Mechanisms of CD47-induced caspase-independent cell death in normal and leukemia cells: link between phosphatidylserine exposure and cytoskeleton organization.

Short title: CD47-induced caspase-independent cell death

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ABSTRACT

Dying cells, apoptotic or necrotic, are swiftly eliminated by professional phagocytes. We previously reported that CD47 engagement by CD47 mAb or thrombospondin induced caspase-independent cell death of chronic lymphocytic leukemic B cells (B-CLL). Here we show that human immature dendritic cells (DCs) phagocyted the CD47 mAb-killed leukemic cells in the absence of caspase 3, 7, 8 and 9 activation in the malignant lymphocytes. Yet, the dead cells displayed the cytoplasmic features of apoptosis including cell shrinkage, phosphatidylserine exposure and decreased mitochondrial transmembrane potential (ΔΨm). CD47 mAb-induced cell death also occurred in normal resting and activated lymphocytes, with B-CLL cells demonstrating the highest susceptibility. Importantly, immature DCs and CD34+ progenitors cells were resistant. Structure-function studies in cell lines transfected with various CD47 chimeras demonstrated that killing exclusively required the extracellular and transmembrane domains of the CD47 molecule. Cytochalasin-D, an inhibitor of actin polymerization and antimycin A, an inhibitor of mitochondrial electron transfer, completely suppressed CD47-induced PS exposure. Interestingly, CD47 ligation failed to induce cell death in mononuclear cells isolated from Wiskott-Aldrich syndrome patients suggesting the involvement of Cdc42/WASP signaling pathway. We propose that CD47-induced caspase-independent cell death be mediated by cytoskeleton reorganization. This form of cell death may be relevant to maintenance of homeostasis and as such might be explored for the development of future therapeutic approaches in lymphoid malignancies.
INTRODUCTION

Apoptosis is a non-inflammatory destruction process, which is essential to the regulation of immune system and homeostasis\(^1,2\). It provides molecular basis for T and B cell development\(^3\), induction of immune tolerance and termination of normal immune response\(^4\). By contrast, necrosis is perceived by the immune system as a danger signal that triggers inflammatory response and acquired immunity. Classically, the activation and function of a set of proteinases, the caspases, is the key event in apoptosis, with mitochondria playing a central role\(^5,6\). A critical event, beside the execution phase of apoptosis, is the engulfment of dying cells by professional APC before the dead cells disgorge their toxic materials in the surrounding milieu\(^7\).

In the past recent years, cell death other than necrosis was reported to occur in the absence of caspase activation in hematopoietic cells; it included glucocorticoïd-induced death of thymocytes, Bax-mediated cell death and death of cell lines induced by growth factor withdrawal\(^8-10\). A growing number of surface molecules were shown to be involved in the induction of this non-classical caspase-independent cell death. Engagement of CD2, CD45, CD47, CD99 and MHC class II and I activated this death process\(^11-17\). Caspase-independent cell death induced by CD47 ligation in B chronic lymphocytic leukemic (B-CLL) cells was characterized by cell shrinkage, exposure of phosphatidylserine (PS), mitochondrial matrix swelling in complete absence of nuclear degradation\(^13\).

CD47 antigen is ubiquitously expressed on hematopoietic and non-hematopoietic cells\(^18\). It serves as a receptor for thrombospondin (TSP) and as a ligand for transmembrane signal regulatory protein (SIRP-\(\alpha\)), mainly expressed on myeloid and neuronal cells\(^19,20\). Through its association with integrins of \(\beta1\), \(\beta2\) and \(\beta3\) families, it initiated heterotrimeric G protein signaling and thus modulated cell motility, leukocyte adhesion and migration, phagocytosis and platelet activation\(^21\). On immune cells, CD47 ligation by soluble mAb was shown to inhibit cytokine production by APC and IL-12 responsiveness by neonatal and adult T cells\(^22-25\). When
immobilized, CD47 mAb costimulated TCR-activated T cells\textsuperscript{26,27}. Thus, the biological consequences of CD47 activation seemingly vary according to: (i) the way the molecule is engaged (ii) the surface molecules it interacts with and, (iii) its conformation and membrane localization, which all depend on the cell type on which CD47 is expressed\textsuperscript{26,28-32}.

The molecular basis for caspase-independent cell death remains elusive. Apoptosis-inducing factor (AIF), released by mitochondria, induced caspase-independent nuclear degradation\textsuperscript{33}. In most of the studies, sole PS externalization in the presence of the broad caspase inhibitor ZVAD-fmk, was used to define caspase-independent cell death pathway. Coexistence of caspase-dependent and independent pathways occurred in various systems including CD2, and CD95 ligation\textsuperscript{11,34,35}. For instance, in CD2-mediated apoptosis of T lymphocytes, the mitochondrial release of AIF preceded the dissipation of transmembrane potential ($\Delta\Psi\text{m}$), the release of cytochrome c (cyt. c) and the caspase-dependent execution phase of apoptosis\textsuperscript{36}.

Our present findings confirm and extend to other cell populations our previous observations that CD47 ligation exclusively induced the cytoplasmic events of apoptosis in B-CLL cells. We here show that dendritic cells recognized and eliminated B-CLL dead cells and present evidence that cytoskeleton rearrangement was a triggering event in $\Delta\Psi\text{m}$ loss and PS exposure after CD47 ligation on human cells.
MATERIALS AND METHODS

Patients
Diagnosis of B-CLL patients included in this study was based on clinical examination and peripheral blood count. CLL was defined as > 5000/µl lymphocytes expressing CD5, CD20 and CD23. Authorized consent forms of patients were obtained before blood collection. Patients with WAS exhibited typical features, i.e. thrombocytopenia with small platelets, eczema and susceptibility to infections, a severe clinical phenotype. In patient “1” A nucleotide insertion at position 147 in exon 2 of the WAS gene was detected. In patient “2” and “3” there was a C to T missense mutation at nucleotide 631 in exon 7 and 155 in exon 1, respectively. In patient “4”, a G to A mutation at position 777 in intron 8 was found. Blood samples were obtained following informed consent of parents.

Cell-lines and transfectants
RAJI is a Burkitt lymphoma, RPMI 8226 and RPMI 8866 are B lymphocytic cell line, Jurkat a T cell hybridoma, KU812 is a granulocytic cell line, U937 and THP-1 are monocytic cell lines, K-562 a chronic myelogenic leukemia cell, OV10 is an ovarian carcinoma transfected with CD47 cDNA\textsuperscript{37} and JinB8 a CD47-negative Jurkat cell line\textsuperscript{26}. The different cDNAs and constructs used in this study were previously described\textsuperscript{26}. Transfected Jurkat, JinB8 and U937 cells were sorted for high transgene expression using a FACSort (Lysys II Software, Becton Dickinson).

Reagents
Recombinant human IL-4, soluble CD40 ligand, and GM-CSF were kindly provided by Immunex Corporation (Seattle, WA) and Dr. D. Bron (Institut Bordet, Brussels, Belgium) respectively. Dr. R-P Sekaly (Université de Montréal, Montreal, Canada) kindly provided polyclonal anti-caspase-3 Ab. Anti-human CD3 (UCH-T1) was provided by P. Beverley
(University College and Middlesex School of Medicine, London, UK). Isotype-matched negative control mAb (mouse IgG1) was prepared in our laboratory.

The other antibodies and reagents used in this study were purchased from manufacturers as indicated below. Anti-CD47 mAbs (clone B6H12): Bioscience (Camarillo, California). Polyclonal anti-caspase-8: Upstate Biotechnology (Lake Placid, New York). Polyclonal antibody against caspase-9 and anti-mouse CD8-α (clone 53-6.7): Pharmingen (San Diego, California). Anti-caspase-7 mAb: Transduction Laboratories (Lexington, Kentucky).

Cell preparation and culture conditions

B-cells were isolated from Chronic Lymphocytic Leukemia (CLL) patients or from tonsils by density gradient centrifugation of heparinized blood or cell suspension respectively, using Lymphoprep (Nycomed, Oslo, Norway) followed by one cycle of rosetting with S-(2 aminoethyl) isothiouronium bromide (Aldrich, Milwaukee) -treated sheep red blood cells to deplete T cells. B cell purity was shown to be > 98% by flow cytometry (FACSort, Becton Dickinson). Highly purified T cells were obtained from monocyte-depleted PBMC from healthy volunteers by rosetting with AET-treated SRBC, followed by treatment of rosette-forming cells with Lympho-Kwik T (One Lambda, Los Angeles, CA) following manufacturer's recommendations. Cell purity was assessed by flow cytometry using PE-conjugated anti-CD3, anti-CD4 or anti-CD8 mAbs (Ancell) and was shown to be > 98%. CD34+ cells were obtained from heparinized cord blood using the “Dynal CD34 progenitor cell selection system” according to the manufacturer's instructions (Dynal Skøyen, Oslo, Norway). Human monocyte-derived immature dendritic cells were prepared exactly as described28.

Highly purified lymphocytes were cultured at 4.10^6/ml, iDC, cell lines and transfectants at 2.10^6/ml in 100 μl of HB101 serum free synthetic medium (Irvine Scientific) or in special medium when indicated, on flat-bottomed 96-wellplates (Nunc) in the presence of soluble or immobilized
mAbs. Plates were pre-coated with anti-CD47 or anti-CD8-α mAbs at 10 µg/ml, in 100 µl 0.1M NaHCO3 pH 9 overnight at 4 °C, then washed and blocked with medium. The following inhibitors were added 15 min before plating cells when indicated. Only cytochalasinD-treated cells were washed before plating. Antimycin A (30 µM) (Sigma). Pertussis Toxin (25-100 ng/ml), CytochalasinD (20 µM) and Rottlerin (5-20 µM) (Calbiochem). Cyclosporin A (5 µM), Aristolochic acid (50 µM) and Bongkrekic acid (50 µM) (Biomol, Plymouth Meeting, PA, USA). For potassium efflux experiments, cells were cultured in 1% FCS Na⁺K⁺free RPMI medium, supplemented with normal or inverted [Na⁺]/[K⁺] ratio as described.

**Phagocytosis assay**

Freshly purified B-CLL cells were stained with PKH26 red fluorescent cell linker (Sigma) according to the manufacturer's instructions before induction of apoptosis by culture for 16 h on immobilized CD47 mAb, hydrocortisone (5x10⁻⁴M), or soluble CD47 mAb as negative control. Cells were washed and given to iDCs (2x10⁵/ml) as a phagocytic meal (10 apoptotic cells per one iDC) for 3 h at 37 °C. Endocytosis of PKH26-stained B cells was determined by FACS after gating on live iDCs.

**Flow cytometry analysis**

CD47 and constructs expression was assessed using two-step procedure. Briefly, cells were incubated for 1 h at 4 °C with biotinylated CD47 mAb (B6H12) or unconjugated anti-CD8-α (53-6.7) or isotype-matched control mAbs (5 µg/ml). After washing, cells were incubated with PE-labeled streptavidin or FITC-conjugated anti-rat mAb (Ancell, London, ON, Canada) and analyzed by flow cytometry (FACSort, Becton Dickinson).
**Assays for apoptosis**

Detection of PS exposure, caspase 3 activity and decrease in \( \Delta \Psi \text{m} \) were performed by flow cytometry using a FACSort (Lysys II Software, Becton Dickinson). *PS exposure*: cells were double-stained with FITC-labeled Annexin-V (R&D system) and propidium iodide (PI) at 2 \( \mu \text{g/ml} \) (Sigma). Decrease of \( \Delta \Psi \text{m} \) was assessed using 3,3'-dihexyloxycarbocyanine iodide (DiOC6 (3)), (Molecular Probes). Caspase 3 activity was detected by flow cytometry in unfixed cells using a fluorogenic substrate (PhiPhlux, OncoImmunium,Gaithersburg,MA). Caspase 3, 7 and 9 cleavage products and caspase 8 expression were analyzed by Western blotting. Briefly, \( 5 \times 10^6 \) cells were directly lysed in hot sample buffer containing 10% of \( \beta \)-mercaptoethanol. Samples were then boiled for 5 min, electrophorezed on 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were probed with indicated antibodies, and immunoreactive products were revealed using ECL (Amersham).

CD47-induced cell death was calculated as follows:

\[
\frac{(\% \text{ of live cells in control}) - (\% \text{ of live cells on CD47 mA})}{(\% \text{ of live cells in control})} \times 100
\]

**Statistical analysis**

The paired Student’s paired \( t \)-test was used for statistical Analysis. *, \( p < 0.05 \).
RESULTS

1. Engulfment by professional phagocytes of CD47 mAb-treated B-CLL cells dying in the absence of caspase activation.

We previously reported that CD47 ligation by immobilized anti-CD47 monoclonal antibodies (CD47 mAb) or its natural ligand, TSP$^{13}$ induced caspase-independent cell death in B-CLL cells. This cell death was characterized by the cytoplasmic features of apoptosis. They included cell shrinking, exposure of phosphatidylserine (PS) to the outer leaflet membrane and as shown in Fig. 1 A, a drop in mitochondrial membrane potential ($\Delta\Psi_m$) as demonstrated by decreased 3,3'-dihexyloxacarbocyanine iodide (DiOC6) staining. This cell death was considered to be caspase-independent since the nucleus of the leukemic dead cells remained intact and PS exposure was not prevented by the presence of the broad caspase inhibitor z-Val-Ala-Asp-(OMe)-fluoromethylketone (zVAD-fmk)$^{13}$. To formally demonstrate that the caspase remained inactive during this process, we performed Western blot analysis (Fig. 1 B) to search for the cleavage products of caspases in CD47 mAb-treated leukemic cells. We failed to detect any cleavage products of caspases-3, 7 and 9 and similar amounts of procaspase-8 were found in immobilized CD47 mAb and control mAb-treated B-CLL cells. The absence of caspase 3/7 activity in individual CD47-treated cells was confirmed by flow cytometry using a fluorogenic caspase substrate (Fig 1C). Hydrocortisone (HC)-treated B-CLL cells displayed caspase 3 activity. Note that B-CLL cells with a low level of spontaneous apoptosis were selected for these experiments.

Both caspase activation and PS exposure have been generally considered as prerequisites for recognition and phagocytosis of cells dying by apoptosis$^{39,40}$. It was therefore important to determine whether CD47 mAb-killed B-CLL cells could signal their death to phagocytes and be eliminated. To test this hypothesis, we performed a phagocytosis assay using human monocytes-derived immature dendritic cells as professional phagocytes$^{41}$. B-CLL cells were
stained by PKH26, a non-toxic red fluorescent cell-linker, and exposed for 16 h to immobilized CD47 mAb or hydrocortisone (HC), a drug which induced caspase-dependent apoptosis in the leukemic cells\textsuperscript{42}. Soluble CD47 mAb did not induce cell death\textsuperscript{13} or Fc-mediated phagocytosis\textsuperscript{23} and was used as negative control. Treated samples were co-cultured with immature DCs for 3 h. Phagocytosis was assessed by flow cytometry by quantifying FL-2 fluorescence after gating on immature DCs (by size scatter). Results indicated that B-CLL killed by immobilized CD47 mAb were phagocytosed as efficiently as HC-treated cells (39% vs 46% FL2 positive cells) (Fig. 1 D). Note that each cell preparation was stained separately with FITC-labeled Annexin-V to evaluate their percentage of Annexin-V-positive cells (39%, 75% and 83% for soluble, immobilized CD47 mAb and HC, respectively).

Taken together, these results indicate that the caspase-independent death signal delivered by CD47 ligation to B-CLL cells is sufficient to allow their elimination by immature DCs.

2. Blood cells susceptibility to CD47-induced cell death.

Since CD47 Ag is ubiquitously expressed on hematopoietic cells, we assessed the in vitro sensitivity of normal blood cells to CD47-induced cell death. From the perspective of a potential therapeutic use of this mAb, we first examined CD34 positive cells since they represent the pool of hematopoietic progenitors\textsuperscript{43,44}. As depicted in Fig. 2 A, CD34\textsuperscript{*} cells remained totally insensitive to CD47 mAb-induced killing (0% of cell-death induction, n=6), despite their high level of CD47 expression. Immature DCs are major players in the clearance of apoptotic cells. Like CD34\textsuperscript{*} cells, they were resistant to CD47-induced cell death (0%, n=4) (Fig. 2 A).

We next observed a differential induction of cell death in normal resting and activated B and T lymphocytes. The percentage of CD47-induced cell death in normal resting B and T cells was 36% (n=7, p<0.01) and 49% (n=14, p<0.01), respectively (Fig. 2 B). B-CLL cells displayed the highest susceptibility (64% (n=10)). Unexpectedly, anti-CD3-stimulated T lymphocytes became
almost insensitive to CD47 mAb-killing, whereas T cell-dependent B cell activation (sCD40L + IL-4) significantly increased the level of death induction (p<0.01). Activation did not modulate CD47 expression as detected by CD47 mAb (clone B6H12) (Fig. 2 B).

Human cell-lines represent the malignant counterpart of lymphoid and myeloid cells at different stage of maturation. In this context, we examined four lymphoid cell lines, (RAJI, RPMI-8226, 8866 and Jurkat), three myeloid cell-lines (U937, THP-1 and K-562), one granulocytic cell-line (KU812) as well as the ovarian carcinoma cell line, OV10\textsuperscript{37}, transfected with CD47 cDNA. As shown in Fig. 3, these cell lines displayed a differential sensitivity to CD47 mAb, with no correlation, as in untransformed cells, with the level of CD47 expression. In addition, the level of CD47-induced cell death induction did not discriminate between specific cell lineage, RAJI and THP-1 and K562 being resistant. Of interest, OV10 CD47 transfectants were efficiently killed by CD47 mAb (44% of death induction, n=5, p<0.001) indicating that CD47 expression was sufficient to confer cell death susceptibility in a cell which did not primarily express the CD47 molecule.

From this data, we conclude that normal as well as transformed cells display differential susceptibility to CD47-induced cell death. CD34\textsuperscript{+} cells and immature DCs are resistant while cell activation differentially modulates the intensity of the response, regardless of the level of CD47 expression.

3. CD47 extracellular and multiply membrane-spanning (MMS) domains are necessary to signal cell death.

As indicated above, Jurkat and U937 cell lines were sensitive to CD47-induced cell death. These cell lines were therefore suitable to perform structure-function studies to determine which portion(s) of the CD47 molecule was dispensable to mediate the cell death signaling. CD47 molecule is made of an extracellular immunoglobulin-like domain (IgV), a five membrane
spanning domain (MMS for multiply-membrane spanning), and a cytoplasmic tail displaying four alternatively spliced isoforms (form 1 to 4 in order of increasing length)\(^{18,45}\). Form 2 of CD47 is predominant in the hematopoietic lineage, while the nearly tailless form 1 (only three amino acids) is expressed in keratinocytes and endothelial cells.

We first examined whether cytoplasmic tail of CD47 was required. We used JinB8 cells, a CD47-negative Jurkat cell line\(^46\), transfected with either of the two native splice forms (hIAP-form 1 and 2) or a chimera, CD8C2, made of the extracellular membrane domain of mouse CD8-\(\alpha\) fused to the cytoplasmic tail of CD47 form 2. Some experiments were performed on U937 cell line transfected with CD8C2 cDNA. Since we observed that Jurkat sensitivity to CD47-induced cell death was augmented by pretreatment with PMA for 24 h, we used PMA-activated cells in the next experiments. Results shown in Fig. 4 B indicated that the cytoplasmic tail of CD47 is not required to signal cell death. First, engagement of hIAP-form 1 and 2 molecules, equally expressed on JinB8, by immobilized CD47 mAb induced similar level of cell death (29% vs. 21% death induction). Secondly, ligation of the chimera CD8C2 by CD8 mAb did not mediate cell death, either in JinB8, U937 or Jurkat (Fig.4 B and data not shown). Of interest, PMA-activated Jurkat-CD8C2 cells did spread when endogenous CD47 or CD8C2 chimera was engaged by immobilized CD47 or anti-CD8 mAb respectively (Fig. 4 C), indicating that the failure to induce cell death was not due to inappropriate chimera ligation.

Previous studies on the CD47 molecule have demonstrated that IgV and MMS domains are generally necessary for CD47-induced cell spreading and costimulation.\(^{26,27,46,47}\) We therefore explored the requirement of the IgV or the MMS domains in CD47-induced cell death. To this end, we used two constructs: IAP/CD7, made of the CD47 IgV domain fused to the membrane and cytoplasmic domains of human CD7 and CD8MC2, made of the extracellular domain of mouse CD8-\(\alpha\) fused to the MMS and cytoplasmic tail of CD47 form 2 (Fig.4 A). The following data indicated that neither CD47 IgV nor MMS domain alone was sufficient to mediate cell death.
signaling. Engagement of CD8MC2 chimera by immobilized anti-CD8 on either U937 or PMA-activated Jurkat transfectants or ligation of IAP/CD7 chimera by CD47 mAb on JinB8 transfectants did not trigger cell death (0%, n=4 and 2%, n=6 respectively). Thus, we conclude that CD47-induced cell death signaling requires both the IgV and MMS domains of the molecule whereas the cytoplasmic tail is not required.

4. PS externalization in CD47-induced cell death is down regulated by K+ efflux impairment and antimycin A treatment.

Cell shrinkage, PS externalization and drop in ΔΨm are major hallmarks of apoptosis. Cell volume loss has been demonstrated to result in potassium (K+) and sodium (Na+) efflux. Furthermore, K+ efflux and drop in ΔΨm are tightly coupled. To examine whether CD47 induced cell death was dependent on K+ efflux; we performed cell cultures in RPMI medium in which Na+/K+ concentrations have been inverted, resulting in K+ efflux impairment by osmotic forces. Under these conditions, CD47-induced PS externalization together with cell shrinkage were delayed following CD47 ligation in B-CLL cells and U937 cell line when compared to that observed in cultures with medium reconstituted with normal Na+/K+ ratio (Fig. 5 A and not shown). ΔΨm was also slightly decreased (V. Mateo and M. Sarfati, unpublished observations).

Classically, PS exposure is dependent on caspase activation, and PS externalization can be blocked by antimycin A, an inhibitor of the mitochondrial respiratory chain. As depicted in Fig. 5 B, antimycin A totally prevented PS exposure in CD47-treated cells while it had no effect on decreased ΔΨm, strongly suggesting that these two events are uncoupled. Mechanisms involved in PS exposure are rather complex and not entirely understood. For example, in some models of apoptosis, long-term PS exposure has been shown to be the net result of increased scramblase (which has no selectivity for the direction of bilayer lipids movement) and decreased translocase activity (which selectively transports PS from the outer leaflet back to the inner
leaflet) activity. PKCδ reportedly activated scramblase in apoptotic cells. We observed CD47-induced cell death in the presence of rottlerin, an inhibitor of new PKC family isoforms, notably of PKCδ (Fig. 5 C). These data exclude a role for PKCδ without ruling out the involvement of other PKC isoforms. Indeed, immobilized CD47 mAb activated PKCδ in T cells.

These results support the hypothesis that CD47-induced cell death involves cell volume dysregulation which is partly associated with K+ efflux, and that PS exposure is intimately linked to cell shrinkage but may be uncoupled from decreased ∆Ψm and is independent of PKCδ activation.

5. CD47-induced cell death involves cytoskeleton rearrangement.

It was previously reported that T and B cell lines as well as B-CLL cells attach to and spread on CD47 mAb-coated surfaces suggesting that CD47 ligation induces a change in cytoskeleton organization. Moreover, immobilized CD47 mAb reportedly induced F-actin polymerization in activated T cells. To assess the role of cytoskeleton in CD47-triggered cell death, we examined the effect of cytochalasinD, an inhibitor of actin polymerization. Treatment with cytochalasinD prevented both PS exposure and ∆Ψm loss in CD47 mAb-treated U937 and B-CLL cells (Fig. 6 A). These data confirm and extend a previous report showing that cytochalasinD inhibited CD47-induced caspase-independent cell death in normal TCR-activated T cells.

The motility of B cell lines plated on CD47 mAb-coated surfaces requires the GTPase Cdc42. Since the Cdc42/WASP pathway is defective in cells from patients with Wiskott-Aldrich syndrome (WAS), we examined the effect of CD47 ligation in PBMC isolated from WAS patients. We tested PBMC of four characterized patients carrying WASP gene mutations; all leading to impaired WASP expression. As depicted in Fig. 6 B and D, we consistently failed to observe PS
exposure and ΔΨm loss following CD47 ligation in WAS PBMC, strongly suggesting a role for the Cdc42/WASP signaling pathway in CD47-induced cell death.

Taken together, these data indicate that cytoskeleton rearrangement is a key event in the triggering of CD47-mediated PS externalization and ΔΨm loss.
DISCUSSION

The present findings indicate that caspase-independent cell death induced by CD47 stimulation is sufficient to trigger a signal for phagocytosis in human dendritic cells. The CD47-induced cell death involves PS exposure, disruption of mitochondrial function and cytoskeleton rearrangement possibly linked to the Cdc42/WASP signaling pathway. This observation challenges the classical concept that both caspase activation and PS exposure triggered by caspases are prerequisites for phagocytosis. Elimination of dead cells in the absence of caspase activation and nuclear degradation has been previously reported. For instance, constitutive but caspase-independent death of platelets, anucleated blood cells, lead to PS exposure and clearance by phagocytes. However, caspase–independent or dependent PS exposure appears to be a critical event to allow engulfment of dead cells by phagocytic cells. A phosphatidylserine receptor was recently identified and cloned and anti-PS receptor mAb inhibited elimination of apoptotic cells. Blocking PS exposure without suppression of mitochondrial collapse and nuclear degradation prevented phagocytosis. But cells dying by delayed necrosis upon exposure to staurosporine and z-VAD-fmK were eliminated without PS externalization, underlying the potential role of other candidates for recognition and elimination of dead cells by APC.

Caspases play a central role in the execution of cell death. These enzymes are recruited and activated by either receptors of TNFR family or by apoptogenic molecules released from the intermembrane space of the mitochondria. Nevertheless, apart from CD47, stimulation of several surface antigens resulted in the activation of caspase-independent PS exposure in human cells. This includes CD2, MHC class I and class II. In contrast to CD47, ligation of these Ags may induce either caspase-dependent or independent death according to the epitope triggered. So far, CD47 ligation by soluble, immobilized or cross-linked mAb recognizing at least
three different epitopes exclusively induced caspase-independent cell death (Ref. 13 and 14, and data not shown).

During apoptosis, mitochondrial changes result in the dissipation of the mitochondrial transmembrane potential ($\Delta \Psi m$). The loss of $\Delta \Psi m$ is generally mediated by opening of the mitochondrial permeability transition pores (PT) and as a consequence, the release of apoptogenic molecules such as AIF and cyt. $c$ with subsequent caspase-independent or dependent nuclear degradation. The engagement of CD47 results in cell shrinkage and $\Delta \Psi m$ loss and this occurs in the absence of caspase activation and DNA degradation likely excluding the involvement of AIF and cyt. $c$. Indeed, AIF release ultimately lead to caspase-independent DNA degradation$^{60}$ and cyt. $c$ to caspase activation$^{61}$. However, inactivation of cyt. $c$ by at least HSP27 binding has been shown to prevent apoptosome formation and caspase activation$^{62}$. We therefore postulate that either cyt. $c$ is inactivated and translocation of AIF to the nucleus impaired or that there is no release of apoptogenic molecules during CD47 stimulation. Our unpublished observations indicate that inhibitors of PTPC opening failed to prevent CD47-induced cell death. They include cyclosporin A in combination with aristolochic acid (which act on cyclophilin D and PLA2 respectively), bongkrekic acid (inhibitor of adenine nucleotide transfer (ANT)) and Ca++ chelators$^{59}$. In support to the latter hypothesis, BCR-mediated apoptosis of immature B cell line resulted in $\Delta \Psi m$ loss but did not induce cyt. $c$ release and caspase activation$^{63}$. Interestingly, cyt. $c$ release may occur in the absence of $\Delta \Psi m$ loss by a direct effect of the proapoptotic molecule Bid/Bik$^{64}$. We also provide evidence that CD47-induced PS exposure may be dissociated from $\Delta \Psi m$ loss but is intimately linked to cell shrinkage. Cell volume decrease during apoptosis is an active mechanism, which was shown to be dependent on K+ channels (reviewed in$^{48,65}$). Maeno et al$^{66}$ observed apoptotic volume decrease in the presence of zVAD-fmk, suggesting that K+ efflux is caspase-independent. Furthermore, it was demonstrated that this phenomenon may occur
upstream of caspase activation. In the present study, mitochondrial inhibitors such as antimycin A, inhibited CD47-induced PS exposure but not dissipation of ΔΨm. Similar observations were made by Zhuang et al., in ectoposide or TPCK-induced apoptosis of THP-1 monocyte cell line56. Of interest, antimycin A alone induced ΔΨm in B-CLL and U937 cell lines. In that respect, antimycin A mimicked BH3 cell-death domain and induced mitochondrial swelling and ΔΨm loss67. Taken together, these results strongly suggest that factors other than cyt. c. released by the mitochondria and caspases may directly or indirectly affect PS exposure in CD47-induced cell death.

As described for CD47-mediated cell spreading and costimulation in T cell lines, both extracellular and multispan transmembrane domains of CD47 molecule were required to induce killing. The short cytoplasmic domain was dispensable. In T cells, ~ 65% of the CD47 molecule is localized in membrane rafts where it regulates TCR-dependent and independent T cell activation. Immobilized CD47 mAb reportedly controlled the activation of heterotrimeric G proteins, triggered F-actin polymerization, and PKCθ translocation27. However, CD47 mAb-induced killing was observed in the presence of G proteins inhibitor (i.e., Pertussis toxin) (V. Mateo and M. Sarfati, data not shown) or new PKC family (δ, ε, η, θ and μ) inhibitor (i.e., rottlerin). PKCθ expression was low in B cell lines largely excluding its involvement in CD47-induced cell death.

Yoshida et al. previously reported that CD47 regulated human B cell mobility through Cdc4252. We observed that engagement of CD47 induced spreading of B-CLL cells. Cdc42 belongs to the small Rho GTPases family, which includes Rho and Rac, known to regulate formation of actin structures in many cell types68. Among others, Cdc42 appears to have a unique role in actin remodeling during T cell activation and endocytosis of immature DC. WASP, uniquely expressed on hematopoietic cells69, is the specific effector of Cdc42. WASP binds Cdc42 and controls actin polymerization by distinct domains70. WAS-immunodeficiency syndrome is characterized by
abnormalities in cytoskeletal function resulting in abnormal chemotaxis, phagocytosis and T cell responses.

Two observations strongly suggest a direct role of cytoskeleton rearrangement in CD47-induced cell death: 1) inhibition of both PS exposure and ΔΨm loss by cytochalasinD, an inhibitor of actin polymerization and 2) absence of CD47-induced death in PBMC of WAS patients. As depicted in a schematic model in Fig. 7, we propose that CD47 ligation induces killing by two non-mutually exclusive pathways. The initial and common event would be the triggering of actin polymerization, perhaps via Cdc42/WASP pathway. This will lead to either mitochondrial changes including matrix swelling and ΔΨm loss, followed by PS exposure (pathway #1) and/or to a direct effect on PS externalization bypassing mitochondria (pathway #2).

When blood cells susceptibility was examined, immature DC, activated T cells and CD34+ precursors appeared to be much less sensitive and virtually resistant to CD47-induced killing. Similar differences in APC susceptibility has been reported in HLA-DR–induced caspase-independent cell death. Pettersen et al. reported that CD47 mAb preferentially induces killing in normal activated but not resting T cells and this was inhibited by cytochalasinD. We failed to induce cell death in anti-CD3-activated T cells. The use of different mAb in the two studies and the regulation of CD47 conformation during T cell activation (M. Sarfati and V. Mateo, unpublished data) may provide one explanation for this discrepancy.

The role of CD47-induced killing in the regulation of immune response remains poorly understood. One may envision that it at least participates to the maintenance of homeostasis. Indeed, apoptosis is a self-destruction process which, in contrast to necrosis, does not lead to inflammatory response and may be involved in the induction of tolerance. Thrombospondin, the natural ligand of CD47, establishes a molecular bridge between apoptotic cells and the APC, facilitating phagocytosis of apoptotic cells. We reported that TSP, like CD47 mAb, induced
caspase-independent cell death via CD47 binding, but also acted on APC to down-regulate the production of the pro-inflammatory molecule IL-12 and prevent DC maturation\textsuperscript{23}. It is important to mention that additional mechanisms inhibit inflammation during phagocytosis of apoptotic cells. They include induction of TGF-\(\beta\) production by apoptotic cells themselves\textsuperscript{73} and by APC upon their interaction with apoptotic cells\textsuperscript{74}. Also, TSP was found to be the main activator of TGF\(\beta\) and reciprocally, TGF\(\beta\) induces TSP production\textsuperscript{75,76}.

CD47 was recently reported to be a marker of self in rodents which prevented clearance of intact red blood cells and lymphoid cells by CD47\(^+\) APC through the engagement of its counterstructure SIRP-\(\alpha\), selectively expressed on APC\textsuperscript{77}. Also, CD47/SIRP-\(\alpha\) interactions negatively regulated APC functions\textsuperscript{28}. However, apoptotic cells (highly expressing CD47\textsuperscript{78}) are known to be efficiently cleared by APC and this is not inhibited by CD47 mAb\textsuperscript{23,79}. Whether CD47 expressed on apoptotic cells has a particular conformation in order not to deliver a negative signal for phagocytosis to APC via SIRP-\(\alpha\) or conversely whether SIRP-\(\alpha\)/CD47 interactions can induce cell death remain open questions.

Taken together, we propose that TSP-induced/enhanced PS exposure via CD47 on lymphoid cells, followed by their elimination by professional phagocytes, represent a process that continuously takes place in peripheral tissues to ensure the maintenance of tissue and host homeostasis. Additionally, the anti-inflammatory activity of TSP combined with its ability to facilitate the clearance of apoptotic cells may further contribute to homeostasis and induction of tolerance by avoiding inappropriate immune response to self-antigens.
LEGEND TO FIGURES

Figure 1. Immature dendritic cells efficiently phagocytosed B-CLL cells killed by CD47-induced caspase-independent cell pathway.

Freshly isolated B-CLL cells were cultured in the presence of soluble or immobilized CD47 mAb (10 µg/ml), control mAb (10 µg/ml) or hydrocortisone (5.10^-4 M). Panel A: cells were double-stained with FITC-labeled Annexin-V and PI (upper panel) or with DiOC6 (lower panel), and analyzed by flow cytometry. Shown are % of dead cells (Annexin V^+ or DiOC6^{low}). Panel B: soluble (lane1) or immobilized (lane2) CD47 mAb-treated cells were lysed and western blot analysis was performed for detection of caspases 3, 7, 8 and 9 cleavage products (as indicated by *). Panel C: Caspase 3 activity was measured after 48 h by flow cytometry using the cell permeable fluorogenic subtrate DEVDase. Panel D: B-CLL cells were stained in red with PKH26 linker before treatment overnight with soluble or immobilized CD47 mAb or HC. Cells were then co-cultured for 3 h with immature dendritic cells (iDC) at 10/1 ratio. The mixture was analyzed by flow cytometry for red fluorescence (FL2) after gating on iDC. Shown are % of iDC that have phagocytosed red dead cells. One representative experiment out of three.

Figure 2. Differential susceptibility of normal human blood cells to CD47-induced cell-death.

Panel A and B: Cord-blood CD34^+ cells (stem cells) (n=6), monocyte-derived iDC (n=4), resting and activated tonsillar B cells (n=4) and peripheral T cells (n=4) were stained for CD47 expression (B6H12 mAb) and analyzed by flow cytometry: isotype-control mAb (open histogram), CD47 mAb (shaded histogram). Panel A: Stem cells and iDC were cultured in the absence or presence of immobilized CD47 mAb (10µg/ml) for 18 h and double-stained with FITC-labeled Annexin-V and PI. Shown are % of viable cells (Annexin-V/PI^+). Panel C: Resting T and B cells were left unstimulated or activated for 48 h with plastic-coated anti-CD3 (3 µg/ml) or
sCD40L (1 µg/ml) + IL-4 (10 ng/ml) respectively and then cultured overnight in the absence or presence of immobilized CD47 mAb. The mean % of CD47-induced cell-death, calculated as indicated in materials and methods were shown.

Figure 3. Susceptibility of human cell lines to CD47-induced cell-death: Lack of correlation between CD47 expression and CD47-induced cell death.

Various cell-lines were stained for CD47 expression using B6H12 mAb: isotype-control mAb (open histogram) and CD47 mAb (shaded histogram). Cells were cultured overnight on immobilized control or CD47 mAb analyzed by flow cytometry. Shown is the mean % of CD47-induced cell-death, calculated as indicated in materials and methods were shown.

Figure 4. Requirement for both extracellular and transmembrane domains for CD47-induced cell-death.

Panel A: Various constructs or/and chimeras of the CD47 molecule (described in “materials and methods”) were transfected into U937, Jurkat or JinB8 (CD47−/−) cell lines. Panel B: Expression of the CD47 products (black histograms) was determined by staining with B6H12 mAb (for hIAP form 1 and 2, IAP/CD7) or anti-mouse CD8α mAb (for CD8-MC2 and CD8-C2). Expression of endogenous CD47 (grey histograms). Cells were cultured overnight on immobilized anti-mouse CD8-α or CD47 mAb (10 µg/ml). * Jurkat and JinB8 transfectants were pretreated with PMA for 24h (2 ng/ml) before killing. CD47-induced cell death was calculated as described in materials and methods. Panel C: Light microscopy of PMA-activated Jurkat cells transfected with CD8-C2 construct. Untreated cells (panel 1), cells cultured for 18h with soluble (panel 2), immobilized (panel 3) CD47 mAb or immobilized anti-mouse CD8α mAb (panel 4).
**Figure 5. Down-regulation of CD47-induced PS-exposure by K⁺ efflux or antimycin A treatment: lack of effect of PKC inhibitor rottlerin.**

Freshly isolated B-CLL cells and U937 cell line were cultured with or without immobilized CD47 mAb. Panel A: Cultures were performed in RPMI medium containing normal (Na⁺) or inverted (Na⁺/K⁺) ratios (K⁺). Shown are % of viable cells (Annexin V⁻/PI⁻). Panel B and C: Cells were cultured in the presence or absence of immobilized CD47 mAb with or without antimycin A (30 µM) or increasing concentrations of rottlerin. Shown is the % of dead cells (Annexin-V⁺ or DiOC₆ low cells). One representative experiment out of 4.

**Figure 6. Link between CD47-induced PS exposure and cytoskeleton rearrangement.**

Panel A: Freshly isolated B-CLL or U937 cell line were cultured overnight in the presence or absence of immobilized CD47 mAb with or without cytochalasin-D (20 µM). Shown is % of dead cells (AnnexinV⁺ or DiOC₆ low cells). One representative experiment out of 4. Panel B-D: PBMC were isolated from 4 WAS patients and cultured in the absence (1) or presence (2) of immobilized CD47 mAb. Annexin-V/PI and DiOC₆ staining (patient #3) (B) and CD47 expression of PBMC from patient #3 (B6H12) (C); % viable cells (Annexin V⁻/PI⁻) in PBMC of 4 WAS patients and one control donor (D).

**Figure 7. Hypothetical model for CD47-induced PS exposure.**

Two non-mutually exclusive pathways leading to PS exposure and initiated by a common triggering event: Cdc42/WASP signaling pathway and F-actin polymerization. Inhibition by cytochalasin-D (orange arrow). Pathway #1 (green arrow): loss in ΔΨm followed by PS externalization. Antimycin A inhibits PS but not ΔΨm loss. Pathway #2 (blue arrow): bypass of mitochondria and direct induction of PS externalization. Elevated extracellular K⁺ slows-down PS...
exposure. Absence of caspase activation and nuclear degradation in CD47-induced cell death. Dotted arrows indicate hypothetical inhibitory pathways.
REFERENCES


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FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
FIGURE 7
Mechanisms of CD47-induced caspase-independent cell death in normal and leukemia cells: link between phosphatidylserine exposure and cytoskeleton organization

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