Caspase-independent type III programmed cell death in chronic lymphocytic leukemia: the key role of the F-actin cytoskeleton

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ABSTRACT

Background

Programmed cell death has been traditionally related with caspase activation. However, it is now accepted that caspase-independent forms of programmed cell death also regulate cell death. In chronic lymphocytic leukemia, CD47 ligation induces one of these alternative forms of cell death: type III programmed cell death. This poorly understood process is characterized by cytoplasmic hallmarks, such as mitochondrial damage. To gain insights into the molecular pathways regulating type III programmed cell death in chronic lymphocytic leukemia, we performed extensive biochemical and cell biology assessments.

Design and Methods

After CD47 triggering, purified B-cells from 20 patients with chronic lymphocytic leukemia were studied by flow cytometry, immunofluorescence and three-dimensional imaging, immunoblotting, electron microscopy, and fibrillar/globular actin measurements. Finally, we subjected CD47-treated chronic lymphocytic leukemia cells to a phagocytosis assay.

Results

We first confirmed that induction of type III programmed cell death is an efficient means of triggering cell death in chronic lymphocytic leukemia. Further, we demonstrated that the signaling events induced by CD47 ligation provoked a reduction in cell size. This alteration is related to F-actin disruption, as the two other cytoskeleton networks, microtubules and intermediate filaments, remain undisturbed in type III programmed cell death. Strikingly, we revealed that the pharmacological modulation of F-actin dynamics regulated this type of death. Finally, our data delineated a new programmed cell death pathway in chronic lymphocytic leukemia initiated by CD47 triggering, and followed by serine protease activation, F-actin rearrangement, mitochondrial damage, phosphatidylserine exposure, and cell clearance.

Conclusions

Our work reveals a key molecular tool in the modulation of cell death in chronic lymphocytic leukemia: F-actin. By assessing the regulation of F-actin and type III programmed cell death, this analysis provides new options for destroying chronic lymphocytic leukemia cells, such as a combination of therapies based on apoptosis regulators (e.g., caspases, Bcl-2, Bax) along with alternative therapies based on type III death effectors (e.g., F-actin).

Key words: actin, apoptosis, caspase-independent cell death, CD47, chronic lymphocytic leukemia, mitochondria.

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal B cells ($CD20^+$, $CD5^+$, and $CD23^+$) in the blood, bone marrow, and peripheral lymphoid organs.[1] This disease is the perfect example of a human malignancy caused by an alteration in the ratio between cell proliferation and programmed cell death (PCD).[2] In fact, CLL was initially considered as a disease derived from an inherent defect in PCD.[3] However, more recent studies showed that the accumulation of leukemia cells is a consequence of deregulation in both proliferation and PCD.[2,4] The elucidation of the molecular pathways controlling PCD is, therefore, fundamental as it may provide new insights into understanding CLL physiopathology. Additionally, a better knowledge of the molecular mechanisms regulating cell death in CLL will help in the design of effective treatments against this leukemia (e.g., providing new targets for the development of specific PCD-based drugs).

In the last decade, the study of PCD focused on caspases, a family of cysteine proteases specifically activated in dying cells.[5] Surprisingly, inhibition of caspases has in fact revealed the existence of caspase-dependent and caspase-independent cell death programs.[6,7] As a consequence, PCD is now classified into type I, type II, or type III.[8-11] Type I PCD, or classical apoptosis, is caspase-dependent[5,12,13] and it can be triggered via *death receptors* along the *extrinsic pathway* or via the mitochondrial *intrinsic pathway*.[14] Type II PCD is morphologically characterized by the appearance of autophagic, double-membraned vacuoles.[15,16] These cytoplasmic vesicles contain cellular organelles, such as mitochondria or endoplasmic reticulum.[10] Type III PCD is the least well understood form of death and occurs without pronounced nuclear chromatin condensation.[17,18] Type II and type III PCD are caspase-independent.

The enormous amount of work performed to characterize type I PCD caspase-dependent apoptosis is related to the fact that most of the currently available chemotherapeutic agents kill tumor cells by triggering this kind of cell death. However, because malignant CLL cells are distinguished by defects in the apoptotic type I PCD machinery (e.g., p53 mutations, higher Bcl-2/Bax ratio) they can be resistant to the cytotoxic action of these drugs. Considering strategies to circumvent this resistance, an important question emerged: is it possible to use alternative caspase-independent PCD pathways to modulate PCD in CLL? The answer is, apparently, yes. In a recent study, we reported that, after CD47-triggering, CLL cells were efficiently killed by a caspase-independent type III PCD program.[17]

CD47 antigen is a widely expressed glycoprotein composed of a single IgV-like extracellular domain, a transmembrane region, and a short intracytoplasmic tail.[19,20] Intense research on this receptor showed that ligation of CD47 by immobilized specific monoclonal antibodies, thrombospondin-1 (TSP-1), or a peptide derived from TSP-1 (4N1K), induces PCD. The CD47-mediated type III PCD pathway is characterized by impairment of the mitochondrial electron transport chain and exposure of phosphatidylserine on the outer leaflet of the plasma membrane.[17,21] Future goals in type III PCD/CLL research are the identification of molecules/events reducing the lifespan of malignant B cells. This will be the basis for the subsequent development of therapeutic agents modulating these factors to control pathological PCD.

In order to identify the key elements involved in type III PCD, we conducted a multi-parametric biochemical and cell biology assessment of purified B-cells from 20 patients with CLL. This assessment revealed that: (i) The F-actin cytoskeleton is a key step in type III PCD; (ii) Pharmacological modulation of F-actin microfilament dynamics activated/abrogated this kind of death. F-actin is, therefore, a target in the modulation of type III PCD in CLL and seems to be an excellent candidate for the future development of PCD-based drugs aimed at caspase-independent cell death; (iii) Type III PCD is a good means of inducing cell death in CLL. Indeed, contrary to type I caspase-dependent PCD, the type III PCD program can be engaged even in CLL cells with a high Bcl-2/Bax ratio. Type III PCD could, therefore, overcome one of the most important problems encountered in cells resistant to type I PCD; (iv) CLL cells killed by type III PCD are efficiently eliminated by macrophages. This kind of death is, therefore, an efficient pathway that allows for the physiological elimination of dead cells; (v) Type III PCD is a highly regulated pathway of cell death in CLL. This is an important issue in the understanding of CLL biology. This death pathway, initiated by CD47 triggering, is followed by serine protease activation, F-actin damage, $\Delta\Psi m$ loss and reactive oxygen (ROS) generation, phosphatidylserine exposure in the outer leaflet of the plasma membrane, and cell engulfment.

Design and Methods

Patients, B-cell purification and culture conditions

After obtaining informed consent, peripheral blood was collected from 20 CLL patients diagnosed according to classical morphological and immunophenotypic criteria[22] (Table 1). The Institutional Ethics Committee at Pitie-Salpetriere Hospital approved this study. Mononuclear cells were purified from blood samples using a standard Ficoll-Hypaque gradient, and B cells were positively or negatively selected by magnetic microbeads coupled either to an anti-CD19 monoclonal antibody (positive selection) or to anti-CD16, CD3, and CD14 monoclonal antibodies (negative depletion) (Miltenyi Biotech). No changes were found in the cell death response of positively or negatively selected cells. B-lymphocytes were cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin). Unless specified, reagents were from Sigma-Aldrich.

Cell death induction and inhibition

To induce CD47-mediated cell death, cells were cultured with soluble TSP-1 (20 μg/mL, Calbiochem) or on pre-coated plates with CD47 monoclonal antibody (5 μg/mL, clone B6H12). In the indicated experiments, cells were treated for 20 h with hydrocortisone (0.5 mM). For

inhibition assays, cytochalasin D, latrunculin A, nocodazole, and colchicine were used at 5 μM, okadaïc acid was used at 100 nM, TPCK at 20 μM, Q-VD.OPh (QVD) at 10 μM, and z-VAD.fmk, z-DEVD.fmk, z-LEHD.fmk, and z-IETD.fmk at 50 μM.

Flow cytometry

We used 0.5 μM tetramethylrhodamineethylester (TMRE, Invitrogen) for ΔΨm assessment, 2 μM hydroethidine (Invitrogen) for detection of reactive oxygen species (ROS), annexin V-allophycocyanin (BD Biosciences) to determine phosphatidylserine (PS) exposure, and propidium iodide (0.5 μg/mL) to assess cell viability. Chymotrypsin-like serine protease cytofluorometric detection was performed with a SerPase™ kit from Imgenex. Data analysis was carried out in a FACScalibur (BD Biosciences) on the total cell population (10,000 cells).

Quantitative real-time reverse transcriptase polymerase chain reaction analysis

Total RNA from control or CLL cells was extracted with Trizol reagent (Invitrogen) according to standard procedures. Samples were examined in an ABI Prism 7000 sequence detector system with TaqMan™ Assays-on-demand™ Gene Expression Products (Applied Biosystems). Data were analyzed using the comparative Ct method following the manufacturer's protocol. The amount of Bcl-2 and Bax mRNA measured in CLL cells was normalized according to an endogenous reference (the human 18S housekeeping gene) and relative to a calibrator (B cells from control donors).

Fibrillar and globular actin assessment

The fibrillar/globular-actin ratio (F-actin/G-actin ratio) was determined by fluorometric assessment.[23] Excitation/emission filters were 485/538 and 544/590 nm for phalloidin-fluorescein isothiocyanate (F-actin, Sigma) and DNase-Alexa 594 (G-actin, Molecular Probes), respectively. One unit refers to the basal F-actin/G-actin ratio measured in 10^6 untreated cells. All reactions were recorded in a Fluoroskan Ascent™ Fluorimeter (Thermo Labsystems).

Phagocytosis assays

U937 monocytes were differentiated into macrophages with 10 ng/mL phorbol 12-myristate 13-acetate (Calbiochem).[24] Fluorescence microscopy assessment was performed with macrophages seeded on glass coverslips, as reported elsewhere.[25] After incubation of macrophages and JinB8 or CLL cells (3 h), coverslips were washed, fixed in 4% PFA for 15 min and stained with Hoescht 33342 before image acquisition. For flow cytometry quantification, macrophages were labeled with mouse monoclonal anti-CD13-allophycocyanin antibody (BD Biosciences), and JinB8 or CLL cells were labeled with 5 μM BODIPY® FL C_5-Ceramide (Molecular Probes) before the phagocytic meal. After 3 h of incubation, cells were analyzed and phagocytosis was scored as the percentage of double-positive cells.

Protein extractions and immunoblot

Mitochondrial and cytosolic fractions were obtained with the help of a kit from Pierce. Cell fractions were lysed in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA. Protein content was determined with the Bio-Rad DC kit and 30 mg of protein were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After blotting, PVDF filters were probed with anti Cox IV (Invitrogen), β-tubulin, or Drp1 (BD Biosciences). All were detected using standard procedures.

Immunofluorescence and imaging

Cells were fixed with 4% PFA and stained for the detection of cytochrome c or Drp1 (BD Biosciences). Proteins were detected according to standard procedures. The quantification of different parameters by fluorescence microscopy was performed in blind testing by at least two investigators, on 150 cells for each data point, and was repeated at least three times for different CLL patients. Images were visualized in an Apotome-equipped Zeiss Axioplan (Axiovert 200M, Zeiss) with an Apochromat 100x/1.4 objective, acquired with a CCD Roper Scientific Coolsnap HQ Camera, and analyzed using Axiovision 4.4 software.

For actin, tubulin, and vimentin, imaging cells were fixed with 4% PFA and maintained in liquid phosphate-

Table 1. Patients' characteristics.

Patient#	Age (yr)	Gender	Binet stage	% IGVH gene identity	% CD38	% ZAP-70	Soluble CD23 (ng/mL)
1	71	M	A	91.8	9	14	47
2	62	M	B	97.4	5	18	70
3	86	M	C	99	9	19	633
4	67	M	A	89	7	15	250
5	72	M	A	89	2	10	75
6	85	F	A	91	3	14	202
7	77	M	A	94.8	9	18	41
8	91	F	A	92.2	8	18	370
9	56	F	A	94.7	5	11	121
10	76	M	A	94	1	15	113
11	78	M	A	92.4	41	40	233
12	75	M	A	94	1	19	86
13	71	F	A	92.6	5	14	190
14	89	F	A	91.8	8	70	7.5
15	78	M	B	92.6	9	14	77
16	80	F	A	87.4	1	17	162
17	74	F	A	97.1	7	63	168
18	84	F	B	93	20	12	60
19	63	M	B	97	9	19	66
20	60	M	A	98.5	9	54	250

Gender: M: male; F: female. Binet stage: Binet stage of disease. % IGVH gene identity: IGVH sequence homology compared with the closest germ line counterpart. The sequences with a germ line homology of 98% or higher are normally considered unmutated and those with a homology of less than 98% mutated. % CD38 and % ZAP-70: protein expression. CD38 and ZAP-70 high expression levels are defined as more than 10% or 20% of positive cells, respectively. Soluble CD23 levels are: controls: <10 ng/mL; stable disease: <70 ng/mL; progressive disease: >70 ng/mL.

buffered saline medium. After staining, cells were loaded in an agar bed to preserve cell volume integrity. Analyses were performed using an inverted laser-scanning confocal microscope Zeiss Axiovert 200M with an Apochromat 100x/1.4 objective. Three-dimensional image acquisition was done with a Z-stack on each 150 nm panel and three-dimensional reconstruction was achieved using the *Iso-surface* module of IMARIS software (Bitplane). Fluorescence quantification was recorded using a single-imaging frame collection and ImageJ 1.34-s software (post-acquisition analysis).

Electron microscopy

Cells were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4) for 2 h at room temperature, washed, and post-fixed in 2% OsO_4 before being embedded in Durcupan™. Analyses were performed as previously described.[17]

Statistical analysis

The significance of differences between experimental data was determined using Student's *t* test for unpaired observations.

Results

Phosphatidylserine exposure, mitochondrial damage, serpase activation, and cell size reduction characterize CD47-mediated type III programmed cell death in chronic lymphocytic leukemia cells

We previously reported that CD47 ligation by an immobilized anti-CD47 monoclonal antibody, or by its natural ligand, thrombospondin-1 (TSP-1), induces type III PCD in CLL cells.[17] This type of death, in which dynamin-related protein-1 (Drp1) and mitochondria play key roles, is exclusively characterized by cytoplasmic hallmarks. They include exposure of PS in the outer leaflet of the plasma membrane (Figure 1A), a decrease in mitochondrial transmembrane potential ($\Delta\Psi m$; Figure 1B), and production of reactive oxygen species (ROS) (Figure 1C).

Morphologically, type III PCD is not marked by signs of nuclear condensation. In contrast, mitochondria undergo striking morphological changes[17] (Figure 1D). Through a new confocal and cytofluorometric assessment we found here that CD47 ligation provoked a significant reduction in cell size (Figure 1E). Untreated CLL cells had a diameter of around 6.585 ± 0.058 μm whereas after 1 h of treatment with anti-CD47 monoclonal antibody the cells could be distinguished into two groups. Cells in the first group (G1; 46% of cells) were of a similar size to untreated cells. The second group (G2; 54% of the total population) showed a 10% decrease in cell diameter (5.656 ± 0.033 μm). The percentage of smaller cells (G2 group) correlated strictly with the percentage of cells showing PS exposure, $\Delta\Psi m$ loss, and ROS production after CD47 ligation (Figures 1A, B, C, and E). Therefore, as has been described for other types of cell death (e.g., type I PCD), our data revealed that the reduction in cell size is a morphological hallmark of type III PCD.

Type III PCD is considered to be caspase-independent since the PS exposure induced by CD47 ligation is not prevented by the presence of either broad or specific caspase inhibitors (Figure 1F). In this way, the main effector caspases, such as caspase-3, -7, or -9, remain inactive proenzymes after CD47 triggering.[17,21,25] In spite of this, type III PCD specifically involves the activation of a family of proteases: chymotrypsin-like serine proteases, a TPCK-inhibitable family of proteases also named serpases (Figure 1G).

Type III programmed cell death, an efficient programmed cell death pathway in chronic lymphocytic leukemia

To give a more translational perspective to the above-described approach, we analyzed the response of a panel of CLL cells to CD47 triggering. Indeed, it seemed important to know whether type III PCD is a general PCD pathway in this form of leukemia. To this end, we first assessed the response of B cells from 20 CLL patients to CD47 ligation (see Table 1 for the patients' characteristics). These B cells have similar CD47 and Drp1 levels. In addition, we analyzed the response of the same cells to treatment with a typical type I caspase-dependent PCD mediator, hydrocortisone. As depicted in Figure 1H, CLL cells had a different response to type I and type III PCD inducers and, when comparing CD47-mediated PCD and hydrocortisone-induced apoptosis, CD47 ligation provided a more efficient means of inducing death in 50% of B cells. In fact, our comparative analysis distinguished two groups of CLL patients. In B cells from 10 patients (group A in Figure 1H), the response to anti-CD47 monoclonal antibody treatment was comparable to that in response to hydrocortisone treatment. In contrast, CD47 ligation appeared to be a more efficient means of inducing PCD in B cells from the CLL patients in group B (Figure 1H, patients # 2, 3, 6, 11, 14, 15, 17, 18, 19, and 20).

To understand the different behavior in the response of CLL cells to hydrocortisone-mediated type I PCD and anti-CD47 monoclonal antibody-mediated type III PCD, we assessed the levels of Bcl-2 and Bax mRNA transcripts by quantitative polymerase chain reaction (Figure 1H, right panel). The Bcl-2 family of proteins that regulates type I PCD does not seem to be involved in the modulation of type III PCD.[17] It, therefore, seems plausible that high ratios of anti-apoptotic Bcl-2/pro-apoptotic Bax proteins regulate hydrocortisone-mediated apoptosis but not anti-CD47 monoclonal antibody-mediated death. This was indeed the case, as B cells displaying high Bcl-2/Bax ratios (≥4.5; group B) were less responsive to hydrocortisone. However, the Bcl-2/Bax ratio had no influence on the response of CLL cells to anti-CD47 monoclonal antibody. These results revealed that, even in apoptotic type I PCD resistant cells, it is possible to cause cell death through the induction of type III PCD. It seems, therefore, that type III PCD is a general pathway in CLL. In this sense, cells from the entire set of patients presented signs of type III PCD, regardless of any prognostic difference.

Overall, our assessment indicates that CD47-mediated PCD could represent a good means of inducing PCD in CLL. This is an important issue when considering this type of cell death in the design of novel PCD-based chemotherapeutic agents.

CD47-mediated programmed cell death involves a morphological alteration in the actin microfilament network

The mitochondrial alterations characterizing type III PCD (Figures 1B, C, and D)[17] led us to extend our field of study to the cytoskeleton. In fact, depolymerization or cleavage of actin, cytokeratins, and other cytoskeletal proteins have been incriminated in the alteration of mitochondrial function and morphology.[13,26]

Previous work showing that CD47-mediated PCD is regulated by actin-associated proteins such as WASP,[25] supports this view, as does the reduced size of cells treated with anti-CD47 monoclonal antibody (Figure 1E).

To determine whether the cytoskeleton was perturbed in CD47-treated cells, we first investigated, through confocal imaging, the cellular distribution and the fluorescence intensity of the three known cytoskeleton networks: actin microfilaments, microtubules, and interme-

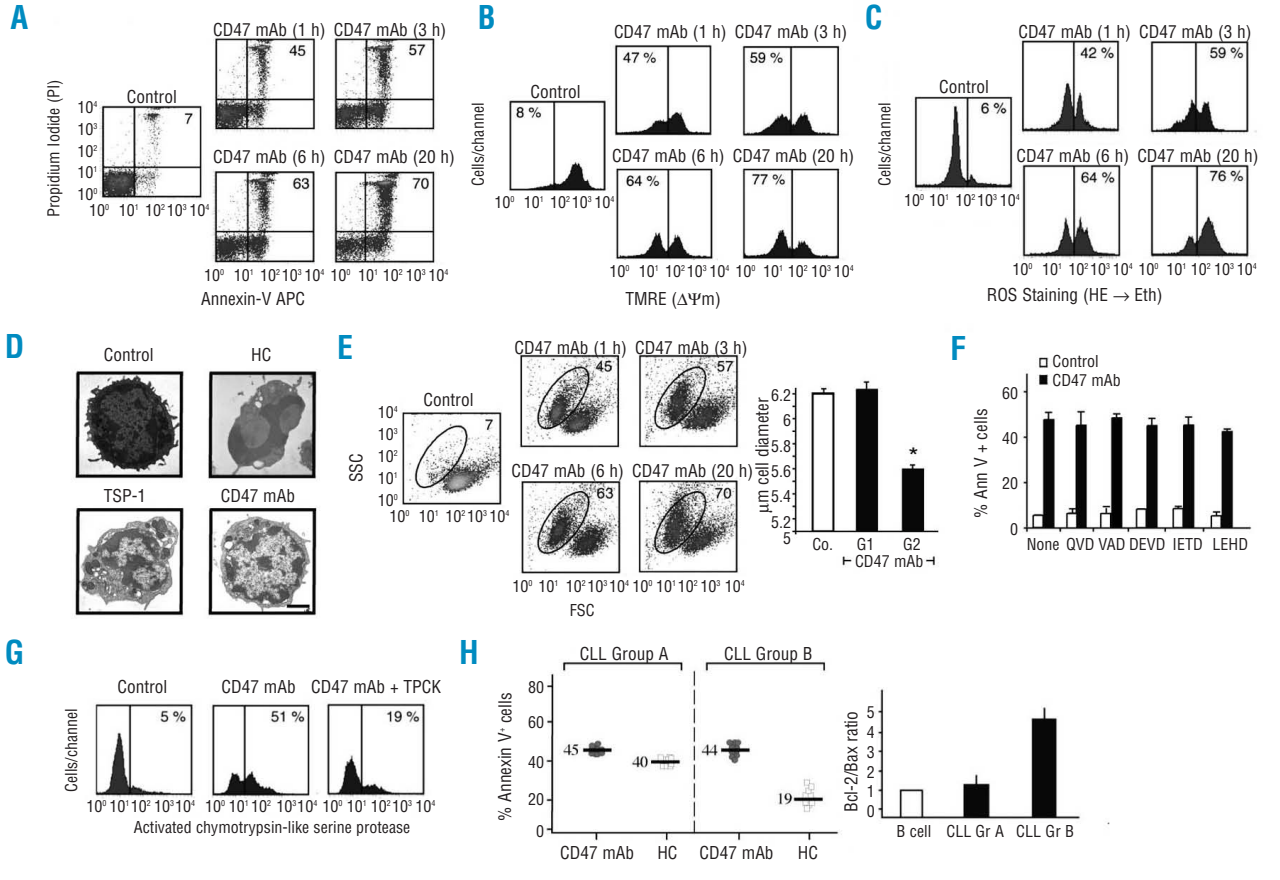

Figure 1. CD47 ligation induces type III PCD. (A) Plasma membrane and cell viability alterations induced by CD47 monoclonal antibody (mAb) ligation at different times. Chronic lymphocytic leukemia (CLL) cells were labeled with annexin V-APC and propidium iodide (PI). Numbers reflect the percentage of annexin V$^+$ and annexin V$^+$/PI$^+$ cells. (B) $\Delta\Psi$m dissipation provoked by CD47 mAb triggering in CLL cells. After the indicated time, cells were labeled with TMRE. The percentages refer to cells with low $\Delta\Psi$m. (C) ROS production provoked by CD47 mAb treatment at different times. CLL cells were labeled with HE, and the cells presenting ROS are shown as a percentage of total cells. Experiments depicted in (A), (B), and (C) were carried out in the set of 20 CLL patients used in Figure 1H below, yielding lower interexperimental variability (<5%). (D) Electron micrographs of CLL cells from a representative CLL donor, left untreated or incubated with hydrocortisone (HC) (type I PCD positive control), TSP-1, or CD47 mAb (1 h). Right panels represent the typical mitochondrial morphology observed after TSP-1 or CD47 mAb treatment. Bar = 1 μm. (E) Changes in cellular size induced by CD47 mAb treatment in CLL cells measured by flow cytometry and immunofluorescence. In flow cytometry panels, the percentages refer to gated cells. FSC: Forward Scatter; SSC: Side Scatter. Note that, after CD47 mAb-treatment, the numbers of cells with reduced size (reduced FSC) increase in a time-dependent manner. In plots, CLL cells untreated (Control) or incubated with CD47 mAb (1 h) were measured, quantified, and plotted as a percentage of total cells. CD47 mAb-treated cells were divided into two groups according to their size: G1 = similar size to untreated cells (46% total cell population); G2 = cells with reduced diameter (54% of total cell population). Cell size was measured in 150 untreated cells and 150 CD47 mAb-treated CLL cells. Data are the means ± SD of five independent experiments. *$p < 0.01$, unpaired Student's *t* test. (F) Assessment of cell death in CLL cells pre-incubated (30 min) with pan-caspase-inhibitors QVD or z-VAD.fmk, or specific inhibitors of caspase-3 and -7 (z-DEVD.fmk), -8 and -10 (z-IETD.fmk), or -9 (z-LEHD.fmk) before induction of death by CD47 mAb (1 h). Results are the means of five independent experiments ± SD. (G) B lymphocytes from a representative CLL donor were left untreated (Control), or incubated with CD47 mAb (1 h) or CD47 mAb + TPCK (20 μM), before assessment of chymotrypsin-like serine protease activity with a green fluorescent SerPase™ kit (FFCK). Note that CD47 triggering provokes positive staining and that TPCK inhibits serpase labeling. Numbers indicate the percentage of cells positively stained. The experiment was repeated four times with a lower variability (<5%). (H) B-lymphocytes from 20 CLL patients (see Table 1) were cultured for 1 h in the presence of CD47 mAb (circles) or 20 h in the presence of the apoptotic inducer HC (squares). The percentage of annexin V$^+$ cells is shown. Median values are shown as black bars. B-cells were divided into two groups according to their relative sensitivity to CD47 mAb and HC (see Results section for details). Right histogram: Bcl-2 and Bax mRNA ratios obtained from healthy (B cell) or CLL B lymphocytes from the two groups defined above. Results are expressed as the mean ± S.E.M.

diate filaments. In untreated CLL cells, the actin network appeared as a fluorescence surrounding the nucleus with a homogeneous distribution, the tubulin network as tubular structures at the periphery of the nucleus, and the vimentin network as uniformly distributed punctuate structures (Figure 2A). CD47 ligation selectively induced a change in actin microfilaments, which were then represented by a discontinuous pattern and formation of actin aggregates (Figure 2A). The other two networks, microtubules (e.g., tubulin) and intermediate filaments (e.g., vimentin), remained undisturbed or only slightly modified (Figure 2A). In a first attempt to quantify these alterations in the cytoskeletal pattern, we measured fluorescence intensity in untreated and anti-CD47 monoclonal antibody-treated CLL cells (Figure 2A). Total fluorescence intensity in the actin network was 490±33 in untreated cells and 248±28 in the CLL-treated cells of reduced size (49% loss of fluorescence). With regards to the tubulin network, total fluorescence intensity in untreated cells was 454±28, and 440±30 in CLL-treated cells. For the intermediate filaments, total fluorescence intensity was 727±36 in untreated cells and 750±46 in CD47-treated cells. Thus, after CD47 ligation, the morphological modifications detected in the actin cytoskeleton were accompanied by a relevant loss in fluorescence intensity. As observed by the absence of alterations in tubulin and intermediate filaments, fluorescence intensity remained unchanged. Additional evidence of the morphological changes induced by CD47 triggering in the actin microfilaments was found using a three-dimensional-reconstruction-based assay (Figure 2B and *Supplementary Movies 1 to 6*). This approach, in which we Z-scanned the entire CLL cell, fully corroborated the discontinuous pattern and the formation of actin aggregates in CLL cells after CD47 triggering.

Actin microfilament rearrangements control CD47-mediated type III programmed cell death

The morphological changes observed in the cytoskeleton of CD47-treated cells led us to evaluate a possible role of this cellular compartment in the regulation of CD47-mediated PCD. A pharmacological analysis demonstrated that actin-dynamic inhibitors such as cytochalasin D or latrunculin A precluded CD47 PCD, while microtubule-interfering agents (e.g., nocodazole, colchicine or taxol) or inhibitors of intermediate filament dynamics (e.g., okadaïc acid) failed to modulate this kind of death (Figure 3A). In the same way, pre-treatment of CLL cells with cytochalasin D eliminated the mitochondrial morphological changes induced by CD47 triggering (Figure 3B). A similar outcome was observed in the entire set of CLL patients described in Figure 1H. Overall, these data reveal a new role for the actin cytoskeleton network in controlling type III PCD.

This important result led us to further characterize this network in type III PCD. We first measured the intracellular F/G-actin ratio (F = fibrillar = polymerized-actin; G = globular = depolymerized-actin)[23] at time intervals in cells treated with TSP-1 or anti-CD47 monoclonal antibody. This approach showed that the F/G-actin ratio decreased in a time-dependent manner with similar kinetics to CD47-mediated death, indicating that CD47 ligation provoked actin depolymerization (Figure 3C). Strikingly, we demonstrated that CD47 triggering not only induced F-actin disruption but also actin degradation (Figure 3D). This certainly explains the loss of fluorescence intensity in the actin cytoskeleton of CD47-treated cells (Figure 2). Interestingly, inhibition of the actin dynamics or blockage of the serpases by TPCK controlled both actin damage and degradation (Figure 3D). Thus, it seems that this family of serine proteases regulates CD47-mediated PCD upstream of the actin cytoskeleton.

Hierarchical relationship between serpases, F-actin damage, and mitochondria in CD47-mediated programmed cell death

The serpase-dependent actin injury depicted in Figure 3 has revealed a new role for this family of proteases and F-actin in controlling type III PCD. Indeed, together with our previous results,[17] our new data strongly suggest a

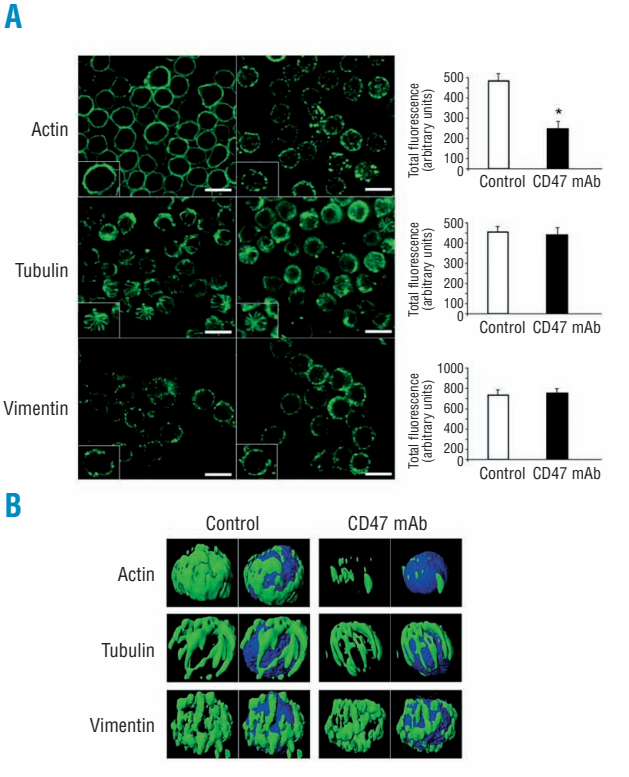

Figure 2. CD47 ligation specifically provokes actin microfilament rearrangements: evidence from fluorescence quantification and 3D-reconstruction. (A) CLL cells were cultured in the absence (Control) or presence of CD47 monoclonal antibody (mAb) for 1 h, and subjected to immunofluorescent detection of actin-microfilaments (actin), microtubules (tubulin), and intermediate filaments (vimentin). Representative micrographs of each stain are shown. Individual cells are representative of the dominant phenotype. Plots depict the statistical analysis of total fluorescence. Bar = 8 μm. This experiment was repeated eight times, yielding comparable results. *, ($p < 0.01$, unpaired Student's *t* test). (B) 3D-reconstruction of CLL cells cultured in the absence (Control) or presence of CD47 mAb as in (a), and subjected to confocal assessment of actin, tubulin, and vimentin. 3D-micrographs for the dominant phenotype of each cytoskeleton stain are shown. The same cell is represented in the absence (left panel)/presence (right panel) of nucleus. Blue = Hoestch 33342 nuclear staining, green = specific network labeled. The experiment was repeated four times with low variability.

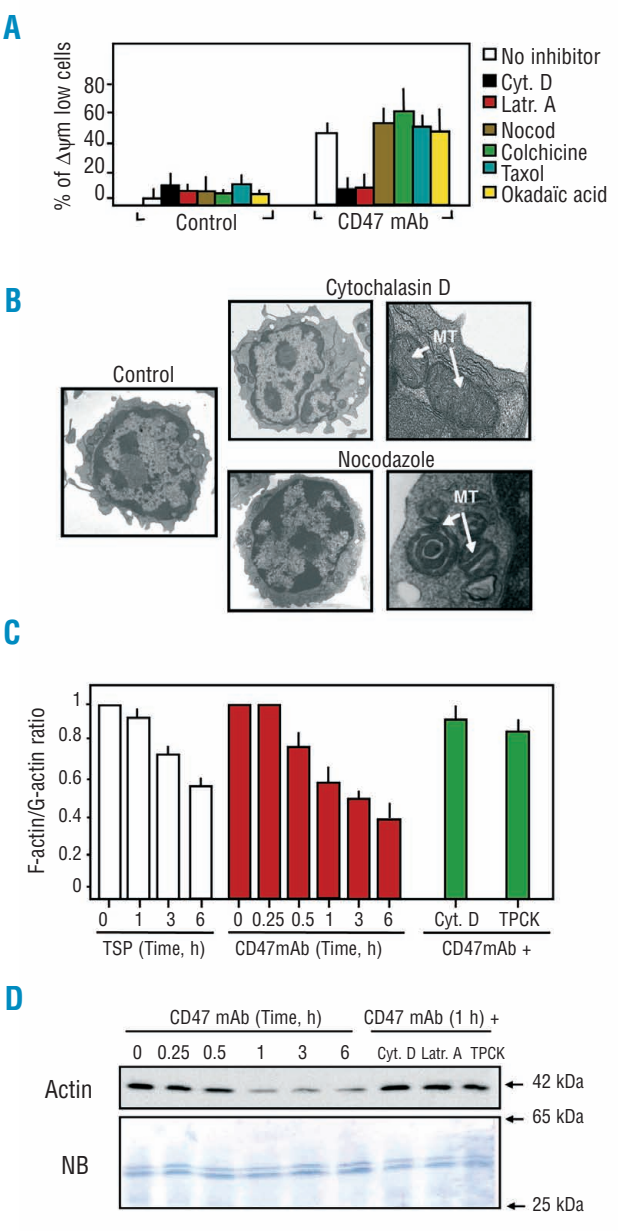

Figure 3. CD47 ligation induces type III PCD via F-actin depolymerization and degradation. (A) ΔΨm dissipation measured in CLL cells treated 1 h with CD47 monoclonal antibody (mAb) in the absence (no inhibitor) or presence of actin-dynamic inhibitors (cytochalasin D and latrunculin A), microtubule-interfering agents (nocodazole, colchicine and taxol), or the intermediate filament disassembler, okadaic acid. Histograms indicate the percentage of cells presenting ΔΨm loss. Data are the mean of five independent experiments ± SD. (B) Electron microscopy views of CLL cells untreated (Control) or pre-incubated (30 min) with cytochalasin D or nocodazole before being treated with CD47 mAb (1 h). Representative mitochondrial micrographs (left panels) show the normal mitochondrial morphology observed after cytochalasin D pre-treatment (upper panel). In contrast, nocodazole did not prevent the mitochondrial modifications provoked by CD47 triggering (lower panel). MT = mitochondria. (C) CLL cells were exposed to CD47 mAb or TSP-1 at different times or pretreated with cytochalasin D or TPCK (30 min) before being treated with CD47 mAb (1 h), and the fluorescence F/G actin ratio was quantified. One unit refers to the basal F/G actin ratio scored in untreated cells. Data are the mean of five independent experiments ± SD. (D) CLL cells were exposed to CD47 mAb at different times or pretreated (30 min) with cytochalasin D, latrunculin A, or TPCK before being treated with CD47 mAb (1 h), and actin was analyzed by immunoblotting in total cell extracts. PVDF membrane was stained with naphtol blue to control protein loading.

hierarchical relationship between serpases, F-actin damage, and mitochondria in this PCD program. To verify this possibility and to determine the sequence of events characterizing CD47-mediated PCD, we used a cytofluorimetric approach. Our working hypothesis was that the serine proteases are activated shortly after CD47 triggering, and before F-actin damage and the mitochondrial alterations representing this type of PCD. We, therefore, chose a suitable time frame (5 to 60 min) to evaluate serpase activation and the modifications in the actin network, and comparatively analyzed CLL cells untreated or treated with anti-CD47 monoclonal antibody. As shown in Figure 4A, serpases were activated as soon as 10-15 min after CD47 triggering. Importantly, at this time point, the actin network still remained undisturbed (Figure 3C). In fact, actin became disrupted only 30 min after CD47 triggering. These results confirm the activation of serpases at an early pre-cytoskeleton step in CD47-mediated PCD. In this way, in contrast to TPCK, neither cytochalasin D nor latrunculin A has an influence on serpase activation (Figure 4A, right panels). However, they inhibit actin degradation (Figure 3D), ΔΨm dissipation (Figure 3A), and the morphological changes induced by CD47 triggering in mitochondria (Figure 3B). Therefore we could propose a sequence of events in which CD47 triggering provokes serpase activation and F-actin rearrangements upstream of the mitochondrial changes characterizing CD47-mediated PCD.

We next sought to integrate our previously reported data on Drp1[17] with the sequence of events described above. In this context, we showed that Drp1 redistribution from the cytosol to mitochondria was actin-related, since pre-treatment of CLL cells with cytochalasin D precluded the mitochondrial relocation of Drp1 (Figure 4B, and C). Nocodazole, which does not inhibit mitochondrial damage or type III PCD, also failed to block the mitochondrial relocation of the protein (Figure 4C). As expected, the serpase inhibitor TPCK alleviated Drp1 mitochondrial redistribution (Figure 4C).

Altogether, these data reveal a sequence of events in which, after CD47 ligation, the activation of serpases is followed by F-actin network disruption, and Drp1 redistribution from the cytosol to mitochondria.

CD47-mediated type III programmed cell death and cell clearance

Type III PCD has often been viewed as an accidental and uncontrolled process of PCD.[10] In this way, its putative role in the elimination of tumor cells is poorly understood. However, despite this view of uncontrolled type III PCD, our results indicate a finely regulated mechanism. Following these new data, a key question emerged: are the cells that die by type III PCD recognized and engulfed by macrophages? To answer this important question, we monitored whether CD47-treated cells are phagocyted by macrophages. CLL cells undergoing hydrocortisone induced (positive control) or CD47-mediated death were cultured with CD13-labeled macrophages and analyzed by immunofluorescence and flow cytometry following a previously described method.[24] This analysis indicated that, like hydrocortisone (a classical type I apoptotic inducer), CD47-induced type III PCD is an efficient path-

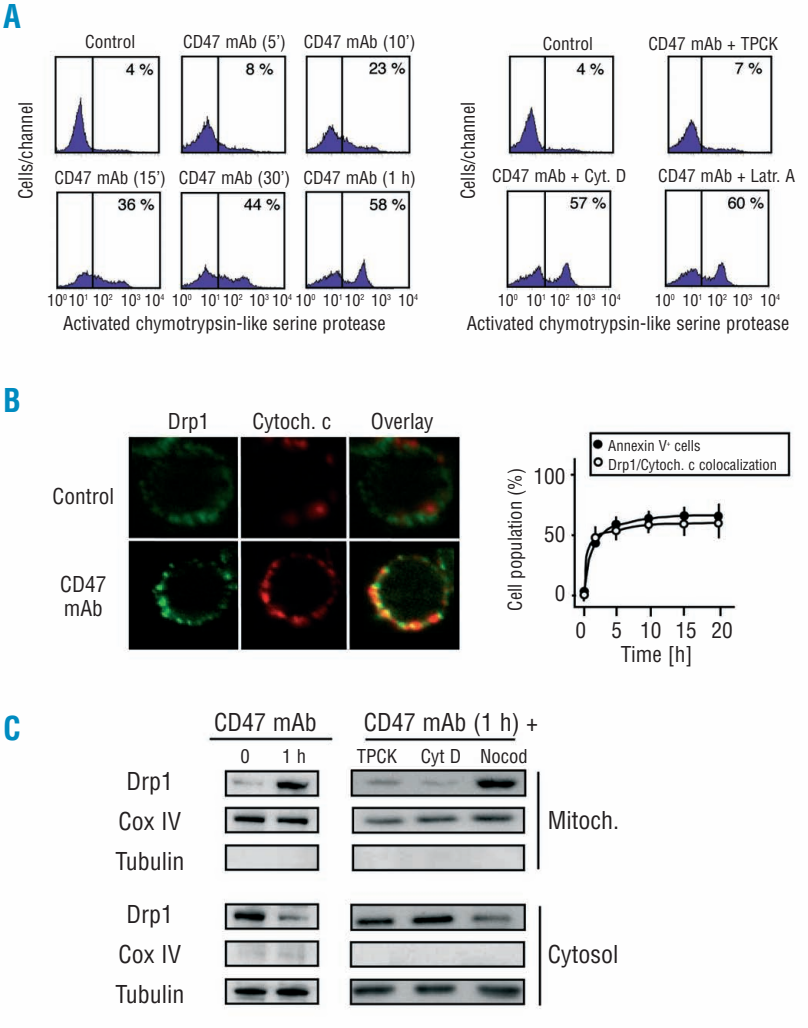

Figure 4. Hierarchical involvement of serine proteases, F-actin, and Drp1 in CD47-mediated type III PCD. (A) B-lymphocytes from a representative CLL donor were left untreated (Control), or incubated with CD47 monoclonal antibody (mAb) at different times before assessment of chymotrypsin-like serine protease activity with a SerPase™ kit as in Figure 1F. Numbers indicate the percentage of cells positively stained. The experiment was repeated four times with a low variability (<5%). In a similar experiment, B-lymphocytes from the same CLL donor were left untreated (Control) or pretreated with TPCK, cytochalasin D, or latrunculin A 30 min before being treated with CD47 mAb (1 h). Numbers indicate the percentage of cells positively stained. Note that only TPCK inhibits serine protease activation. The experiment was repeated three times with a low interexperimental variability (<5%). (B) CLL cells were left untreated (Control) or treated with CD47 mAb for 1 h, and stained for the detection of Drp1 and cytochrome c (Cytoch. c, used as a mitochondrial marker), before being examined by fluorescent microscopy. Representative cells show that Drp1 has a cytosolic distribution in control cells, whereas it co-localizes with cytochrome c in CD47-treated cells. The number of cells presenting positive annexin V labeling (measured by flow cytometry), or Drp1/cytochrome c colocalization was quantified and plotted as a percentage of total cells. Data are the means ± SD of five independent experiments. (C) Drp1 mitochondrial redeployment detected by immunoblotting. CLL cells were left untreated, treated with CD47 mAb for 1 h, or pre-incubated with TPCK (20 μM), cytochalasin D, or nocodazole (30 min) before being treated with CD47 mAb (1 h). Cells were subjected to subcellular fractionation and mitochondrial and cytosolic fractions were blotted for Drp1 immunodetection. Fractionation quality and protein loading were verified by the distribution of the specific subcellular markers: Cox IV for mitochondria and tubulin for cytosol.

way enabling dying cells to be recognized and engulfed (Figures 5A and B). Control experiments performed with caspase and serpase inhibitors, modulators of actin dynamics, a CD47$^{-/-}$ cell line (JinB8),[27] and a double approach showing that the number of dying cells is strictly related to the number of engulfed cells, all confirmed that CD47-mediated PCD is an efficient pathway for eliminating dying cells (Figures 5B and C).

Discussion

Drug resistance limits the effectiveness of existing medical treatments and is a major challenge in the current research on PCD. In the last few years, PCD-based pharmacological therapies have been mainly focused on the apoptosis type I PCD pathway and the main regulators of this type of cell death (e.g., caspases or the Bcl-2 family of proteins). Theoretically, modulation of PCD using caspase/Bcl-2 regulators could be an effective treatment for cancer. Unfortunately, most of these studies are still in the stage of preclinical development because of the relatively low efficacy of the agents. This is certainly related to the resistance developed by tumor cells against type I PCD. Hence, there is enormous interest in designing new therapeutic agents to modulate key molecules involved in non-apoptotic or caspase-independent PCD pathways. Emerging knowledge about these death pathways have revealed new potential strategies for medical therapy. In this sense, type III PCD is a very promising target in CLL. On the one hand, our data confirm that it is a general PCD pathway. On the other hand, this death pathway seems to be independent of the main apoptotic proteins altered in CLL, such as Bcl-2, Bcl-XL, Mcl-1, Bax, Bak, or Bim.[17]

Through a large assessment, we have described here a newly discovered sequence of events characterizing type III caspase-independent PCD. Briefly, we have shown that ligation of the CD47 receptor leads, by means of serpases, to F-actin injury and the translocation of Drp1 from the cytosol to mitochondria (Figure 6). Importantly, F-actin

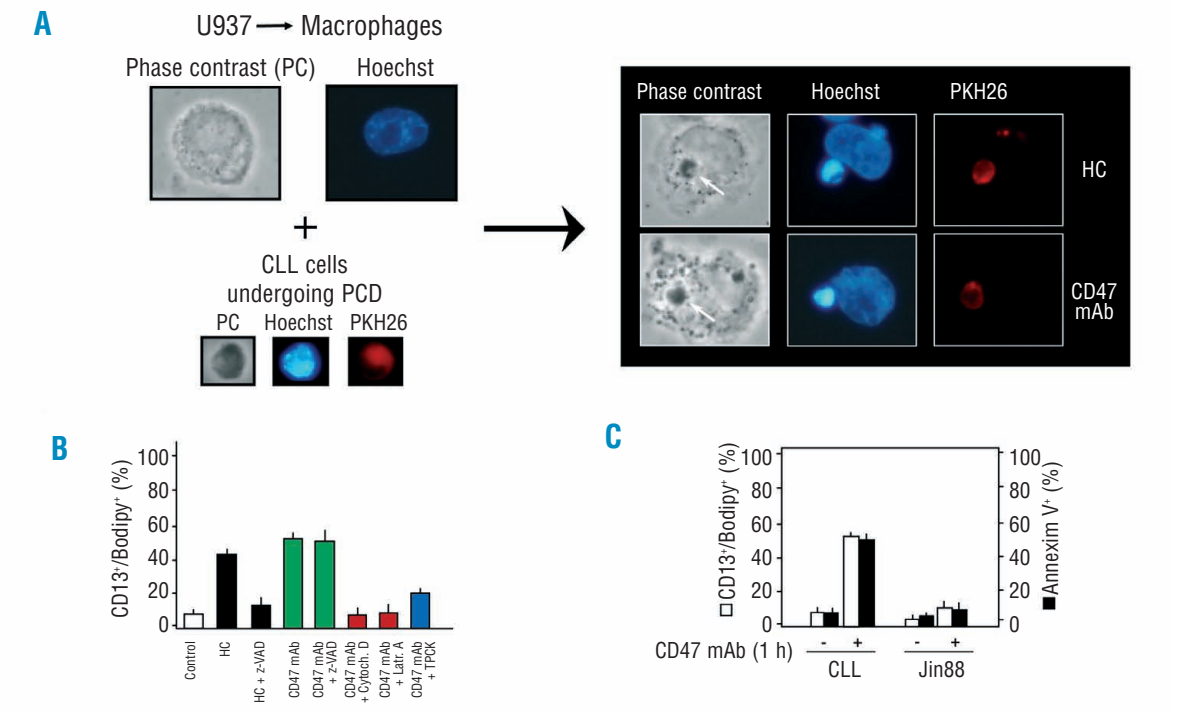

Figure 5. CD47-mediated type III PCD induces cell death clearance. (A) CLL cells were stained in red with PKH26 linker before treatment with hydrocortisone (HC) or CD47 monoclonal antibody (mAb) (1 h). Cells were then cultured for 3 h with U937 cells differentiated to macrophages at a 1:1 ratio, and the mixture analyzed by immunofluorescence. Cells were stained with Hoeschst 33342 to visualize nuclei and chromatin condensation. Phase contrast was used to localize cells. White arrows mark engulfed CLL cells. (B) Quantification of CLL cell engulfment. In a similar experiment to (A), CLL cells were left untreated or treated with HC, HC + z-VAD.fmk, CD47 mAb (1 h), CD47 mAb (1 h) + z-VAD.fmk, or pretreated with cytochalasin D, latrunculin A, or TPCK (20 μM) before being treated with CD47 mAb for 1 h, and stained with BODIPY® FL C5-Ceramide (green) and macrophages were labeled with anti-CD13-APC antibody (red). After co-culture, the mixture was analyzed by flow cytometry and the double positive cells (macrophages containing apoptotic cells) quantified and expressed as a percentage. Values are shown as the mean ± SD (n=6). (C) Relationships between CLL or JinB8 cell engulfment and death. Cells were stained in green with BODIPY® FL C5-Ceramide before being left untreated or treated with CD47 mAb (1 h). Cells were then cultured with macrophages differentiated from U937 cells labeled with anti-CD13-APC antibody and analyzed as in (B). Values are shown as the mean ± SD (n=5). Alternatively, cells were labeled with annexin V-APC to assess cell death. The percentage of annexin V+ cells was recorded and expressed as a histogram. Note that, in contrast to CLL cells, JinB8 (a CD47$^{-/-}$ cell line) cells are resistant to CD47 mAb ligation.

rearrangement is a key step in type III PCD. At least three main observations support this assertion. First, depolymerization of the actin network is a constant feature in this type of PCD. Second, inhibitors of actin dynamics (e.g., latrunculin A or cytochalasin D) abolish the hallmarks of this type of PCD (e.g., mitochondrial alterations, PS exposure, and cell clearance). In contrast, inhibitors of the other two cytoskeleton networks (e.g., nocodazole or okadaïc acid) do not prevent type III PCD. Finally, CD47 triggering selectively induces a change in actin microfilaments, while the other two networks, microtubules and intermediate filaments, remain undisturbed. Importantly, F actin disruption/degradation and the control mediated by the serpases are essential to an understanding of the interplay between the multiple type III PCD signals.

Our results place serpases and cytoskeleton rearrangements at an early pre-mitochondrial step in caspase-independent type III PCD and indicate a hierarchical relationship between serpases, F-actin rearrangement, and Drp1 redistribution from the cytosol to mitochondria. A more specific analysis could now be developed to understand the relationship between serpases, actin, Drp1, and other proteins previously implicated in CD47-mediated PCD, such as WASP,[25] protein kinase C θ,[28] or protein kinase A.[29]

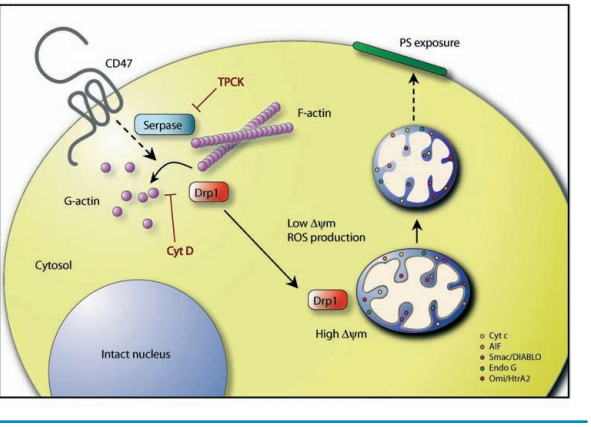

Figure 6. Schematic model for CD47-mediated type III PCD. Ligation of the CD47 receptor led, by means of the serpases, to F-actin depolymerization and translocation of dynamin-related protein 1 (Drp1) from the cytosol to mitochondria, where the protein induced mitochondrial damage characterized by ΔΨm collapse, ROS production, and no release of proapoptotic proteins from the intermembrane space.[17] These alterations are followed by phosphatidylserine (PS) externalization and the efficient clearance of dying cells. Serpase inactivation by TPCK, and actin dynamics modulation by Cyt D (cytochalasin D), block this PCD pathway. ROS indicates reactive oxygen species; Cyt c cytochrome c; AIF apoptosis inducing factor; Endo G endonuclease G.

In this context, it is also interesting to highlight the resemblances between CD47 and CD99 cell death programs. Both receptors induce a rapid caspase-independent PCD related to actin cytoskeleton dynamics.[30]

Despite the marked differences characterizing type I and type III PCD, it is interesting to note that our work revealed that these two types of death share common biochemical features, namely outer leaflet exposure of PS in the plasma membrane, alterations to mitochondria, production of ROS, and cell engulfment. From the standpoint of PCD, the common biochemical features in type I and III PCD indicate that these two pathways leading to cell death could be different facets of similar PCD processes. Thus, unraveling the mechanisms governing type III PCD could lead to a better understanding of PCD in CLL.

Finally, our observations indicated that the CD47/TSP-1 link could play a role in the elimination of tumor cells. TSP-1, secreted by macrophages and dendritic cells, may contribute to the elimination of tumor cells and even to the maintenance of immune B-cell homeostasis. Indeed, rapid recognition and clearance of dying cells by phagocytes play pivotal roles in the control of immune responses and resolution of inflammation.[18,31] Great progress has been made during the last few years in identifying the molecules on the surface of type I apoptotic cells, but it is still not clear how type III PCD dying cells are recognized by macrophages. It seems that these cells are internalized by a macropinocytotic mechanism[32] and that PS exposure mediates recognition and engulfment. Our data seem to confirm this assertion and indicate that PS-mediated clearance could be a general mechanism irrespective of the way a cell dies. Certainly, the cell death system that we have identified is an interesting way for deciphering the precise molecular mechanisms of recognition and phagocytic uptake of cells killed by caspase-independent apoptosis.

Future goals in research associating CLL and PCD include the development of therapeutic agents that provoke leukemic cell death. Our experiments, performed in B cells from 20 CLL patients, show that CD47 ligation provokes PCD rapidly and with a high efficacy. Importantly, our molecular data further imply the existence of a biochemical type III PCD pathway that could be regulated to target CLL cells. The induction of type III PCD can, therefore, be used in CLL as a potential means of bypassing otherwise blocked PCD pathways. Moreover, in a complementary approach, chemotherapeutic efficacy could also be improved by combining therapies that target more than one single cell death effector (e.g. caspases and F-actin).

We can propose four strategies for using type III PCD and, more specifically, F-actin in the enhancement of the cell death response in CLL: (i) Based on previous studies carried out in other cellular systems, a first interesting therapeutic approach to regulating type III PCD in CLL is the modulation of F-actin dynamics. For example, addition of jasplakinolide, a drug that stabilizes the actin cytoskeleton and induces accumulation of large F-actin aggregates, increases PCD in human Jurkat T cells and interleukin-2 (IL-2)-dependent lymphocytes.[33] Incubation of cells with cytochalasin D also modulates PCD in actinomycin-induced cell death.[34] Similarly, down-regulation of the actin protein, gelsolin, stabilizes the actin cytoskeleton and regulates apoptosis.[33] In this system, treatment with cytochalasin D protects cells from death. (ii) In addition to the actin stabilization/depolymerization possibility, a second approach to regulating F-actin-mediated PCD is the simple alteration of F- or G-actin status. This is sufficient to induce death.[36] (iii) Recent data suggest that alterations in the activity of actin regulatory proteins, such as coronin, gelsolin, β-thymosins, and cofilin, play crucial roles in the regulation of PCD in mammals.[36] These proteins are potential targets to provoke PCD through F-actin disruption. (iv) Residing in the F-actin cytoskeleton,[37] Drp1 translocated to mitochondria where this protein provoked death after CD47 triggering.[17] This result indicates that the Drp1-cytoskeleton link was disturbed after CD47-ligation. Thus, interference in Drp1-cytoskeleton binding could be used to enhance Drp1 redistribution from the cytoskeleton to mitochondria and, consequently, to enhance type III PCD.[38]

Given the growing understanding of the complexity of the PCD phenomenon, it is becoming clear that targeting only caspase pathways is often not sufficient (or efficient) for the treatment of diseases with disorders in proliferation/death equilibrium, such as CLL. A comprehensive analysis of caspase-independent cell death pathways thus offers a new challenge in the design of drugs targeting PCD more broadly. Regarding type III PCD, our results demonstrated that it is possible to provoke PCD in cells with a deficient mechanism of apoptotic (type I) cell destruction. This is good news, implying that specific type III PCD pathways can be targeted in CLL *independently* of classical caspase-dependent apoptotic pathways. This will certainly help in avoiding ineffective treatments. In any case, investigating the various cell death cascades will potentially lead to a deeper understanding of the physiopathology of hematologic diseases, such as chronic lymphocytic leukemia.

Authorship and Disclosures

SB, LC, MB, PS, GR, CV, VJY, and JEE designed and performed research and analyzed data; MR and SB provide vital reagents; MS and HMB provided vital reagents and revised the manuscript; SAS designed research, analyzed data, and wrote the manuscript.

The authors reported no potential conflicts of interest.

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