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CD47 promotes both phosphatidylserine-independent and phosphatidylserine-dependent phagocytosis of apoptotic murine thymocytes by non-activated macrophages

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ABSTRACT

The ubiquitously expressed cell surface glycoprotein CD47 on host cells can inhibit phagocytosis of unopsonized or opsonized viable host target cells. Here we studied the role of target cell CD47 in macrophage uptake of viable or apoptotic murine thymocytes. As expected, IgG-opsonized viable CD47$^{-/-}$ thymocytes were taken up more efficiently than equally opsonized Wt thymocytes. However IgG-opsonized apoptotic thymocytes from Wt and *CD47*$^{-/-}$ mice were taken up equally. Although uptake of apoptotic thymocytes by non-activated bone marrow-derived macrophages was phosphatidylserine (PS)-independent, while uptake by non-activated resident peritoneal macrophages was PS-dependent, both macrophage populations showed a reduced uptake of non-opsonized apoptotic *CD47*$^{-/-}$ thymocytes, as compared with the uptake of apoptotic Wt thymocytes. This difference was only seen with non-activated macrophages, and not with β-1,3-glucan-activated macrophages. CD47 promoted binding of thymocytes to macrophages, which did not require F-actin polymerization. CD47 became clustered on apoptotic thymocytes, both co-localized with or separated from, clustered PS and cholesterol-rich GM-1 domains. Thus, CD47 does not inhibit, but rather support, both PS-independent and PS-dependent uptake of apoptotic cells in the murine system. This mechanism only comes into play in non-activated macrophages.

Introduction

Apoptotic cells or apoptotic bodies are cleared by phagocytic cells, mainly macrophages [1,2], which involves many phagocytic receptors, bridging molecules, and "eat-me" markers on apoptotic cells [3,4]. The best characterized "eat me" marker is phosphatidylserine (PS), normally distributed only in the inner leaflet of the plasma membrane, but rapidly exposed on the cell surface of apoptotic cells [3,4]. Several phagocyte receptors implicated in apoptotic cell clearance (e.g. integrins, scavenger receptors or lectins), may or may not depend on PS-recognition. Recently, BAI1 and TIM-4 were identified as novel PS-binding phagocytic receptors [5–7], but PS is also recognized by bridging molecules (e.g. protein S, Gas6, MFG-E8, or β2-GPI), interacting with a number of phagocytic receptors [4]. Whether PS-dependent or PS-independent mechanisms are used for apoptotic cell uptake seems to depend on macrophage tissue origin and/or activation status. Thus, apoptotic cell uptake is PS-independent in non-activated bone marrow-derived macrophages (BMM) [8], whereas resident peritoneal macrophages (RPM) and inflammatory-activated BMM mostly rely on PS-dependent uptake mechanisms [6,8].

Activation of macrophage phagocytosis seems to be determined by the balance between the relative signaling strength of activating and inhibitory signals [9]. One inhibitory system controlling macrophage uptake of host cells is based on the interaction between the ubiquitously expressed cell surface glycoprotein CD47 on target host cells, and the inhibitory macrophage receptor signal regulatory protein alpha (SIRPα) [9,10]. This interaction prevents phagocytosis of normal viable red blood cells (RBCs), lymphocytes, or platelets, and blood cells from CD47-deficient mice are rapidly cleared by macrophages in Wt recipient mice [10–12]. The CD47/SIRPα-interaction also inhibits Fcγ and complement receptor-mediated phagocytosis of RBC or platelets [9,12]. CD47 on apoptotic human cells is clustered into patches, segregated away from clustered pro-phagocytic ligands such as PS or calreticulin [13], suggesting that redistribution of clustered CD47 could prevent the phagocytosis-inhibitory signals normally generated by CD47 on viable cells in contact with macrophages [13]. Intriguingly, it was shown that the CD47/SIRPα-interaction could mediate tethering of apoptotic cells to the phagocyte [14], opening up the possibility that CD47 could have opposite effects in regulating macrophage phagocytosis of viable or apoptotic T cells in the murine system. Therefore, we here further investigated this possibility, and investigated the role of apoptotic cell CD47 in regulating PS-dependent or PS-independent phagocytosis by non-activated, or activated macrophages.

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Materials and methods

Antibodies and reagents. Rat anti-murine CD3 and CD45 were from BD Pharmingen, USA. Cy3 goat anti-mouse IgG was from Jackson ImmunoResearch Europe Ltd., UK. The anti-CD47 mAb miap430 (mouse IgG1), derived by immunizing $CD47^{-/-}$ Balb/c mice with human CD47-Fc fusion protein, found to cross-react with mouse CD47, was purified from hybridoma supernatants [15]. Annexin V-FITC was from ImmunoTools, Germany. 5-Chloromethylfluorescein diacetate (CMFDA) was from Molecular Probes, USA. 1-Palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleyl-sn-glycero-3-phospho-L-serine (PS), and cholesterol were from Avanti Polar Lipids, USA. Dulbecco's Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagles Medium (DMEM), Alexa 647-conjugated cholera toxin-subunit (CtB), penicillin, streptomycin, and fetal calf serum (FCS) were from Invitrogen, Sweden. Vectashield with DAPI was from Vector Labs, UK. All other reagents were from Sigma–Aldrich, USA.

Mice. Two to four months old female $CD47^{-/-}$ C57BL/6 mice [16], back-crossed to C57BL/6 (Jackson Laboratory, USA) for 21 generations, and their homozygous wild-type (Wt) littermates were from our own breeding colony. The mice were housed in a barrier facility, according to local guidelines. All experiments were approved by the Local Animal-Ethics Committee.

Isolation and culture of macrophages. BMM were generated as previously described [12], by culture in bacterial-plastic dishes for 6 days in DMEM with 10% heat-inactivated FCS, 100 U/mL penicillin and streptomycin (DMEM/10% FCS) supplemented with 15% L929-cell supernatant (as a source of M-CSF). Mature macrophages were cultured in DMEM/10% FCS without M-CSF for 24 h before being used in experiments. Activated BMM were generated by incubating mature BMM with 100 μg/ml β-1,3-glucan for 48 h [8]. RPM were isolated by peritoneal lavage of euthanized mice, using 8 ml sterile DMEM/10% FCS. BMM or RPM were plated (1×10^5) on 11 mm round sterile glass cover-slips. Plated cells were cultured for 2 h at 37 °C, after which non adherent cells were removed by gentle washing.

Preparation of thymocytes and induction of apoptosis. Thymuses from Wt or $CD47^{-/-}$ mice were minced to yield a single cell suspension, and thymocytes were incubated for 3 h at 37 °C and 5% CO_2 with 1 μM Dexamethasone (Dex) in DMEM/10% FCS. For IgG-opsonization, viable and Dex-treated thymocytes were incubated with anti-CD3 (1 μg/10^6 cells), or anti-CD45 (0.25 μg/10^6 cells) for 30 min at +4 °C, as previously described [17], washed with PBS, and used in phagocytosis experiments. Equal levels of opsonization were confirmed by flow cytometry.

Preparation of liposomes. Unilamellar phospholipid liposomes were prepared essentially as described by Hoffmann et al. [18]. Lipid stock solutions in cholesterol and methanol (90:10) were mixed in disposable glass test tubes and evaporated to dryness under a stream of nitrogen. Dry lipids were dissolved in DMEM and sonicated for 20 min in a water bath sonicator. PC liposomes contained an 80:20 M ratio of PC to cholesterol, and PS liposomes contained a 30:50:20 M ratio of PS to PC to cholesterol. In phagocytosis experiments, each sample received 100 nmol of total lipids.

In vitro phagocytosis experiments. Thymocytes at a 10:1 ratio were incubated with adherent macrophages for 1 h at 37 °C and 5% CO_2 and then placed on ice. Non-ingested thymocytes were removed by two washes in cold PBS and the cells were fixed and stained using May-Grunewald/Giemsa staining [19]. Phagocytosis was evaluated in a blinded way, using light microscopy, and expressed as a phagocytosis index ([number of phagocytosed targets/total number of macrophages] × 100). At least 200 macrophages were counted on each cover-slip with duplicate cover-slips in each test-group.

In vivo phagocytosis experiments. Thymocytes were labeled with CMFDA for 45 min at room temperature, treated with 1 μM Dex for 3 h, washed, resuspended in sterile physiological saline, and 1×10^7 thymocytes were injected intraperitoneally (i.p.) in Wt mice. After 30 min, peritoneal cells were isolated by peritoneal lavage, and analysed by flow cytometry (FACscan, Becton Dickinson), gating on $F4/80^+$ cells. Data are presented as a phagocytosis index ([mean fluorescence intensity of CMFDA-positive macrophages] × [per cent phagocytosing macrophages]).

Binding experiments. Binding of apoptotic cells to macrophages was studied essentially as previously described [20], using macrophages adherent to glass cover-slips, preincubated in the presence or absence of 10 μg/ml cytochalasin D for 30 min at 37 °C. Thymocytes were added at a 10:1 ratio, followed by a 15 min co-incubation at 37 °C and 5% CO_2. Cover-slips were then dipped five times respectively in two tubes containing cold PBS, followed by May-Grunewald/Giemsa fixation/staining. Binding was evaluated using light microscopy and expressed as number of bound thymocytes/100 macrophages.

Immunofluorescence microscopy. Viable or Dex-treated thymocytes were incubated with mAb miap430, Annexin V-FITC, and Alexa 647-CtB in Ca^{2+}-containing binding buffer for 30 min on ice. Cells were then fixed in 2% paraformaldehyde for 30 min, washed three times in PBS, and incubated with Cy3-conjugated goat anti-mouse IgG. Washed cells were adhered to poly-L-lysine-coated cover-slips, and mounted in Vectashield with DAPI. Cells were analyzed using laser-scanning confocal microscopy (Leica TSP-2, Heidelberg, Germany). To avoid bleed through, FITC, Cy3 and Alexa 647 were sequentially scanned using excitation wavelengths of 488 nm, 534 nm, and 633 nm, respectively. Images were analyzed using Leica LCS software (Leica, Heidelberg, Germany).

Statistics. Statistical analyses were performed by using two-tailed Student's *t*-test for paired or unpaired analysis. All results are expressed as means ± SEM.

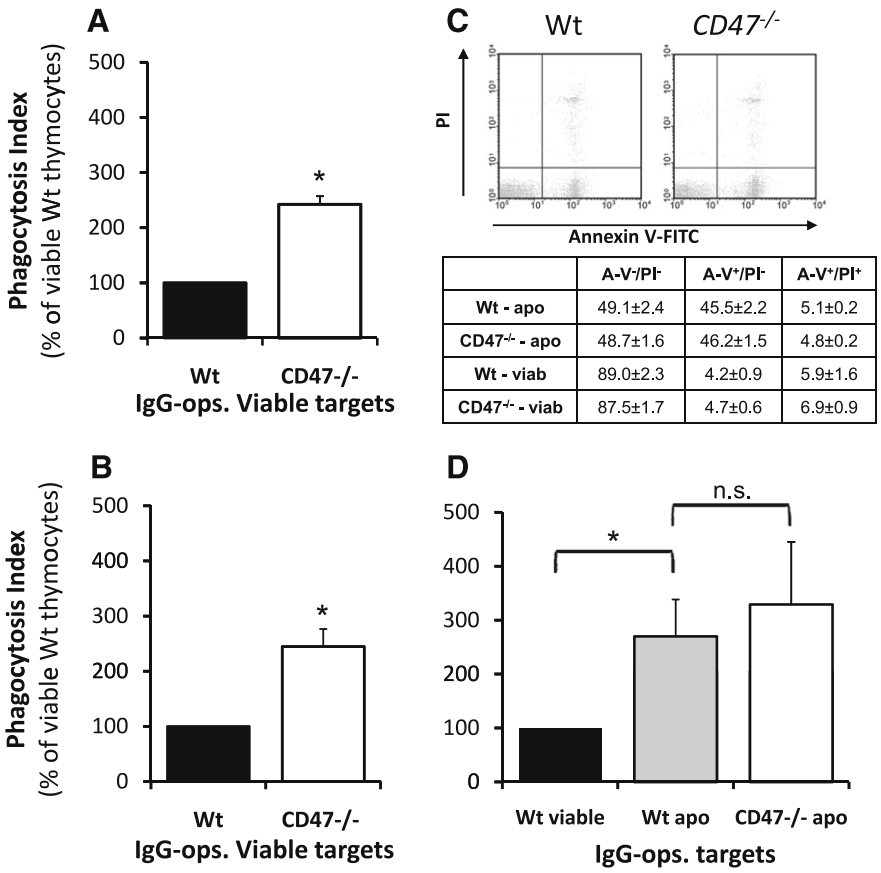
Results

CD47 inhibits FcγR-mediated uptake of viable but not apoptotic thymocytes

We first studied if CD47 regulated phagocytosis of IgG-opsonized viable T cells. As expected, $CD47^{-/-}$ thymocytes opsonized with anti-CD45 IgG (Fig. 1A) or anti-CD3 IgG (Fig. 1B) were phagocytosed significantly more than equally opsonized Wt thymocytes ($P < 0.05$). We next studied uptake of IgG-opsonized apoptotic thymocytes. Apoptosis in $CD47^{-/-}$ or Wt thymocytes was induced by incubation with 1 μM Dex for 3 h, resulting in about 46% Annexin V^+/PI^- thymocytes, with only about 5% Annexin V^+/PI^+ cells, with no difference between the two genotypes (Fig. 1C). Phagocytosis of IgG-opsonized apoptotic Wt thymocytes was significantly increased, as compared with IgG-opsonized viable Wt cells, and to a level not significantly different from that using IgG-opsonized apoptotic $CD47^{-/-}$ thymocytes (Fig. 1D). Equal opsonization of viable and apoptotic thymocytes was confirmed by flow cytometry (data not shown). These experiments suggested that CD47 on apoptotic thymocytes could have a reduced ability to inhibit phagocytosis.

CD47 promotes both PS-independent and PS-dependent uptake of apoptotic thymocytes

We next studied phagocytosis of unopsonized apoptotic thymocytes by RPM or BMM, which differ in their need for PS-recognition. Phagocytosis by non-activated RPM was PS-dependent (Fig. 2A) [6], whereas phagocytosis by non-activated BMM was



	A-V⁻/PI⁻	A-V⁺/PI⁻	A-V⁺/PI⁺
Wt - apo	49.1±2.4	45.5±2.2	5.1±0.2
CD47$^{-/-}$ - apo	48.7±1.6	46.2±1.5	4.8±0.2
Wt - viab	89.0±2.3	4.2±0.9	5.9±1.6
CD47$^{-/-}$ - viab	87.5±1.7	4.7±0.6	6.9±0.9

Fig. 1. CD47 on viable but not apoptotic thymocytes can inhibit phagocytosis. CD47 inhibits phagocytosis of viable anti-CD45 (A) or anti-CD3-opsonized (B) thymocytes by non-activated BMM ($n = 3–5$). (C) Treatment with 1 μM Dex for 3 h induces apoptosis to a similar degree in Wt and *CD47*$^{-/-}$ thymocytes. The table shows mean ± SEM from 5 to 6 independent experiments. (D) CD47 inhibits phagocytosis of IgG-opsonized viable thymocytes, but not IgG-opsonized Dex-treated thymocytes, by non-activated BMM. The phagocytosis index of IgG-opsonized viable CD47$^{+/+}$ thymocytes was 17.8 ± 5.0 targets/100 macrophages, and set to 100 % ($n = 4$). Equal levels of opsonization between Wt and *CD47*$^{-/-}$ thymocytes, and between viable and Dex-treated thymocytes, was confirmed by flow cytometry (not shown). $^{*}P < 0.05$, using Student's *t*-test for paired comparisons.

not (Fig. 2D) [8]. Uptake of apoptotic *CD47*$^{-/-}$ thymocytes by RPM was reduced by 32.9 ± 9.6%, as compared with apoptotic Wt thymocytes ($P < 0.02$; Fig. 2B), with 27.8 ± 6.7% fewer macrophages ingesting at least one apoptotic *CD47*$^{-/-}$ thymocyte ($P < 0.02$; data not shown). Similarly, uptake of apoptotic *CD47*$^{-/-}$ thymocytes by BMM was reduced by 34.5 ± 8.3%, as compared with apoptotic Wt thymocytes ($P < 0.01$; Fig. 2E), with a 22.5 ± 9.8% reduction in macrophages ingesting at least one apoptotic *CD47*$^{-/-}$ thymocyte ($P < 0.05$; data not shown). Using CMFDA-labeled apoptotic thymocytes, we found that apoptotic *CD47*$^{-/-}$ thymocytes were taken up significantly less than apoptotic Wt thymocytes by RPM also *in vivo* (Fig. 2C). Non-activated and activated macrophages also use different uptake mechanisms for uptake of apoptotic cells [4,21,22]. Thus, we next studied uptake of apoptotic thymocytes by non-activated, or β-1,3-glucan-activated BMM. In contrast to that in non-activated macrophages, we found equal uptake of apoptotic cells of both genotypes in β-1,3-glucan-activated BMM (Fig. 2F). Thus, CD47 on the apoptotic cell promotes both PS-dependent and PS-independent uptake, but only in non-activated macrophages.

Reduced binding of apoptotic CD47$^{-/-}$ *thymocytes to macrophages*

Since CD47 could function to tether apoptotic T cells to macrophage cell lines [14], we investigated if the reduced uptake of apoptotic *CD47*$^{-/-}$ thymocytes was due to reduced binding to primary macrophages. Apoptotic *CD47*$^{-/-}$ thymocytes bound significantly less to BMM, as compared with apoptotic Wt thymocytes (62.9 ± 6.7% inhibition; $P < 0.05$; Fig. 3A). To investigate if the actin cytoskeleton was involved in this process, macrophages were preincubated in the presence of cytochalasin D, which disrupts actin filaments and prevents phagocytosis. Albeit binding of apoptotic Wt thymocytes to cytochalasin D-treated macrophages was reduced by 40–50% (Fig. 3B), the binding of apoptotic *CD47*$^{-/-}$ thymocytes was reduced to virtually the same extent, relative to apoptotic Wt thymocytes (68.9 ± 5.6% inhibition; $P < 0.05$; Fig. 3C), as seen in the absence of cytochalasin (Fig. 3A). Thus, CD47-promoted binding of apoptotic thymocytes to macrophages does not require F-actin polymerization in the macrophage.

CD47 is clustered on apoptotic thymocytes and may co-localize with phosphatidylserine in GM-1-positive lipid rafts

We next studied the distribution of CD47 on viable or apoptotic thymocytes, and its possible co-localization with PS. In confocal mid-sections of apoptotic cells, CD47 was evenly distributed on viable (annexin V^{-}) thymocytes, but had a clustered appearance on apoptotic (annexin V^{+}) thymocytes (Fig. 4A). As expected [24], PS was also distributed in patches on the surface of apoptotic thymocytes (Fig. 4A). Interestingly, CD47 and PS were either co-localized in patches on the apoptotic cell surface (Fig. 4A—Apoptotic type I), or this co-localization was more limited (Fig. 4A—Apoptotic

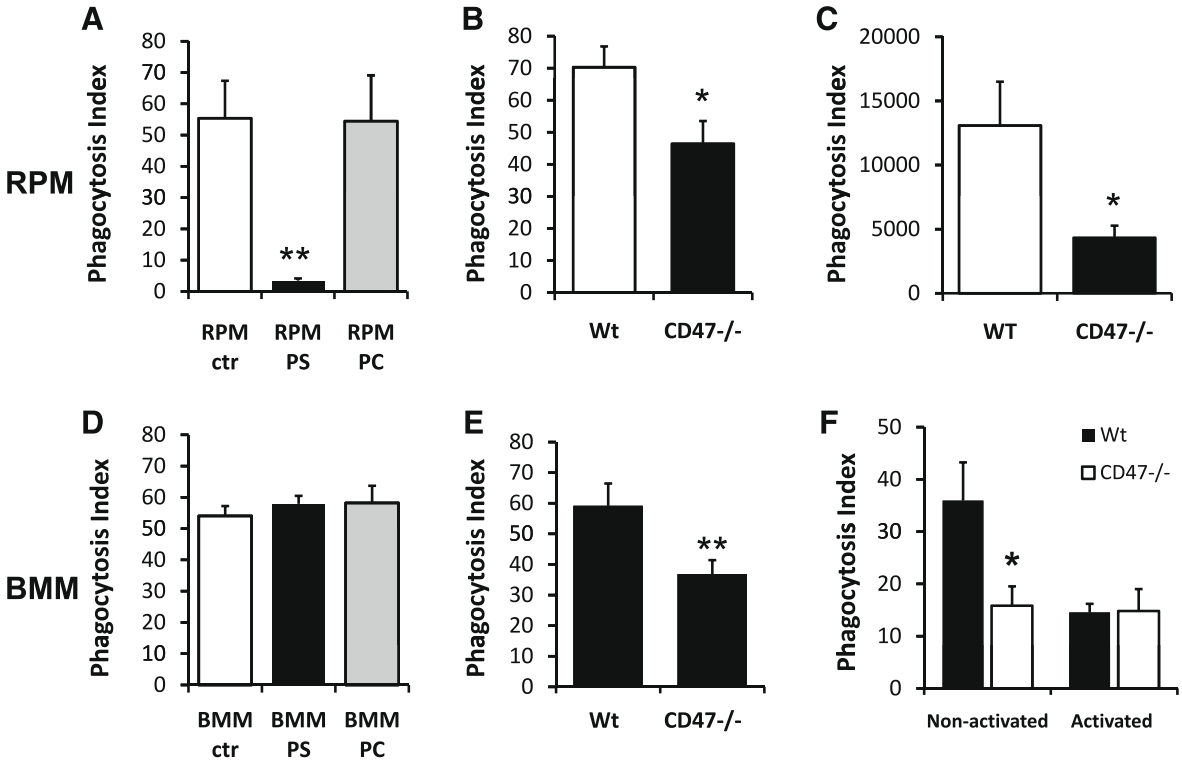


Fig. 2. Apoptotic cell CD47 promotes both PS-independent and PS-dependent phagocytosis in non-activated macrophages. (A) Phagocytosis of Dex-treated thymocytes by RPM is inhibited by PS liposomes, but not by PC liposomes ($n = 3$). (B) Phagocytosis of apoptotic *CD47*$^{-/-}$ thymocytes by RPM is less efficient than uptake of apoptotic Wt thymocytes ($n = 6$). (C) Reduced uptake of CMFDA-labeled apoptotic *CD47*$^{-/-}$ thymocytes *in vivo* ($n = 6$). (D) Phagocytosis of Dex-treated thymocytes by BMM is not affected by PS or PC liposomes ($n = 3$). (E) Apoptotic *CD47*$^{-/-}$ thymocytes are phagocytosed less efficient by BMM than apoptotic Wt thymocytes ($n = 6$). (F) Similar uptake of Wt (black bars) or *CD47*$^{-/-}$ thymocytes (open bars) by β-1,3-glucan-activated BMM. ($n = 5$). $^{*}P < 0.05$ or $^{**}P < 0.01$, using Student's *t*-test for paired comparisons.

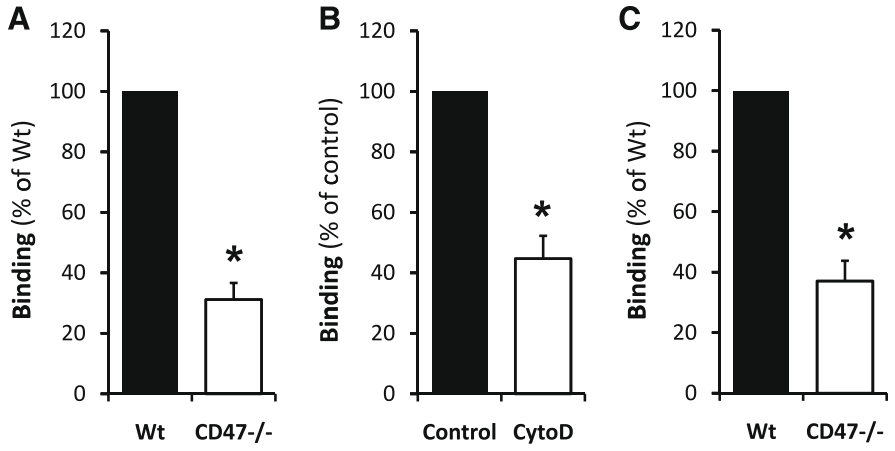


Fig. 3. Target cell CD47 is important for binding of apoptotic thymocytes to BMM. (A) Reduced macrophage binding of apoptotic *CD47*$^{-/-}$ thymocytes to non-activated BMM adherent to glass cover-slips. The number of thymocytes bound/100 macrophages was determined by light microscopy, and binding is expressed in per cent of that using Wt thymocytes ($n = 3$). (B) Cytochalasin D (Cyto D, 10 μg/ml) reduces the binding of apoptotic Wt thymocytes to macrophages ($n = 3$). (C) Reduced binding of apoptotic *CD47*$^{-/-}$ thymocytes to macrophages in the presence of 10 μg/ml Cyto D ($n = 3$). $^{*}P < 0.05$, using Student's *t*-test for paired comparisons.

type II). CD47 has been shown to be present in cholesterol-rich membrane domains [25]. Indeed, in viable thymocytes, CtB^{+} cholesterol-rich GM-1 domains and CD47 were both evenly distributed in the plasma membrane, showing an overlapping pattern (Fig. 4A). However, in apoptotic thymocytes, GM-1 domains were clustered into patches, which co-localized with CD47 in Apoptotic type I cells, but less so in Apoptotic type II cells (Fig. 4A). In analysis of *z*-stacks through the apoptotic cells, clustered CD47 was found either co-localized with PS-clusters, or in areas free of PS, on the same cell (Fig. 4B).

Discussion

We found that CD47 on viable thymocytes inhibited FcγR-mediated phagocytosis, but that CD47 on unopsonized apoptotic thymocytes instead promoted both PS-dependent and PS-independent phagocytosis by non-activated macrophages. CD47 on the apoptotic cells was important in mediating binding of the apoptotic thymocytes to the macrophages. On apoptotic thymocytes, CD47 was clustered into patches in the plasma membrane, which were either co-localized or separated from clustered PS.

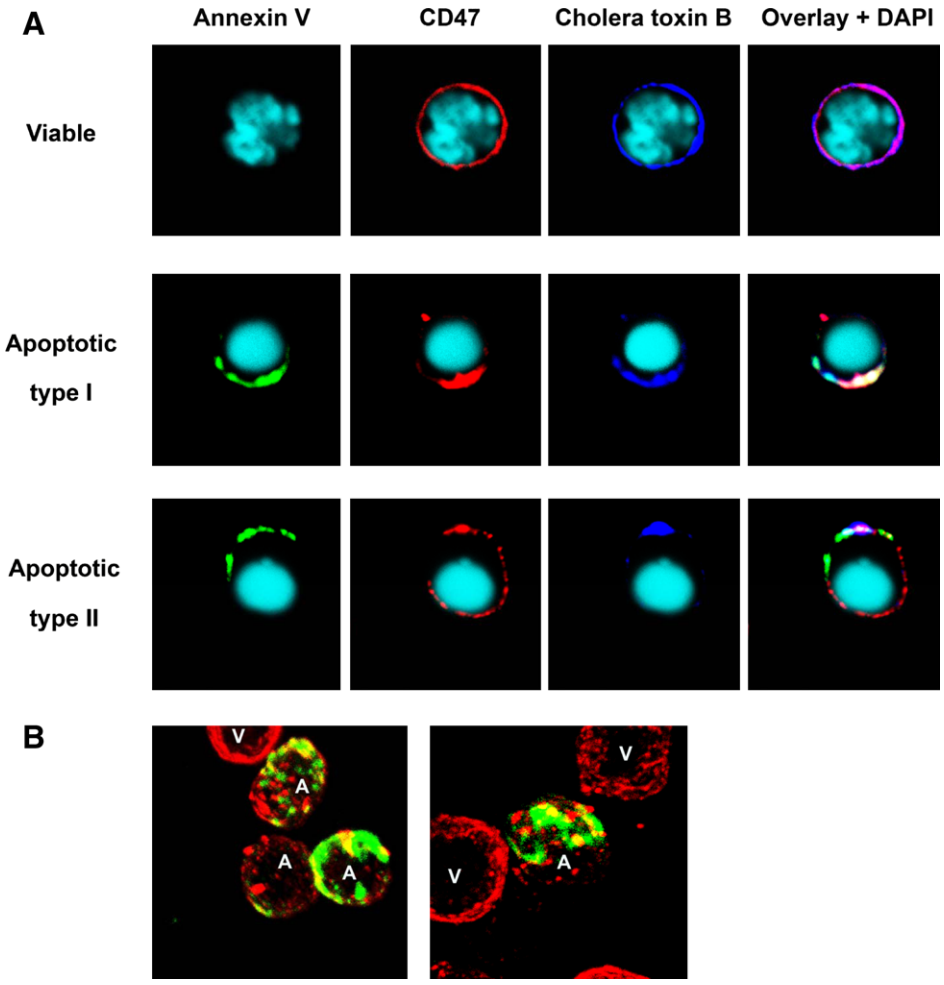


Fig. 4. CD47 is aggregated, and may co-localize with clustered PS and GM-1 domains, on apoptotic thymocytes. (A) The distribution of CD47, PS, and cholera toxin B (CtB)$^+$ GM-1 domains, are shown in confocal sections of representative viable (Annexin V$^-$), or apoptotic (Annexin V$^+$) cells. Both CD47 and CtB$^+$ GM-1 domains are evenly distributed on the surface of viable cells. In some apoptotic cells, clustered PS to a large extent overlapped with clustered CD47 and CtB$^+$ GM-1 domains (Apoptotic type I). In other apoptotic cells, clustered CD47 showed a minimal overlap with PS and CtB$^+$ GM-1 domains (Apoptotic type II). (B) Confocal *z*-stacks, obtained by scanning focal sections 0.5 μm apart throughout the cells, showed that clustered CD47 may be present in membrane domains overlapping with or segregated away from clustered PS. Shown are representative cells from at least 3 independent experiments. V = viable cells, A = apoptotic cells.

A large number of receptors on the phagocyte and ligands on the apoptotic cells have been shown to be involved in apoptotic cell uptake [4,24], where the uptake mechanisms involved are dependent on the macrophage phenotype [23]. Uptake by non-activated BMM is independent of PS-recognition, whereas activation for 48 h with β-1,3-glucan induces PS-dependent uptake mechanisms [8,26]. Also in thioglycollate-elicited murine peritoneal macrophages, uptake is dependent on PS-recognition [26]. We found that CD47 on apoptotic thymocytes is important to promote phagocytosis in non-activated macrophages, but not in β glucan-activated macrophages. This finding is supported by the finding that apoptotic CD47-deficient murine T cell lymphoma WR19L cells were phagocytosed by thioglycollate-elicited peritoneal macrophages to the same extent as CD47 Wt apoptotic thymocytes, whereas the mouse macrophage cell line BAM3 showed reduced PS-dependent uptake of the WR19L cells [14]. Thus, CD47 on the apoptotic cell may be less important to facilitate uptake by inflammatory-activated macrophages. As was previously suggested [14], the importance of apoptotic cell CD47, in relation to the macrophage phenotype, may indeed be dependent on the exact contribution of other receptor–ligand interactions or bridging molecules involved in apoptotic cell uptake. It is therefore interesting to note, that CD47 promoted both PS-independent and PS-dependent uptake by non-activated macrophages *in vitro*, and by RPM *in vivo*. Furthermore, that CD47 rather promotes than inhibits uptake of apoptotic cells in the murine system, raised the question of whether CD47 could at all function as a "marker of self/don't eat me-signal" on lymphocytes [14]. We think that our present data clearly show that CD47 indeed negatively regulates phagocytosis of viable thymocytes, but that this function of CD47 may be lost on apoptotic thymocytes.

We found a changed distribution of CD47 on the cell surface during apoptosis. On viable cells, CD47 was evenly distributed, whereas it is clustered into patches during apoptosis. Despite this redistribution of CD47 on apoptotic cells, SIRPα fusion protein binding to apoptotic cells does not seem to be affected (Nilsson and Oldenborg, unpublished data; [14]). In apoptotic human neutrophils or Jurkat cells, we found that clustered PS was co-localized with cholesterol-rich GM-1 domains in the plasma membrane, but clustered CD47 did neither co-localize with GM-1, nor with PS [13]. However, according to our present results, clustered CD47 may be co-localized with both PS and GM-1 in certain domains on the surface of apoptotic murine T cells, suggesting a possible species difference. In addition, plasma membrane cholesterol may indeed be important for the clustering of CD47, since we found that cholesterol-depletion with cyclodextrin reduced the clustering of

CD47 and PS in Dex-treated murine thymocytes (Nilsson and Oldenborg, unpublished observation). Although the exact mechanisms behind clustering of CD47 on apoptotic cells are still unclear, it is interesting to speculate that the clustering of CD47 may be required for its function to mediate binding to the phagocyte, while avoiding the phagocytosis-inhibitory role played by CD47 on viable cells.

Conclusion

CD47 on viable thymocytes prevents their uptake by primary murine macrophages, a function which is lost on apoptotic thymocytes, where CD47 instead mediates binding to the phagocyte and facilitates efficient uptake. CD47 promotes both PS-dependent and PS-independent apoptotic cell uptake by non-activated macrophages, but not by activated macrophages. On apoptotic thymocytes, CD47 becomes clustered into patches which may or may not co-localize with clustered PS. Clustering of CD47 may be one mechanism that mediates the dual role of CD47 to inhibit phagocytosis of viable cells and stimulate uptake of apoptotic cells.

Acknowledgments

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