase-9, thereby inhibiting its activation [33]. It is unclear whether C5a can directly phosphorylate pro-caspase-9 through the PI 3-K signaling pathway. In separate experiments, we used high-doses of staurosporine to maximally activate caspase-9 and observed that pre-exposure to C5a markedly reduced caspase-9 activity. Although this finding does not unravel the intrinsic mechanism by which C5a inhibits

apoptosis, it does support the hypothesis that C5a can inhibit caspase-9 activation following potent apoptotic stimuli.

Finally, studies on the signal transduction pathway involved in C5a-mediated neuronal protection suggest that C5a inhibits programmed cell death via receptor-dependent phosphorylation of the ERK2 pathway and direct inhibition of caspase-3 activity [34]. It remains to be determined whether this caspase-9–independent pathway is operative in human neutrophils. Such a mechanism would explain the discrepancy in our results between the inability to demonstrate marked reduction in caspase-9 activity by C5a alone, contrasting with the ability of this molecule to inhibit staurosporine-induced caspase-9 activation. We speculate that C5a stimulates several survival signaling pathways that result in delayed apoptosis.

In summary, this study demonstrates that C5a inhibits neutrophil apoptosis via a PI 3-K-dependent pathway (Fig. 6). This may be a protective mechanism by which complement activation improves neutrophil survival and function in the inflammatory milieu. In a recent update on the molecular control of neutrophil apoptosis, two out of five studies demonstrated $Bcl_{XL}$ expression by human neutrophils [35]. Additional studies will be required to examine the effect of C5a on neutrophil-associated MAPK, mainly the ERK pathway, and to see whether in human neutrophils, Bad phosphorylation does result in its dissociation from $Bcl_{XL}$.

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