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Cell-Surface Calreticulin Initiates Clearance of Viable or Apoptotic Cells through *trans*-Activation of LRP on the Phagocyte

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Summary

Apoptotic-cell removal is critical for development, tissue homeostasis, and resolution of inflammation. Although many candidate systems exist, only phosphatidylserine has been identified as a general recognition ligand on apoptotic cells. We demonstrate here that calreticulin acts as a second general recognition ligand by binding and activating LDL-receptor-related protein (LRP) on the engulfing cell. Since surface calreticulin is also found on viable cells, a mechanism preventing inadvertent uptake was sought. Disruption of interactions between CD47 (integrin-associated protein) on the target cell and SIRPα (SHPS-1), a heavily glycosylated transmembrane protein on the engulfing cell, permitted uptake of viable cells in a calreticulin/LRP-dependent manner. On apoptotic cells, CD47 was altered and/or lost and no longer activated SIRPα. These changes on the apoptotic cell create an environment where "don't eat me" signals are rendered inactive and "eat me" signals, including calreticulin and phosphatidylserine, congregate together and signal for removal.

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Introduction

Removal of apoptotic cells in multicellular organisms is critical for development, tissue remodeling, maintenance of homeostasis, and response to injury. Rapid and efficient apoptotic-cell clearance is crucial to prevent cell lysis and the release of proinflammatory and antigenic autoimmune components. Clearance of apoptotic cells appears to occur by highly conserved mechanisms and specific signaling pathways (Hoffmann et al., 2001; Ogden et al., 2001). The uniqueness of this process has led us to suggest the name efferocytosis (from effero—to carry to the grave, to bury) (deCathelineau and Henson, 2003). Many different cell types are capable of efferocytosis, including both professional (macrophages or immature dendritic cells) and so-called nonprofessional phagocytes (fibroblasts and endothelial and epithelial cells). Apoptotic-cell removal in vivo usually occurs in situ by near neighbors and generally without induction of a local tissue response.

A wide variety of phagocyte (efferocyte) cell-surface receptors and soluble bridging molecules have been implicated in recognition and removal of apoptotic cells (Savill et al., 2002). Surprisingly, much less is known about the apoptotic-cell-surface structures that are being recognized. We earlier showed that they expose phosphatidylserine (PS) and that this was involved in their removal, as well as in the induction of anti-inflammatory and anti-immunogenic responses (Fadok et al., 2001a; Hoffmann et al., 2005). Although other surface changes have been implicated in apoptotic-cell recognition (see Dini, 2000; Moffatt et al., 1999), no other ligand with such a wide distribution between cell types and across animal phyla has yet been identified.

Calreticulin is a highly conserved, 46 kDa protein earlier considered to be an obligate endoplasmic reticulum protein. The localization of calreticulin within the cell is broad and is still not completely clear. It is found in the ER/SR membranes (Ostwald and MacLennan, 1974) and seems to localize to the nucleus (Burns et al., 1994). It is also found on the surface of most mammalian cells (Arosa et al., 1999; Sadasivan et al., 1996; White et al., 1995) and was earlier shown to be identical to a key cellular receptor for C1q (Stuart et al., 1997). Cellular stress, including apoptosis, induces expression of many stress proteins, including calreticulin (Heal and McGivan, 1998); this may lead to increased amounts of calreticulin on the cell surface (Jethmalani et al., 1997), although how calreticulin gets there is not fully understood. It may remain associated with proteins that it chaperones, such as MHC class 1, or, alternatively, its KDEL retrieval sequence may be cleaved (Arosa et al., 1999).

In the ER, calreticulin participates in calcium homeostasis and acts as a protein chaperone (Krause and Michalak, 1997; Sadasivan et al., 1996). On the cell surface, it binds thrombospondin and induces focal adhesion disassembly (Orr et al., 2003a). This effect is mediated through the LDL-receptor-related protein (LRP, also known as CD91 or the α2-macroglobulin receptor)

(Orr et al., 2003b). Our own previous studies have implicated calreticulin in apoptotic-cell removal, as it can act as a "receptor," in conjunction with LRP, on the efferocyte for the collagenous tails of collectin family members (Ogden et al., 2001; Vandivier et al., 2002). These ligands for calreticulin can stimulate cells through a *cis* interaction with LRP on the same responding cell.

However, the wide distribution of calreticulin on cell surfaces also raised the possibility that this molecule on apoptotic cells could directly bind and stimulate LRP on a responding cell, i.e., acting in *trans* mode. Accordingly, the potential for calreticulin to serve as another general ligand for recognition and clearance of apoptotic cells is addressed and supported by the data reported herein.

Calreticulin is found on the surface of viable cells; why then does it not induce their uptake? We provide evidence that surface calreticulin is increased and redistributed during apoptosis, possibly enhancing stimulation of LRP on the efferocyte. In addition, the process of apoptosis is shown to induce alterations of active regulatory signals present on viable cells that normally prevent their removal.

CD47 (integrin-associated protein, IAP), is widely distributed on cells and binds to a number of proteins (Brown and Frazier, 2001; Cant and Ullrich, 2001), including SIRPα, a heavily glycosylated transmembrane protein with an immunoreceptor tyrosine-based inhibition motif (ITIM) domain. Ligation of SIRPα leads to tyrosine phosphorylation (Kharitonenkov et al., 1997) and subsequent activation of inhibitory tyrosine phosphatases such as SHP-1 and SHP-2. Interaction of CD47 with SIRPα blocks IgG or complement-induced phagocytosis (Oldenborg et al., 2001). Moreover, nonopsonized erythrocytes from *CD47*$^{-/-}$ mice are recognized and rapidly eliminated in wild-type recipients and are phagocytosed by wild-type macrophages in vitro (Oldenborg et al., 2000). For these reasons, we examined the possible role for the CD47-SIRPα system in preventing uptake of calreticulin-expressing viable cells and for its likely inactivation during apoptosis to allow removal of apoptotic cells.

Results

Calreticulin (CRT) on Apoptotic Cells Is a Ligand for Their Removal

Pretreatment of apoptotic fibroblasts, neutrophils, or Jurkat T cells with a blocking antibody against calreticulin (followed by washing) reduced their ingestion (Figure 1A). Fibroblasts were used as phagocytic cells to extend the observations to nonprofessional phagocytes as well as to avoid Fc receptor (FcR) effects. Furthermore, apoptotic CRT-deficient mouse embryonic fibroblasts (MEFs) were not engulfed by either macrophages or fibroblasts (Figure 1B). Since these cells underwent apoptosis to the same degree as wild-type (Figure 1C) and showed no changes in PS exposure (see below and see also Figure S2 in the Supplemental Data available with this article online), the difference in uptake can be attributed to the absence of calreticulin. Direct addition of exogenous calreticulin to either viable or apoptotic *CRT*$^{-/-}$ MEFs restored surface calreticulin levels to those observed on wild-type cells (Figure 1D) and restored the ability of apoptotic cells to be ingested by murine thioglycollate-elicited peritoneal macrophages, human monocyte-derived macrophages (HMDMs), and nonprofessional phagocytes (Figure 1E and Figure S1). The presence of a calreticulin-expressing cell could not itself passively restore staining on CRT-deficient MEFs (Figure S3). Heat shock protein 90 (Hsp90), another ligand for LRP, was used as a control and was shown to be ineffective. Additionally, factor Va blocking of PS on wild-type or *CRT*$^{-/-}$ MEFs reconstituted with calreticulin reduced efferocytosis of both cell types (data not shown), showing normal activity of the PS system.

A role for calreticulin in apoptotic-cell clearance in vivo was supported by examination of 13.5 day embryos from *CRT*$^{-/-}$ mice (the deletion is embryonic lethal; Mesaeli et al., 1999). In the wild-type and heterozygote embryos, apoptotic cells (stained for active caspase 3) were observed predominantly within macrophages (F4-80 positive) (Figure 1F), whereas increased numbers of noningested apoptotic cells were observed in the *CRT*$^{-/-}$ embryos (Figure 1G). In addition, apoptotic wild-type and *CRT*$^{-/-}$ MEFs were injected into the peritoneum of normal mice. Lavage after 1 hr showed that *CRT*$^{-/-}$ MEFs were not cleared as efficiently as controls (Figure 1H and Figure S4).

Direct Stimulation with Calreticulin Induces Macropinocytosis and Efferocytosis through LRP

Three additional lines of evidence support direct stimulation of engulfing cells by exogenous calreticulin. First, calreticulin induces macropinocytosis (Figure 2A), a process related to efferocytosis (Hoffmann et al., 2001). Hsp90 was ineffective. Second, bystander uptake (Hoffmann et al., 2001; Ogden et al., 2001) was directly stimulated by soluble calreticulin (Figures 2B and 2C). This was accompanied by the incorporation of lucifer yellow, a water-soluble dye used to measure extracellular fluid internalization and indicating formation of spacious phagosomes. Third, in keeping with the known requirement for Rac-1 in mammalian and nematode apoptotic-cell uptake, exogenous calreticulin directly induced activation of Rac-1 in J774 macrophages (Figure S5). In further support of the role for calreticulin, apoptotic *CRT*$^{-/-}$ MEFs reconstituted with exogenously added calreticulin were now able to stimulate membrane ruffling and macropinocytosis in macrophages (Figure 2D and Movies S1 and S2).

Cell-surface calreticulin is known to bind and form complexes with LRP on that same surface, i.e., to act in a *cis* mode (Orr et al., 2003b). This made phagocyte LRP the prime candidate also for this *trans*-stimulation by calreticulin on the apoptotic cell. Thus, a ligand-blocking antibody against LRP was found to substantially reduce apoptotic-cell uptake (to the levels observed with *CRT*$^{-/-}$ targets) (Figure 2E). An antibody against the intracellular domain of LRP (which did not alter LRP activity) or an irrelevant surface protein, CD45, had no effect. To determine if exogenous calreticulin directly interacted with LRP, wild-type and *LRP*$^{-/-}$ MEFs were examined for binding of exogenous calreticulin by flow cytometry (Figure 2F). In contrast to wild-type cells, not only did *LRP*$^{-/-}$ MEFs show reduced calretic-

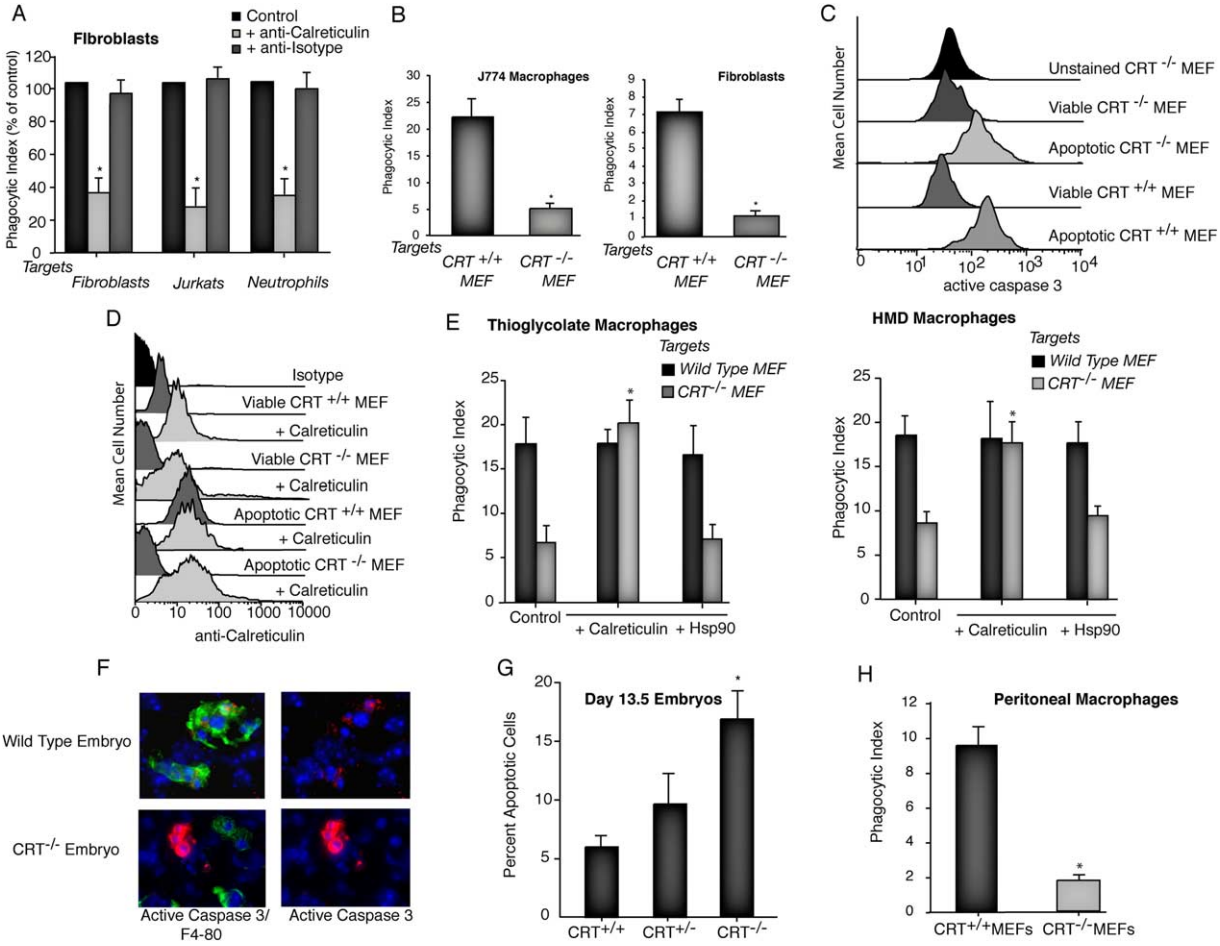

Figure 1. Calreticulin on Apoptotic Cells Is Required for Their Engulfment

Error bars are ±SD. *p < 0.05 versus control.

(A) Blockade of surface calreticulin on apoptotic Jurkat T cells, human neutrophils, or murine MEFs with anti-CRT antibody inhibited uptake into MEFs without affecting their viability or the viability of the phagocyte.

(B) Engulfment of apoptotic *CRT*$^{-/-}$ MEFs by J774 macrophages or fibroblasts was significantly decreased in comparison with uptake of wild-type MEF cells.

(C) Demonstration of comparable apoptosis in *CRT*$^{-/-}$ or wild-type. Apoptosis was assessed with a Phi-Phi Lux reagent and analyzed for peptide cleavage by flow cytometry.

(D) Calreticulin can be reconstituted on the surface of *CRT*$^{-/-}$ MEFs. Wild-type and *CRT*$^{-/-}$ MEFs were stained for calreticulin after pretreatment (followed by washing) with exogenously added calreticulin. Isotype control is of apoptotic wild-type MEFs and is representative of all samples.

(E) Reconstitution of calreticulin on the *CRT*$^{-/-}$ cells restored their ability to be ingested. Apoptotic wild-type and *CRT*$^{-/-}$ cells were coated with either calreticulin or Hsp90 and assessed for uptake into murine thioglycollate-elicited macrophages or human monocyte-derived macrophages (HMDMs).

(F) CRT-deficient embryos exhibit decreased numbers of apoptotic cells associated with macrophages. Day 13.5 embryos were sectioned and stained for active caspase 3 (red) or a macrophage cell marker F4-80 (green) and DAPI (blue) and were analyzed for interactions.

(G) Apoptotic bodies in wild-type and calreticulin heterozygote and knockout embryos were quantified.

(H) Apoptotic *CRT*$^{-/-}$ or wild-type MEFs were instilled into the murine peritoneum for 60 min. After 60 min, lavagates were assessed for phagocytosis.

ulin on their surface at baseline, but upon addition of calreticulin there was no enhancement of surface levels.

Receptor-associated protein (RAP) has been used extensively as an antagonist in the study of LRP and blocks LRP signaling to a large variety of ligands (Bu, 2001; Obermoeller-McCormick et al., 2001; Orr et al., 2003a). RAP inhibited the engulfment of *CRT*$^{-/-}$ MEFs reconstituted with calreticulin to the same degree as seen with anti-LRP antibody (Figure 2G). This indicated that calreticulin on target cells binds directly to macrophage LRP. To further support direct interaction of calreticulin and LRP, pretreatment of macrophages with RAP inhibited calreticulin-induced macropinocytosis (Figure 2H) and activation of Rac-1 (Figure S5). Importantly, *LRP*$^{-/-}$ MEFs stimulated with calreticulin were unable to undergo macropinocytosis (Figure S6). *LRP*$^{-/-}$ MEFs were inefficient at ingesting apoptotic cells and

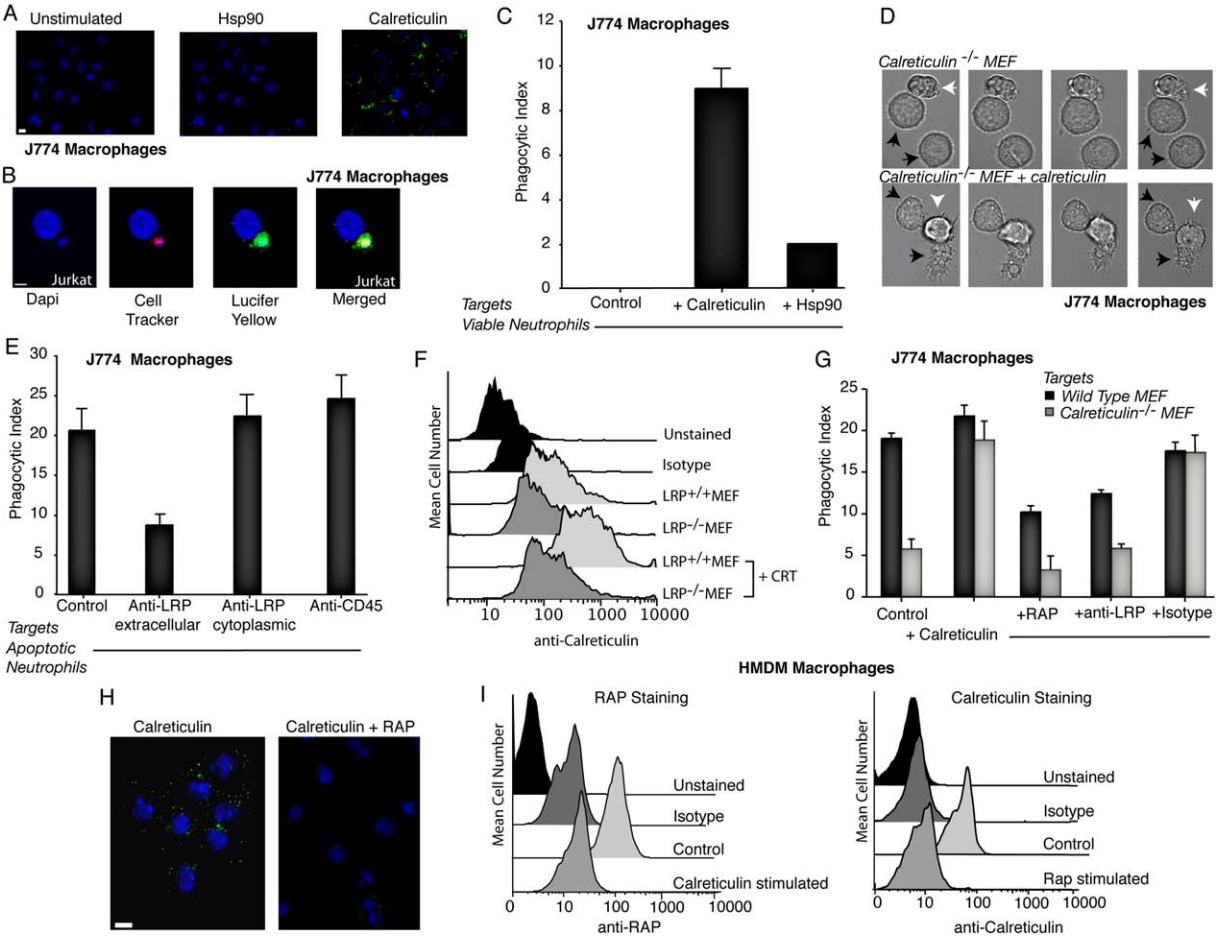

Figure 2. Direct Addition of Calreticulin Stimulates Macrophage Ruffling, Macropinocytosis, and Bystander Uptake

Error bars are ±SD.

(A) Macropinocytosis: macrophages were stimulated with calreticulin or Hsp90 as a control in the presence of lucifer yellow (LY) for 20 min. Nuclei are shown in blue (DAPI) and LY uptake in green.

(B) Stimulation with soluble calreticulin initiates engulfment of viable neutrophils preadhered to J774 macrophages (see Experimental Procedures).

(C) Bystander uptake of preadhered viable Jurkat T cells into macrophages stimulated with soluble calreticulin involves engulfment into spacious phagosomes with accompanying LY. The Jurkat T cells are red (cell tracker red), and LY is shown in green.

(D) Calreticulin on the surface of target cells promotes ruffling, macropinocytosis, and engulfment in macrophages. Apoptotic *CRT*$^{-/-}$ MEFs alone or reconstituted with calreticulin were incubated with J774 macrophages, and live images were captured (see also Movies S1 and S2). Shown are representative 15 s frames from a 30 min movie. Black arrows identify J774 macrophages; white arrows identify the apoptotic MEFs.

(E) Ingestion was inhibited with a blocking anti-LRP antibody. Macrophages were treated with antibodies against either the intra- or extracellular domains of LRP as well as the irrelevant surface protein CD45 for 30 min before examination of apoptotic-cell uptake.

(F) Wild-type and *LRP*$^{-/-}$ MEFs were analyzed for surface calreticulin expression by flow cytometry in either the presence or absence of calreticulin. Isotype controls were identical; the one shown is for apoptotic wild-type MEFs and is representative of all isotypes performed.

(G) Preincubation of J774 macrophages with RAP, an inhibitor of LRP, or the LRP-blocking antibody inhibited engulfment of apoptotic *CRT*$^{-/-}$ MEFs reconstituted with calreticulin. Percent inhibition of RAP and anti-LRP for calreticulin-coated wild-type MEFs was 65 ± 5.8 and 63.3 ± 4, respectively. This was similar to 76% ± 5% and 76% ± 6% for the *CRT*$^{-/-}$ MEFs.

(H) Addition of RAP prevented calreticulin-induced macropinocytosis. J774 macrophages pretreated with RAP were stimulated with calreticulin in the presence of lucifer yellow (green) before fixation and processing for ingested LY, nuclei (blue).

(I) Treatment of macrophages with calreticulin reduced surface antibody staining for RAP. HMDMs stimulated in the presence or absence of calreticulin and then stained with anti-RAP antibody were examined by FACS. In reverse, treatment of HMDMs with RAP reduced staining for surface calreticulin. Isotypes presented are HMDMs treated with calreticulin or RAP, respectively, and were equivalent for all samples.

therefore could not be used as phagocytes in these experiments.

The mechanisms by which calreticulin activates LRP are unknown. In the *cis* mode, thrombospondin was suggested to induce calreticulin-LRP association (Orr et al., 2003b). On the other hand, in keeping with a suggestion by Li et al. (2000), we hypothesized that calreticulin may stimulate LRP by displacing normally suppressive RAP. To examine this possibility, macrophages were stained for surface RAP after incubation with calreticulin. Addition of calreticulin significantly reduced the amount of surface RAP, and, reciprocally, RAP re-

duced the levels of calreticulin (Figure 2I), supporting a competitive effect on LRP binding sites. In addition, only the full-length form of calreticulin and a mutant form lacking the thrombospondin binding domain reduced the amount of surface RAP (Figure S8). A fragment of calreticulin containing only the thrombospondin binding region had no effect on RAP levels and was unable to restore engulfment of *CRT*$^{-/-}$ MEFs (Figure S7). While these experiments support direct stimulation of efferocyte LRP by calreticulin, we also questioned the possible contribution of thrombospondin. This has been suggested to participate in apoptotic-cell clearance (Savill et al., 1992) and is known to ligate calreticulin to stimulate LRP in a *cis* mode on the same responding cell (Orr et al., 2003b). Importantly, *CRT*$^{-/-}$ MEFs reconstituted with calreticulin or mutated forms lacking the thrombospondin binding domain restored uptake into macrophages. However, a peptide fragment of calreticulin containing only the thrombospondin binding domain had no effect (Figure S7).

Calreticulin Distribution on Apoptotic Cells

A potential problem with this model is that calreticulin is found on the surface of most cells, whether or not they are apoptotic, without stimulating uptake. To explain the selective recognition and effect of calreticulin on apoptotic versus viable cells, we suggest alterations in the amount and distribution of calreticulin during apoptosis as well as alterations in "don't eat me" signals that normally prevent uptake of viable cells. Examination of a variety of cell types with different apoptosis inducers showed that the overall amounts of calreticulin on the cell surface increased during apoptosis (shown for fibroblasts and Jurkat T cells in Figure 3A). Intriguingly, for both cell types, a subpopulation showed a high-intensity staining with anti-calreticulin. This did not correlate exactly with the proportion that was apoptotic at that time point or with membrane permeability. Less than 5% were propidium iodide permeable, and these showed only low-intensity staining for calreticulin (data not shown). The potential relationship between increased calreticulin exposure and the degree of apoptosis is shown for neutrophils in Figure 3B. Surface calreticulin increased as cells became more apoptotic (Figure 3B), and this correlated with decreased CD47 levels (Figure S10) and an increased ability to become engulfed (phagocytic indexes were 3, 6, 10, and 15, respectively). Additionally, surface calreticulin became markedly redistributed into patches on the cell surface of apoptotic neutrophils (Figure 3C), a phenomenon seen for all cells so far studied and that may contribute to calreticulin's ability to stimulate LRP on the efferocyte. Furthermore, exogenous calreticulin on the surface of apoptotic *CRT*$^{-/-}$ cells was seen in patches, but less so if they were viable (Figure 3D). At this point, we cannot rule out a possible change in calreticulin structure or exposure on the apoptotic cell that may make it a more effective LRP stimulus.

Ingestion of Viable Cells in the Absence of CD47-SIRPα "Don't Eat Me" Stimulation

An additional explanation for calreticulin (or indeed other ligand) gain of function could be that apoptotic cells lose normally inhibitory signals that inhibit calreticulin-LRP stimulation. A likely candidate is CD47 on the target interacting with SIRPα on the efferocyte. As shown in Figure 4A, viable *CD47*$^{-/-}$ erythrocytes or leukocytes were readily taken up into macrophages and nonprofessional phagocytes by processes similar to apoptotic-cell removal. Leukocytes were engulfed into spacious phagosomes with accompanying extracellular fluid (Figure 4B), and engulfment of erythrocytes was blocked by amiloride (Figure S9), which inhibits macropinocytosis and efferocytosis but not FcR-mediated phagocytosis (deCathelineau and Henson, 2003; Hoffmann et al., 2001). In addition, inhibition of CD47 on target cells with a Fab'_2 antibody and blockade of SIRPα on the engulfing cell each led to uptake of viable human neutrophils (Figures 4C and 4D). Engulfed cells were identified as viable by maintenance of normal nuclear architecture and lack of activated caspase 3 (Figures 4E and 4F). Accompanying this uptake induced by blockade of CD47-SIRPα "don't eat me" signals was extensive induction of membrane ruffling (Movies S3 and S4).

The uptake appeared dependent on LRP and calreticulin since *CD47*$^{-/-}$ erythrocytes incubated with *LRP*$^{-/-}$ MEFs were not ingested, compared to wild-type MEFs (Figure 5A). By plating macrophages on surfaces coated with anti-LRP antibody to "modulate" the LRP beneath the engulfing cells and away from the targets, suppression of uptake was again observed (Figure 5B). This treatment does not suppress uptake through the Fc receptor (Ogden et al., 2001). Similar data were obtained with viable *CD47*$^{-/-}$ leukocytes as targets (data not shown). Additionally, antibody blockade of calreticulin on the *CD47*$^{-/-}$ target cells as well as inhibition of macrophages with RAP prevented uptake to the same degree (Figure 5C). Importantly, blockade or absence of CD47 did not alter PS exposure (Figure 5D), i.e., PS and its recognition processes did not appear to be required.

Redistribution of Calreticulin and PS Away from CD47 on the Apoptotic-Cell Surface

CD47 is present on most cells and must presumably be removed or inactivated during apoptosis to allow their remarkably efficient clearance. Examination of a variety of apoptotic-cell types (e.g., neutrophils and fibroblasts) revealed an overall decrease in detectable CD47 (Figures 6A and 6B) that correlate with the degree of apoptosis (Figure S10). By contrast, murine lymphocytes or human Jurkat T cells did not show a decrease in CD47 (Figure 6C) but rather an increase in surface patching during apoptosis (Figure 6D). Patching occurred on other apoptotic-cell types whether or not the total surface levels were reduced, and, most importantly, the CD47 became segregated away from calreticulin (Figure 6E and data not shown).

Redistribution of surface CD47 could reduce its overall ability to bind SIRPα on the efferocyte. However, we did not find a significant difference in binding of a soluble SIRPα-Fc construct to viable or apoptotic Jurkat T cells or neutrophils (data not shown). On the other hand, stimulation of the SIRPα by CD47 on the apoptotic cell was altered. Viable but not apoptotic Jurkat T cells induced macrophage SIRPα phosphorylation to levels seen following direct addition of a CD47-Fc li-

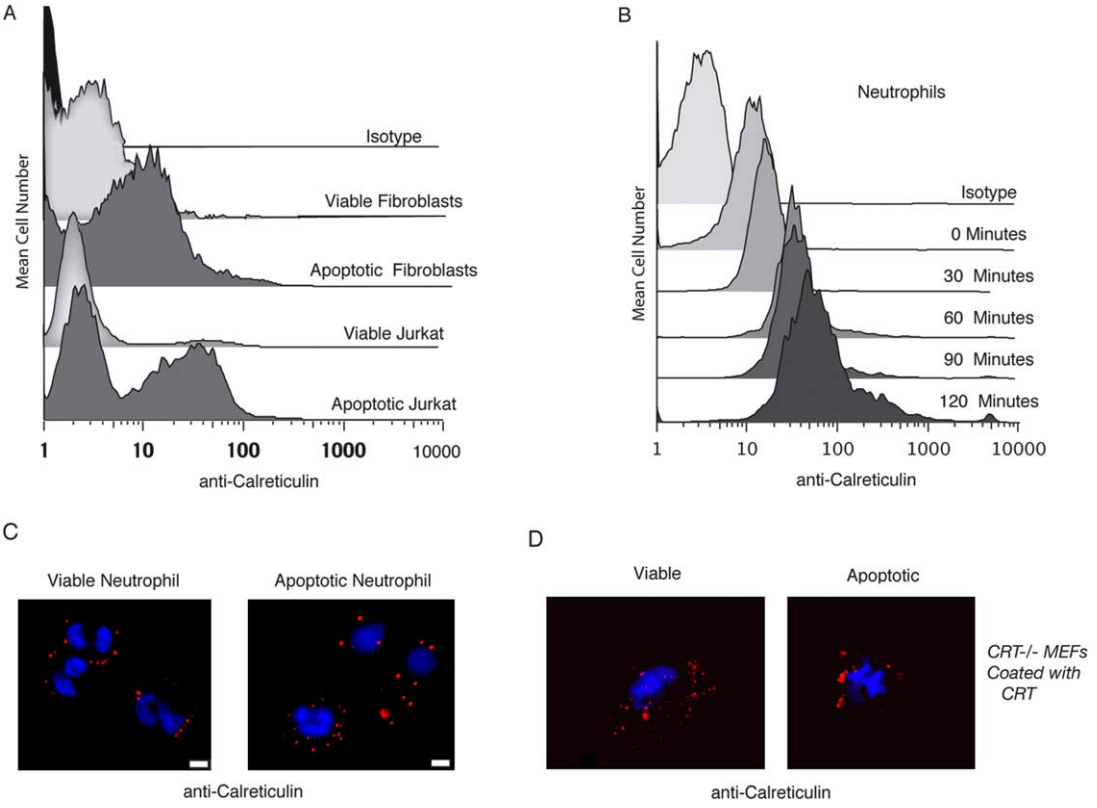

Figure 3. Calreticulin Is Upregulated and Redistributed on Apoptotic Cells

(A) Surface expression of calreticulin was increased on apoptotic fibroblasts and Jurkat T cells as measured by FACS analysis with an anti-calreticulin antibody. PI-positive cells were excluded (5%). The isotype control is of apoptotic fibroblasts and is representative of all isotype samples.

(B) Neutrophils were induced to undergo apoptosis for various times and calreticulin levels assessed by flow cytometry. Apoptosis at the various time points was 5%, 18%, 31%, 52%, and 67%. Isotype control represents neutrophils undergoing apoptosis for 120 min.

(C) The pattern of calreticulin staining changes as cells undergo apoptosis, becoming distinctly more patched. Viable and apoptotic neutrophils were stained with an anti-calreticulin antibody, fixed, and examined. Calreticulin staining is shown is red and nuclei in blue.

(D) Exogenous addition of calreticulin to $CRT^{-/-}$ MEFs also displayed the patched surface staining changes on $CRT^{-/-}$ MEFs as they became apoptotic.

gand even though the CD47 on the latter was not reduced in total amount (Figure 6F). Viable neutrophils also induced SIRPα phosphorylation (Figure 6F). The target cells did not contribute any phosphorylated SIRPα (data not shown). SIRPα acts through stimulation of the tyrosine phosphatases SHP-1 and 2, and its activation of SHP-1 can inhibit Fc-mediated phagocytosis (Okazawa et al., 2005). Accordingly, pretreatment of macrophages with a SHP-1 inhibitor promoted uptake of viable neutrophils, while a control SHP-2 inhibitor had little effect (Figure 6G). Furthermore, stimulation of SIRPα with the soluble CD47-Fc blocked apoptotic-cell uptake (Figure 6H).

Calreticulin Associates with PS on Apoptotic Cells

The redistributed CD47 segregated away from calreticulin on the apoptotic cell (Figure 6E), suggesting a separation of "don't eat me" signaling from those inducing uptake. Apoptotic wild-type and $CRT^{-/-}$ MEFs were shown to expose PS to comparable degrees (Figure 7A). Examination of the apoptotic-cell surfaces, as shown in Figure 7B, demonstrated that a significant proportion of the two "eat me" ligands, PS and calreticulin, were found together in patches on the apoptotic-cell membrane and therefore away from CD47. Additionally, apoptotic cells that exposed PS displayed higher levels of calreticulin (Figure S11). PS and calreticulin also colocalized with markers for cholesterol-rich, GM-1 ganglioside-containing "rafts" (Figure 7C). Thus, the "don't eat me" CD47 became excluded from the "eat me" calreticulin and PS domains on the apoptotic cell (Figure S12). CD47 has also been reported to reside within cholesterol-rich domains. Thus, its segregation away from GM-1-, PS-, and calreticulin-containing regions suggests either its loss from such domains or its association with cholesterol-rich regions containing different glycosphingolipids. At this point, neither the structures on the cell to which endogenous or exogenous calreticulin is binding nor the mechanism for its exposure is known. Nevertheless, our data support a model in which PS becomes exposed in cholesterol-rich domains (Frasch et al., 2004) along with calreticulin and, together with calreticulin, provides cooperative stimuli for engulfment.

To help elucidate the complex interaction between the "eat me" PS and calreticulin signals and "don't eat me" CD47, the human monomyelocytic PLB-985 cell line was employed. Undifferentiated, these cells undergo normal apoptosis but do not expose PS, whereas after differentiation they recover this ability (Fadok et al., 2001b). Uptake of undifferentiated apoptotic PLB-985 cells is decreased compared to differentiated, PS-exposing cells, supporting the role of PS in engulfment (Fadok et al., 2001b). Undifferentiated PLB-985 cells do exhibit surface calreticulin, which is both upregulated and redistributed during apoptosis (Figure 7E). This might indicate a primary role for PS in stimulating efferocytosis. However, the apoptotic undifferentiated PLB-985 cells neither downregulated nor redistributed their surface CD47 (Figure 7F), and, if cells were pretreated with blocking anti-CD47 Fab'_2 antibody, they were now readily ingested (Figure 7D), even though they were still deficient in PS exposure. Blockade of calreticulin also prevented uptake (data not shown). As expected, the differentiated apoptotic PLB-985 cells were ingested and not only exposed PS in a normal fashion but also exhibited decreased levels and redistribution of CD47 (Figure 7F and Figure S13). These observations support a key role for calreticulin in efferocytosis and also raise the intriguing question of a relationship between PS exposure and the loss or patching of CD47 (Figure 7G).

Discussion

Identifying the ligands on apoptotic cells that are involved in their recognition and engulfment has proven challenging. The data presented herein demonstrate that, in addition to phosphatidylserine, calreticulin serves as a crucial recognition and clearance ligand on apoptotic cells. It appears to mediate engulfment of apoptotic cells by activating the internalization receptor, LRP, on the phagocyte. Increasingly, LRP fits the role of a key signaling receptor for efferocytosis. It is highly conserved; the LRP homolog in *Drosophila* has also been implicated in apoptotic-cell clearance (Manaka et al., 2004), and the intracellular domains of mammalian LRP and *C. elegans* CED1 can be exchanged (Su et al., 2002). Not surprisingly, deletion of LRP or calreticulin in mice disrupts development and is lethal. We show that calreticulin activation of LRP stimulates Rac-1 and drives engulfment (efferocytosis) of apoptotic cells.

The experiments also suggest that calreticulin does not need to be bound by a ligand (such as thrombospondin or collectins) to engage and stimulate LRP. Rather, we suggest (see also Li et al., 2000) that the observed displacement of RAP from the LRP by calreticulin may lead to release of the LRP from a RAP-induced latent or inhibited state.

The role for surface calreticulin in recognition of apoptotic cells is supported by its upregulation and redistribution during apoptosis prior to postapoptotic membrane permeability. A wide variety of professional and nonprofessional phagocytes were unable to ingest $CRT^{-/-}$ cells unless calreticulin was restored on their surface. It is not at this point clear what structures the calreticulin binds to on either viable or apoptotic cells. Although not explored rigorously, restoration of calreticulin on apoptotic cells appeared to be saturated (Figure 1D), implying limited availability of binding sties. More calreticulin bound to apoptotic versus viable $CRT^{-/-}$ cells and, in the former, in distinct patches. The segregation of calreticulin into GM-1-positive areas indicates that the binding partner may redistribute into rafts.

In addition, the sites of calreticulin exposure or binding significantly, but not exclusively, corresponded with localized areas of PS exposure, supporting a combined role for each in optimal apoptotic-cell recognition and induction of uptake. It seems likely that calreticulin alone can stimulate uptake of apoptotic cells but, in normal circumstances, PS and calreticulin act together to drive optimal efferocytosis. On the other hand, PS appears to drive the anti-inflammatory consequences of apoptotic-cell recognition, whereas LRP stimulation is, if anything, proinflammatory (Gardai et al., 2003). We suggest a balance between these secondary effects, with the PS anti-inflammatory response usually dominant.

Apoptotic cells have been noted in $CRT^{-/-}$ embryos (Rauch et al., 2000) and herein. Clearance in vivo is usually highly efficient, and detection of free apoptotic cells may be evidence for a greatly increased rate of apoptosis or, more likely, clearance defects. These two possibilities cannot be distinguished in the $CRT^{-/-}$ mice, and loss of multifunctional calreticulin might increase apoptosis as well as decrease clearance (although $CRT^{-/-}$ MEFs can be cultured and do not show increased apoptosis in vitro; Figure 1C and Rauch et al., 2000). To further support a role for calreticulin in vivo, clearance of $CRT^{-/-}$ MEFs in the peritoneum of a normal mouse was found to be defective compared to wild-type control.

Calreticulin is also found on the surface of most viable cells examined, including erythrocytes (data not shown). Accordingly, if it is a key ligand for apoptotic-cell recognition, its apparent inactivity on such viable cells needs explanation. Upregulation and patching of calreticulin during apoptosis may enhance its effects but are unlikely to be the complete explanation. On the other hand, removal and/or inactivation of "don't eat me" signals during apoptosis could allow calreticulin and other ligands to now induce their uptake. We show here that direct activation of SIRPα with CD47 inhibits efferocytosis of apoptotic cells and that viable cells expressing normal amounts and distribution of CD47 stimulate SIRPα phosphorylation in the phagocytes and are not ingested. By contrast, apoptotic cells lose their ability to activate SIRPα and are engulfed. The mechanisms for the observed loss, redistribution, and inactivation have not been elucidated. However, the CD47 did become redistributed into patches that were segregated away from the calreticulin and PS "eat me" stimuli. In addition, many cell types (though not all) did show decreased staining for CD47 after they became apoptotic.

In contrast, Tada et al. (2003) proposed that CD47 is required for removal of apoptotic cells, although with a limited spectrum of phagocytic cells. A possible explanation is that bridging molecules found in plasma can use CD47 to tether apoptotic cells to phagocytes via

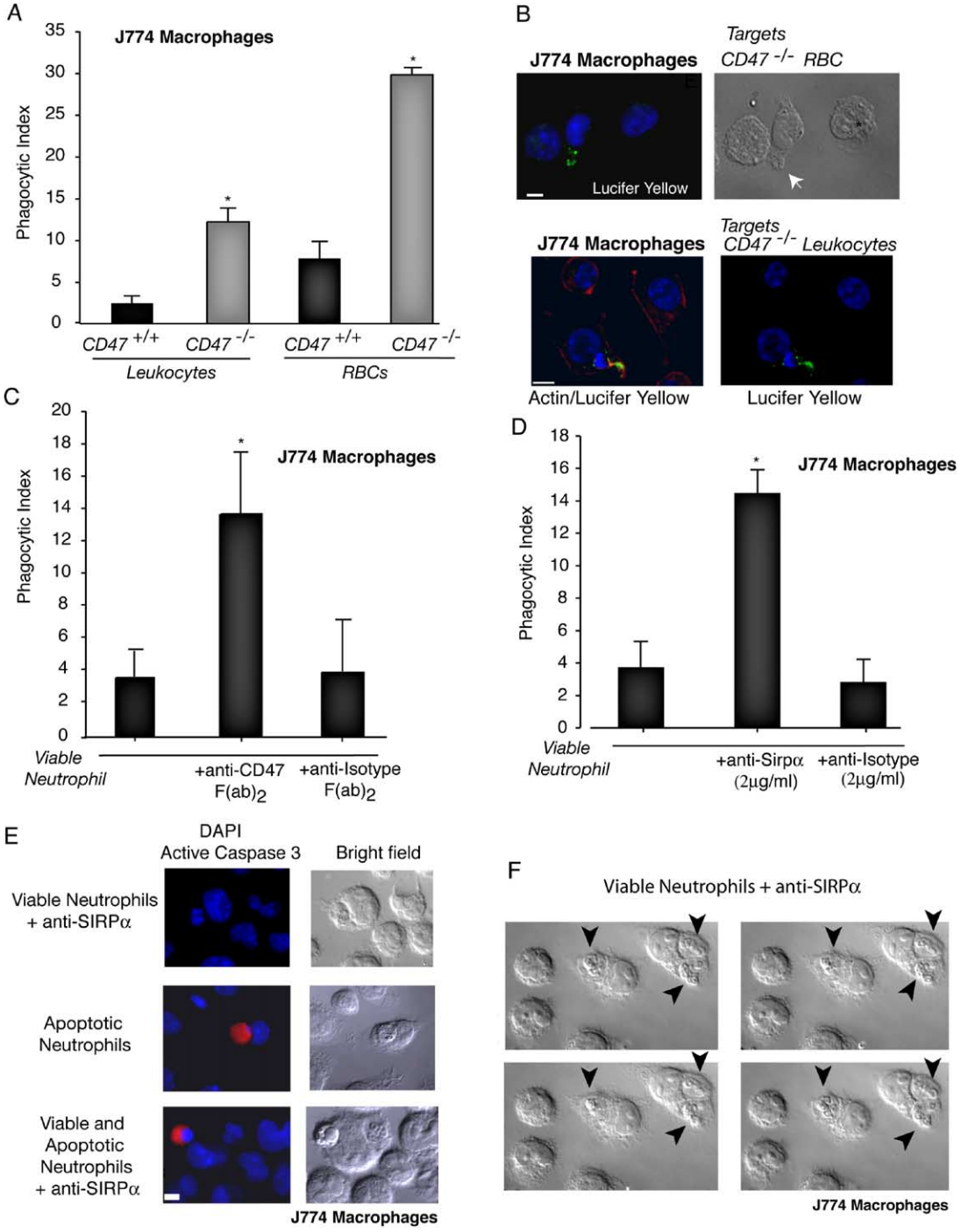

Figure 4. CD47 Is a Potent "Don't Eat Me" Signal on Viable Cells

Error bars are ±SD. *p < 0.05 versus control.

(A) Viable leukocytes and RBCs lacking CD47 are ingested by macrophages. Wild-type or *CD47*$^{-/-}$ leukocytes or RBCs isolated from whole blood were incubated with J774 macrophages and examined for uptake.

(B) Engulfment of *CD47*$^{-/-}$ RBCs and leukocytes incubated with J774 macrophages was accompanied by concurrent ingestion of lucifer yellow, indicating the formation of spacious phagosomes. J774 macrophage nuclei are stained blue (DAPI); the lucifer yellow, green; F-actin, red. The cell engulfed in the J774 macrophage is a viable mouse neutrophil. The white arrow on the DIC points to a *CD47*$^{-/-}$ RBC within the phagosome of a J774 macrophage.

(C and D) Blockade of CD47 on the target cells (C) or of SIRPα on the macrophages (D) resulted in viable-cell engulfment. Viable human neutrophils were treated with an anti-CD47 Fab′$_2$ antibody or an isotype control and incubated with J774 macrophages followed by washing before addition to the macrophages. Alternatively, the macrophages were incubated with an anti-SIRPα antibody or an isotype control prior to the addition of viable neutrophils.

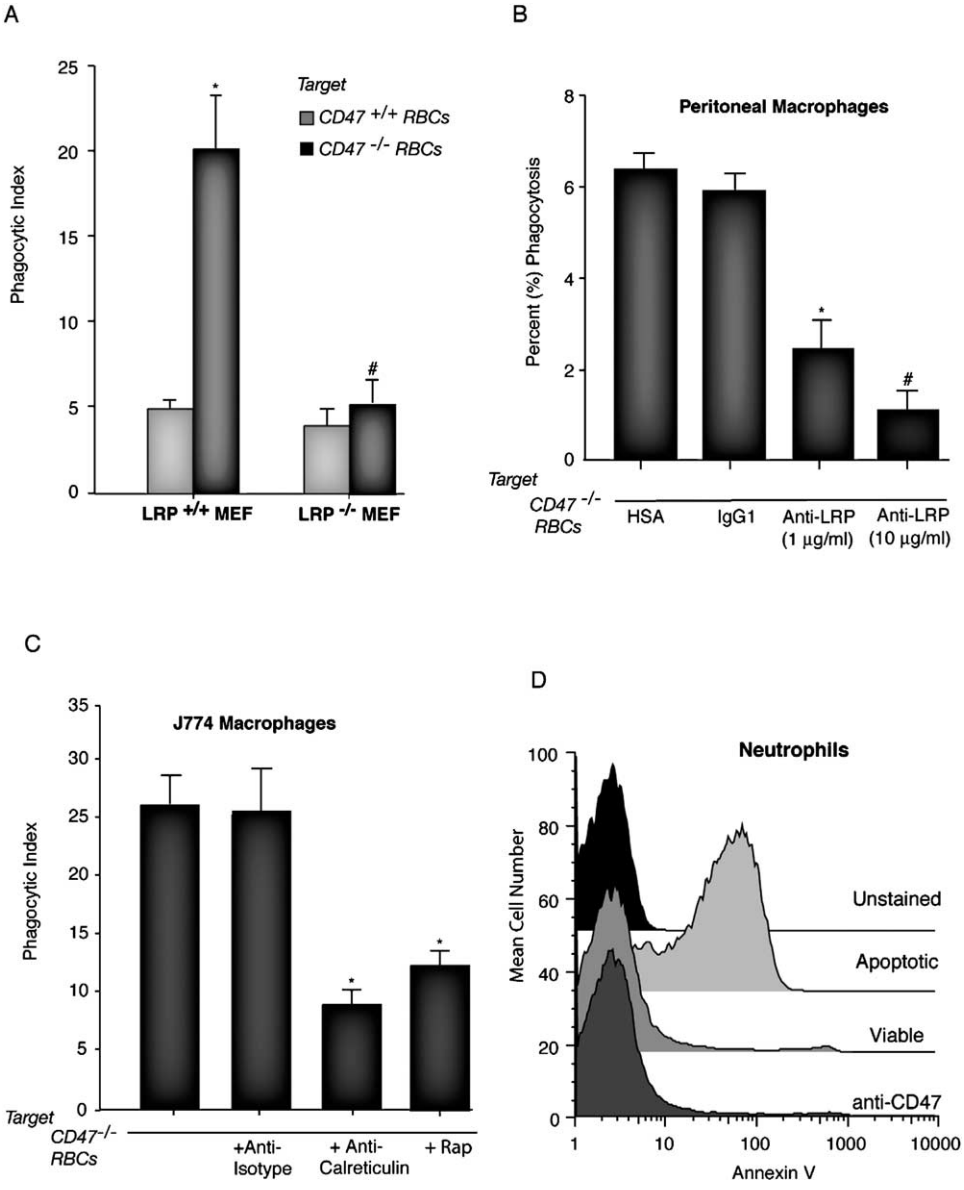

Figure 5. When CD47 or SIRPα Is Absent or Inactivated, Calreticulin on the Viable Cell Interacting with LRP on the Efferocyte Induces Engulfment

Error bars are ±SD. *p < 0.05 versus control; #p < 0.05 versus experimental sample.

(A) *LRP*$^{-/-}$ MEFs were unable to ingest *CD47*$^{-/-}$ RBCs.

(B) Sequestration of LRP beneath the macrophages by plating them on anti-LRP antibody prevented uptake of *CD47*$^{-/-}$ RBCs.

(C) Blocking calreticulin on *CD47*$^{-/-}$ RBCs by preincubation with anti-CRT antibody and addition of RAP to block LRP on the macrophages each prevented engulfment of the RBCs.

(D) To exclude a possible involvement of phosphatidylserine in uptake of CD47-inhibited viable cells, blocking Fab′$_2$ anti-CD47 (which induces uptake) was shown not to induce exposure of PS on viable neutrophils, i.e., staining with annexin V was negative.

their secondary bridging to CD36 or α_v integrins. In the presence of serum, apoptotic CD47-deficient leukocytes (i.e., with no CD47 at all) were not engulfed as well as CD47-expressing cells. However, in the absence of serum, although ingestion was less, the presence or absence of CD47 had no effect on phagocytosis (A.S.

(E) Demonstration of viable-cell uptake. J774 macrophages were pretreated with anti-SIRPα for 20 min prior to the addition of viable or apoptotic neutrophils or were incubated with apoptotic neutrophils without pretreatment. Samples were stained for anti-active caspase 3 (red) and DAPI (blue) to show uptake of cells with intact nuclei and lacking active caspase 3.

(F) To show complete uptake of viable cells, confocal sections were taken in series through the macrophages in 1 μm intervals. Panels are arranged from the upper cell surface to the bottom. Black arrows indicate viable neutrophil nuclei.

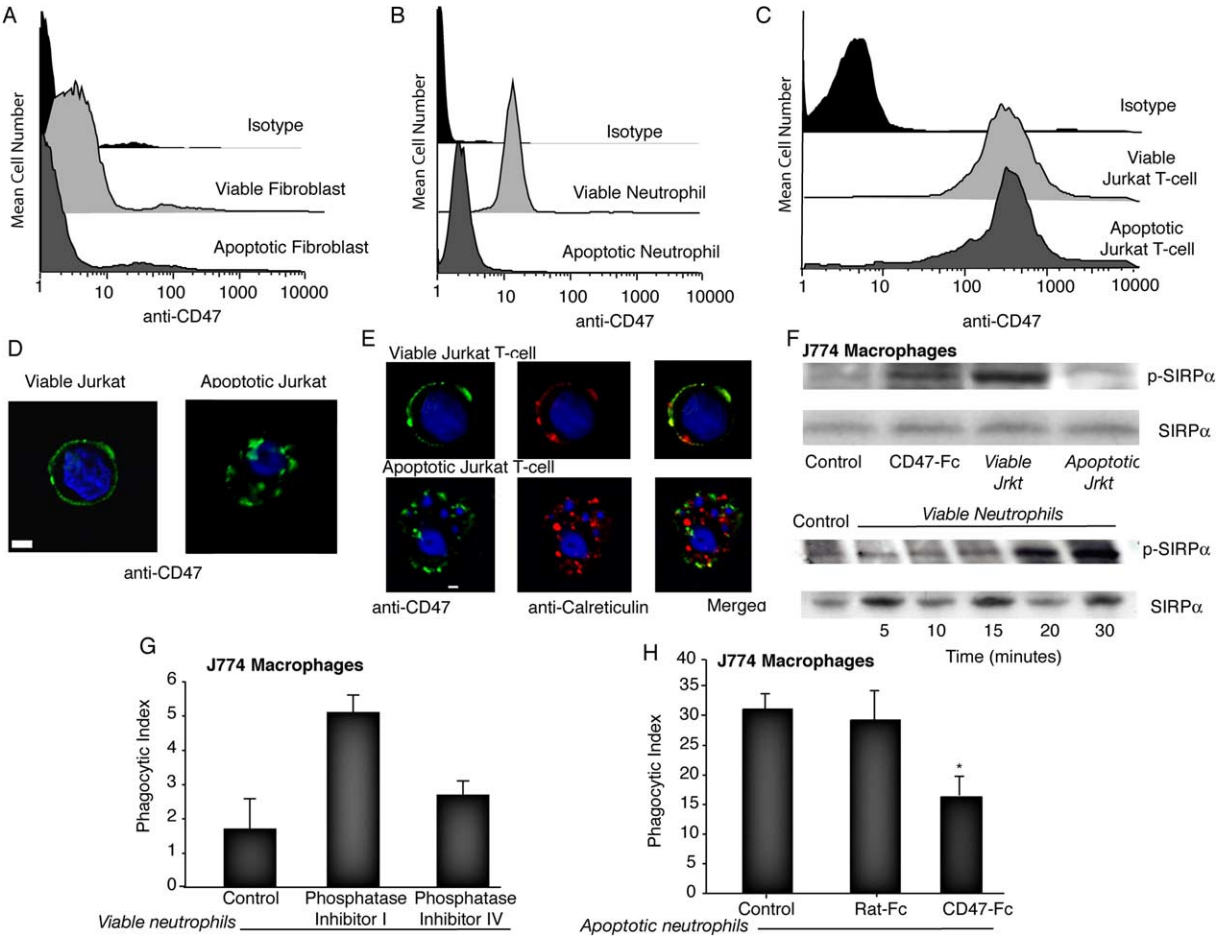

Figure 6. Changes in CD47 Amount, Distribution, and Signaling Capacity during Apoptosis

Error bars are ±SD. *p < 0.05 versus control.

(A–C) Viable and apoptotic cells were stained for surface CD47 and examined by flow cytometry. CD47 levels were found to be lower on apoptotic fibroblasts and neutrophils (A and B) but not Jurkat T cells (C).

(D) On the other hand, during apoptosis of Jurkat T cells (and in fact of all the cell types examined), CD47 was found to distribute into patches (stained green).

(E) Calreticulin (red) and CD47 (green) were seen to segregate away from each other on apoptotic cells.

(F) Viable but not apoptotic cells stimulated SIRPα phosphorylation in macrophages. J774 macrophages were stimulated with a CD47-Fc ligand as control as well as viable or apoptotic Jurkat T cells for 30 min before examination of SIRPα phosphorylation. Additionally, macrophages were stimulated with viable neutrophils for various times.

(G) Inhibitors of the tyrosine phosphatase SHP-1 blocked the "don't eat me" pathways and allowed uptake of viable neutrophils. Protein tyrosine phosphatase inhibitor I (42 μM), a SHP-1-specific inhibitor, or protein tyrosine phosphatase inhibitor IV (10 μM), a control inhibitor, was incubated with the macrophages for 20 min prior to the addition of target cells.

(H) Activation of the "don't eat me" signaling by stimulation of macrophage SIRPα with a CD47-Fc construct or a rat Fc control for 20 min prior to washing and addition of apoptotic neutrophils prevented their uptake.

and P.-A.O., unpublished data). Thus, CD47 does not seem to be required for apoptotic-cell removal but may enhance uptake by serum factors that may act as bridges or perhaps block CD47 signaling to SIRPα.

An implication of the loss of "don't eat me" signals during apoptosis is that, if they are deliberately blocked, viable cells may now be recognized and ingested through calreticulin effects even if they do not expose PS. In fact, since PS often becomes exposed during cell activation (Callahan et al., 2000; Frasch et al., 2004), a normal inhibitory "don't eat me" signal may be important, and its loss, therefore, may contribute to combined PS- and calreticulin-mediated engulfment.

The studies support a role for CD47 as a "don't eat me" signal, but it should be noted that CD47-deficient mice are viable even though they exhibit abnormal cell removal in vivo, are lymphatopenic, and show defects in host defense (Lindberg et al., 1996). Given the presumed importance of preventing casual removal of viable cells, we suggest the existence of additional "don't eat me" signals that act in a compensatory role in *CD47*$^{-/-}$ mice. Supporting this theory, wild-type or *CD47*$^{-/-}$ T cells and bone-marrow cells were infused into irradiated immunodeficient recipients, and bone-marrow transfers of *CD47*$^{-/-}$ T cells were unable to engraft or reconstitute wild-type recipients (Blazar et al.,

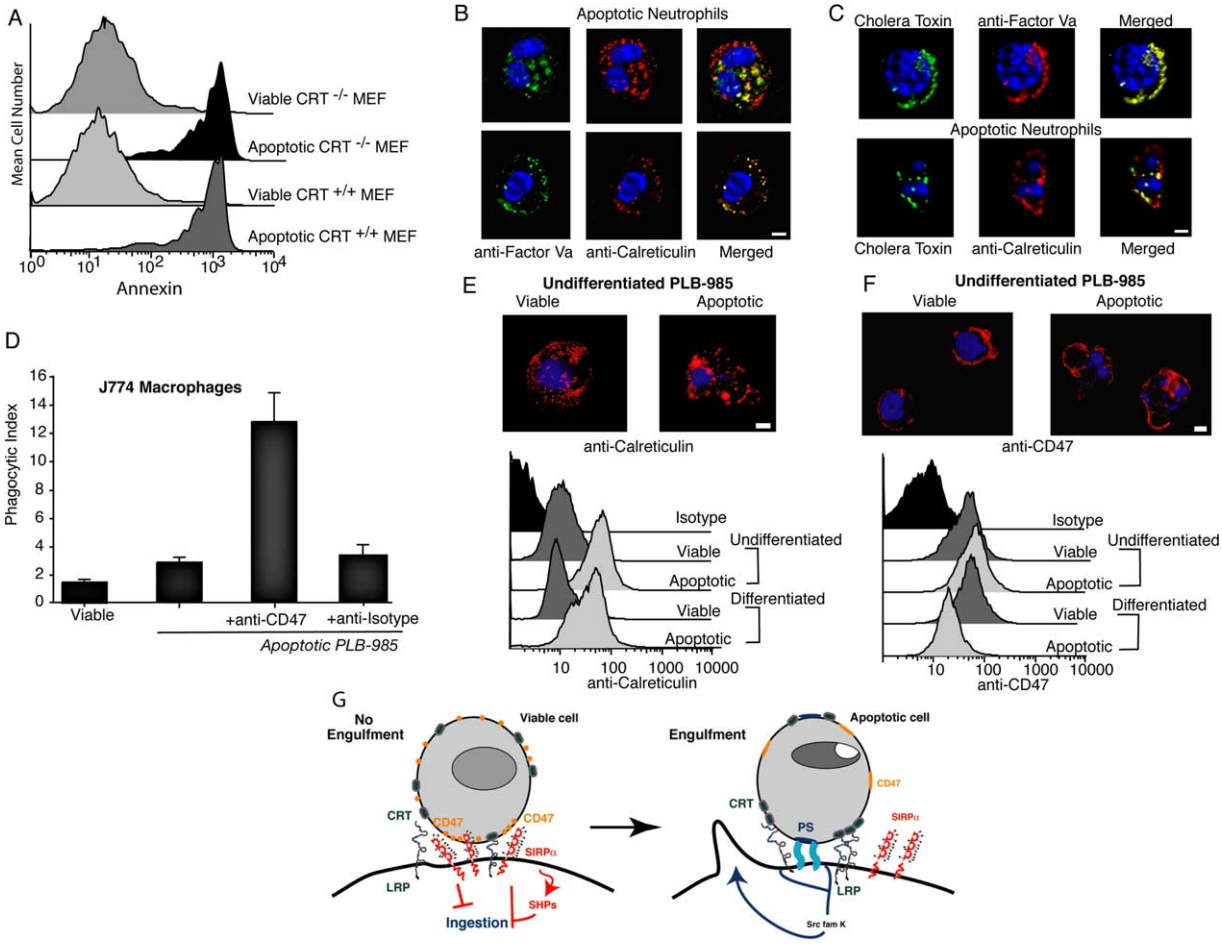

Figure 7. Calreticulin Associates with PS on Apoptotic Cells

Error bars are ±SD.

(A) Apoptotic *CRT*$^{-/-}$ and *CRT*$^{+/+}$ MEFs did not show differences in PS exposure as assessed by binding of annexin V.

(B) Calreticulin and phosphatidylserine colocalized on the surface during apoptosis. Apoptotic neutrophils were stained for calreticulin (red) or phosphatidylserine using factor Va and an anti-factor Va antibody (green).

(C) Phosphatidylserine and calreticulin localized to GM-1-positive sites. Apoptotic neutrophils were labeled with cholera toxin B (green) and then stained for calreticulin or phosphatidylserine (red).

(D) Apoptotic undifferentiated PLB-985 cells (which do not expose PS and are poorly ingested) were rendered capable of being engulfed after pretreatment with Fab′$_2$ anti-CD47 to block their "don't eat me" signals.

(E) Undifferentiated PLB-985 cells exhibited normal upregulation and redistribution of calreticulin as they became apoptotic.

(F) Undifferentiated PLB-985 cells did not redistribute or lose CD47 during apoptosis. Differentiated PLB-985 cells exhibited decreased surface CD47 during apoptosis.

(G) A cartoon representation of surface changes that occur as a cell becomes apoptotic. Although viable-cell calreticulin may engage LRP on the efferocyte cell surface, the CD47-SIRPα signaling pathway inhibits engulfment. Following apoptosis, calreticulin is upregulated and patches to better stimulate LRP on the efferocyte. PS is also exposed at this point, and the SIRPα-CD47 signaling system is rendered ineffective.

2001), i.e., wild-type splenic dendritic cells and macrophages removed almost all of the CD47-deficient cells within 1 day of infusion. Alternatively, *CD47*$^{-/-}$ BM was able to rescue *CD47*$^{-/-}$ but not *CD47*$^{+/+}$ recipients, indicating that the phagocytes in the CD47-deficient mouse could not recognize and remove the *CD47*$^{-/-}$ cells. It seems likely that there is significant redundancy in "don't eat me" ligands and signaling pathways that have not yet been fully explored.

The observations described herein raise two additional points. Much emphasis has been placed on redistribution of potential ligands on the apoptotic-cell surface that may be involved in stimulating or inhibiting apoptotic-cell engulfment. We believe this to be a critical process in determining the stimuli that apoptotic cells deliver to the efferocyte and the ultimate fate of the apoptotic cells. The mechanisms that mediate this redistribution are at present unknown, although we have suggested a possible connection between altered phospholipid asymmetry and the loss, redistribution, or inactivation of CD47 seen on apoptotic cells. In addition, the finding that viable cells are readily ingested if "don't eat me" signals are disrupted raises the intriguing and challenging possibility that recognition and re-

moval by efferocytosis is a default process in multicellular organisms that is actively prevented by inhibitory ligands on viable cells.

Experimental Procedures

Reagents

Lucifer yellow and antibody were obtained from Molecular Probes (Eugene, Oregon). Calreticulin from either bovine liver (90% purity, Sigma, St. Louis) or recombinant GST-fusion proteins was expressed and purified as described (Orr et al., 2003a). Native purified calreticulin as well as recombinant proteins bind calcium at levels found in tissue culture media. Hsp90 (MBL International, Woburn, Massachusetts) and calreticulin were used at 1 μg/ml, and RAP (Research Diagnostics Inc., Flanders, New Jersey) was used at an inhibitory concentration of 250 nM. Anti-N-terminal calreticulin (Affinity Bioreagents Inc., Golden, Colorado) was used at 1 μg/ml. Anti-active caspase 3 (Cell Signaling Technology, Beverly, Massachusetts) was used at 1:50, and FITC-conjugated F4-80 (Caltag, San Diego, California) was used at 1:100. Anti-CD47 monoclonal antibody (mAb B6H12; IgG1) was purified from hybridoma supernatants by ammonium-sulfate precipitation and protein G chromatography using HiTrap columns (Amersham Biosciences, Piscataway, New Jersey). Phi Phi Lux was used according to the manufacturer's recommendations (Calbiochem, San Diego, California). An LRP ligand-blocking antibody or control cytoplasmic antibody (a generous gift from Dr. Dudley Strickland, the University of Maryland Hospital) was used at 50 μg/ml.

Cell Culture and Induction of Apoptosis

See Supplemental Data.

Stimulation of Macropinocytosis

Macrophages were stimulated with calreticulin or Hsp90, *CD47*$^{-/-}$ or wild-type erythrocytes, and/or leukocytes in the presence of lucifer yellow. Cells were washed, fixed, and permeabilized with 0.1% Triton-X for 15 min and incubated with a 1:500 dilution of anti-lucifer-yellow antibody, washed, and counterstained with FITC anti-rabbit IgG antibody.

Phagocytosis Assays

Phagocytosis assays were performed as previously described (Hoffmann et al., 2001). In some experiments, viable human neutrophils were coated with Fab′$_2$ monoclonal antibody against CD47 (2 μg/ml) (Fab′$_2$ fragments of mAb B6H12 were obtained by pepsin digestion and removal of remaining Fc fragments by protein A chromatography) or an isotype control for 30 min prior to washing and feeding to macrophages. Alternatively, the macrophages were pretreated with 2 μg/ml of anti-SIRPα antibody (anti-SIRPα, P84) or isotype control for 30 min and washed before addition of viable human neutrophils. Amiloride (Sigma) was added at 3 mM in serum-free DMEM for 15 min prior to the addition of target cells. SHP-1 inhibition experiments were performed with J774 macrophages pretreated with 45 μM protein tyrosine phosphatase inhibitor I or 10 μM phosphatase inhibitor IV (Calbiochem), a SHP-2 and PTP protein tyrosine phosphatase inhibitor, prior to the addition of viable neutrophils. MEFs were plated at 5 × 10^5 for 24 hr prior to addition of targets. *CRT*$^{-/-}$ MEFs were coated with calreticulin or Hsp90 on ice for 30 min and washed twice prior to addition to phagocytes. CD47-wild-type and -deficient erythrocytes were added to macrophages or fibroblasts at a ratio of 15:1, incubated for 90 min, and assayed as previously described (Vandivier et al., 2002). Treatment of targets with an anti-calreticulin (2 μg/ml) or control antibody for 30 min at 4°C was followed by washing. For stimulated phagocytosis assays (bystander uptake, Hoffmann et al., 2001), J774 macrophages were plated in 24-well plates at 5 × 10^4 cells/well for 2 days. Viable neutrophils were allowed to adhere for 20 min prior to stimulation with calreticulin or Hsp90. In all cases, uptake of targets was measured as described (Fadok et al., 1993). The inherent variability of professional and nonprofessional phagocytes in ingesting apoptotic cells lends to the differences observed in the phagocytic indexes.

Surface Modulation Experiments

These were performed as described (Ogden et al., 2001) with 1% HSA (Pharmacia, Sweden), 1 μg/ml or 10 μg/ml mouse anti-human CD91 (α chain) monoclonal antibody (BioMac, Leipzig, Germany), or control antibody (Santa Cruz Biotech, Heidelberg, Germany).

Videomicroscopy

J774 macrophages were cultured on Delta T dishes (Bioptechs) for 2 days and washed before addition of apoptotic target MEFs at a ratio of 10:1. After 30 min on a 37°C heated stage, they were analyzed using a 60× oil objective on an Olympus IX70 microscope. Pictures were recorded every 15 s for 30 min using Tillvision software. Anti-SIRPα antibody (1 μg/ml) or control was added 30 min before viable targets.

Surface Staining

Cells were washed twice and transferred to 4°C in PBS containing 0.5% serum and 2% BSA. Antibodies or controls were added at 1 μg/ml for 1 hr before washing and fixation (4% paraformaldehyde and 3% sucrose). A second antibody at 1:100 was added for an additional 30 min on ice before washing and prepared for flow cytometry or microscopy. For GM-1 (5.0 μg/ml, Sigma) staining, cells were treated with cholera toxin B for 15 min on ice prior to antibody addition. Anti-factor Va experiments were performed according to Frasch et al. (2004). Microscope images were collected with Slidebook software (Intelligent Imaging Innovation [3I], Denver) on a Leica DMRXA microscope at 62× magnification. Scale bars are all equal to 5 μm. All microscopy samples were processed with the appropriate isotype control, and these were demonstrated to have no significant staining. Examples of this can be seen for CD47 and calreticulin in the flow experiments in Figures 1D, 2F, and 6A–6C and Figure S14.

Rac Activity Assay

Rac activity assays were performed as per the manufacturer's instructions (Upstate Biotechnology). In short, 10 million J774 macrophages were plated for 2 days and stimulated with calreticulin for 20 min or RAP for 20 min prior to calreticulin stimulation. Samples were lysed and active Rac was isolated using Sepharose bound Pak. Lysates were incubated for 45 min, washed, boiled, and run on a 12% SDS-PAGE gel.

SIRPα Phosphorylation

J774 macrophages were plated at 5 × 10^5 for 2 days before addition of targets. Cells were washed, lysed, and SIPRα immunoprecipitated (Upstate Biotechnology). Samples were run on a 7% gel and probed with an anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). To ensure equal loading, the membrane was stripped and reprobed with an anti-SIRPα antibody.

Embryo Processing

Day 13.5 embryos were harvested from heterozygote mating and typed via Western blot. Embryos were fixed in 4% paraformaldehyde overnight, frozen in OTC, and stored at –80°C. Sequential sections were stained for active caspase 3 according to manufacturer's specifications and were washed twice, and a Cy3 secondary at 1:500 dilution was added along with FITC-conjugated anti-F4-80 antibody. Sections were incubated for 1 hr at 4°C, washed, mounted with OPDA containing a 1:1000 dilution of DAPI, and visualized on a Leica DMRXA microscope using Slidebook software. Quantification of apoptotic cells was performed using at least ten random high-power fields from five different slides and multiple embryo samples.

In Vivo Experiments

In vivo apoptotic-cell clearance was performed as described (Stuart et al., 2005). The peritoneums were lavaged 60 min after instillation of the apoptotic cells. Total cell counts were made for each mouse. A cytospin slide of lavaged cells was stained with modified Wright's Giemsa (Leukostat; Fisher) and evaluated for macrophage phagocytosis of apoptotic cells and the total number of apoptotic cells recovered.

Statistics

All experiments were performed at least three times. Statistical analysis and p values were carried out using the JMP statistical program (SAS Institute, Cary, North Carolina). The Tukey-Kramer and Dunnett's parametrical tests were used for single and multiple comparisons, respectively.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, 14 figures, and 4 movies and can be found with this article online at http://www.cell.com/cgi/content/full/123/2/321/DC1/.

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