*The Journal of* **Immunology**

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# Proteinase 3, the Autoantigen in Granulomatosis with Polyangiitis, Associates with Calreticulin on Apoptotic Neutrophils, Impairs Macrophage Phagocytosis, and Promotes Inflammation

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Proteinase 3 (PR3) is the target of anti-neutrophil cytoplasm Abs in granulomatosis with polyangiitis, a form of systemic vasculitis. Upon neutrophil apoptosis, PR3 is coexternalized with phosphatidylserine and impaired macrophage phagocytosis. Calreticulin (CRT), a protein involved in apoptotic cell recognition, was found to be a new PR3 partner coexpressed with PR3 on the neutrophil plasma membrane during apoptosis, but not after degranulation. The association between PR3 and CRT was demonstrated in neutrophils by confocal microscopy and coimmunoprecipitation. Evidence for a direct interaction between PR3 and the globular domain of CRT, but not with its P domain, was provided by surface plasmon resonance spectroscopy. Phagocytosis of apoptotic neutrophils from healthy donors was decreased after blocking lipoprotein receptor-related protein (LRP), a CRT receptor on macrophages. In contrast, neutrophils from patients with granulomatosis with polyangiitis expressing high membrane PR3 levels showed a lower rate of phagocytosis than those from healthy controls not affected by anti-LRP, suggesting that the LRP-CRT pathway was disturbed by PR3-CRT association. Moreover, phagocytosis of apoptotic PR3-expressing cells potentiated proinflammatory cytokine in vitro by human monocyte-derived macrophages and in vivo by resident murine peritoneal macrophages, and diverted the anti-inflammatory response triggered by the phagocytosis of apoptotic cells after LPS challenge in thioglycolate-elicited murine macrophages. Therefore, membrane PR3 expressed on apoptotic neutrophils might amplify inflammation and promote autoimmunity by affecting the anti-inflammatory "reprogramming" of macrophages. *The Journal of Immunology*, 2012, 189: 2574–2583.

G ranulomatosis with polyangiitis (GPA), formerly called Wegener's granulomatosis, is a systemic disease characterized by granulomatous inflammation of the upper and lower respiratory tract, glomerulonephritis, and necrotizing vasculitis (1). GPA is associated with anti-neutrophil cytoplasmic autoantibodies (ANCAs) directed against proteinase 3 (PR3) (2).

In vitro, ANCA bind to their cognate Ag to trigger neutrophil adhesion to endothelial cells and activation. Described first in azurophilic granules, its main storage compartment, PR3 is also present in the secretory vesicles and on the plasma membrane (3). The peculiarity of PR3 membrane expression is its heterogeneity (4): the percentage of membrane PR3-expressing neutrophils varies among individuals but is genetically determined. Notably, a high percentage of membrane PR3-expressing positive neutrophils is observed in patients with chronic inflammatory diseases such as ANCA-associated vasculitis. In addition, unlike other neutrophil granule proteins, PR3 is coexternalized with phosphatidylserine (PS) during neutrophil apoptosis because of its association with phospholipid scramblase 1 and impaired macrophage phagocytosis (5). We recently reported that PR3, which is a peripheral membrane protein, has a hydrophobic patch essential for its membrane anchorage and for its proinflammatory activity (6). Accordingly, a hydrophobic patch-deficient PR3 mutant was not externalized on apoptosis and did not impair phagocytosis (6).

Phagocytosis of apoptotic cells, also known as efferocytosis, prevents the release of intracellular contents and unwanted immune responses to self-Ags derived from dying cells (7). During inflammation, neutrophils are massively recruited at the inflammation site where they play a fundamental role in pathogen destruction and then undergo apoptosis. Apoptotic neutrophils are recognized and ingested by macrophages, thereby inducing an anti-inflammatory phenotype and promoting the resolution of inflammation (7). Apoptotic cell uptake involves many phagocyte receptors that bind molecules expressed at the apoptotic cell surface called "eat-me"

signals. These signals include the adsorption of soluble proteins (such as C1q and thrombospondin) from outside the cell, and the translocation of molecules such as PS and calreticulin (CRT) from inside the cell to the surface, as well as the suppression of "don't-eat-me" signals such as CD47 (8). The best characterized eat-me signal is PS, exposed at the cell surface in the early stages of apoptosis. PS is recognized by phagocyte receptors, among them TIM4 and BAI1 (7, 8). Unlike necrotic cells, apoptotic cell recognition induces the release from macrophages of anti-inflammatory mediators such as TGF-β, IL-10, and PGE$_2$, and inhibits proinflammatory cytokine secretion, such as TNF-α and IL-8 (9).

It is now commonly accepted that CRT, originally characterized as an endoplasmic reticulum chaperone protein (10), is also a majoreat-me signal expressed at the surface of apoptotic cells, including neutrophils (11). Accordingly, decreased CRT membrane expression reduced apoptotic cell uptake by phagocytes (12). One of the main receptors of CRT is the low-density lipoprotein receptor-related protein (LRP), also called CD91, found at the surface of phagocytes (13–15). In this article, we provide evidence that PR3 externalized at the membrane of apoptotic neutrophils can bind and possibly interfere with CRT function, thus impairing phagocytosis of apoptotic neutrophils and promoting inflammation.

## Materials and Methods

### Human neutrophil isolation and stimulation, and the induction of apoptosis

Blood donors gave their written informed consent to participate in this study, which was approved by the INSERM Institutional Review Board and the Ethics Committee of Cochin Hospital (Paris, France). Human neutrophils from healthy donors (Etablissement Français du Sang, Paris, France) and from 10 ANCA-associated vasculitis patients were isolated from EDTA-anticoagulated blood, using density-gradient centrifugation and Polymorphprep (Nycomed), as described previously (3). The patients, who fulfilled the criteria for GPA (16), were admitted to the Department of Internal Medicine at Cochin Hospital. All of them exhibited anti-PR3 ANCA, and active disease assessed with the Birmingham vasculitis activity score (≥3) (17).

To trigger in vitro degranulation, we preincubated neutrophils with cytochalasin B (10 μg/ml; Sigma) in HBSS (Life Technologies) for 5 min, and then stimulated them with 1 μM fMLF (Sigma) for 10 min (5). To induce physiological apoptosis, we resuspended the neutrophils at $2 \times 10^6$/ml in RPMI-1640 (Life Technologies) with 10% FCS and incubated them for 15 h at 37°C. PS externalization was quantified by PE-Annexin V labeling after necrotic 7-amino-actinomycin D (7-AAD)–labeled cells had been excluded from the analysis using a FACScan flow cytometer (Cell Quest software and Becton Dickinson Immunocytometry Systems), as described previously (18).

### Transfection and activation of rat basophilic line cells and induction of apoptosis

Rat basophilic cell line (RBL) cells were cultured as described previously (18, 19). Recombinant human PR3 or the membrane-anchorage–deficient PR3 mutant, PR34H4A, was expressed in stably transfected RBL cells as described previously (6, 19). RBL/CD177 and RBL/CD177-PR3 were obtained by transfecting RBL or RBL/PR3 cells with pcDNA3-neomycin containing CD177 cDNA (GeneCopoeia) using the AMAXA system as described previously (6). Cells were selected on their neomycin resistance and cloned. Apoptosis was induced by 16-h incubation at 37°C with 2 μg/ml gliotoxin (Sigma), as described previously (6). Degranulation was induced with 2 μM A23187 calcium ionophore and was evaluated by measuring β-hexosaminidase activity in the degranulation supernatant, using the chromogenic substrate $p$-nitrophenyl-$N$-acetyl-β-$D$-glucosaminide (Sigma), as described previously (18). To study protein traffic, we treated RBL cells ($10^6$/ml) for 1 h with 10 μg/ml brefeldin A (BFA) to block the endoplasmic reticulum to Golgi complex transport. Apoptosis or degranulation was then triggered by exposure to gliotoxin for 6 h or A23187 calcium ionophore for 30 min, respectively.

### Flow cytometry analysis of PR3, CRT, CD177, and LRP, and CD66b membrane expression

After FcR blocking, $5 \times 10^5$ cells were incubated for 30 min with one of the following Abs: mouse monoclonal anti-PR3 (clone CLB12.8; 2 μg/ml; Sanquin), rabbit polyclonal anti-CRT (clone PA3-900; 5 μg/ml; Thermo-Scientific), mouse monoclonal anti-CRT (clone SPA 601; 30 μg/ml; Stress-Gen), mouse monoclonal anti-hCD177 (1.2 μg/ml; Serotec), mouse monoclonal anti-LRP (6 μg/ml; Becton Dickinson), or mouse monoclonal anti–CD66b-FITC (25 μg/ml; Immunotech), followed by incubation with fluorochrome-conjugated anti-mouse IgG or anti-rabbit IgG (Immunotech).

### Confocal microscopy analysis of immunofluorescence labeling of PR3 and CRT

Neutrophils were induced to adhere to poly-L-lysine–precoated coverslips, then fixed and permeabilized with streptolysin-O as described previously (5). After saturation in PBS with 1% BSA, cells were incubated for 45 min with the mouse anti-PR3 or rabbit anti-CRT Abs and incubated for 30 min with Alexa 488-conjugated anti-mouse IgG or Alexa 555-conjugated anti-rabbit IgG (13 μg/ml; Molecular Probes). Slides were analyzed using confocal microscopy with a Leica TCS SP5 confocal scanning laser microscope.

### Coimmunoprecipitation experiments and Western blot analysis

Neutrophils ($5 \times 10^6$) were lysed in 100 μl lysis buffer consisting of 50 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 1% digitonin, 50 mM HEPES buffer, and the anti-proteinases PMSF (4 mM), leupeptin (400 μM), pepstatin A (400 μM), and EDTA (2 mM) for 20 min at 4°C. The mixture was then centrifuged to obtain the soluble fraction. Six micrograms of one of the following Abs: isotypic IgG1 (control; Immunotech), rabbit IgG (Beckman Coulter) mouse anti-PR3 mAb, or rabbit anti-CRT were mixed with the neutrophil lysate, together with 70 μl Sepharose-protein G beads (Amersham) and 400 μl lysis buffer on a rotating wheel at room temperature for 30 min. The beads were then incubated with 20 mM dimethyl-pimelimidate-2 (Pierce) in 200 mM carbonate buffer, at pH 9, for 30 min. The cross-linking solution was quenched with 50 mM Tris for 15 min followed by two lavages with 50 mM glycine in PBS-Tween, 0.65%, pH 2.7. Ab-coupled beads were incubated for 2 h at 4°C with 100 μl cell lysate. Bound material was eluted in 50 μl Laemmli sample buffer five times and analyzed with Western blotting, as described previously (5). PR3 and CRT were detected using a rabbit polyclonal anti-PR3 peptide (5) and a rabbit polyclonal anti-CRT Ab using the SuperSignal West Pico detection kit (Pierce).

### Surface plasmon resonance spectroscopy

Analyses were carried out using surface plasmon resonance (SPR) spectroscopy with a BIAcore 3000 instrument (BIAcore; GE Healthcare). Purified PR3 (1 mg/ml in distilled water; Athens Research & Technology) was incubated with 2 mM DFP for 30 min at room temperature. CRT immobilization running buffer consisted of 10 mM HEPES, 145 mM NaCl, and 5 mM EDTA (pH 7.4). Recombinant CRT, the CRT P domain (residues 189–288), and the CRT globular domain (residues 1–187 fused by a three-residue linker to residues 285–400) were produced as described previously (15) and were diluted in format buffer (pH 3) to 50, 15, and 40 μg/ml, respectively. They were then immobilized on a CM5 sensor chip (GE Healthcare) using the BIAcore amine coupling kit. Binding of PR3 to immobilized CRT was measured at a flow rate of 20 μl/min in a running buffer (20 mM HEPES, 150 mM NaCl, 1 mM CaCl$_2$, and 0.005% surfactant P20, pH 7.4). Surfaces were regenerated by an injection of 20 μl of 10 mM NaOH. The specific binding signal shown was obtained by subtracting the background signal, routinely obtained by injection of the sample over an activated-deactivated surface. Data were analyzed by global fitting to a 1:1 Langmuir binding model of the association and dissociation phases for several concentrations of PR3 simultaneously, using the software BIAevaluation, version 3.2 (GE Healthcare), giving a statistic $\chi^2$ value <2, as described previously (15). The apparent equilibrium dissociation constants, $K_D$, were calculated from the ratio of the dissociation and association rate constants, $k_d/k_a$.

### Phagocytosis of apoptotic RBL cells by human monocyte-derived macrophages

Human monocytes were isolated from blood using Ficoll-Paque and were plated at $3 \times 10^6$ cells/well in six-well plates as described previously (5). After 1-h incubation at 37°C, nonadherent cells were removed, and monocytes were cultured in Iscove's DMEM-10% autologous serum at 37°C in a 5% CO$_2$ atmosphere to obtain monocyte-derived macrophages (20). After

7 d, the culture medium was removed and human monocyte-derived macrophages were incubated with TAMRA-labeled apoptotic RBL cells (at a ratio of 1:1) in 2 ml Iscove's DMEM for 2 h, as described previously (6), to quantify the cytokines secreted in culture supernatants with the RayBio human inflammation Ab array or the Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. For the phagocytosis of apoptotic neutrophils from healthy donors and vasculitis patients, macrophages were incubated with a mouse monoclonal anti-LRP Ab (America Diagnostica) for 1 h, then with TAMRA-labeled apoptotic neutrophils (at a ratio of 10:1) in 2 ml Iscove's DMEM for 2 h. After phagocytosis, the macrophages were washed twice with PBS/EDTA (2 mM), and phagocytosis was analyzed using flow cytometry (21). Cells doublets were gated out by comparison of forward scatter signal pulse height (FSC-H) and area (FSC-A) to preclude the analysis of apoptotic cells in contact with rather than phagocytosed by macrophages. Apoptotic neutrophils and macrophages were identified by their distinct laser scattering properties. The percentage of macrophages was considered to represent the percentage of macrophages that had phagocytosed TAMRA-labeled apoptotic neutrophils.

### Phagocytosis of apoptotic RBL cells by resident peritoneal macrophages in vivo

Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Cochin Institute. Apoptotic RBL clearance by resident peritoneal macrophages was studied in 8-wk-old C57bl6 mice (Charles Rivers) after an i.p. injection of $2 \times 10^6$ TAMRA-labeled apoptotic RBL cells. After 30 min, peritoneal cells were recovered by lavage with ice-cold PBS containing 25 U heparin and 2 mM EDTA, and phagocytosis was assessed after macrophage immunolabeling (21) with an FITC-coupled rat anti–mouse-F4-80 Ab (AbD Serotec) and analyzed using flow cytometry. The percentage of double-positive macrophages was considered to represent the percentage of macrophages that had phagocytosed TAMRA-labeled apoptotic RBL cells.

### Phagocytosis of apoptotic RBL cells by thioglycolate-induced peritoneal macrophages ex vivo

Peritoneal macrophages from C57Bl6 mice were mobilized by i.p. injection of thioglycolate (3%; BBL Thioglycolate Medium, Brewer Modified; BD). At 72 h, macrophages were plated (500,000 macrophages/well) in DMEM supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 10 mM HEPES buffer, 10 mM nonessential amino acids, 4 mM L-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-ME for 6 h. Purity assessed after multiple washing for adherent cells with F4/80-allophycocyanin (clone BM8; eBioscience) was >90%. Apoptosis of RBL cells was induced by UV-B (7 min, $\lambda = 312$ nm, 0.68 $\mu$W/cm$^2$) for 24 h and was evaluated using flow cytometry after Annexin V-PE/7-AAD (BD) labeling. Apoptotic cells (typically, the percentage was >90%) were then added to macrophages (ratio of 2:1), and phagocytosis could proceed for 24 h. The culture medium, so-called phagocytosis supernatant, was collected and frozen at −80°C until further analysis. Macrophages were next stimulated by LPS (10 ng/ml, O55B5; Sigma) during 24 h to collect the culture medium, so-called stimulation supernatant, and were frozen at −80°C. Mouse TNF-α was measured by ELISA kits according to the manufacturer's protocol (Mouse TNF-α Platinum ELISA; eBioscience).

### PR3 binding on macrophages

Human monocyte-derived macrophages ($2 \times 10^6$ cell/well) were washed with PBS and preincubated with a mouse monoclonal anti-LRP Ab (America Diagnostica) or with a control isotype at a concentration of 1 μg/ml. Purified PR3 (4 μg; Athens Research and Technology) was then added and the mixture was incubated for 3 h at 4°C. PR3 binding to the macrophage cell surface was then analyzed with FITC-conjugated anti-PR3 for 30 min using flow cytometry.

### Statistical analysis

Statistical analyses were performed using Prism GraphPad 5.01 software. Comparisons were made using the Student t test or the nonparametric Mann–Whitney test, when appropriate. Differences were considered significant when $p < 0.05$.

## Results

### PR3 and CRT are colocalized on neutrophil membrane on apoptosis

We first examined the colocalization of PR3 with CRT in neutrophils under basal conditions and after the induction of physiological

apoptosis, using immunofluorescence labeling after streptolysin-O permeabilization to preclude the detection of the PR3 localized in granules, as described previously (5). This confocal microscopy analysis provided evidence that, in neutrophils, PR3 and CRT were partially localized in the cytosol, but also on membranes (Fig. 1A). PR3 and CRT colocalization increased significantly after physiological apoptosis induction, suggesting modification of subcellular protein localization facilitating the PR3–CRT interaction. Immunoprecipitation experiments performed in neutrophil lysates using the anti-PR3 Ab and validated by Western blot analysis using anti-PR3 and anti-CRT (Fig. 1B) supported the notion of PR3–CRT association. The coimmunoprecipitation of CRT with PR3 was confirmed in RBL/PR3 cells (Fig. 1B), which we have used previously to study membrane PR3 (5, 6, 18). PR3 binding on different domains of immobilized CRT was then analyzed using SPR spectroscopy (Fig. 1C). Recombinant CRT, the CRT P domain, and the CRT globular domain designed to preserve the CRT structural domain limits (15, 22) were immobilized on a CM5 sensor chip.
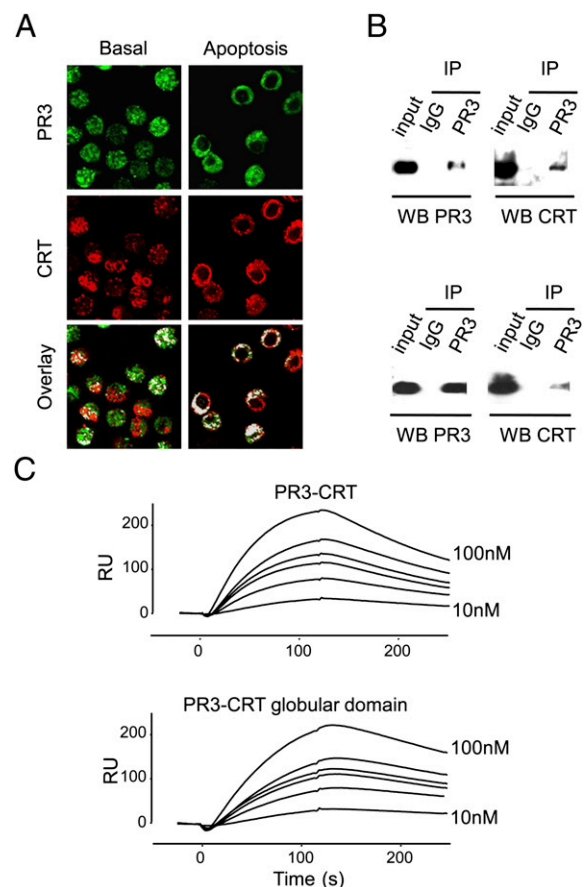


**FIGURE 1.** Colocalization and molecular association of PR3 and CRT in neutrophils. (**A**) Confocal microscopy analysis of PR3 (green) and CRT (red) immunofluorescence in human neutrophils after streptolysin-O permeabilization under basal conditions and after the induction of physiological apoptosis (original magnification ×40). (**B**) Immunoprecipitation of PR3 with a mouse monoclonal anti-PR3 (CLB12.8) or control IgG1 in human neutrophils (*upper panel*) and in RBL/PR3 cells (*lower panel*). Immunoprecipitates were analyzed with Western blot (WB) to detect PR3 (at 30 kDa) and CRT (at 55 kDa). (**C**) SPR analysis of PR3 binding to CRT (*upper panel*) and CRT globular domain (*lower panel*)-immobilized surfaces with a $K_D$ of $4.1 \times 10^{-8}$ and $3 \times 10^{-8}$ M, respectively. A dose–response effect was observed when using increasing soluble PR3 concentrations with 10 (*lower curve*), 30, 50, 70, 90, and 100 (*upper curve*) nM for CRT; and 10, 30, 50, 60, 90, and 100 nM for CRT globular domain. Association and dissociation curves were recorded for 120 s.

The kinetic parameters of CRT recognition were determined by recording sensorgrams at varying PR3 concentrations from 10 to 100 nM. PR3 bound to recombinant CRT with a $K_D$ of 41 nM (Fig. 1C). Notably, PR3 bound directly to the CRT globular domain with a $K_D$ of 30 nM (Fig. 1C) but failed to associate with the CRT P domain (data not shown), thereby constituting a suitable negative control. The two values of $K_D$ are in the same range, thus suggesting that PR3–CRT interaction occurred mainly, if not exclusively, through the globular domain of CRT.

As described previously (4), a subset of freshly isolated neutrophils expressed PR3 at their surface. Fig. 2A shows that 60% of the neutrophils expressed PR3 at their surface under basal conditions, but no CRT could be detected. Constitutive apoptosis triggered by 15-h incubation at 37°C, evaluated by Annexin V and 7-AAD labeling (Fig. 2B), showed an increase in PR3 (as previously described in Ref. 5) and CRT membrane expression (Fig. 2A), from $7.9 \pm 2.8\%$ CRT$^+$ neutrophils before the induction of apoptosis, to $40.2 \pm 5.4\%$ ($n = 10$) after. In contrast, after stimulation with fMLF (1 μM) in the presence of cytochalasin B, a condition triggering degranulation and increased PR3 membrane expression (5), no increase in CRT expression was observed (Fig. 2C). However, as expected, compared with basal conditions, cytochalasin B combined with fMLF increased membrane expression of CD66b (Fig. 2D), a protein stored within specific granules, and of PR3 (Fig. 2C). It was concluded that coexternalization of PR3 and CRT is specific to the apoptotic condition and did not result from the mobilization of neutrophil granules.
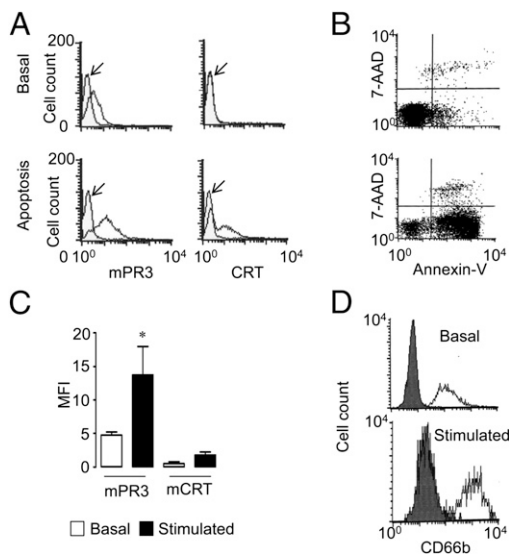
## PR3 interacts with CRT in RBL/PR3 cells on apoptosis and is independent of degranulation

In contrast with neutrophils, neither PR3 nor CRT was expressed at the cell surface of resting RBL/PR3 cells (18). However, after gliotoxin-induced apoptosis, the majority of the cells externalized PS and expressed both PR3 and CRT at their surface. The percentages of cells expressing PR3 and CRT were $87 \pm 7\%$ and $58 \pm 12\%$ ($n = 7$), respectively (Fig. 3A). Similarly to PR3 (6, 18), CRT was coexternalized with PS on apoptosis (Fig. 3B), and double immunolabeling of PR3 and CRT showed that they are coexternalized at the cell surface (Fig. 3C). Gliotoxin-induced PR3 and CRT surface expression were not significantly affected by the BFA toxin, which prevents trafficking between the endoplasmic reticulum and the Golgi complex (Fig. 3D), thus suggesting that the classical granule-mediated secretory pathway was not involved in these processes. It is noteworthy that BFA treatment did not modify the percentage of cells that were Annexin V$^+$, being $34.5 \pm$

**FIGURE 2.** CRT and PR3 are coexternalized during neutrophil apoptosis. (**A**) Flow cytometry analysis of PR3 (mPR3, *left column*) and CRT (mCRT, *right column*) membrane expression in basal (*upper row*) and apoptotic (*lower row*) neutrophils. Control IgG labeling is shown by an arrow. This experiment is representative of seven performed on different donors. (**B**) Flow cytometry analysis of PS externalization using Annexin V and 7-AAD labeling in basal (*upper row*) and apoptotic (*lower row*) neutrophils as in (A). (**C**) PR3 and CRT membrane expression on basal and fMLF-stimulated neutrophils. Flow cytometry analysis of PR3 and CRT membrane expression was performed in the absence (basal) or in the presence of cytochalasin and fMLF. The MFIs are depicted in the histograms on the right. Results are given in terms of MFI units of PR3 and CRT membrane expression. Values are the means $\pm$ SEM, $n = 4$; *$p < 0.05$ using a Mann–Whitney $U$ test. (**D**) Flow cytometry analysis of CD66b at the membrane of neutrophils. Results of a representative experiment showing CD66b membrane expression on neutrophils under basal conditions (*upper panel*) and after degranulation (*lower panel*).
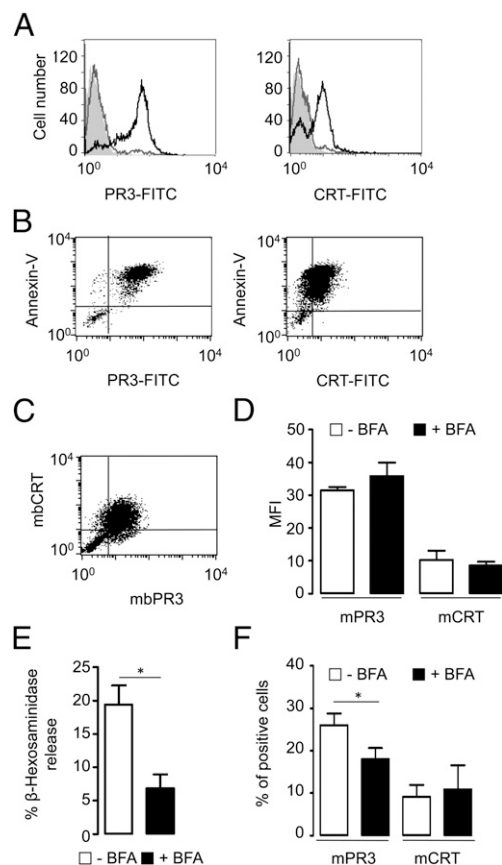
**FIGURE 3.** Coexternalization of CRT and PR3 occurred in RBL/PR3 cells during apoptosis but not degranulation. (**A**) PR3 (*left panel*) and CRT (*right panel*) externalization determined by flow cytometry analysis in RBL/PR3 cells after gliotoxin-induced apoptosis. Isotypic control is shaded gray. (**B**) Results of flow cytometry analysis of PS externalization membrane expression in RBL/PR3 cells after induction of apoptosis (labeled with Annexin V-PE) and PR3 (*left panel*) or CRT (*right panel*). (**C**) Double immunolabeling of PR3 and CRT membrane expression in RBL/PR3 cells during gliotoxin-induced apoptosis. (**D**) Effect of BFA (10 μg/ml for 1 h) on PR3 and CRT membrane expression after gliotoxin-induced apoptosis in RBL/PR3 cells. Data are MFI and are expressed as mean $\pm$ SEM, $n = 4$. (**E**) Effect of BFA (10 μg/ml for 1 h) on β-hexosaminidase release. Data are expressed as mean $\pm$ SEM, $n = 4$; *$p < 0.05$, using a Mann–Whitney $U$ test. (**F**) PR3 and CRT membrane expression after induction of degranulation with calcium ionophore A23187 (2 μM) for 30 min ($n = 4$). Data are expressed as mean $\pm$ SEM, $n = 4$; *$p < 0.05$, using a Mann–Whitney $U$ test.

9% and 32.5 $\pm$ 11% ($n = 3$) in the absence and presence of BFA, respectively. In contrast, ionophore A23187-induced β-hexosaminidase release (Fig. 3E) and PR3 membrane expression (Fig. 3F) were sensitive to BFA because these processes were granule mediated, according to analysis using an enzymatic assay and flow cytometry, respectively. As expected, no increase in CRT membrane expression was observed after ionophore A23187-induced degranulation in the presence or absence of BFA (Fig. 3F), thus corroborating the belief that the coexposure of CRT and PR3 on apoptotic cell membrane was independent of granule mobilization.

*CD177 is not associated with membrane PR3 on apoptosis*

In neutrophils, PR3 is expressed on the same neutrophil subset as CD177 (also called NB1) (23, 24), a GPI-anchored glycoprotein. CD177 has been suggested to be a PR3 "receptor" involved in PR3 constitutive membrane expression in neutrophils, and we hypothesized that the lack of constitutive PR3 membrane expression of RBL/PR3 cells could be due to the absence of CD177 expression in these cells. As expected, stably CD177-transfected RBL (RBL/CD177; Fig. 4A) and RBL/PR3 (RBL/PR3/CD177) cells (data not shown) expressed CD177 on the membrane at the basal state. Notably, CD177 expression was unchanged after gliotoxin-induced apoptosis in RBL/CD177 cells. Moreover, similarly to RBL/PR3, PR3 membrane expression was not observed under basal conditions in RBL/PR3/CD177 cells (Fig. 4B), thus demonstrating that CD177 expression at the plasma membrane did not result in constitutive PR3 membrane expression. Again, CD177 expression did not modulate the increase in PR3 membrane expression observed after gliotoxin-induced apoptosis (Fig. 4B), thus strongly suggesting that apoptosis-induced PR3 membrane expression was independent of CD177 expression in RBL cells.

*Membrane PR3 impairs macrophage phagocytosis of neutrophils from patients with GPA through an LRP-dependent mechanism*

As previously described by our group (4) and confirmed by others (25–27), the percentage of PR3-expressing neutrophils was higher
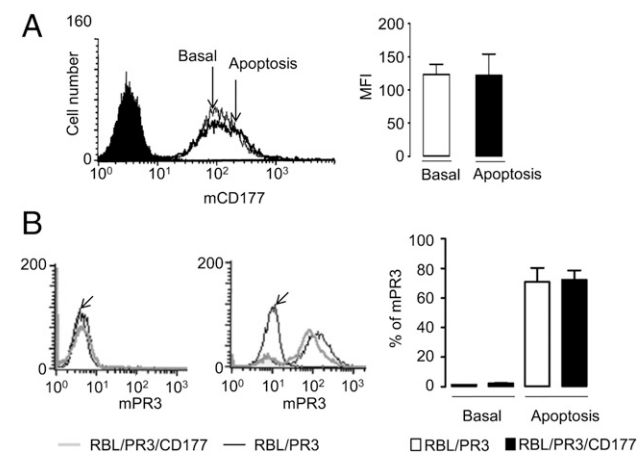
in GPA patients than in healthy control subjects, being 65.1 $\pm$ 8.5% and 39.2 $\pm$ 9.9% ($n = 9$), respectively, in this study. No difference was observed between apoptosis-induced PS externalization in neutrophils from healthy control subjects and from GPA patients (Fig. 5A). Interestingly, the percentage of PR3-expressing neutrophils was related to the percentage expressing CRT after apoptosis (Fig. 5B), and the phagocytosis of apoptotic neutrophils was significantly lower in GPA patients than in healthy control subjects (Fig. 5C). Blocking the CRT receptor by anti-LRP on macrophages from healthy control subjects significantly decreased macrophage phagocytosis. However, the low levels of LRP expression on macrophages compared with those on monocytes (Fig. 5D) might explain the modest phagocytosis inhibition observed. Remarkably, anti-LRP failed to show any effect on the phagocy-
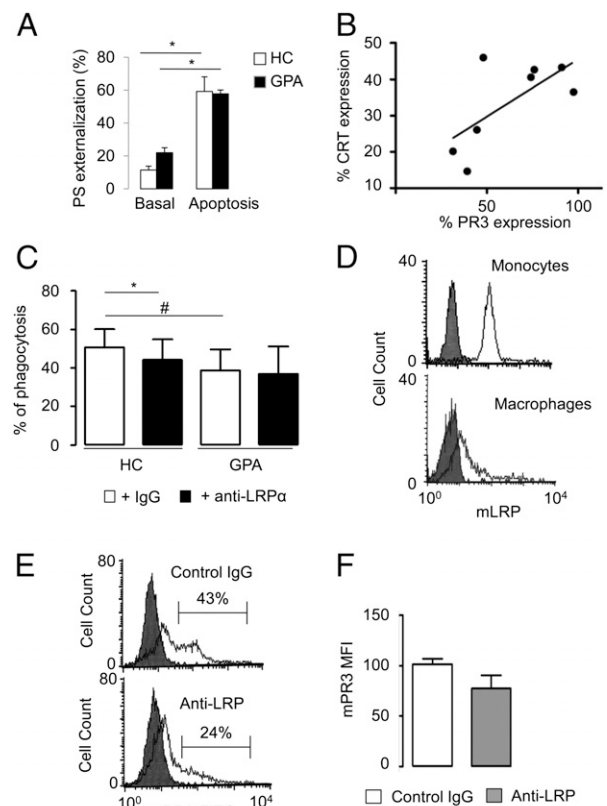


**FIGURE 5.** PR3 impaired the phagocytosis of apoptotic neutrophils of GPA patients through the CRT–LPR pathway. (**A**) Percentage of neutrophils showing PS externalization in the basal state and after physiological apoptosis (16 h at 37°C) in healthy control subjects (HC; $n = 10$) and GPA patients ($n = 10$) evaluated by Annexin V labeling and analyzed by flow cytometry. Data are expressed as mean $\pm$ SEM. *$p < 0.05$, Student $t$ test. (**B**) Relationship between the percentages of neutrophils expressing membrane CRT and PR3 after apoptosis. (**C**) Percentage of phagocytosis of apoptotic neutrophils analyzed by flow cytometry in HC and GPA patients. Macrophages were preincubated by an anti-LRPα Ab or a control IgG for 1 h. Results are expressed as the mean $\pm$ SEM of six healthy donors and five GPA patients. *$p < 0.05$, paired Student $t$ test; #$p < 0.05$, Student $t$ test. (**D**) Flow cytometry analysis of membrane expression of LRP in monocytes and monocyte-derived macrophages. Isotypic control is shaded gray. This experiment is representative of four performed on different donors with similar results. (**E**) Effect of anti-LRP on the exogenous binding of PR3 on macrophages. Flow cytometry analysis of membrane-bound PR3 on macrophages after incubation with soluble purified PR3 in the presence or absence of anti-LRP. This experiment is representative of three performed on different donors with similar results. (**F**) Effect of anti-LRP on PR3 binding on macrophages. Membrane-bound PR3 was detected as in (E). The MFI of PR3+ macrophages are depicted in the histograms. Results are expressed as the mean $\pm$ SEM, $n = 3$.



**FIGURE 4.** CD177 (NB1) expression in RBL/PR3 cells during apoptosis. (**A**) Flow cytometry analysis of CD177 membrane expression in RBL/CD177 cells under basal conditions or after gliotoxin-induced apoptosis. Isotypic control is shaded black. A representative experiment is shown (*left panel*), and the MFI are depicted (histograms in *right panel*). Results are MFI units of CD177 membrane expression and are expressed as means $\pm$ SEM, $n = 3$. (**B**) Flow cytometry analysis of PR3 membrane expression in RBL/PR3 and RBL/PR3/CD177 cells in the basal state and after gliotoxin-induced apoptosis. A representative experiment is shown (*left panel*), and the percentage of PR3+ cells are depicted (histograms in *right panel*). Results are expressed as the mean $\pm$ SEM, $n = 3$.

tosis of apoptotic neutrophils from GPA patients (Fig. 5C), presumably because the CRT–PR3 association interfered with the CRT–LRP recognition pathway. CRT could be "blocked" by PR3, thus precluding its binding to LRP, which remained "free." It could also be suggested that PR3 (already bound to CRT on the apoptotic cell) associates directly to LRP expressed on macrophages, resulting in the "blockade" of the receptor. To test this latter hypothesis, we added exogenous purified PR3 to macrophages in the presence of anti-LRP or a control IgG. PR3 binding to macrophage surface was observed in IgG-treated macrophages, and the percentage of PR3$^+$ macrophages was significantly decreased in the presence of anti-LRP (Fig. 5E). In addition, the mean fluorescence intensity (MFI) of anti-LRP–treated macrophages was lower than those treated with IgG (Fig. 5F), thus suggesting that PR3 or PR3–CRT complexes might also interact directly with LRP to impair its LRP function.

*Proinflammatory cytokines are secreted by macrophages after RBL/PR3 phagocytosis in vitro and in vivo*

Cytokine analysis of supernatants from macrophages after phagocytosis of apoptotic RBL or RBL/PR3 cells showed a global increase in inflammatory cytokines and chemokines after phagocytosis of apoptotic RBL/PR3 cells compared with RBL cells (Fig. 6A). Measurement of cytokine concentrations using specific ELISA showed an increase in the secretion of MIP-1β, TNF-α, IL-6, and IL-8, together with a decrease in the anti-inflammatory IL-10 (Fig. 6B). To determine whether PR3 could inhibit phagocytosis of apoptotic cells in vivo, TAMRA-labeled apoptotic RBL or RBL/PR3 cells were injected into the mouse peritoneal cavity, and macrophage phagocytosis was quantified by flow cytometry on double-stained macrophages (Fig. 7A). Apoptotic RBL/PR3 cells were less phagocytosed than RBL/PR34H4A cells expressing a membrane-anchorage–deficient PR3 mutant (6) or RBL cells in vivo (Fig. 7B), suggesting that the decrease in phagocytosis was due to the presence of PR3 at the surface of apoptotic RBL cells (Fig. 7B). Again, cytokine array analysis of the peritoneal lavage fluid provided evidence that the decreased clearance of apoptotic PR3-expressing cells triggered an increase in proinflammatory cytokines such as MIP-1α, IL-6, and MCP-1, and a decrease in the secretion of anti-inflammatory cytokines such as IL-10, compared with apoptotic RBL cells (Fig. 7C). To study the physiological

relevance of this potential "reprogramming activity" of PR3-expressing apoptotic cells on macrophages at the site of inflammation, we studied TNF-α secretion in response to LPS ex vivo, in thioglycolate-elicited macrophages after phagocytosis of apoptotic cells expressing or not expressing PR3 (Fig. 8A). These experiments first confirmed that apoptotic RBL/PR3 cells triggered an increased TNF-α secretion by macrophages as compared with the TNF-α secreted by apoptotic control RBL cells. It should be noted that in the absence of apoptotic cell phagocytosis, elicited macrophages collected after a 24-h thioglycolate treatment produced almost no TNF-α. Notably, LPS stimulation of elicited macrophages triggered a strong TNF-α secretion (Fig. 8B) that is dramatically decreased by the phagocytosis of apoptotic RBL cells, thus confirming the anti-inflammatory effect of the phagocytosis of apoptotic cells on macrophages. Remarkably, phagocytosis of PR3-expressing apoptotic cells significantly decreased this anti-inflammatory effect, thereby counteracting the anti-inflammatory potential of apoptotic cells and perpetuating inflammation (Fig. 9).

## Discussion

In the pathophysiological context of GPA, characterized by the presence of anti-PR3 ANCA that can potentially bind their cognate Ag, PR3 membrane expression constitutes a critical event that can modulate the state of activation or the fate of neutrophils (1, 2). The pattern of PR3 membrane expression was found to be more complex than previously thought because of the presence of several PR3 pools within neutrophils that might fulfill different functions (28). Although the main storage pool of PR3 is in the azurophilic granules (3), PR3 is constitutively expressed at the plasma membrane of neutrophils, and the percentage of neutrophils expressing PR3 is increased in patients with ANCA-associated vasculitis, including GPA (4). Intriguingly, PR3 was expressed at the plasma membrane in the early stages of neutrophil apoptosis in association with molecules that are involved in the recognition of apoptotic cells such as PS. Apoptosis-induced PR3 membrane expression was not enzymatically active (6) but was associated with impaired phagocytosis of apoptotic cells by macrophages. Because this biological function was independent of PR3 enzymatic activity (5), it was hypothesized that it might be related to new PR3 partner protein(s) such as CRT.

**FIGURE 6.** Proinflammatory cytokine secretion by macrophages after phagocytosis of RBL and RBL/PR3 cells. (**A**) Analysis of the pattern of cytokines secreted into supernatants collected after phagocytosis of apoptotic RBL and RBL/PR3 cells by macrophages using a cytokine array. The experiment was performed three times giving similar results. (**B**) Quantification of MIP-1β, TNF-α, IL-8, and IL-10 secretion from supernatants measured using specific ELISA. Results are given as means ± SEM, $n = 4$; *$p < 0.05$, using a Student $t$ test.
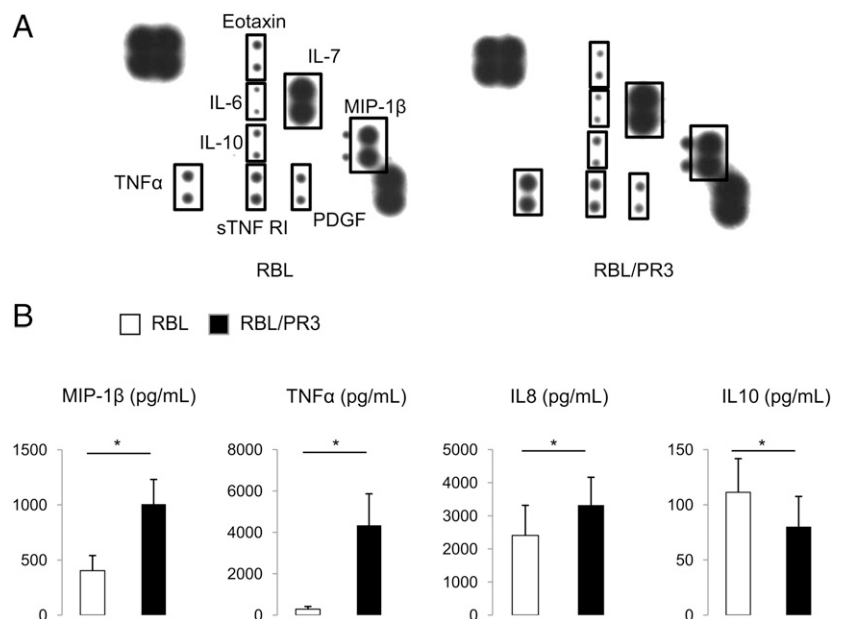
**FIGURE 7.** In vivo phagocytosis of apoptotic RBL cells. Apoptotic RBL, RBL/PR3, and RBL/4H4A cells labeled with TAMRA were injected into mice peritoneal cavity. After 30 min, the mice were sacrificed, and i.p. macrophages were collected and labeled with a FITC-conjugated anti-F4/80 Ab. (**A**) Gating strategy used to differentiate RBL cells (R1), macrophages (R3), and phagocyted RBL cells in macrophages (R2). (**B**) Quantification of phagocytosis by flow cytometry analysis. Data are the percentages of macrophages that have phagocytosed apoptotic RBL cells. Results are expressed as the mean ± SEM, $n = 12$ mice/group. $*p < 0.05$, using a Mann–Whitney $U$ test. (**C**) Cytokine secretion in i.p. lavage fluids evaluated using a cytokine array. Results are given as the means ± SEM, $n = 4$; $*p < 0.05$, using a Student $t$ test.
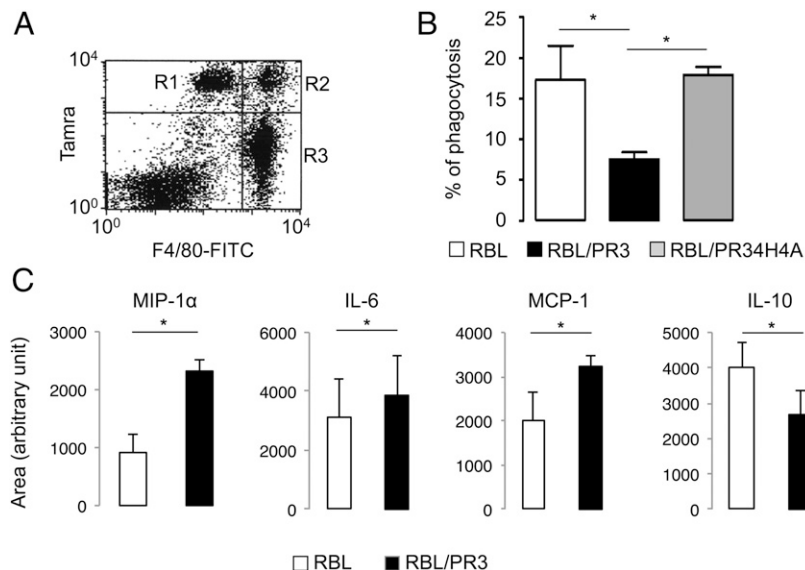


CRT is well-known as a calcium-binding chaperone protein mainly located in the endoplasmic reticulum, involved in calcium homoeostasis and in the folding of newly synthesized proteins (10). CRT also plays a role in many other biological systems, including functions outside the endoplasmic reticulum, indicating that the protein is a multiprocess molecule (29). Its involvement in the recognition and immunogenicity of apoptotic cells has recently been elucidated (11). In this study, we provide evidence that PR3 is associated with CRT, described as an eat-me signal on the apoptotic cell. The intracellular trafficking pathway involved in apoptosis-induced PR3 and/or CRT expression is not clear but did not require granule mobilization. BFA did not block the externalization of either PR3 or CRT during apoptosis. Indeed, BFA resulted in only a slight decrease in CRT surface expression in RBL/PR3 cells, in contrast with a previous study in which it was reported that BFA blocked CRT membrane expression during apoptosis in cancer cells treated with chemotherapy (11). Remarkably, we observed that the coexternalization of PR3 with CRT occurred only under apoptotic conditions and not during neutrophil degranulation. CRT has been shown to interact directly with PS in other cell types (15). Under apoptotic stress conditions, cytosolic CRT increased and associated with PS, maintaining PS on the cytosolic side of the cell membrane (12). PR3 thus belongs to a membrane protein complex together with phospholipid scramblase 1 that is assembled at the plasma membrane concomitantly with PS externalization on neutrophil apoptosis. We have shown in this study, using SPR spectroscopy, that CRT interacts specifically with PR3 through its globular domain and not via its P domain. The fact that the globular domain of CRT, which recognizes PR3, is also involved in PS and C1q binding (14, 30, 31), which is critical in apoptotic cell recognition (9, 32), provides further evidence of the important functional role of the CRT–PR3 interaction. The way in which these molecules interact with each other, to contribute to cell–cell contact and regulation of the signaling pathways, remains to be investigated. However, it is noteworthy that the membrane protein CD177, reported to be a protein that can associate with PR3 at the neutrophil plasma membrane, was not upregulated on apoptosis and was not part of this complex in RBL cells. Nonetheless, it was confirmed in this study that exogenous purified PR3 can specifically bind to CD177 expressed on resting RBL/CD177 cells, whereas no PR3 binding was observed on wild type RBL cells (data not shown), thus confirming earlier results (23, 24) showing that CD177 is a receptor for exogenous PR3.
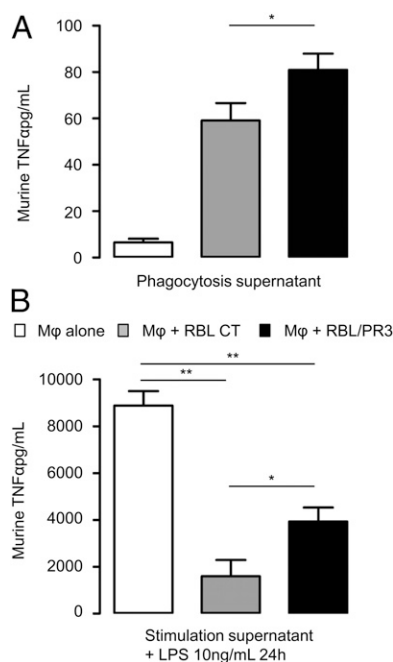
### CRT–PR3 association disables the LRP-CRT–mediated phagocytic pathway

CRT is not a transmembrane protein but is exposed at the surface of apoptotic cells (11, 12, 15) and interacts with LRP at the macrophage surface (13), which may serve as a signaling partner (11, 33). PR3 directly binds to CRT at the neutrophil cell surface, and PR3–CRT association could decrease CRT access to LRP on macrophages, thus impairing phagocytosis. In line with this



**FIGURE 8.** Ex vivo TNF-α secretion by thioglycolate-elicited macrophages after phagocytosis of apoptotic RBL cells and LPS stimulation. (**A**) Apoptotic RBL and RBL/PR3 cells were added to peritoneal macrophages ex vivo during 24 h, and secreted murine TNF-α was measured by ELISA in "phagocytosis supernatants." Macrophages alone were used as controls. (**B**) Macrophages were next stimulated by LPS (10 ng/ml) during another 24-h period, and TNF-α was measured in the "stimulation supernatants." Results are expressed as the mean ± SEM, $n = 5$ mice. $*p < 0.05$, $**p < 0.01$, using a Mann–Whitney $U$ test.

thinking, we hypothesized that PR3 could directly interact with LRP, thus hampering in a steric way the binding of CRT. It turned out that anti-LRP impaired the binding of soluble PR3 to macrophages, thus suggesting (but not demonstrating) that a direct association between PR3 and LRP (or LRP-containing complexes) could occur. It also should be mentioned that CRT is also expressed at the surface of macrophages, and that homotypic CRT–CRT interactions have also been described during the phagocytosis of apoptotic cells (14, 22). It thus could be foreseen that PR3–CRT association might interfere with this CRT–CRT homotypic interaction (Fig. 9). The relevance of the PR3–CRT association was underlined by the characterization of neutrophils from GPA patients showing a high percentage of neutrophils expressing PR3. Evidence was provided that a blocking monoclonal anti-LRP Ab decreased the phagocytosis of apoptotic neutrophils from healthy donors but not from patients with GPA. It has to be noted that the inhibitory effect of blocking LRP was very modest in our assay and might be because of the low level of LRP expression on macrophages differentiated with autologous serum. Although LRP is the only CRT receptor described so far, it is possible that other "partner proteins" that bind CRT on macrophages may be involved, and that PR3 interacts with other eat-me signals or eat-me signal receptors. For instance, CRT is also known to act in conjunction with LRP on macrophages as a receptor for collectins such as C1q, or the surfactant proteins A and D (34, 35). Consequently, the PR3–CRT interaction could affect these pathways through mechanisms not yet characterized. Our findings could allow new therapeutic drugs to be found to facilitate the resolution of inflammation.

### Phagocytosis of apoptotic PR3-expressing cells induced a proinflammatory response in vitro and in vivo

Phagocytosis of apoptotic neutrophils by macrophages does not trigger the release of inflammatory mediators (36). In fact, the low inflammatory mediator release associated with apoptotic cell clearance is more than the passive avoidance of inflammation (9).
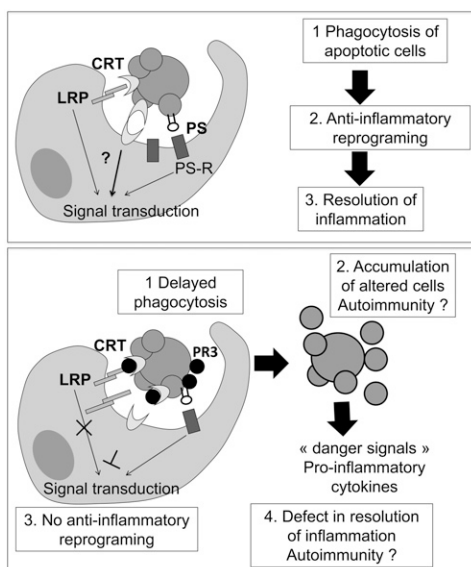


**FIGURE 9.** Potential pathophysiologic consequences of the impairment of clearance by macrophages of apoptotic cells expressing PR3. The CRT/LRP pathway is one of the recognition systems involved in efferocytosis. PR3 could interfere with this pathway or with homotypic CRT–CRT interaction. PR3 could favor autoimmunity mechanisms through a combined defect of clearance of dying cells and of anti-inflammatory reprogramming of macrophages. PS-R, PS-receptor.

Apoptotic neutrophils, like all other apoptotic cells, are potently immunosuppressive (37) because they trigger an anti-inflammatory response in macrophages characterized by anti-inflammatory cytokines such as IL-10 and TGF-β. Hence one salient observation of this study was that the phagocytosis of apoptotic PR3-expressing cells enhanced the secretion of proinflammatory cytokines such as TNF-α and IL-8, chemokines such as MIP-1β, and decreased IL-10 secretion, thus promoting inflammation. Likewise, in vivo experiments performed in mice confirmed that apoptotic RBL/PR3 cells injected i.p. into mice were less phagocytosed by macrophages. This effect was dependent on the presence of PR3 at the cell surface because the effect was not observed when RBL cells expressed PR34H4A, a PR3 mutated in the hydrophobic amino acids responsible for its membrane anchorage (6). Moreover, increased synthesis of proinflammatory cytokines such as TNF-α and IL-6, and chemokines such as MIP-α was observed together with decreased IL-10 secretion in the peritoneal lavage fluids after phagocytosis of apoptotic RBL/PR3 cells, compared with controls, thus promoting inflammation. Recent studies have proposed that efferocytosis could eventually transform macrophages to another phenotype that is postulated to promote macrophage regulatory properties at remote sites (38). Accordingly, we provide evidence that the phagocytosis of apoptotic RBL/PR3 cells is not as anti-inflammatory as the phagocytosis of control RBL cells in response to an LPS stimulation, thus strongly suggesting that PR3 expressed on apoptotic cells can counteract the "anti-inflammatory effect" of macrophages reprograming after phagocytosis of apoptotic neutrophils (38). Another aspect of crucial importance would be to evaluate the fate of macrophages that have ingested PR3-expressing cells and compare it with that of macrophages that have ingested control cells, to establish whether they can recirculate, as has been recently reported (39). Our findings further corroborate the notion that an increase in membrane PR3 expression constitutes a proinflammatory factor that can certainly affect the immune response associated with apoptotic cell clearance (Fig. 9). Because phagocytosis of apoptotic cells by macrophages functions as a mechanism for the maintenance of tolerance and the reduction of inflammation (40), it is important to explore whether PR3 can divert this mechanism and promote autoimmunity (38).

### Role of macrophages in the modulation of autoimmunity in GPA

The mechanisms leading to autoimmunity in GPA are still unknown, but several theories have been proposed including the superantigen theory, Ag mimicry, and the complementary PR3 theory (41). As exemplified in lupus, autoimmunity can be enhanced by a dysregulated apoptotic cell clearance. Animals displaying a genetic defect in bridging molecules such as C1q (42) and MFG-E8 (43) suffer from a defective phagocytosis of apoptotic cells. GPA patients have leukocytoclastic vasculitis characterized by infiltration and accumulation of unscavenged apoptotic or necrotic neutrophils in the tissues around the vessels (44). Whether this persistence of apoptotic neutrophils results from perturbed neutrophil apoptosis itself or macrophage clearance is not yet clear, and it may be a combination of both. Neutrophils from patients with ANCA-associated vasculitis have been found to exhibit enhanced rates of TNF-α–induced apoptosis compared with those in healthy control subjects (45). Moreover, ANCA-treated primed neutrophils have been found to be less phagocytosed by macrophages than control IgG-treated neutrophils (45). In addition, apoptotic neutrophils expressing PR3 can be opsonized by ANCA, resulting in enhanced recognition and subsequent uptake by macrophages via Fc-receptor interaction. This process led to a macrophage proinflammatory response with increased IL-8 and IL-1 synthesis, thus perpetuating inflammation by

recruiting more neutrophils. Nonetheless, some results suggest that the regulation of constitutive apoptosis in neutrophils from patients with chronic inflammatory diseases such as rheumatoid arthritis may be disturbed, and that they have a lower rate of apoptosis (46). Along the same lines, we have recently reported high levels of the cytoplasmic proliferating cell nuclear Ag, thus reflecting a pro-survival state in neutrophils from GPA patients (47). Although in this study no difference was observed in PS externalization in patients with ANCA-associated vasculitis, it has been recently reported that neutrophils from these patients had a delayed apoptosis (48). Regardless of the initiating mechanisms, unscavenged apoptotic neutrophils could release their intracellular content, explaining why patients with ANCA-associated vasculitis develop autoimmunity against neutrophil proteins released in tissues (49). We have shown in this study that PR3 is unique, because it is the only autoantigen whose expression at the membrane of apoptotic cells directly influences the phagocytosis by macrophages and their subsequent immune response, thus suggesting a potential relationship between macrophage clearance of apoptotic neutrophils and the occurrence of autoimmunity in GPA (50). Moreover, different subpopulations of macrophages (51) undertake different functions, and varied macrophage receptor expression from different anatomical locations has been clearly demonstrated. The phenotypic variation within the macrophage pool may serve to tailor the type of response to apoptotic cells. Because the macrophage is decisive in the net response to cell death, further characterization of their phenotypes in different pathophysiologic situations, for instance, within the granuloma typical of GPA (1, 52), might be necessary.

## Acknowledgments

## Disclosures

The authors have no financial conflicts of interest.

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