

Proliferating cell nuclear antigen acts as a cytoplasmic platform controlling human neutrophil survival

Véronique Witko-Sarsat,^{1,2} Julie Mocek,^{1,2} Dikra Bouayad,^{1,2} Nicola Tamassia,³ Jean-Antoine Ribeil,^{4,5} Céline Candalh,^{1,2} Noélie Davezac,⁷ Nathalie Reuter,^{8,9} Luc Mouthon,^{1,2,10} Olivier Hermine,^{5,6} Magali Pederzoli-Ribeil,^{1,2} and Marco A. Cassatella³

¹Institut National de la Santé et de la Recherche Médicale Unité 1016, 75014 Paris, France

²Institut Cochin, Université Paris Descartes, 75014 Paris, France

³Department of Pathology and Diagnostics, Section of General Pathology, University of Verona, 37134 Verona, Italy

⁴Service de Biothérapie, ⁵Centre National de la Recherche Scientifique Unité Mixte de Recherche 8147, and ⁶Service d'Hématologie, Hôpital Necker-Enfants Malades, Université Paris Descartes, 75014 Paris, France

⁷Centre National de la Recherche Scientifique Unité Mixte de Recherche 5241, Université Paul Sabatier, 31000 Toulouse, France

⁸Computational Biology Unit, Bergen Center for Computational Science and ⁹Department of Molecular Biology, University of Bergen, NO-5020 Bergen, Norway

¹⁰Service de Médecine Interne, Hôpital Cochin, 75014 Paris, France

Neutrophil apoptosis is a highly regulated process essential for inflammation resolution, the molecular mechanisms of which are only partially elucidated. In this study, we describe a survival pathway controlled by proliferating cell nuclear antigen (PCNA), a nuclear factor involved in DNA replication and repairing of proliferating cells. We show that mature neutrophils, despite their inability to proliferate, express high levels of PCNA exclusively in their cytosol and constitutively associated with procaspases, presumably to prevent their activation. Notably, cytosolic PCNA abundance decreased during apoptosis, and increased during *in vitro* and *in vivo* exposure to the survival factor granulocyte colony-stimulating factor (G-CSF). Peptides derived from the cyclin-dependent kinase inhibitor p21, which compete with procaspases to bind PCNA, triggered neutrophil apoptosis thus demonstrating that specific modification of PCNA protein interactions affects neutrophil survival. Furthermore, PCNA overexpression rendered neutrophil-differentiated PLB985 myeloid cells significantly more resistant to TNF-related apoptosis-inducing ligand- or gliotoxin-induced apoptosis. Conversely, a decrease in PCNA expression after PCNA small interfering RNA transfection sensitized these cells to apoptosis. Finally, a mutation in the PCNA interdomain-connecting loop, the binding site for many partners, significantly decreased the PCNA-mediated antiapoptotic effect. These results identify PCNA as a regulator of neutrophil lifespan, thereby highlighting a novel target to potentially modulate pathological inflammation.

CORRESPONDENCE

Véronique Witko-Sarsat:
veronique.witko@inserm.fr

Abbreviations used: 7-AAD, 7-aminoactinomycin D; ATRA, all-trans retinoic acid; B2m, β_2 -microglobulin; CDK, cyclin-dependent kinase; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; DMF, dimethylformamide; IP, immunoprecipitation; MD, molecular dynamic; MGG, May-Grünwald Giemsa; MPO, myeloperoxidase; mRNA, messenger RNA; NADPH, nicotinamide adenine dinucleotide phosphate; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand.

Neutrophils are terminally differentiated effector cells, whose principal function is to migrate to sites of inflammation, where they exert anti-infectious and proinflammatory effects (Witko-Sarsat et al., 2000; Henson, 2005; Theilgaard-Mönch et al., 2006). However, a growing body of evidence shows that neutrophils can modulate the inflammatory process (Nathan, 2006). Notably, they can synthesize a wide variety of cytokines involved in immunoregulation (Cassatella, 1999). However, once

their effector role is terminated, their temporally regulated apoptosis, followed by phagocytosis by tissue macrophages, is necessary for successful inflammation resolution (Serhan and Savill, 2005; Fox et al., 2010). Thus, neutrophil apoptosis is pivotal in inflammation resolution,

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although the molecular mechanisms are not completely understood (Simon, 2003). Unlike macrophages or dendritic cells, neutrophils do not proliferate and have a short lifespan. Mcl-1 has been shown to be the main Bcl-2 homologue controlling neutrophil apoptosis (Derouet et al., 2004). Yet, they express several cell cycle regulatory proteins, such as CDK2, p27 (Klausen et al., 2004), and survivin (Altzner et al., 2004), which, in view of the close relationships between proliferation and apoptosis, neutrophils might use to regulate their own survival.

Proliferating cell nuclear antigen (PCNA) is a crucial factor in DNA synthesis and repair, initially characterized as the auxiliary protein of DNA polymerases δ and ϵ (Moldovan et al., 2007). Although all of the PCNA functions described to date exclusively reflect its nuclear localization, within the last few years, many proteins have been found to interact with PCNA, including various enzymes and regulatory proteins such as cyclin-dependent kinases (CDKs; Koundrioukoff et al., 2000) or the CDK inhibitor p21/waf1 (Waga et al., 1994). As a corollary, PCNA has been accorded roles in cellular pathways other than replication, e.g., nucleotide-excision repair, mismatch repair, the cell cycle, and apoptosis, thus acting as a cellular communicator connecting all of these important cellular processes. In the search for additional cell cycle regulatory proteins expressed in neutrophils, we have identified an unexpected antiapoptotic role for PCNA in neutrophils, which, interestingly, express PCNA exclusively in their cytosol. Based on the knowledge that PCNA functions within the nuclei of proliferating cells as a platform for protein interaction, we sought to characterize the molecular interactions between cytosolic PCNA and potential partners in neutrophils. Our findings reveal that procaspases are novel PCNA-associated proteins and, as a result, identify PCNA as a new key player that positively regulates neutrophil survival.

RESULTS

Mature human neutrophils express PCNA exclusively in their cytoplasm

Western blot analysis of neutrophil lysates readily detected PCNA in amounts comparable with those in lymphocytes but less than those in the PLB985 promyelocytic cell line (Fig. 1 A). PCNA could be detected as a single 36-kD band in neutrophils and HeLa cells, using either the anti-PCNA rabbit pAb Ab5 or the mouse mAb PC10 (Fig. S1 A). PCNA specificity was confirmed using purified recombinant PCNA as an antibody competitor (Fig. S1 A). Surprisingly, subcellular fractionation of neutrophils showed high PCNA contents only in the cytosol and not in the nucleus or granules (Fig. 1 B). The quality of our fractionation procedure was validated by the detection of specific markers: β -actin, elastase, and lamin B for cytosol, granules, and nuclei, respectively. Furthermore, the exclusive cytoplasmic localization of PCNA in neutrophils and its nuclear localization in HeLa cells were also illustrated by immunofluorescence labeling (Fig. S1 B). Finally, Western blot and confocal microscopy analysis showed that

in monocytes and lymphocytes, PCNA was located in both the nuclear and cytoplasmic compartments (Fig. S1, C and D), thus indicating that neutrophils are peculiar leukocytes in which PCNA is exclusively cytoplasmic.

Nuclear to cytoplasmic PCNA relocalization occurs during granulocyte differentiation

PCNA subcellular localization was also studied by confocal microscopy after PCNA immunolabeling during the course of *in vitro* granulocyte differentiation of human CD34⁺ cells, isolated from umbilical cord blood and cultured with IL-3 and G-CSF. Complete granulocyte maturation was evaluated by morphological analysis after May-Grünwald Giemsa (MGG) staining (unpublished data). Before inducing differentiation, PCNA was detectable almost exclusively in the nucleus of CD34⁺ cells, whereas 7 d after IL-3-G-CSF treatment, the protein exhibited a mixed cytoplasmic and nuclear distribution (Fig. 1 C). On day 13, most cells had multilobular nuclei with PCNA located in the cytoplasm, similar to that observed in mature peripheral neutrophils. Quantitative analysis (Fig. 1 C, histograms), consisting of counting the cells with nuclear, cytoplasmic, or mixed nuclear-cytoplasmic localization, confirmed this redistribution of PCNA from the nucleus to the cytoplasm of differentiated neutrophils. This relocalization during granulocyte differentiation was also seen in NB4 promyelocytic cells after all-trans retinoic acid (ATRA)-induced differentiation into granulocytes (Fig. S2). Finally, we examined PCNA localization in myeloid cells isolated from human BM aspirates, more specifically in both promyelocytes (identified by myeloperoxidase [MPO] immunolabeling) and mature neutrophils (identified by CD35 immunolabeling; Fig. 1 D). Similarly to that observed in granulocyte-differentiated CD34⁺ cells, PCNA was nuclear in BM granulocytic progenitors but became exclusively cytoplasmic upon differentiation to mature neutrophils (Fig. 1 D).

PCNA is targeted to proteasomal degradation during neutrophil apoptosis while it is stabilized by G-CSF

As indicated by its name, the level of PCNA is modulated during the cell cycle and is particularly high during the S phase of proliferating cells (Moldovan et al., 2007). We show in this study that PCNA expression also seems to be modulated in nonproliferating neutrophils but as a function of their survival status. Indeed, PCNA expression decreased in apoptotic neutrophils, independently of the death pathway, as it could be observed during constitutive and, more markedly, after anti-Fas- or gliotoxin-potentiated apoptosis (Fig. 2 A). The apoptotic process was evaluated by the degree of phosphatidylserine externalization (Fig. 2 B), which, as expected, confirmed a significant increase in annexin-V⁺ and 7-aminoactinomycin D (7-AAD)⁻ cells after 15-h incubation at 37°C (Fig. S3 A), the increase being further enhanced by an anti-Fas mAb or gliotoxin. Similarly, time-dependent increases in caspase-8 (Fig. 2 C) and caspase-3 (Fig. 2 D) activities were observed in neutrophils during constitutive

apoptosis, which was again further enhanced after anti-Fas or gliotoxin exposure. Finally, neutrophil apoptosis was also demonstrated by mitochondrial depolarization after 3,3'-dihydroxycarbocyanine iodide (DiOC₆) labeling (Fig. S3 B). Thus, the decreased PCNA expression (Fig. 2 A) was related to the degree of apoptosis, regardless of the test or experimental

conditions used. Notably, the proteasome inhibitors PS-341 (Fig. 2 E) and MG132 (Fig. S4 A) reversed the apoptosis-induced PCNA degradation and, consequently, significantly inhibited neutrophil death. Interestingly, PS-341 also reversed the PCNA degradation taking place after 6-h neutrophil incubation with anti-Fas mAb or gliotoxin (Fig. 2 F), implying

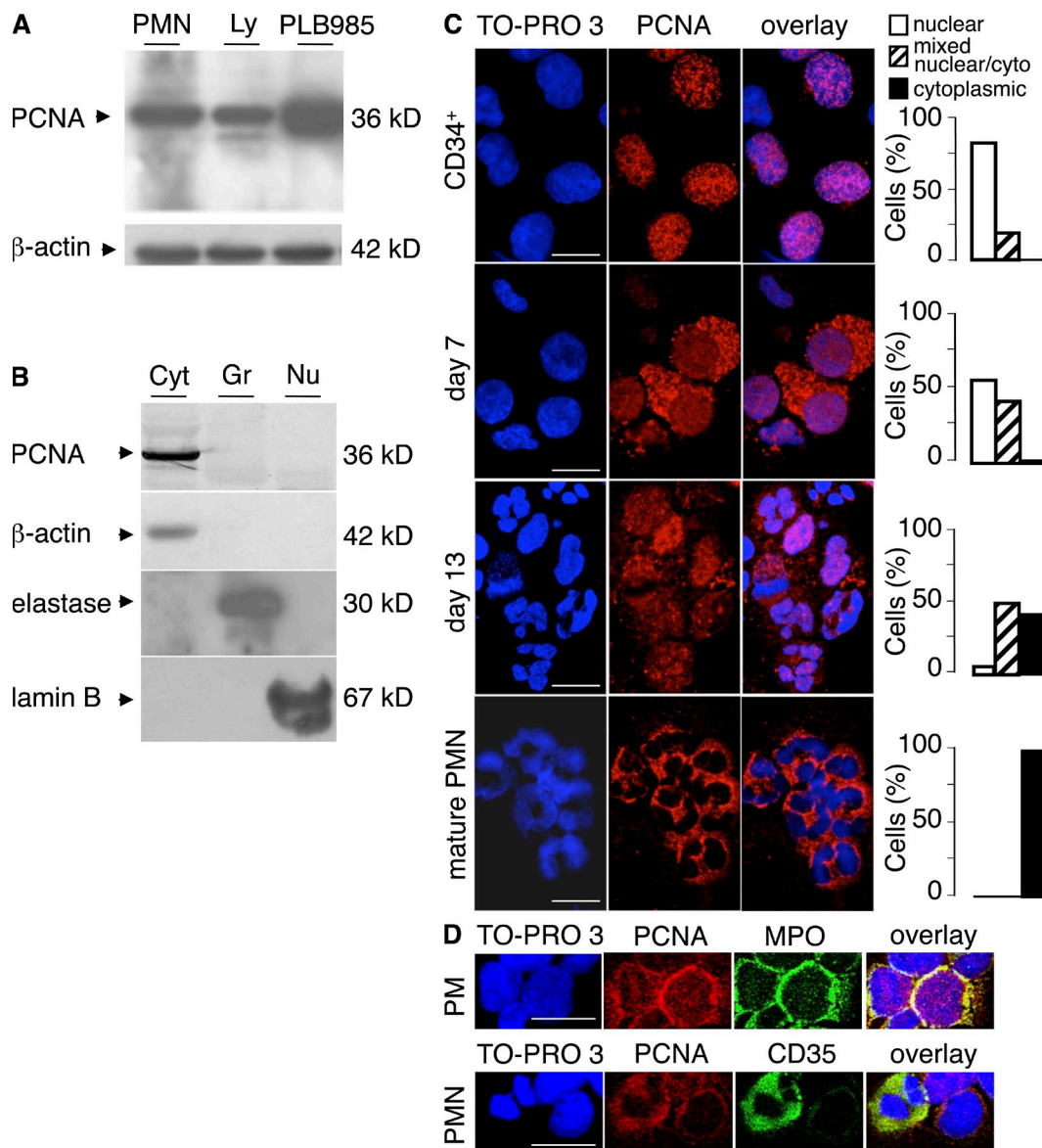


Figure 1. PCNA is expressed exclusively in the cytosol of mature neutrophils. (A) PCNA immunodetection in neutrophils (PMN), lymphocytes (Ly), or PLB985 promyelocytic cells. 50,000 cells/lane were analyzed using PC10 mAb as the primary antibody; anti- β -actin served as the loading control. (B) PCNA expression in different neutrophil subcellular compartments with β -actin, human neutrophil elastase, or lamin B serving as control markers for the cytosolic (Cyt), granular (Gr), and nuclear (Nu) fractions, respectively. The SDS-PAGE gel was run using 50 μ g protein/lane, and PCNA was detected with the PC10 mAb. (C) Immunofluorescence analysis by confocal microscopy of PCNA localization in human CD34⁺ cells before (CD34⁺) and at different times (7 or 13 d) during granulocyte differentiation and in mature neutrophils using the rabbit pAb Ab5 and TO-PRO 3 iodide for nuclear labeling. Bar graphs show percentages of cells exhibiting nuclear, mixed nuclear–cytoplasmic, or cytoplasmic PCNA localization, as determined by counting the cells under the microscope. (D) Immunofluorescence analysis by confocal microscopy of PCNA localization in human BM cells after sorting on a Percoll gradient. Cells from band 3, containing myeloblasts and promyelocytes (PM; top), were labeled with MPO. In contrast, cells from band 1, containing the mature neutrophils as shown by their typical nuclear morphology (PMN; bottom), were labeled with anti-CD35. A–D show representative experiments of three yielding the same results. Bars, 10 μ m.

that proteasome-mediated PCNA degradation occurs in both the death receptor and mitochondrial apoptotic pathways. Accordingly, PCNA is ubiquitinated in neutrophil cytosol after 1-h incubation at 37°C, as indicated by PCNA detection after immunoprecipitation (IP) using an antiubiquitin mAb (Fig. S4 B). Furthermore, chymostatin, a cathepsin inhibitor, had no effect on the decrease in PCNA occurring during constitutive neutrophil apoptosis (Fig. S4 C). The observation that PCNA levels were closely linked with neutrophil survival was corroborated by the stable PCNA levels maintained by G-CSF, which has been described as extending neutrophil longevity (Maianski et al., 2004). In fact, analysis of PCNA expression kinetics revealed that the decrease

observed after 6-h incubation at 37°C was totally prevented by G-CSF, as PCNA protein levels remained elevated until 15 h (Fig. 3 A). Such G-CSF-induced PCNA level stabilization was dose dependent and clearly associated with prolonged neutrophil survival (Fig. 3 B). Incubating neutrophils with G-CSF for up to 21 h did not modify PCNA messenger RNA (mRNA) expression or transcription, as assessed by real-time RT-PCR and primary transcript real-time RT-PCR, respectively, but up-regulated *BLyS* gene expression (Fig. 3 C; Scapini et al., 2003). These results were confirmed using different pairs of PCNA gene-specific primers, together with one of the four different housekeeping genes: *B2m* (β_2 -microglobulin), *GAPDH*, *PPIB* (*peptidyl-propyl isomerase B*),

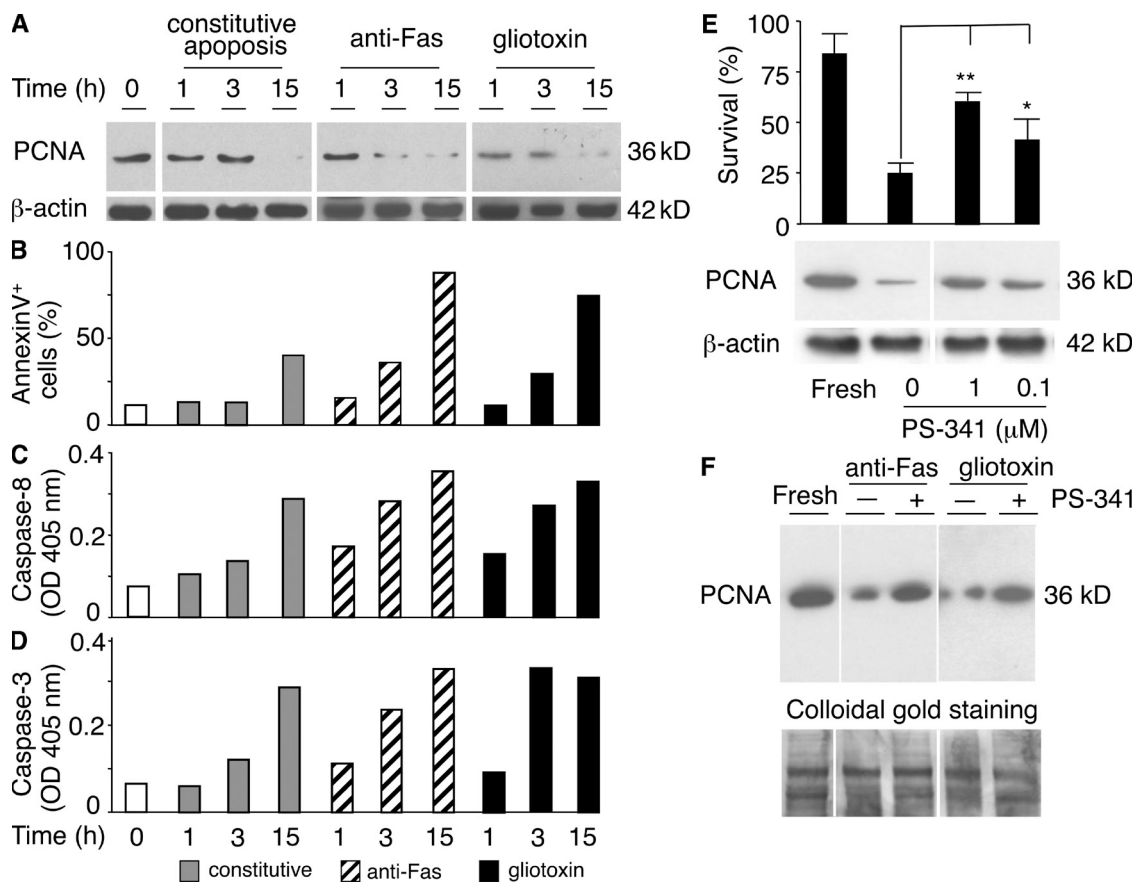


Figure 2. PCNA is degraded by the proteasome during neutrophil apoptosis. (A) PCNA expression kinetics in neutrophils incubated at 37°C for 1, 3, or 15 h alone (constitutive apoptosis), with 10 ng/ml anti-Fas mAb, or with 0.1 μ g/ml gliotoxin. Western blot analysis of neutrophil cytosolic fractions (50 μ g/lane) using the anti-PCNA PC10 mAb. β -Actin immunoblotting served as a loading control on the same membrane. (B) Flow cytometric measurement of phosphatidylserine externalization on neutrophils cultured as in A and labeled with annexin-V-FITC and 7-AAD to assess apoptosis and necrosis, respectively. (C and D) Spectrophotometric determination of caspase-8 (C) and caspase-3 (D) activities in neutrophil lysates using their respective specific chromogenic IETD-pNA and DEVD-pNA substrates. (E) Effect of the proteasome inhibitor PS-341 at the indicated concentrations on survival (top) and on PCNA expression in neutrophils (bottom) cultured as in A. Apoptosis was obtained by incubating neutrophils at 37°C for 15 h, and neutrophil survival was evaluated as the percentage of annexin-V⁻ 7-AAD⁻ neutrophils, to exclude cell apoptosis and necrosis, and compared with freshly isolated neutrophils (fresh). Values are means \pm SEM of eight independent experiments performed in duplicate (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test). A representative PCNA immunoblot obtained under the same experimental conditions is shown on the bottom. (F) Effect of 1 μ M PS-341 on PCNA expression in neutrophils incubated for 6 h at 37°C with anti-Fas or gliotoxin and compared with freshly isolated neutrophils. The percentages of annexin-V⁻ 7-AAD⁻ neutrophils used to evaluate neutrophil survival were 60 and 85% in anti-Fas-treated neutrophils (without and with PS-341) and 55 and 80% in the gliotoxin-treated neutrophils (without and with PS-341), compared with 99% in untreated neutrophils. The bottom panel shows colloidal gold staining of the membrane used as a loading control. A–D and F show results from representative experiments that were performed at least four times and yielded identical results.

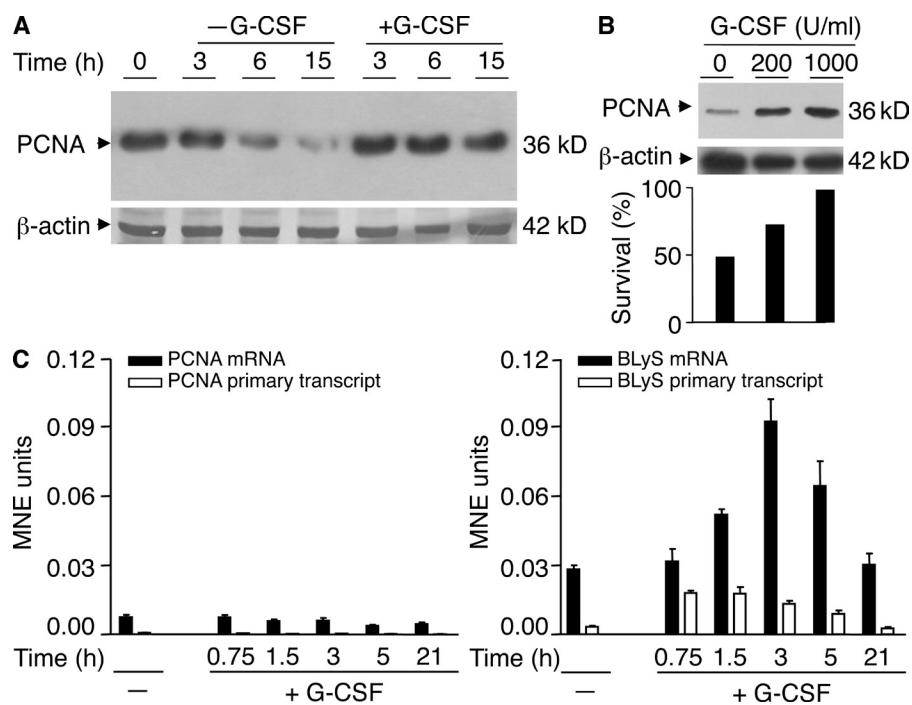


Figure 3. Stable PCNA protein levels are maintained in neutrophils exposed to G-CSF in vitro without transcriptional regulation. (A) Neutrophils were cultured with and without 1,000 U/ml G-CSF at 37°C. After 0, 3, 6, or 15 h of incubation, cells were lysed, and Western blot analysis was performed with the PC10 mAb, and anti- β -actin served as the loading control. (B) Neutrophils were incubated for 15 h at 37°C in the absence (0) or in the presence of 200 or 1,000 U/ml G-CSF and analyzed as described in A. The percentages of viable neutrophils, i.e., annexin-V⁻ 7-AAD⁻ cells under the same conditions, are shown in the histogram below the blot. (C) Total RNA was extracted from neutrophils cultured with and without 1,000 U/ml G-CSF for the times indicated, and PCNA, BlyS, and β 2m mRNA expression was measured by real-time RT-PCR and primary transcript real-time RT-PCR. Their expression is given as mean normalized expression (MNE) after normalization to β 2m in triplicate reactions for each sample. Error bars represent SEM. The data presented in A–C are from one representative experiment of three.

and *GNB2L1* (guanine nucleotide-binding protein, β polypeptide 2-like 1; unpublished data). These observations were also confirmed by Northern blotting experiments (unpublished data).

PCNA involvement in positively regulating neutrophil survival was further confirmed by examining neutrophils isolated from the blood of healthy donors treated with G-CSF for 5 d. Indeed, the PCNA content in these neutrophils, assessed by immunoblotting, was markedly higher than that before G-CSF treatment (Fig. 4 A). This higher neutrophil cytoplasmic PCNA expression in G-CSF-treated donors, also confirmed by confocal microscopy analysis (Fig. 4 B), was unequivocally associated with a decrease in the ability of these neutrophils to undergo constitutive apoptosis (Fig. 4 A). In keeping with the in vitro data, no PCNA mRNA induction was observed in neutrophils from G-CSF-treated patients, unlike what was observed for IL-1ra mRNA (Fig. 4 C).

To test whether PCNA could be also up-regulated during systemic inflammation, neutrophils were isolated from patients with either Wegener's granulomatosis, a necrotizing vasculitis associated with antineutrophil cytoplasmic antibodies at the time of a disease flare, or with sepsis. Remarkably, PCNA protein levels were increased in neutrophils isolated from both types of patients compared with controls (Fig. 4, D and E) without, however, any increase in the corresponding mRNA transcripts (Fig. 4 F). Finally, we also observed that mouse neutrophils isolated from either BM or peripheral blood expressed PCNA exclusively within their cytoplasm (Fig. S5 A). In addition, BM neutrophils displayed decreased levels of both PCNA and procaspase-3 upon apoptosis triggered by gliotoxin (Fig. S5 B), indicating

that PCNA is associated with neutrophil survival not only in humans but also in mice.

A p21 peptide known to interfere with PCNA partners counteracts PCNA-mediated neutrophil antiapoptotic effects

PCNA has no intrinsic enzymatic activity as its biological role relies on its ability to mediate associations with different partners (for reviews see Warbrick, 2000; Maga and Hubscher, 2003). Most PCNA interactions occur at the interdomain-connecting loop. The protein with the highest currently known affinity for PCNA is the CDK inhibitor p21/waf1, which blocks the binding pocket, which explains its dominant role in inhibiting cell replication and other PCNA-regulated functions. Therefore, we tested the effect of an RYIRS-tagged p21 peptide corresponding to the residues 141–160 (so-called carboxyp21), which was previously demonstrated to be able to penetrate inside the cells (Dong et al., 2003) and interfere with PCNA partners in proliferating cells (Warbrick, 2006). In mature neutrophils, p21/waf1 is barely detectable under basal conditions (Klausen et al., 2004; unpublished data), thereby excluding its involvement in the regulation of cytosolic PCNA antiapoptotic activity. A modified carboxyp21 peptide that does not bind PCNA was also designed using molecular dynamic (MD) simulations (not depicted) and used as a control (Fig. 5 A). Immunofluorescence analysis of FITC-conjugated carboxyp21 peptide-treated neutrophils showed a strong cytoplasmic pattern of fluorescence (Fig. 5 B), thus confirming the efficiency of the cytoplasmic peptide delivery (Dong et al., 2003). Accordingly, carboxyp21, which is supposed to bind to PCNA and compete with PCNA partners, triggered neutrophil apoptosis, as

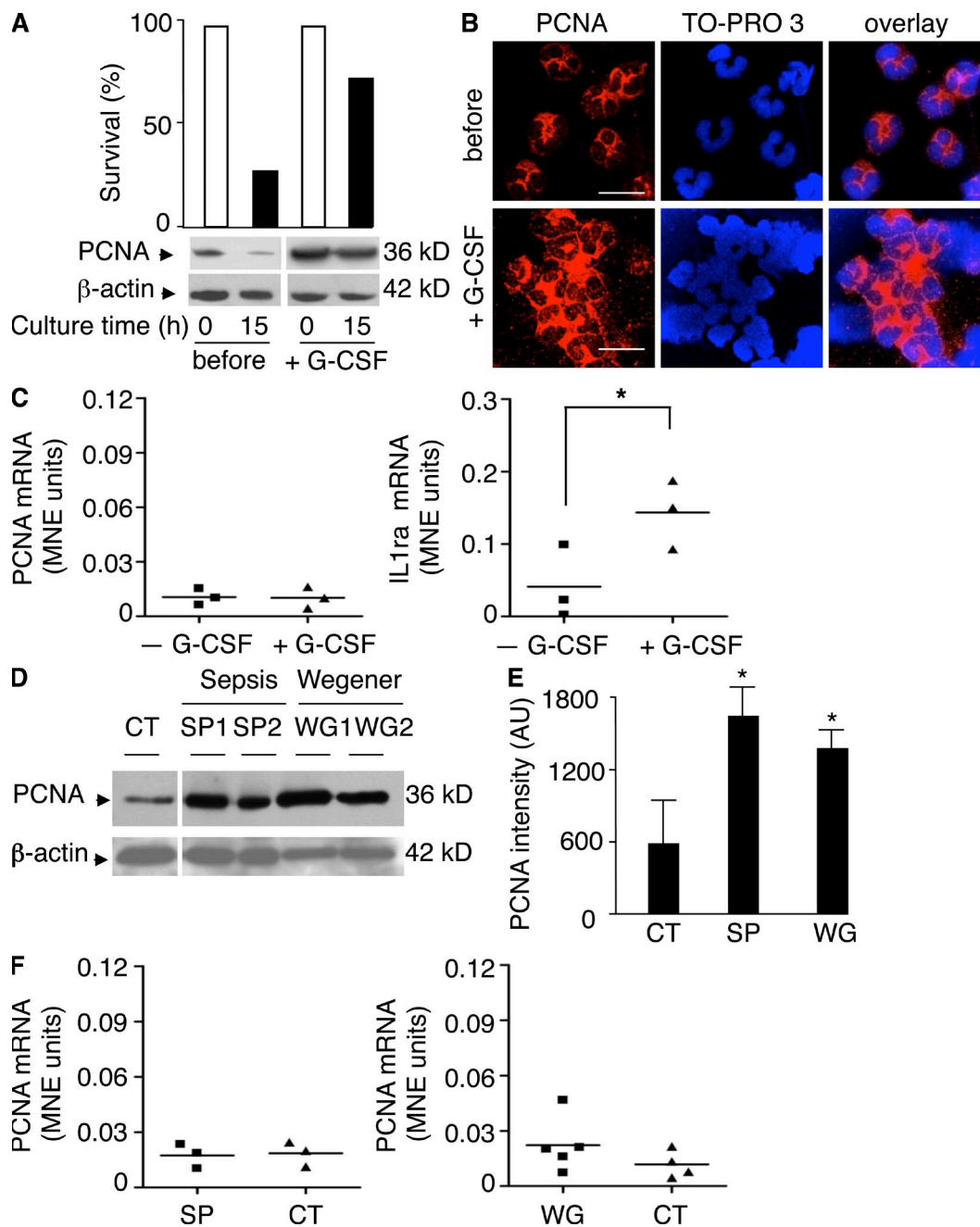


Figure 4. High PCNA protein levels in neutrophils isolated from G-CSF-treated donors and from patients with systemic inflammation.

(A) Neutrophils from a representative G-CSF-treated donor were analyzed either before or during (+G-CSF) cytokine exposure, with PCNA immunodetected in lysates from either freshly isolated (0) or 15-h cultured cells. The percentages of viable neutrophils after culture as in Fig. 3 B are shown in the histogram above the blot. (B) Immunofluorescence analysis by confocal microscopy of neutrophil PCNA detected with the Ab5 pAb. Neutrophils were isolated from a donor before or during (+G-CSF) *in vivo* G-CSF treatment. Data in A and B are from one G-CSF-treated donor, representative of four different donors. Bars, 10 μ m. (C) Total RNA was extracted from neutrophils isolated from healthy donors or G-CSF-treated patients and assayed in triplicates. PCNA and IL-1ra mRNA was normalized to β 2m expression and expressed as mean normalized expression (MNE) units. mRNA analysis was performed in three donors before and during G-CSF treatment (*, $P < 0.05$). (D) Representative Western blots showing neutrophil lysates prepared just after cell isolation from a control subject (CT) or from two patients with sepsis (SP1 and SP2) or Wegener's granulomatosis (WG1 and WG2). (E) Densitometric analysis of PCNA expression in healthy subjects (CT; $n = 7$), patients with sepsis (SP; $n = 5$), and patients with Wegener's granulomatosis (WG; $n = 8$). Data are expressed as arbitrary units (AU; means \pm SEM; *, $P < 0.05$; Student's *t* test). (F) Analysis of PCNA mRNA expression in neutrophils isolated from sepsis ($n = 3$) and Wegener's granulomatosis ($n = 5$) patients or healthy donors (CT; $n = 7$). (C and F) Horizontal bars represent the mean value.

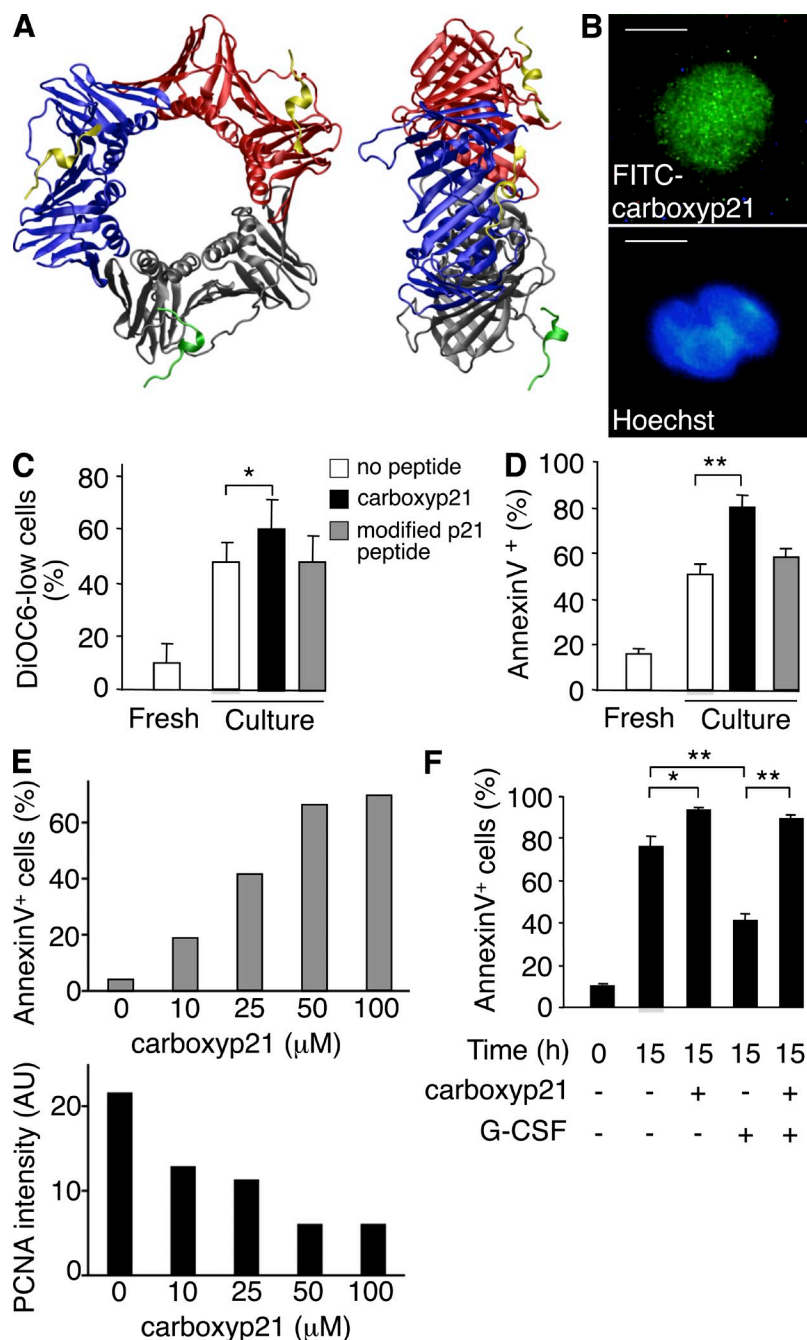


Figure 5. Potentiation of neutrophil apoptosis by carboxyp21, a PCNA-competing peptide. The synthetic carboxyp21 and a control modified p21 peptide (whose charged amino acids, identified as crucial for binding to PCNA, were modified to prevent its binding to PCNA) were incubated with neutrophils to evaluate their effect on apoptosis. (A) Structure of PCNA (blue, red, and gray are used to distinguish the three monomers) bound to two carboxyp21 (yellow) and one modified p21 peptide (green). The structure represented was obtained at the end of the MD simulation. The secondary structure elements are highlighted by a ribbon representation, and two different views are presented: from above the ring (left) and from the side (right). (B) Neutrophils were incubated with 50 μM FITC-conjugated carboxyp21 peptide for 1 h and then analyzed by fluorescence microscopy. The nucleus was visualized by Hoechst staining. The panels show one representative experiment of four. Bars, 5 μm . (C and D) Neutrophils were cultured for 6 h at 37°C alone or with 50 μM carboxyp21 or modified carboxyp21. The percentages of apoptotic neutrophils were assessed as depolarized mitochondria after DiOC₆ labeling (C) or phosphatidylserine externalization after annexin-V labeling (D). Basal apoptosis was assessed before incubation (fresh). Data are means \pm SEM of five independent experiments (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test). (E) Neutrophils were exposed to increasing carboxyp21 concentrations for 3 h, and apoptosis was measured by annexin-V labeling. The PCNA expression in the same samples was evaluated by Western blot analysis, and the bands were quantified by densitometric scanning. Data are from one representative experiment of four. AU, arbitrary unit. (F) Neutrophils were incubated with or without 1,000 U/ml G-CSF in the presence or absence of 50 μM carboxyp21 for 15 h before determining the percentage of apoptotic cells by annexin-V labeling. Data are means \pm SEM of five independent experiments (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test).

indicated by higher percentages of cells with depolarized mitochondria after DiOC₆ labeling, whereas no such activity was observed with the modified carboxyp21 peptide (Fig. 5 C). The proapoptotic effect of carboxyp21 was also demonstrated by a significantly increased percentage of cells with externalized phosphatidylserine after annexin-V labeling (Fig. 5 D). Note that carboxyp21-triggered apoptosis paralleled a dose-dependent decrease in PCNA expression (Fig. 5 E). This decreased PCNA cytoplasmic expression after treatment with the carboxyp21 was further confirmed using immunofluorescence labeling. Indeed, nuclear morphological

changes, typical of neutrophil apoptosis, were associated with a decreased cytoplasmic PCNA expression (Fig. S6). Moreover, carboxyp21 significantly inhibited G-CSF-induced neutrophil survival (Fig. 5 F), once again strongly suggesting that PCNA has a regulatory role in neutrophil survival. Based on these findings, we concluded that carboxyp21 triggers neutrophil apoptosis via a cell cycle-independent mechanism, probably by competing with cytoplasmic PCNA partners.

PCNA overexpression delays the apoptosis induction in differentiated PLB985, a cellular model of neutrophils

Neutrophils are short-lived, nonproliferating primary cells that are not suitable for transfection experiments. Therefore, promyelocytic cell lines, such as PLB985, which can differentiate into neutrophils upon dimethyl-formamide (DMF) treatment, constitute valuable cell models mimicking mature neutrophils (Pedruzzi et al., 2002). As observed in NB4 cells

(Fig. S2), PCNA displays a nuclear to cytoplasmic relocalization after granulocytic differentiation (Fig. S7 A). Accordingly, PLB985 cells were stably transfected with pcDNA3PCNA (PLB985-PCNA), as confirmed by Western blot analysis (Fig. 6, A and B). After DMF treatment, no differences were observed in the expression of CD11b (Fig. S7 B) or in nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity (Fig. S7 C). As expected, no superoxide was produced before differentiation, whereas significant chemiluminescence was triggered by PMA both in control and PCNA-overexpressing PLB985. Thus, PCNA overexpression did not affect granulocyte differentiation or responsiveness. As previously described, apoptosis could be triggered in PLB985 cells either via the death receptor pathway using TNF-related apoptosis-inducing ligand (TRAIL) or via the mitochondrial pathway using gliotoxin (Moriceau et al., 2009). After DMF-induced differentiation, PLB985 cells overexpressing PCNA were protected against TRAIL- or gliotoxin-induced apoptosis compared with differentiated PLB985 transfected with the control plasmid, as judged by DNA fragmentation (Fig. 6 C), caspase-3 activation (Fig. 6 D), or chromatin condensation (Fig. 6 E). In Fig. 6 D, the pro-caspase-3 band at 32 kD was still present in PCNA-transfected

PBL985, whereas it was hardly detectable in control PLB985. Likewise, the cleaved caspase-3 at 17 kD was more pronounced in controls as compared with PCNA-transfected cells showing both the 23- and 17-kD fragment. In addition, either mitochondrial depolarization (Fig. 6 F) or caspase-8 activity (Fig. 6 G) was significantly lower in PLB985-PCNA after constitutive TRAIL- or gliotoxin-induced apoptosis compared with controls.

Charge mutations in the PCNA interdomain-connecting loop, which is also the p21-interacting domain corresponding to residues 120–132 in the PCNA sequence, were then used to determine whether this PCNA domain was involved in its antiapoptotic activity. PLB985 cells expressing an interdomain-connecting loop-mutated PCNA were therefore generated. PCNA antiapoptotic activity assessed by mitochondrial depolarization was significantly reduced in PLB985 expressing the interdomain-connecting loop-mutated PCNA compared with PLB985-PCNA (Fig. 6 F). Likewise, PLB985 stably transfected with this mutant PCNA also exhibited increased caspase-8 activity compared with PLB985-PCNA after TRAIL- or gliotoxin-induced apoptosis (Fig. 6 G). In the absence of apoptotic stimulus, PCNA offered protection against differentiation-induced apoptosis, which might

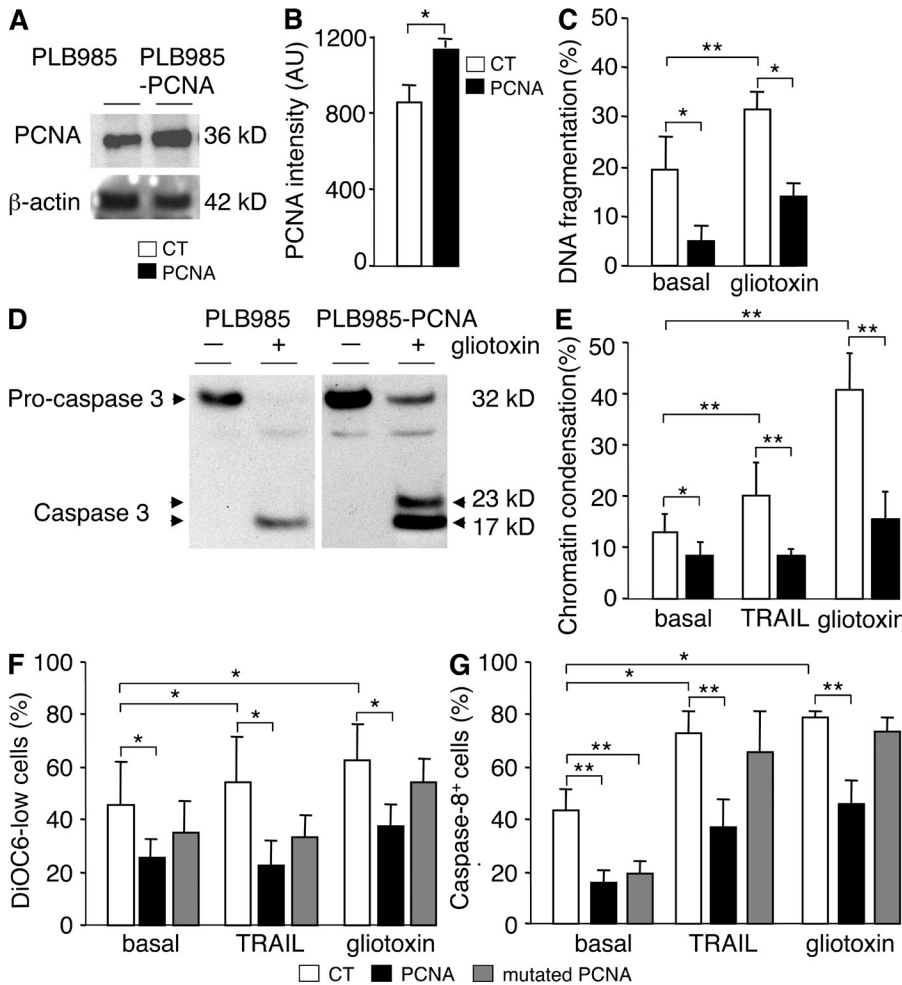


Figure 6. Stable PCNA transfection protects neutrophil-differentiated PLB985 myeloid cells from apoptosis. (A) Cytosolic PCNA expression in control (CT; pcDNA3-transfected cells) and pcDNA3PCNA-transfected PLB985 cells, as detected by Western blot analysis using the Ab5 pAbs and β -actin as loading control. (B) Quantification of PCNA expression after densitometry scanning and analysis by ImageJ software. Data are means \pm SEM of six independent experiments (*, $P < 0.05$; Student's *t* test). AU, arbitrary unit. (C–G) DMF-differentiated control or PCNA- or mutated PCNA-transfected PLB985 cells were incubated with or without 2 μ g/ml gliotoxin or 10 ng/ml TRAIL for 15 h to induce apoptosis. (C) Percentage of cells in the sub-G1 phase showing DNA fragmentation after propidium iodide labeling. (D) Caspase-3 expression by Western blot analysis in DMF-differentiated PLB985 cells after gliotoxin-induced apoptosis. Data are from one representative experiment that was performed four times, yielding identical results. (E) Percentage of cells showing chromatin condensation after Hoechst labeling. (F) Percentage of cells with mitochondrial depolarization after DiOC₆ labeling. (G) Percentage of cells with caspase-8 activation using a fluorescent IETD-based substrate. Data from C and E–G are means \pm SEM of at least four independent experiments (*, $P < 0.05$; **, $P < 0.01$; Student's *t* test).

reflect physiological neutrophil apoptosis. However, the mutated PCNA had roughly the same antiapoptotic effect, thus suggesting that in this case, PCNA domains other than that of the interdomain-connecting loop might also be important in the PCNA antiapoptotic effect. Collectively, these findings strongly suggest that some PCNA protein partners could bind to the PCNA interdomain-connecting loop to mediate the observed antiapoptotic effect. These observations also corroborate the results showing that carboxyp21 triggered neutrophil apoptosis by binding specifically to this PCNA site.

PCNA small interfering RNA (siRNA) sensitized DMF-differentiated PLB985 to apoptosis

In a final series of experiments, PLB985 cells were stably transfected with PCNA siRNA to knock down PCNA gene expression, as confirmed by Western blot analysis (Fig. 7, A and B). Notably, the siRNA procedure was performed on days 3 and 4 after DMF treatment, before analysis of the apoptosis rate on day 5. At this time point, all PCNA was cytoplasmic in DMF-differentiated PLB985 cells (Fig. S7 A). No effect of PCNA siRNA was observed on CD11b expression ($66.5 \pm 7.7\%$ vs. $60.9 \pm 6.3\%$ CD11b-expressing PLB985 cells for scrambled and PCNA siRNA, respectively), thus suggesting that it did not affect the differentiation process. PCNA siRNA but not scrambled siRNA slightly but significantly enhanced the constitutive apoptosis that PLB985 cells undergo after differentiation, as assessed by mitochondria depolarization (Fig. 7 C). Moreover, PCNA siRNA also dramatically potentiated the gliotoxin- or TRAIL-induced apoptosis of PLB985 cells (Fig. 7 C), further supporting the antiapoptotic activity of PCNA.

PCNA associates with procaspase-3, procaspase-8, procaspase-10, and procaspase-9 and interferes with activation of the latter

Finally, we performed co-IP experiments using neutrophil cytosols to identify potential cytosolic PCNA partners, which could contribute to its antiapoptotic activity. Mcl-1 is one of the most important Bcl-2 homologues expressed in neutrophils

(Derouet et al., 2004), the expression of which decreases during apoptosis. Because it was previously described as a PCNA partner in proliferating cells (Fujise et al., 2000), we investigated whether PCNA could bind Mcl-1. Although Mcl-1 could be successfully immunoprecipitated, no physical association was detected between PCNA and Mcl-1 or other Bcl-2 homologues, e.g., the proapoptotic proteins Bax or Bid (unpublished data). In contrast, using the same PCNA co-IP protocol, procaspase-3 (Fig. 8 A), procaspase-8 (Fig. 8 B), procaspase-9 (Fig. 8 C), and procaspase-10 (Fig. 8 D) could be detected using specific mAbs. In addition, the interaction was confirmed in the reciprocal IP experiments using anti-procaspase IP and anti-PCNA for the Western blot analysis (Fig. S8, A–D). Moreover, results from double immunolabeling with anti-PCNA and anti-procaspase performed on neutrophils strongly suggest that PCNA colocalizes with procaspase-3, procaspase-8, procaspase-9, and procaspase-10 (Fig. S8, A–D, right). Notably, the amount of procaspase-9 coimmunoprecipitated by an anti-PCNA mAb decreased when neutrophil cytosolic fractions were preincubated with the p21 peptide compared with the mutated p21 peptide (Fig. 8 E). The latter finding indicates that the p21 peptide displaces the procaspase-9–PCNA interaction, which in turn suggests that the interconnecting loop domain is involved in the interaction between PCNA and procaspase-9. To further validate such a hypothesis, caspase-9 activation was investigated in PLB985 cells overexpressing PCNA or the interdomain-connecting loop-deficient PCNA mutant. After 5 d of DMF exposure, the caspase-9 activity was elevated in contrast to undifferentiated cells. This caspase-9 activity corresponds to physiological apoptosis of aged neutrophils. Notably, this differentiation-induced caspase-9 activation was significantly lower in PLB985-PCNA than in control PLB985 cells, thus confirming the antiapoptotic effect of PCNA. In contrast, no protective effect against differentiation-induced apoptosis was observed in PLB985 cells expressing the mutant PCNA (Fig. 8 F). Collectively, our findings strongly suggest that the interdomain-connecting loop is involved in the PCNA–procaspase-9 interaction. Therefore, we hypothesized that the cytosolic PCNA–procaspase

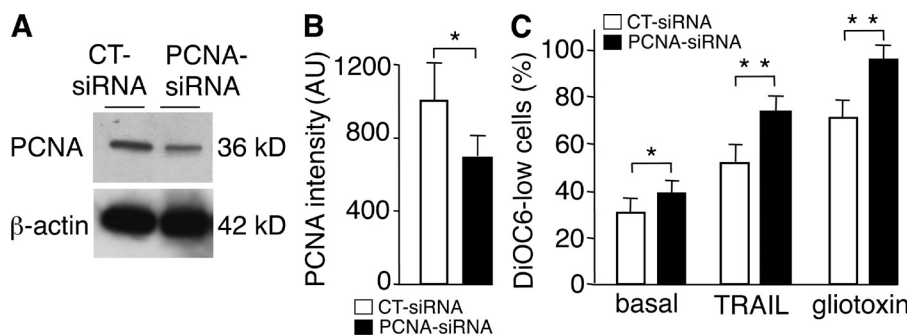


Figure 7. Knocking down PCNA expression by siRNA sensitizes DMF-differentiated PLB985 cells to apoptosis. DMF-differentiated PLB985 cells were transfected twice with control (CT) siRNA or with PCNA siRNA on days 3 and 4 after DMF. (A) Cytosolic PCNA expression was evaluated by Western blot analysis using the Ab5 pAb (β -actin expression was used as loading control). (B) Quantification of PCNA expression by densitometric scanning using ImageJ software. Data are means \pm SEM of six independent experiments (*, $P < 0.05$; Student's t test). (C) Effect of

PCNA siRNA on the percentage of apoptotic PLB985 cells as measured by mitochondrial depolarization after DiOC₆ labeling. PLB985 cells were incubated with 2 μ g/ml gliotoxin ($n = 6$), 10 ng/ml TRAIL ($n = 5$), or in basal conditions ($n = 10$) for 15 h. Data are means \pm SEM of n independent experiments (*, $P < 0.05$; **, $P < 0.01$; Student's t test).

interaction might prevent their activation. This was tested by evaluating procaspase-9 cleavage in an *in vitro* assay, using neutrophil cytosol containing the apoptosome components, i.e., procaspase-9 and Apaf-1 (apoptotic protease-activating factor 1), as previously described (McStay et al., 2008). The addition of exogenous purified cytochrome *c* and dATP triggered procaspase-9 cleavage (37 kD) in a time-dependent manner, as indicated by Western blot analysis of caspase-9 (Fig. 9 A). However, when purified recombinant PCNA was added to the mixture, procaspase-9 cleavage was delayed, with maintained levels of the 46-kD fragment of procaspase-9 but reduced 37-kD fragment detection, thus demonstrating that purified PCNA impairs procaspase-9 activation. Ovalbumin tested at the same concentration did not affect procaspase-9 activation and thus served as a control (unpublished data). Notably, PCNA was also cleaved in parallel with the activation of caspase-9 (Fig. 9 B), thus explaining why the inhibitory effect on procaspase-9 activation by PCNA decreases with the time. Finally, cytosols from neutrophils isolated from G-CSF-treated donors that contained high PCNA

levels (Fig. 9 C, right) showed an impaired capacity to activate procaspase-9 *in vitro* as compared with cytosols from control neutrophils (Fig. 9 C, left).

DISCUSSION

Regulation of the neutrophil lifespan provides a fine balance between their function as host defense effector cells and a safe turnover of these potentially harmful cells. Neutrophil apoptosis is essential for the resolution of inflammation, and delayed apoptosis and compromised neutrophil clearance are, in fact, hallmarks of chronic inflammation (Simon, 2003; Fox et al., 2010). Therefore, the neutrophil has a carefully orchestrated lifespan, which requires the intervention of a pre-established intracellular clock with successive steps and key control points, which are still undefined.

In this study, we have discovered that PCNA, a well-known nuclear protein involved in DNA replication and repair and considered one of the central molecules responsible for the life or death decision in proliferating cells only (Moldovan et al., 2007), displays all of the features of a master

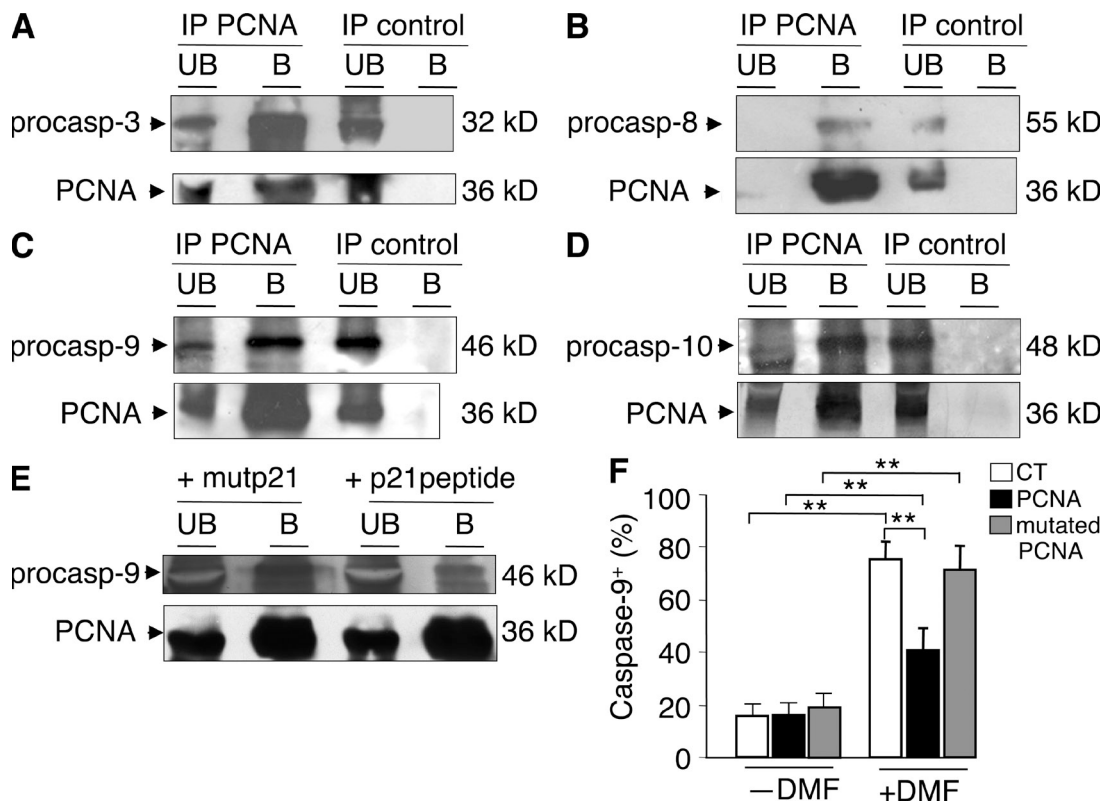


Figure 8. Co-IP experiments identify procaspase-3, procaspase-8, procaspase-9, and procaspase-10 as PCNA partners. Co-IP experiments were performed using neutrophil cytosol. Unbound material (UB) and bound (B) immunoprecipitated proteins were analyzed by Western blot analysis. (A–D) In IP, PCNA pAb (IP PCNA) or empty beads (IP control) were used, whereas in Western blot analysis, PC10 anti-PCNA was used (to ascertain the presence of PCNA) together with one of the following: anti-procaspase-3 mAb (A), anti-procaspase-8 mAb (B), anti-procaspase-9 mAb (C), or anti-procaspase-10 mAb (D). (E) Co-IP experiments performed as in C using PCNA containing neutrophil cytosol incubated with 100 μM carboxyp21 or the mutated p21 peptide (used as a control). Both unbound material and bound immunoprecipitated proteins were analyzed by Western blot analysis using anti-procaspase-9 and anti-PCNA (PC10). A–E show data from representative experiments that were performed at least four times, yielding identical results. (F) Caspase-9 activity in PLB985 cells overexpressing wild-type or mutated PCNA compared with controls (CT). CD11b⁺-caspase-9⁺ cells were measured by flow cytometry before and after DMF-induced differentiation. Data are means ± SEM of four independent experiments (**, P < 0.01; Student's *t* test).

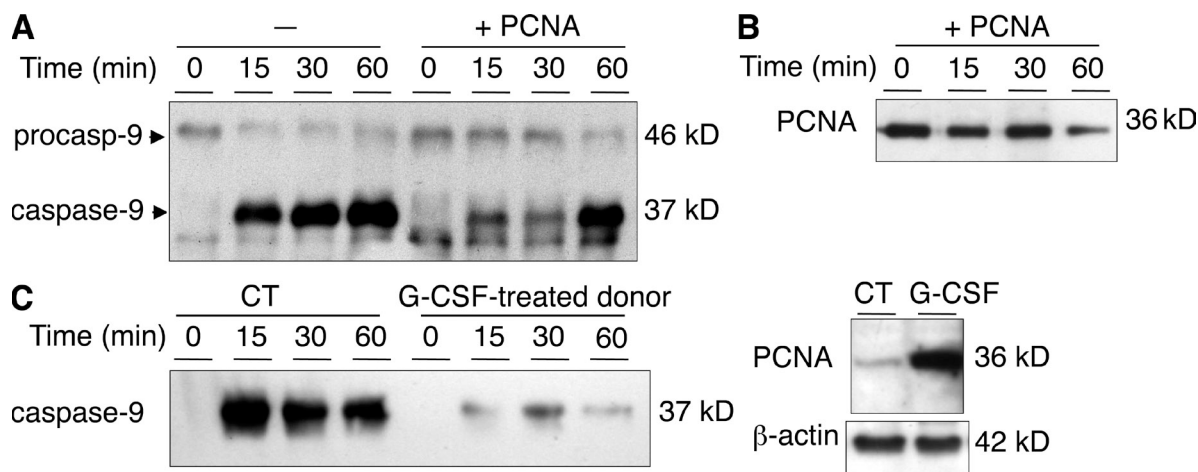


Figure 9. PCNA impaired pro-caspase-9 activation in vitro. (A) Kinetics of immunodetection of cleaved caspase-9 after an in vitro procaspase-9 activation assay performed using neutrophil cytosol, 50 μ M cytochrome c, and 1 mM ATP, without and with 50 μ M recombinant PCNA. (B) Kinetics of PCNA immunodetection using PCNA pAb performed in the same in vitro assay as in A in the absence of exogenous recombinant PCNA to detect only endogenous PCNA. (C) Kinetics of cleaved caspase-9 immunodetection as in A in cytosol of neutrophils isolated from G-CSF-treated donors or controls (CT). PCNA Western blot analysis confirmed higher PCNA levels in the G-CSF-treated donors (right). A–C show data from representative experiments that were performed at least four times, yielding identical results.

regulator of neutrophil survival. Indeed, we observed that although circulating mature neutrophils contain elevated amounts of PCNA, their PCNA contents change as a function of their viability status. In neutrophils undergoing apoptosis, PCNA was subjected to proteasome-mediated degradation, regardless of whether the trigger signaling cascade passed through the extrinsic (death receptors) or the intrinsic pathway (mitochondria). Although the proteasome seems to play a pivotal role in PCNA degradation, other proteolytic pathways might be involved to modulate PCNA levels during apoptosis.

In contrast, PCNA levels are stably maintained by factors that prolong neutrophil survival, e.g., G-CSF. Notably, modulation of PCNA contents, at least in human neutrophils, appears to involve only posttranscriptional events because variations in PCNA mRNA expression were not detected during in vitro or in vivo G-CSF treatment. In addition, neutrophils isolated from G-CSF-treated donors exhibited both prolonged survival, as previously described (Basu et al., 2002), and also high PCNA contents, in keeping with the major role of PCNA in neutrophil survival. PCNA gene induction has not been reported to occur in neutrophils isolated from donors treated with G-CSF in vivo (Drewniak et al., 2009), which is consistent with our findings. Furthermore, PCNA levels in neutrophils isolated from patients with sepsis or necrotizing vasculitis were much higher than those isolated from healthy individuals, and no PCNA mRNA up-regulation was observed. These findings support the notion that neutrophil PCNA levels vary during systemic inflammation related (sepsis) or not (Wegener's granulomatosis) to an infection. Whether or not cytoplasmic PCNA is absolutely required for neutrophil survival cannot, at present, be explored because of the lack of viable PCNA knockout animals

(Roa et al., 2008). Similarly, it is currently unknown whether or not cytoplasmic PCNA is associated with the survival of differentiated cells from nonmyeloid origin.

According to numerous studies, PCNA functions in the nucleus as a remarkable adaptor molecule that binds to numerous proteins (for review see Warbrick, 2000). Indeed, several authors have established that trimeric PCNA plays a central role as a moving platform along the DNA molecule (for review see Maga and Hubscher, 2003), which acts as a communication center for a variety of cell nuclear processes, such as DNA replication, nucleotide excision repair, postreplication mismatch repair and apoptosis. Therefore, it is clear that one key to understanding the molecular mechanisms whereby PCNA exerts its antiapoptotic activity in neutrophils is to identify its partners. However, in noncycling cells such as neutrophils, PCNA antiapoptotic activity should theoretically proceed via mechanisms not involving any nuclear function. Indeed, one salient feature revealed in our study was the exclusive localization of PCNA in the neutrophil cytosol, and we documented its nucleus to cytosol relocalization in the later stages of neutrophil maturation. The underlying mechanisms, either in terms of nucleocytoplasmic transport or sequestration mechanisms to retain PCNA within the cytoplasm compartment, remain to be deciphered. Notably, we have clearly demonstrated that in primary neutrophils, PCNA is constitutively associated with procaspase-3, procaspase-8, procaspase-9, and procaspase-10, presumably sequestering them within the cytosol to prevent their activation. How such interactions occur at the molecular level remains to be elucidated, but it can be foreseen that posttranslational modifications of PCNA, e.g., acetylation, phosphorylation (Naryzhny and Lee, 2004), or sumoylation (Moldovan et al., 2007), already described in other cell types could regulate PCNA association with its neutrophil

partners. Notably, neutrophils contain extremely low amounts of cytochrome *c* but exhibit high Apaf-1 levels and very high caspase-9 activity during physiological apoptosis (Murphy et al., 2003). Thus, procaspase-9 sequestration by PCNA seems to be a very efficient way to inhibit apoptosis. Accordingly, *in vitro* procaspase-9 activation was delayed either by adding exogenous recombinant PCNA or in neutrophil cytosol from G-CSF-treated donors containing high PCNA levels. However, we cannot exclude that association with other cytoplasmic proteins might mediate the antiapoptotic effect of PCNA in neutrophils.

In proliferating cells, PCNA is strongly controlled by the tumor suppressor protein p21/waf1, which was initially identified as a potent CDK inhibitor (Waga et al., 1994). Structural experiments indicated that p21/waf1 directly blocks the surface region required for polymerase binding (Waga et al., 1994). Biochemical experiments broadened that concept by showing that p21/waf1 is an effective competitor for other PCNA partners (Warbrick, 2006). Indeed, synthetic peptides carrying the consensus sequence for binding to the PCNA interdomain-connecting loop, such as the carboxyp21 peptide (residues 120–132 on the PCNA sequence) used in our study, stop progression through the cell cycle and induce apoptosis when transfected into proliferating cells (Dong et al., 2003). We have shown that carboxyp21 (a) directly triggers neutrophil apoptosis and concomitant PCNA degradation and (b) impairs the capacity of G-CSF to prolong neutrophil survival, thus clearly proving that PCNA is absolutely essential for the G-CSF antiapoptotic effect in neutrophils. Previous *in vitro* studies have shown that the C-terminal region of p21, which contains the PCNA-interacting domain, can competitively block the reassociation of the polymerase complex with PCNA in proliferating cells (Mattock et al., 2001b; Warbrick, 2006). However, the carboxyp21 peptide also contains 4 aa at the C terminus that function as a site for CDK association, thus suggesting that its effect might be caused by CDK inhibition (Mattock et al., 2001a). However, in this study, we show that a p21 peptide mutated at the PCNA-interacting domain, with an intact CDK-interacting domain at the C terminus, does not display the same degree of apoptosis potentiation observed with the wild-type p21 peptide. Therefore, even if the p21 peptide might theoretically promote apoptosis via CDK inhibition at higher concentrations, we concluded that in neutrophils, the effect of the p21 peptide, at least at the concentrations that we have tested, is mainly caused by its binding to PCNA.

Interestingly, inhibition of CDK by the synthetic inhibitor roscovitine triggers neutrophil apoptosis and, consequentially, promotes inflammation resolution *in vivo* (Rossi et al., 2006). Thus, these authors have provided evidence that CDK activities, so far restricted to cell cycle regulation, can control apoptosis in nonproliferating cells like neutrophils. Roscovitine has also been shown to improve the resolution of the inflammation after pneumococcal infection and accelerated recovery (Koedel et al., 2009) and to reverse the late apoptosis observed in neutrophils from cystic fibrosis patients (Moriceau et al., 2010). Furthermore, although several cell cycle proteins are down-regulated during neutrophil differentiation, mature neutrophils expressed

high levels of CDK2 and the CDK inhibitor p27 (Klausen et al., 2004). Indeed, during inflammation, neutrophils are able to up-regulate survivin, a member of the IAP (inhibitor of apoptosis protein) family, which acts as a link between apoptosis and control of mitogenic progression in proliferating cells (Altzner et al., 2004). Thus, it becomes increasingly apparent that cell cycle regulatory proteins other than PCNA might be implicated in controlling neutrophil lifespan.

Most studies on neutrophils, viewed as potential cellular targets in inflammation, attempted to block their migration and influx. However, another aspect of neutrophil biology that could be targeted, albeit often underestimated, is the modulation of neutrophil survival (Hallett et al., 2008). In this regard, an in-depth understanding of the specific and highly regulated molecular mechanisms controlling neutrophil survival/death is required to intervene efficiently and effectively. The results reported in this paper are notable, not only because they demonstrate that PCNA acts as a cytoplasmic platform pulling the strings of neutrophil survival, but also because they reveal new ways of thinking about neutrophils and their intracellular pathways in the context of inflammation or neutropenia.

MATERIALS AND METHODS

Blood and BM cell isolation, differentiation of CD34⁺ precursors, and cell culture. Blood or BM donors gave their written informed consent to participate in this study, which was approved by the Institut National de la Santé et de la Recherche Médicale Institutional Review Board and the Ethics Committee of Hôpital Necker-Enfants Malades and Hôpital Cochin (Paris, France). Human neutrophils from healthy (Etablissement Français du Sang, Paris, France) or G-CSF-treated healthy donors (10 µg/kg for 5 d to induce hematopoietic stem cell mobilization; Service de Biothérapie, Hôpital Necker-Enfants Malades) were isolated from EDTA-anticoagulated blood, using density-gradient centrifugation through Polymorphprep (Nycomed). Neutrophils were isolated from the blood of vasculitis and septic patients hospitalized in the Service de Médecine Interne, Hôpital Cochin. Patients with sepsis ($n = 5$) had documented gram-positive or -negative infection, body temperature $>38.5^{\circ}\text{C}$, and C-reactive protein >100 mg/ml. Patients with antineutrophil cytoplasmic antibody-associated vasculitis ($n = 8$) fulfilled the criteria for Wegener's granulomatosis (Leavitt et al., 1990). They had anti-proteinase 3 antibodies and active disease, as assessed by a Birmingham vasculitis activity score of ≥ 3 (Luqmani et al., 1994). Neutrophil subcellular fractionation was performed to obtain separate cytosolic, granular, and nuclear fractions, as previously described (Borregaard et al., 1983). BM cell separation was performed by Percoll gradient centrifugation as previously described (Cowland and Borregaard, 1999). Monocytes and lymphocytes were isolated on Ficoll gradient as previously described (Kantari et al., 2007). Differentiation of CD34⁺ cells into granulocytes was induced as previously described (Hino et al., 2000), with some minor modifications. In brief, CD34⁺ cells were isolated from cord blood and then cultured with 10 ng/ml stem cell factor, 10 ng/ml IL-3, and 100 ng/ml IL-6 for 7 d. CD34⁻ cells were isolated and then incubated with 10 ng/ml G-CSF, 100 ng/ml stem cell factor, and 10 ng/ml IL-3 for 13 d to promote granulocyte differentiation. MGG staining was used at different times to monitor differentiation. Peripheral blood neutrophils and mature neutrophils from femur BM were isolated using a Percoll density gradient as previously described (Mócsai et al., 2002).

HeLa, PLB985, and NB4 promyelocytic cell lines were cultured in RPMI supplemented with 10% fetal calf serum. NB4 cells were induced to differentiate with 1 µM ATRA (Sigma-Aldrich) for 5 d, and granulocyte differentiation was validated by CD11b expression and by morphological

analysis after MGG staining. PLB985 cell granulocyte differentiation was induced by exposure to 0.5% DMF for 5 d and validated as for NB4. Functional analysis of NADPH-oxidase activity was performed with lucigenin-amplified chemiluminescence in a single-photon luminometer (AutoLumat LB953; Berthold Technologies). The chemiluminogenic substrate, lucigenin (10,10-dimethyl-9,9-biacridium dinitrate), was used to selectively measure NADPH-oxidase-dependent extracellular superoxide anion formation. In brief, 100 μ l containing 5×10^5 cells, neutrophils, or PLB985 cells was placed in polystyrene tubes containing 100 μ l of 0.2 mM lucigenin and 50 μ l of stimulus, either HBSS or PMA (16 μ M final concentration). The luminescence was measured in duplicate and expressed as integrated total counts in 40 min.

Immunofluorescence labeling and confocal microscopy analysis.

Immunolabeling of neutrophils, HeLa, CD34⁺, PLB985, or NB4 cells to study PCNA subcellular localization was performed as previously described (Kantari et al., 2007). Cells were fixed in PBS containing 3.7% formaldehyde (Sigma-Aldrich) for 20 min on ice and permeabilized with 0.25% Triton X-100 for 5 min at room temperature, followed by ice-cold methanol for 10 min, incubated with rabbit pAb diluted 1:25 (Ab5; EMD) for 45 min, followed by biotinylated rabbit IgG diluted 1:100 (Dako) for 30 min, and then by streptavidin-coupled Alexa Fluor 555 diluted 1:200 (Invitrogen) for 30 min. The nuclei were stained with TO-PRO 3 iodide (10 μ M solution; Invitrogen) or with 5 μ g/ml Hoechst (Sigma-Aldrich) for 30 min. For colocalization with PCNA, anti-CD35 (mouse IgG1; clone J3D3; diluted 1:50; Beckman Coulter) or anti-MPO (mouse IgG2a; clone MPO-7.17; dilution 1:200; PeliCluster; Red Cross; diluted 1:100) or anti-procaspase-8 (EMD), anti-procaspase-3, or anti-procaspase-9 or anti-procaspase-10 (Santa Cruz Biotechnology, Inc.) mAbs were used as primary antibodies, followed by an Alexa Fluor 488-conjugated anti-mouse mAb (diluted 1:100; Invitrogen). Slides were analyzed by confocal microscopy with a confocal scanning laser microscope (LSM-5 version 3.2 SP2; Carl Zeiss, Inc.) equipped with an argon laser and helium-neon lasers using a Plan-Apochromat 63 \times NA 1.40 oil immersion objective lens at room temperature. Green fluorescence was observed with a 505–530-nm band-pass emission filter under 488-nm laser illumination, and red fluorescence was observed with a 488–560-nm-long-pass emission filter under 543-nm laser illumination. Pinhole diameters were set to get 0.8- μ m-thick optical slices, and images were collected every 0.4 μ m along the z axis. Imaging of Alexa Fluor 488, Alexa Fluor 555, Hoechst, and TO-PRO 3 iodide fluorescence was obtained using the multitrack mode. The fluorescence was analyzed using ImageJ software version 1.42d (National Institutes of Health).

Analysis of apoptosis. Neutrophil apoptosis was triggered by incubating neutrophils at 37°C alone (constitutive apoptosis) or with 10 ng/ml anti-Fas mAbs (Beckman Coulter) or 0.1 μ g/ml gliotoxin (Sigma-Aldrich; Ward et al., 1999) for the indicated times. Neutrophil apoptosis was evaluated using phosphatidylserine externalization after annexin-V and 7-AAD labeling (Kantari et al., 2007), mitochondrial depolarization after DiOC₆ labeling (Moriceau et al., 2009), and by caspase-3 and caspase-8 activities measured by spectrophotometry (BioVision) in neutrophil lysates. In DMF-differentiated PLB985 cells, apoptosis was triggered by 2 μ g/ml gliotoxin or by 10 ng/ml recombinant TRAIL (R&D Systems; Yin et al., 2005), and caspase-8 and caspase-9 activities were determined after gating on CD11b⁺ cells using the Caspase-Glow assay (Promega). Apoptosis-induced chromatin condensation and DNA fragmentation were assessed after DNA staining with 5 μ g/ml of the blue fluorescent dye Hoechst and propidium-iodide staining, respectively (Goepel et al., 2004). Carboxyp21 (KRRQTSMTDFYHSKRRLIF-SRYIRS) corresponding to the p21/waf1 C terminus sequence (residues 141–160) and containing a RYIRS C-terminal extension to facilitate its cell entry, as previously demonstrated (Dong et al., 2003), was synthesized and purified (GeneCust). A modified peptide, in which the charged amino acids responsible for its binding to PCNA have been changed according to dynamic simulation experiments (KRRQTGETDFDHAKAALIFSRYSIRS), served as control. An FITC-conjugated carboxyp21 peptide was used to

study its subcellular localization. Neutrophils were incubated for 1 h with the fluorescent peptide (at the indicated concentrations) and were induced to adhere on poly-L-lysine-precoated coverslips as previously described (Kantari et al., 2007). Fluorescence microscopy was performed with a microscope (DMRD; Leica) and a camera (model DP11P; Olympus). The effects of PS-341 (LC Laboratories) and MG132 were tested on neutrophil apoptosis and PCNA expression.

Real-time RT-PCR and primary transcript real-time RT-PCR.

Real-time RT-PCR and primary transcript real-time RT-PCR were performed as described by Tamassia et al. (2008) using gene-specific primer pairs (Invitrogen) available in the public database RTPPrimerDB under the following entry codes: human PCNA (7839), PT-PCNA (7840), BLYS/TNFSF13B (7841), PT-BLYS (7842), IL-1ra (3544), GAPDH (3539), PPIB (7786), GNB2L1 (8211), and β 2m (3534). The reaction conditions were identical for all primer sets, as follows: 50°C for 2 min, 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. β 2m was chosen as a normalizing gene because of its stable expression levels in leukocytes. The data were analyzed with QGene software and are expressed as mean expression units after β 2m normalization. The same RNA samples were processed in the absence of RT and served as controls for genomic DNA contamination. Northern blot analysis was conducted as previously described (Rossato et al., 2007).

Plasmid vector construction and recombinant PCNA expression.

Recombinant PCNA was produced in *Escherichia coli* using the plasmid PETPCNA containing the human PCNA cDNA (gift from B. Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and purified as previously described (Waga et al., 1994). To obtain PCNA overexpression in PLB985 cells, PCNA cDNA was subcloned into the expression vector pCDNA3/neo (Invitrogen) between the HindII and NotI restriction sites. Site-directed mutagenesis was performed using the QuikChange method (Agilent Technologies) to obtain PCNA mutated on charged amino acids within the known p21-interacting sequence located at the interdomain-connecting loop (PCNA-D120A-D122A-E124A-E130A-E132A). All cDNA sequences were confirmed by direct sequencing. PLB985 cells were transfected using the Amaxa system (Lonza), according to the manufacturer's instructions. In brief, 2×10^6 cells were resuspended in 100 μ l of cell line solution with 1 μ g of plasmid (control pcDNA3-neomycin, pcDNA3-PCNA, or pcDNA3-mutated PCNA). The cells were then electroporated and transferred to culture plates. Transfected cells were cloned and selected based on their resistance to 1 mg/ml neomycin.

For PCNA siRNA experiments, PLB985 cells were treated with DMF to induce granulocytic differentiation and transfected with 1 μ M siRNA (Applied Biosystems) twice at a 24-h interval (day 3 and day 4 after DMF treatment) using the Amaxa system as described in the previous paragraph. FITC-conjugated control siRNA, used to detect intracellular siRNA by flow cytometry and to monitor transfection efficiency, showed that the fluorescent siRNA was taken up in >90% of the PLB985 cells. Constitutive, gliotoxin- and TRAIL-induced apoptosis was evaluated by mitochondria depolarization after DiOC₆ labeling after gating on CD11b⁺ cells after control scrambled siRNA and PCNA siRNA transfection. PLB985 cells transfected with specific PCNA siRNA or control siRNA were also lysed to analyze PCNA expression by Western blotting using the rabbit anti-PCNA pAb.

Western blot analysis and co-IP experiments. Neutrophils were sonicated, and cytosolic proteins were analyzed using either the PC10 mAb or the Ab5 pAb anti-PCNA, according to standard immunoblot procedures (Moriceau et al., 2009). Co-IP experiments were performed using neutrophil cytosols obtained by cavitation (Borregaard et al., 1983). 500 μ g cytosol was mixed with 50 μ l protein G and Ab5 pAb and incubated for 30 min at 4°C under shaking. The sample was loaded onto a column containing Sepharose-coated magnetic beads (Miltenyi Biotec) and washed with a stringent washing buffer (50 mM Tris, 500 mM NaCl, 1% NP-40, and

0.5% Triton X-100). The column was washed twice with this buffer, twice with a similar buffer containing 300 mM NaCl and, finally, five times with a hyposaline buffer (20 mM Tris, pH 7.5). The sample was eluted from the column with 30 μ l of 5 \times sample buffer and analyzed by Western blotting using different mAbs to potential partners. PCNA was detected on the Western blot with the PC10 mAb, and procaspases were detected with anti-procaspase-8 mAb, mouse anti-procaspase-10, anti-procaspase-9, or anti-procaspase-3 mAbs, followed by horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:5,000; Nordic Immunology), using the SuperSignal West Pico detection kit (Thermo Fisher Scientific). For the reciprocal co-IPs, procaspase-3, procaspase-8, procaspase-9, and procaspase-10 were immunoprecipitated with their corresponding mAbs, and PCNA was detected by Western blotting using the rabbit pAb anti-PCNA (Ab5). An antiubiquitin mAb (Santa Cruz Biotechnology, Inc.) was used for immunoprecipitating ubiquitinated proteins, and detection of PCNA was performed using the rabbit pAb anti-PCNA (Ab5). Mouse anti-lamin B (EMD) mAb as well as rabbit anti- β -actin (EMD) and antielastase (EMD) pAbs were also used.

In silico design of the modified p21 peptide using dynamic simulations.

To design a modified p21 peptide that does not bind to PCNA and could be used as a control peptide, we first inventoried the PCNA-p21 interactions at the atomic level of detail based on the trajectory obtained from 20-ns-long MD simulations of PCNA complexed with three carboxyp21 peptides (RQTSMTDFYHSKR). We then identified the p21 peptide amino acids forming strong interactions with PCNA and subsequently ran new MD simulations of PCNA complexed with the modified p21 peptides (RQTGETDFDHAKA). The initial set of coordinates used for the MD simulations was determined from the x-ray structure of PCNA complexed to the peptide SAVLQKKITDYFHPKK (Protein Data Bank ID 1VYJ). Three simulations of the PCNA trimer were run as follows: (1) MD1, PCNA trimer with a p21 peptide (RQTSMTDFYHSKR) interacting with each of the three monomers (labeled A, C, and E in the x-ray file); (2) MD2, PCNA with three modified peptides (RQTGETDFDHAKA); and (3) MD3, PCNA with two p21 peptides (on PCNA monomers A and C) and one modified peptide (on PCNA monomer E). The simulation was performed as previously described (Hajjar et al., 2006), except that the production runs lasted for 20 ns. The systems contain \sim 100,000 atoms, including explicit water molecules, proteins, and counter-ions. Analysis of the trajectories obtained, performed as previously described (Hajjar et al., 2006), revealed the loss of strategic interactions between PCNA and the modified p21 peptide, providing evidence that it could barely bind PCNA (Fig. 5 A). Interactions between the carboxyp21 and PCNA remained stable during the 20 ns of the simulations.

Statistical analysis. Statistical analysis was performed using the StatView (SAS Institute Inc.) software package. Comparisons were made using the Student's *t* test. Differences were considered significant when $P < 0.05$. Similar results were obtained when using the nonparametric Mann-Whitney test.

Online supplemental material. Fig. S1 shows the specificity of the anti-PCNA antibodies (Ab5 and PC10) and that PCNA has a nuclear and a cytosolic localization in circulating lymphocytes and monocytes. Fig. S2 shows the nuclear to cytosolic PCNA relocation during ATRA-induced granulocytic differentiation in the NB4 cell line. Fig. S3 shows apoptosis assessment using annexin-V-FITC and 7-AAD labeling and using DiOC₆ staining. Fig. S4 shows the effect of proteasome and cathepsin inhibitors on PCNA expression. Fig. S5 shows that mouse neutrophils express cytosolic PCNA that decreases during apoptosis. Fig. S6 shows that cytosolic PCNA can be detected by immunofluorescence in fresh but not in apoptotic neutrophils. Fig. S7 shows that the nuclear to cytosolic PCNA relocation also occurs during DMF-induced granulocytic differentiation in PLB985 cells. Fig. S8 shows the reverse IP between PCNA and procaspase-3, procaspase-8, procaspase-9, and procaspase-10, as well as colocalization studies between PCNA and these procaspases using immunofluorescence. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092241/DC1>.

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