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P2X₇ Is a Scavenger Receptor for Apoptotic Cells in the Absence of Its Ligand, Extracellular ATP

Ben J. Gu,*† Bernadette M. Saunders,‡ Steven Petrou,† and James S. Wiley*†

Phagocytosis of apoptotic cells is central during development and tissue remodeling. Our previous study has shown that the P2X₇ receptor regulates phagocytosis of nonopsonized particles and bacteria. In this study, we demonstrate that P2X₇ also mediates phagocytosis of apoptotic lymphocytes and neuronal cells by human monocyte-derived macrophages under serum-free conditions. ATP inhibited this process to a similar extent as observed with cytochalasin D. P2X₇-transfected HEK-293 cells acquired the ability to phagocytose apoptotic lymphocytes. Injection of apoptotic thymocytes into the peritoneal cavity of wild-type mice resulted in their phagocytosis by macrophages, but injection of ATP prior to thymocytes markedly decreased this uptake. In contrast, ATP failed to inhibit phagocytosis of apoptotic thymocytes in vivo by P2X₇-deficient peritoneal macrophages. The surface expression of P2X₇ on phagocytes increased significantly during phagocytosis of either beads or apoptotic cells. A peptide screen library containing 24 biotin-conjugated peptides mimicking the extracellular domain of P2X₇ was used to evaluate the binding profile to beads, bacteria, and apoptotic cells. One peptide showed binding to all particles and cell membrane lipids. Three other cysteine-containing peptides uniquely bind the surface of apoptotic cells but not viable cells, whereas substitution of alanine for cysteine abolished peptide binding. Several thiol-reactive compounds including N-acetyl-L-cysteine abolished phagocytosis of apoptotic SH-SY5Y cells by macrophages. These data suggest that the P2X₇ receptor in its unactivated state acts like a scavenger receptor, and its extracellular disulphide bonds play an important role in direct recognition and engulfment of apoptotic cells. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BODIPY 630/650-SE, 6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaz-a-s-indacene-3-y1)sulfonyloxy)acetyl)aminohexanoic acid, succinimidyl ester; CMTMR, 5-((and-6)-(((4-chloro-2-methylbenzoyl)amino)tetramethylrodamine; CytD, cytochalasin D; GSH, reduced glutathione; NMMHC, nonmuscle myosin H chain; OxATP, oxidized ATP; PI, phosphatidylinositol; PS, phosphatidylserine; P2X<sup>−/−</sup>, P2X<sup>−/−</sup> gene-deficient mice; P2X<sup>−/−</sup>-ED, recombinant P2X<sub>7</sub>, extracellular domain; SR, scavenger receptor; SSC, side scatter; Tim, T cell Ig and mucin-domain containing molecule; YG, Yellow-Green.

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inflammation. Understanding how a phagocytic pathway is regulated may suggest its role in certain organs and tissues; for example, pathways that are active in the absence of serum may have particular relevance in the CNS.

Recent findings from our group and others (14, 15) have suggested a direct involvement of the purinergic P2X7 receptor in phagocytosis of nonopsonized particles and bacteria. We showed that overexpression of the P2X7 receptor greatly augmented the phagocytosis of nonopsonized beads and heat-killed bacteria by transfected HEK-293 cells, whereas blocking P2X7 expression by small interfering RNA significantly reduced the phagocytic ability of human monocytic cells. The P2X7 receptor has other unique features consistent with that of an SR. First, P2X7 on the surface of monocytic cells is tightly associated with nonmuscosin H chain (NMMHC)-IIA (16), the ATPase of which provides the energy for cytoskeletal rearrangements required for particle engulfment; second is the diversity of foreign particles recognized by P2X7—expressing cells, such as latex beads and live and heat-killed bacteria, all in the absence of serum (14); and third is the ability of extracellular ATP to terminate P2X7-mediated phagocytosis and change the role of P2X7 from silent removal of particles to activation of well-recognized proinflammatory pathways (14, 17, 18).

We have extended these studies to show that under serum-free conditions, P2X7 on the monocyte/macrophage surface mediates the uptake of apoptotic cells through a pathway not only involving recognition of exposed PS, but also involving thiol-disulfide reactions with cysteines in the extracellular domain of P2X7 (P2X7-ED). Thus, thiol-reactive compounds inhibited uptake of apoptotic cells, whereas short peptides mimicking P2X7-ED bound to the apoptotic cell surface. Extracellular ATP inhibited P2X7-mediated phagocytosis and switched the downstream signaling events from phagocytic cup formation to the well-recognized secretion of proinflammatory cytokines.

Materials and Methods

Materials

ATP, BzATP, oxidized ATP (OxATP), cytochalasin D (CytD; a classic inhibitor of F-actin polymerization and phagocytosis), tetramethylammonium hydroxide, polyinosinic acid, and 7-aminoactinomycin D (7-AAD) were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotides and peptides were synthesized by Sigma-Genosys. The recombinant human IFN-γ and Mini-complete Protease Inhibitor mixture were from Roche Applied Science (Mannheim, Germany). For use as an agonist in vitro, ATP was dissolved in KCl buffer (145 mM KCl, 5 mM KOH, and 10 mM HEPES [pH 7.5]) at a stock concentration of 100 mM and neutralized with 18% (w/v) tetramethylammonium hydroxide to pH 7. For in vivo studies, ATP was dissolved in 0.5 M Na2HPO4 at a stock concentration of 100 mM, diluted to 5 mM working concentration with 100 mM NaCl, and passed through a 0.22-μm filter. CellTracker Orange 5-(and-6)-[(4-chloromethyl)benzoyl] aminoetarmethyloctadecane (CMTRMR) and 6-((4,4-difluoro-5-(2-thienyl) -4-butyrimidole-3-yl)carbonyl) N-(2-((2-morpholino)ethyl) succinimidy1 ester (BODIPY 560/560-E) were from Invitrogen (Carlsbad, CA). Vector pAcGFP-N1 and pDsRed-monomer-N1 were from Clontech (Mountain View, CA). Wild-type P2R27 knock out was originally a gift from Dr. Gary Buell (Glaxo Institute for Molecular Biology, Geneva, Switzerland) and was reconstructed into vectors in our laboratory. MatTek culture dishes with collagen coated 1.0-mm slides were from MatTek (Ashland, MA). Murine anti-human P2X7 receptor mAb (clone L4; kindly provided by Dr. Gary Buell and Ian Chessell, Glaxo Institute for Applied Pharmacology, Cambridge, U.K.) (19) and IgG1, isotype control mAb (clone WMD7; kindly provided by Dr. Linda Belland, Millennium Institute, Westmead, NSW, Australia) were purified from clone L4 hybridoma supernatants by chromatography on Protein A Sepharose Fast Flow (GE Healthcare) as described previously (20). The 6xHis-tagged recombinant P2X7-ED (rP2X7-ED) was prepared as previously described (14). Sheep anti-human P2X7 polyclonal Ab against a nonhomologous extracellular epitope of the human P2X7 receptor has been described (21). Fluoresbrite yellow-green carboxylate microspheres (1 μm Yellow-Green [YG] beads) and 25-kDa linear polyethyl enylenimine were from Polysciences (Warrington, PA). Membrane Lipid Strips were from Echelon, Frontier Scientific (Logan, UT). The Quick Change Site-Directed Mutagenesis Kit was purchased from Stratagene. The Capto Q and Con A Sepharose 4B columns were from GE Healthcare.

Sources of cells

Human PBMC were separated by density gradient centrifugation over Ficoll-Hypaque, washed once in RPMI 1640 medium, and resuspended in HEPES-buffered NaCl medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES [pH 7.5]), plus 5 mM glucose, 0.1% BSA, and 0.1 mM CaCl2). Human embryonic kidney cell line HEK-293 was cultured in RPMI 1640 medium containing 10% FCS and 5 μg/ml gentamicin. For treatment with Abs, cells (2–4 × 105) were incubated with Ab in a 100 μl NaCl medium at various concentrations followed by dilution to 1 ml and the addition of beads. The study was approved by the Human Research Ethics Committee of Sydney West Area Health Service (06/058) and Eastern Health, Melbourne (E05-1011). Informed consent was provided according to the Declaration of Helsinki.

Phagocytosis of YG beads in vitro

Phagocytosis of beads was assessed by a flow cytometry method as previously described (14). Briefly, PBMC (4 × 106/ml) or HEK-293 cells (2 × 106/ml) were resuspended in 1.0 ml Na medium with 0.1 mM CaCl2. All samples were stirred and temperature controlled at 37°C using a Time Zero module. Five-microliter YG beads were added, and cells were analyzed at 500 events/s on an FACSCalibur flow cytometer (BD Biosciences) and gated by forward and side scatter (SSC) and by cell type-specific Abs (CD14 for monocytes or DsRed intensity). The linear mean channel of fluorescence intensity for each gated subpopulation over successive 10-s intervals was analyzed by WinMDI software and plotted against time. The area under the YG bead uptake curve in the first 6.5 min was calculated using a Microsoft Excel function (Microsoft) and taken as the phagocytosis ability.

Phagocytosis of apoptotic lymphocytes or SH-SYSY cells in vitro

Phagocytosis was assayed as previously described (22). Briefly, human mononuclear cells were isolated from 400 ml peripheral blood by centrifugation over Ficoll. The monocytes were further separated from lymphocytes by plastic adhesion and cultured in RPMI 1640 medium plus 10% FCS and 100 U/ml IFN-γ overnight. Cells were washed twice with Na medium (145 mM NaCl, 5 mM KCl, 1 mg/ml BSA, 5 mM glucose, 0.5 mM CaCl2, and 10 mM HEPES [pH 7.5]); macrophages (1.5 × 106 in 1 ml) were treated with various reagents or fixed with 2% paraformaldehyde. The autologous lymphocytes were kept in RPMI 1640 medium plus 10% FCS or autologous serum before labeling with 5 μM CFSE for 2 h. Cells were washed once and incubated in complete medium with 1.0 mM staurosporine overnight. Viable lymphocytes were then separated by centrifugation over Ficoll and washed twice with Na medium. The proportion of apoptotic cells was assessed using Annexin V and 7-AAD dual staining and ranged from 60–85% of the total cell population (data not shown). SH-SYSY cells were kept in RPMI 1640 medium plus 20% FCS before labeling with 5 μM CFSE for 2 h and then induced into apoptosis with 0.2 mM staurosporine overnight. After three washes with Na medium, apoptotic autologous lymphocytes or apoptotic SH-SYSY cell suspension was added to pretreated adherent macrophages with a target cell/phagocyte ratio of 5:1. The cell mixture was incubated at 37°C for 2 h or 3 h with gentle shaking, and cells were collected by trypsin/EDTA digestion. Samples were vortexed thoroughly and fixed at 4°C before analysis of CMTRMR-gated macrophages by flow cytometry. During this process, any targets attached to macrophages do not remain attached, although attached cells can be enumerated by static microscopic examination (22). In addition, cell doublets were also excluded by SSC-A and SSC-H gating. For measuring P2X7 surface expression, cells were collected before and after phagocytosis of apoptotic lymphocytes and resuspended in PBS with 0.1% NaN3 followed by incubation with Alexa 647-conjugated anti-P2X7 mAb (clone L4) at 4°C for 30 min. Cells were vigorously stirred and fixed before analyses by flow cytometry on CMTRMR- CFSE-gated macrophages.

Changes of P2X7 surface expression during phagocytosis of YG beads

Fresh isolated PBMC (1 × 106/ml) were collected before and after phagocytosis of 1 μM YG (5 μ/ml cells) beads and resuspended in PBS with 0.1% NaN3 followed by incubation with Alexa 647-conjugated anti-
P2X7 mAb (clone L4) at 4°C for 30 min. Cells were vigorously stirred and fixed for flow cytometric analysis. The linear mean channel fluorescence intensity of Alexa 647 was measured on gated CFSE+ monocytes.

**Transfection of HEK-293 cells**

HEK-293 cells do not express P2X receptors in subconfluent cultures. A total of 10 μg plasmid DNA or 3 μg plain vector was incubated in serum-free Opti-MEM I medium for 5 min followed by incubation with 25-kDa linear polyethylenimine (20 μl 1 mg/ml stock, diluted with Opti-MEM I medium) (23) for 20 min at room temperature. The solution was transfected into subconfluent monolayers of HEK-293 cells (~1.5 × 10⁶ in 3 ml Opti-MEM I with 5% FCS). After 72 h, transiently transfected cells were collected by mechanical scraping, or stably transfected cells were maintained in complete RPMI 1640 medium containing 0.8 mg/ml G-418. For imaging studies, HEK-293 cells were cultured and transfected on collagen-coated 27-mm MatTek culture dishes (MatTek).

**Phagocytosis of apoptotic HPB cells in vitro**

Human T leukemia cell line HPB cells were incubated with 10 μM CFSE for 2 h followed by two washes and further incubation with 1 μM staurosporine overnight in serum-free RPMI 1640 medium. Cells were washed once and resuspended in Na medium containing 0.5 mM Ca²⁺ at 5 × 10⁶/ml. Subconfluent HEK-293 cells (~0.5 × 10⁶) stably transfected with pDsRed-monomer-N1 or hp2X7-pDsRed-N1 were incubated with 5 μM BODIPY 630/650-SE overnight in RPMI 1640 medium with 10% FCS and 0.8 mg/ml G-418 in six-well plates. Cells were washed three times and covered with 1 ml Na medium containing 0.5 mM Ca²⁺. HEK-293 cells were then incubated with ATP (1 mM) or CytD (20 μM) for 15 min followed by the addition of 0.5 ml CFSE-labeled apoptotic HPB cell suspension. The cell mixture was incubated at 37°C for 4 h with gentle shaking, after which 1 ml trypsin/EDTA solution was added to each well. Cells were collected, vigorously stirred, and fixed with 2% paraformaldehyde at 4°C for analysis by flow cytometry on gated BODIPY+/DsRed+ HEK-293 cells.

**Phagocytosis of apoptotic thymocytes in vivo by resident murine peritoneal macrophages**

P2X7 gene-deficient mice (P2X7⁻/⁻) were a kind gift from Dr. Chris Gabel (Pfizer, Groton, CT) (24); they were backcrossed 10 times onto a C57BL/6 background and maintained under specific pathogen-free conditions in the Centenary Institute Animal Facility. The wild-type control specific...
pathogen-free C57BL/6 were purchased from the Animal Research Centre (Perth, WA, Australia). Genotype was confirmed by PCR analysis. All experiments were conducted with ethical approval from the Sydney University Animal Ethics committee. The thymuses were removed from C57BL/6 mice and dispersed on a soft mesh. The single cells were resuspended in RPMI 1640 medium with 10% FCS and cultured overnight with 0.5 μM staurosporine and 5 μM CFSE. Cells were washed three times and resuspended in PBS at 1.0 × 10^6/ml. The apoptotic rate was assayed using Annexin V and 7-AAD dual staining. Mice were injected i.p. with either 1 ml PBS or 5 mM ATP followed 30 min later by injection of 1 ml apoptotic thymocyte suspension. Mice were sacrificed after 60 min, and peritoneal exudates were collected. Cells were immediately fixed with 2% paraformaldehyde at 4°C and stained with PE-Cy5.5–conjugated anti-mouse GR-1 mAb and Alexa Fluor 700-conjugated anti-mouse CD11b mAb at 4°C and washed twice. Cells were vigorously stirred before analysis on a BD LSR II flow cytometer (BD Biosciences). Cell doublets were excluded by SSC-A and SSC-H gating.

Peptide screen
Short peptides identical in sequence to the P2X7-ED were synthesized by Sigma-Aldrich and tagged with biotin on N termini. The peptides were first dissolved in dimethylformamide (20 mg/ml) and then in acetonitrile/H2O (50%) (10 mg/ml) and further diluted in PBS containing 1% BSA (total volume is 1 ml). Particles were washed twice and resuspended in 100 μl HRP-labeled streptavidin (Jackson ImmunoResearch Laboratories; 1:2000 diluted with PBS containing 1% BSA) for 30 min. After two washes, 500 μl SuperSignal West Pico (Pierce) was added, and the chemiluminescence was measured by a Glo 2020 luminometer (Promega).

Peptide and P2X7-ED binding assay
SH-SY5Y, HPB, and human lymphocytes were induced to apoptosis with 0.2–0.5 μM staurosporine for 6 h. Cells were resuspended in Na+ medium with 150 mM NaCl and incubated with biotinylated short peptides mimicking the P2X7-ED at a concentration of 10 μg/ml for 30 min, followed by two washes and incubation with FITC-conjugated streptavidin, allophycocyanin-conjugated Annexin V, and 7-AAD (5 μg/ml). All events were gated on the low 7-AAD staining population. For binding assay with P2X7-ED, apoptotic cells were incubated with P2X7-ED at a concentration of 15 μg/ml for 30 min, followed by two washes and incubation with anti-5xHis mAb, and subsequently with FITC-conjugated goat anti-mouse IgG, allophycocyanin-conjugated Annexin V, and 7-AAD. All events were gated on the low 7-AAD staining population.

Membrane lipid-binding assay
The assays were performed according to the manufacturer’s instruction. Briefly, Membrane Lipid Strips were blocked with 0.1% BSA and 1% skim milk in PBS for 2 h. Twenty-four peptides (500 μg/ml, 150 μl each) mimicking the P2X7-ED were added to 2 ml PBS containing 0.1% BSA to cover the strips. After 1 h incubation, the membranes were washed three times with PBS containing 0.05% Tween-20, followed by incubation with streptavidin-HRP (1:2000) in PBS with 0.1% BSA for 1 h. After three washes, SuperSignal West Pico (Pierce) was added, and the chemiluminescence was captured by a Bio-Rad GelDoc (Bio-Rad).

Site-directed mutagenesis
To remove nine consecutive amino acids in sequential regions of the P2X7-ED, the Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used to perform the PCR, and the full-length hP2X7-DsRed-monomer-N1 (16) was used as the template. The P2RX7 deletions were constructed using pairs of complementary mutagenic primers. The PCR was performed according to the manufacturer’s instruction except the annealing temperatures were set at 43°C, 52°C, and 58°C instead. After digestion of the template DNA with Dpn I, intact newly synthesized DNA containing the desired deletion was transformed into XLBlue competent cells. All deletions were confirmed by sequencing.

Results
Extracellular ATP inhibits the phagocytosis of apoptotic cells
Our previous studies have shown that P2X7 has a tight molecular association with NMMHC-IIA in monocyte cells (16), and this complex regulates phagocytosis of nonopsonized beads and live and dead bacteria (14). These data suggested a nonspecific scavenger role for this receptor, and we investigated whether P2X7 is also involved in phagocytosis of apoptotic cells. Monocytes were isolated from fresh blood by multiple cycles of plastic adhesion and differentiated to macrophages, which greatly upregulates P2X7 (25). Lymphocytes from the same donor were induced into apoptosis with staurosporine. After seeding the apoptotic lymphocytes on top of macrophage layer, macrophages started to engulf the apoptotic lymphocytes. The z-stack confocal images in Fig. 1A showed three lymphocytes within a macrophage, indicating the intracellular location of engulfed lymphocytes. The phagocytosis of apoptotic lymphocytes by autologous macrophages was confirmed by multicolor flow cytometry. Following a 3-h coculture, 40–70% of macrophages became positive for CFSE, confirming the engulfment of CFSE-labeled lymphocytes by these macrophages. The number of lymphocytes phagocytosed varied from one to five per macrophage (Fig. 1B). However, this marked uptake was inhibited by pretreatment of macrophages with CytD, a classical phagocytic inhibitor, OxATP (an irreversible antagonist for P2X7 receptors), or by fixation (Fig. 1B–D). ATP also showed an inhibitory effect on the phagocytosis of apoptotic cells. Pretreatment of macrophages with 1 mM ATP for 15 min significantly reduced phagocytosis of apoptotic lymphocytes to the low levels observed with CytD or OxATP pretreatment in 15 randomly selected normal subjects (p < 0.01, paired t test) (Fig. 1B, 1C). Because ATP and CytD gave similar reductions in phagocytosis as observed at 4°C (Fig. 1B), we conclude that ATP

FIGURE 2. Phagocytosis of apoptotic thymocytes in vivo by murine peritoneal macrophages. C57BL/6 mice (wild-type [WT] and P2X7−/−, top panels) were injected i.p. with either 1 ml PBS or 5 mM ATP followed 30 min later by injection of 1 ml CFSE-labeled apoptotic thymocytes. The mice were sacrificed after 60 min, and peritoneal exudates were collected. Cells were stained with allophycocyanin-conjugated anti-mouse CD11b and PE-Cy5–conjugated anti-mouse GR1 mAb at 4°C for 20 min before being analyzed by a BD LSR II flow cytometer (BD Biosciences) in gated CD11b+ GR1− macrophage singlet population (bottom panel). *p < 0.001; n = 5.
like CytD is inhibiting the engulfment process rather than adhesion of lymphocyte to macrophage (26). Polynosinic acid, a conventional inhibitor for class A SR, did not alter the phagocytosis of apoptotic lymphocytes by macrophages (data not shown).

**Inhibitory effect of ATP on phagocytosis of apoptotic cells is mediated via P2X7**

Our previous study has shown that activation of P2X7 by ATP leads to dissociation of NMMHC from the P2X7 membrane complex and subsequent attenuation of phagocytosis of beads and bacteria by monocytes (14, 16). In this study, the inhibitory effect of ATP on phagocytosis of apoptotic cells was dose dependent with significant inhibition of phagocytosis at 0.5–1.0 mM ATP. However, low concentrations of ATP (0.2 mM) failed to show a significant inhibitory effect, as shown in a previous study (27). BzATP, a more potent P2X7 agonist, at a concentration of 0.1 mM showed a similar effect to 1 mM ATP (Fig. 1B). In contrast, pretreatment of macrophages with 0.2 mM UDP or UTP did not affect the phagocytosis of apoptotic lymphocytes (Fig. 1D). Pretreatment of apoptotic cells with the rP2X7-ED also inhibited the phagocytosis of these cells by macrophages (Fig. 1D), suggesting the direct involvement of P2X7 in this process.

**P2RX7−/− mice do not show ATP inhibition of phagocytosis**

Injection of apoptotic thymocytes into the peritoneal cavity of mice leads to their phagocytosis in vivo by resident peritoneal macrophages (Fig. 2A). When ATP was infused into the peritoneal cavity 30 min prior to the thymocytes, the highly phagocytic macrophage population reduced to half that in wild-type C57BL mice (Fig. 2B). This dramatic effect contrasted with the results in P2RX7−/− mice. In these gene-deleted mice, the highly phagocytic population comprised most of the macrophages present, and ATP had little effect in removing this population (Fig. 2B). This result shows that ATP can inhibit phagocytosis of apoptotic cells both in vivo and in vitro, and this inhibitory effect is mediated via the P2X7 receptor.

**P2X7 expression on HEK-293 cells confers the ability to phagocytose apoptotic cells**

We have previously shown that transfection with P2X7 constructs conferred increased phagocytic ability on HEK-293 cells, a kidney cell line with poor phagocytic ability. In this study, we transfected HEK-293 cells with DsRed-tagged P2X7 and incubated these cells for 3 h with apoptotic HPB cells, a human leukemic T cell line. The confocal images in Fig. 3A show that DsRed-tagged P2X7

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**FIGURE 3.** Phagocytosis of apoptotic HPB cells by HEK-293 cells transfected with P2X7. **A**, Confocal images showing a HEK-293 cells transfected with DsRed-tagged P2X7 (red) phagocytosed two CFSE-labeled HPB cell and captured another one. Images were acquired by an Olympus IX81 confocal microscope (Olympus) (original magnification ×600). **B**, A typical flow cytometry histogram showing phagocytosis of CFSE-labeled HPB cells by DsRed mock (left panel) and P2X7-DsRed–transfected (right panel) HEK-293 cells. HEK-293 cells were labeled with BODIPY 630/650-SE first and pretreated with 1 mM ATP or 20 µM CytD for 15 min prior to incubation with HPB cells. BODIPY+/DsRed++ HEK-293 cells were gated for analysis.
was expressed on the surface and intracellular organelles of a transfected HEK-293 cell, as well as the phagosome membrane surrounding two CFSE-labeled HPB cells engulfed by this HEK-293 cell (Fig. 3A, yellow arrows). Phagosome localization of P2X7 has been shown by others (15). In our study, accumulation of P2X7 is clearly seen at the point of attachment between a captured HPB cell and the HEK-293 cell (Fig. 3A, blue arrow), strongly supporting a role of P2X7 in recognition of apoptotic cells. The phagocytic ability of P2X7-transfected HEK-293 cells was confirmed by flow cytometry assay. Following a 3-h coculture of CFSE-labeled apoptotic HPB cells with BODIPY 630/650-SE–labeled HEK-293 cells, a significant increase of CFSE-positive HEK-293 cells was found in cells transfected with P2X7-DsRed but not in mock-transfected cells (Fig. 3B). CytD completely inhibited the phagocytosis by P2X7-transfected HEK-293 cells to the same level observed with mock-transfected cells (Fig. 3B). Pretreatment of P2X7-transfected HEK-293 cells with ATP also abolished the engulfment of apoptotic HPB cells (Fig. 3B).

**Upregulation of P2X7 surface expression on monocytes and macrophages during phagocytosis**

Although phagocytosis of nonopsonized beads by monocytes is rapid, engulfment of apoptotic cells is slower, and incubations of 3 to 4 h were chosen to study the changes in expression of P2X7 on the monocyte surface. A 2-fold increase of P2X7 surface expression was found in the subpopulation of monocytes after these cells had phagocytosed five or more beads, as compared with the basal value or monocytes with only weak phagocytic ability (Fig. 4A–C). This increase was apparent as soon as 15 min after phagocytosis commenced and persisted for at least 4 h (Fig. 4C).

**Deletions in the P2X7-ED alter phagocytosis of apoptotic cells**

To confirm the involvement of P2X7 extracellular motifs in phagocytosis, we made eight deletion constructs of P2X7 tagged with DsRed in the C terminus, in each of which nine contiguous amino acids in P2X7-ED were deleted. The deletion constructs, together with pDsRed-monomer vector and DsRed-tagged full-length P2X7, were transfected into HEK-293 cells, and the phagocytic ability of these P2X7-transfected cells was examined. Surface expression of these deletion constructs was confirmed because cells expressing high density of deletion constructs showed similar phagocytic ability to YG bead as cells transfected with full-length P2X7 (Supplemental Fig. 1). Among the eight constructs of P2X7, deletion of residues 117–125 and 306–314 showed significant effects, both reducing the phagocytosis of apoptotic HPB cells to 20–30% of wild-type values (Fig. 5). These results provide further supportive evidence for the involvement of P2X7-ED in phagocytosis of apoptotic cells.

**Peptides with unique P2X7 sequences bind apoptotic cells**

To further explore which region of P2X7 could directly interact with particles, a total of 24 biotin-labeled peptides mimicking the
Peptides in the 115–162 region of P2X7-ED bind to apoptotic cells but not viable cells

To evaluate whether P2X7 could directly recognize different apoptotic cells, we measured the binding of the short peptides to three different apoptotic cell types. These target cells included human neuroblastoma cells SH-SY5Y, T-lymphoblastoid HPB cells, and unfractionated human lymphocytes from peripheral blood. Peptides 5, 7, 8, 11, 22, and 23, which have shown binding to apoptotic HPB, were chosen, and peptide 24 was used as the negative control. Peptides 5, 7, and 8 bound to the surface of all three types of early apoptotic cells (7-AAD negative, Annexin V positive) but not to Annexin V-negative viable cells (Figs. 6C, 7A).

In contrast, peptides 11, 22, and 23 only showed weak or absent binding to these three types of apoptotic cells (Fig. 6C). It is notable that none of the 24 peptides bound to Annexin V-negative live cells. Moreover, the rP2X7-ED also bound to apoptotic SH-SY5Y cells but not viable cells (Fig. 6D), demonstrating that P2X7 motif responsible for binding of apoptotic targets is accessible in this 300-residue recombinant protein. The importance of the P2X7 115–162 region was confirmed by binding studies of shorter peptides (9 or 10 residues) with P2X7 sequences that covered segments of this region. Three of these short peptides containing P2X7 sequence 117–125, 125–133, and 134–143 showed strong binding to apoptotic cells, whereas peptides 151–159 and 147–156 showed moderate binding to apoptotic targets (Fig. 6E). Each of these five short peptides contained a cysteine residue, whereas no binding was observed for peptide 138–147, which contained no cysteine (Fig. 6E). It is noteworthy that like peptides 5, 7, and 8, those 9- or 10-mer short peptides that bound to Annexin V-positive apoptotic cells did not bind to Annexin V-negative viable cells.

A P2X7 extracellular motif recognizes cell membrane lipids

Phospholipids, in particular PS exposure on the exterior membrane, are a hallmark of apoptotic cells. To assess whether different P2X7 extracellular motifs can recognize cell membrane lipids, each of the 24 peptides was incubated with membrane lipid strips. The result showed that peptide 22 bound strongly to phosphatidylinositol (PI), PI (4)-phosphate, PI (4,5)-bisphosphate, and PL (3,4,5)-triphosphate, with moderate binding to 3-sulfogalactosylceramide (sulfatide), cardiolipin, phosphatidylglycerol, triglyceride, diacylglycerol, phosphatidic acid, and PS (Fig. 6B). The other 23 peptides, including the three peptides that bound uniquely to apoptotic cells, did not show binding to any of the lipid species (Fig. 6B).

Intermolecular disulfide bonds are critical for P2X7 to recognize apoptotic cells

Because each of the three binding peptides (5, 7, and 8) contains one or two cysteine residues, it is possible these peptides form intermolecular disulfide bonds with newly expressed protein on the surface of apoptotic cells. This possibility was supported by the effect of 5 mM DTT, which almost completely abolished the binding of peptides 5, 7, and 8 to the apoptotic cell surface (Fig. 7A). In contrast, the binding of the cysteine-free peptide 22 to the apoptotic cell surface was not affected by DTT (Fig. 7A). To further assess the role of disulfide bonding in recognition of apoptotic cells, the cysteine residues in peptides 5, 7, and 8 were replaced with alanine. Alanine substitution in peptide 5 completely abolished binding to apoptotic cells, whereas for peptides 7 and 8, which contain two cysteines, replacement of either cysteine did not significantly alter the binding capacity (Fig. 7B). However, replacement of both cysteines by alanine completely abolished the binding of these two peptides to apoptotic SH-SY5Y or HPB cells (Fig. 7B). The effect of small thiol-reactive compounds on the phagocytosis of apoptotic lymphocytes or SH-SY5Y cells by human monocyte-derived macrophages was then studied. Pretreatment of macrophages with either 5 mM reduced glutathione (GSH) or 1 mM N-acetyl-L-cysteine (a precursor of GSH) almost completely abolished the phagocytosis of apoptotic lymphocytes or SH-SY5Y cells by macrophages (Fig. 7C). As shown above for apoptotic lymphocytes (Fig. 1), P2X7 on macrophages directly mediated phagocytosis of apoptotic neuronal SH-SY5Y cells: pretreatment of macrophages with ATP or OXATP inhibited this phagocytosis to a similar extent as observed with pretreatment with CytD (Supplemental Fig. 3).
CaCl₂ and incubated with P2X₇-ED at a concentration of 10 μg/ml. Cells were washed and incubated with HRp-labeled streptavidin for 30 min followed by chemiluminescence assay. A Cell Membrane Lipid Strip binding assay shows that only peptide 22 (aa 306–320) binds to a number of the cell membrane lipids (bottom panel). The remaining peptides (peptides 1–21, 23, and 24) show no binding to any of the cell membrane lipid spots (one example image is shown in the top panel). C, Flow cytometry density plots shows only four peptides (5, 7, 8, and 22) bind to the apoptotic lymphocyte surface but not to viable cells (negative binding profiles for peptides 11 and 23 are shown as examples; the remaining peptides are not shown). Human lymphocytes were induced to apoptosis with 0.5 μM staurosporine. Cells were resuspended in Na⁺ medium with 3 mM CaCl₂ and incubated with peptides as indicated at a concentration of 10 μg/ml for 30 min, followed by two washes and incubation with FITC-conjugated streptavidin, allophycocyanin-conjugated Annexin V, and 7-AAD. All events were gated on a low 7-AAD population to exclude necrotic cells. D, Flow cytometry density plots showing rP2X₇-ED binds to apoptotic cell surfaces but not to live cells. Human neuronal cells SH-SY5Y cells were induced to apoptosis with 0.2 μM staurosporine. Cells were resuspended in Na⁺ medium with 3 mM CaCl₂ and incubated with P2X₇-ED at a concentration of 15 μg/ml for 30 min, followed by two washes and incubation with anti-5xHis mAb and subsequently with FITC-conjugated goat anti-mouse IgG, allophycocya- nin-conjugated Annexin V; and 7-AAD. All events were gated on low 7-AAD population to exclude necrotic cells. E, Fine mapping of the 115–162 region of P2X₇ by binding of short peptides. The binding of biotin-labeled peptides with sequences shown on the x-axis was measured to apoptotic SH-SYSY and HPB cells. The FITC-labeled streptavidin was added, and relative binding was analyzed by flow cytometry. To ensure only binding to apoptotic cells was measured, all fluorescent events were collected on gated Annexin V⁺/7-AAD⁻ data. Cells were presented as normalized mean value.

Discussion

In a previous study, we showed that the P2X₇-NMMHC-IIA complex expressed endogenously on monocyctic cells or following transfection of HEK-293 cells regulates the engulfment of both bacteria and latex beads in the absence of serum. In this study, we show that the range of nonopsonized targets recognized by P2X₇ includes apoptotic lymphocytes that are engulfed by autologous monocytic or neuronal cells with P2X₇-ED inhibited the phagocytosis of these apoptotic cells by macrophages. A third line of evidence is that pretreatment of phagocytes with ATP inhibited further phagocytosis in vitro and in vivo, but not in P2X₇ knockout mice. These results reveal a direct role of P2X₇ in phagocytosis of apoptotic cells, in addition to its role in regulating uptake of nonopsonized beads and heat-killed and live bacteria (14).

The present data also suggest that P2X₇, in the unactivated state, can function as an SR. SR must attach to the cytoskeleton to allow internalization of the attached particle, and it is notable that the P2X₇ membrane complex contains multiple cytoskeletal proteins including nonmuscle myosin and actin (16, 28) required for membrane blebbing (29) and internalization of a wide variety of nonopsonized particles including apoptotic cells. P2X₇ receptors have a similar topology and tissue distribution as class B SR (SR-BI and CD36) (30), with two transmembrane domains and intracellular amino and C termini, but there is little or no sequence homology between P2X₇ and either CD36 or SR-BI (31). Astrocytes express SR-BI, whereas microglia express low levels of surface of the target cell. Pretreatment of apoptotic lymphocytes or neuronal cells with P2X₇-ED inhibited the phagocytosis of these apoptotic cells by macrophages. A third line of evidence is that pretreatment of phagocytes with ATP inhibited further phagocytosis in vitro and in vivo, but not in P2X₇ knockout mice. These results reveal a direct role of P2X₇ in phagocytosis of apoptotic cells, in addition to its role in regulating uptake of nonopsonized beads and heat-killed and live bacteria (14).
CD36, and both of these molecules have been proposed to play a role in the clearance of apoptotic cells in the CNS (31, 32). P2X7 also has a similar distribution in the nervous system, with expression in astrocytes and neurons of spinal cord, and a particularly high expression in microglia (33–36). This novel phagocytic function for the P2X7 membrane complex may represent a previously unidentified mechanism of removing apoptotic cells within specialized compartment of the body such as the CNS.

Extracellular ATP inhibits P2X7-mediated phagocytosis

Exposure of human macrophages to extracellular ATP for 15 min greatly reduced the subsequent phagocytosis of apoptotic cells by monocyte-derived macrophages, as well as by P2X7-transfected HEK-293 cells. ATP is a physiological ligand for several purinergic receptors, but our data with the murine peritoneal macrophages showed that the reduction of phagocytosis of apoptotic thymocytes in vivo following i.p. infusion of ATP was observed only in wild-type mice but not in P2X7 gene-deleted animals. However, the total phagocytic ability of peritoneal macrophages was similar for wild-type and P2X7 knockout animals, presumably due to upregulation of other SR, an effect that was also observed in CD204 (class A SR type I, II)-deficient mouse and CD36 knockout mouse (32, 37).

Moreover, the inhibitory effect of ATP was dose dependent because 0.1 mM BzATP (a potent P2X7 agonist) was equally potent to 1.0 mM ATP in inhibiting P2X7-mediated phagocytosis of apoptotic cells. We propose that the mechanism by which ATP inhibits phagocytosis of nonopsonized particles, including apoptotic cells, is due to the slow dissociation of myosin IIA from the P2X7 membrane complex as described in our previous studies (14, 16). Fig. 1C and 1D also shows that OxATP inhibits phagocytosis of autologous apoptotic lymphocytes. OxATP is the only antagonist we found that effectively inhibits both P2X7 channel/pore function and phagocytosis of apoptotic cells. The mechanism is unknown, but it presumably attaches in the ATP binding pocket of P2X7 and causes conformational change and/or blocks the recognition of particles. However, any OxATP-induced conformational change is insufficient to open the P2X7 ionic channel because this analog is an irreversible inhibitor of P2X7-mediated permeability changes. It is noteworthy that the P2Y6 receptor agonist UDP, which increases phagocytosis of beads (38), had no effect on phagocytosis of apoptotic lymphocytes by macrophages.

Upregulation of P2X7 surface expression after phagocytosis

We and others have shown that most cellular P2X7 receptors are located intracellularly rather than on the cell surface (15, 20, 39). In this study, an increase of P2X7 surface expression on phagocytes was observed following phagocytosis of either apoptotic cells or beads, similar to the upregulation of the P2X7 receptor on THP-1 cells after phagocytosis of beads (40). Endoplasmic reticulum membrane is known to contain significant amounts of P2X7 (41), so the observed increase of P2X7 surface expression may occur via a general process of direct association and fusion of endoplasmic reticulum membrane to the surface membrane of macrophages during early phagocytosis (41). Meanwhile, this upregulation of P2X7 would further enhance the P2X7-mediated phagocytosis as the recognition of nonopsonized particles by...
P2X 7 shows strong dependence on the density of P2X 7 expressed on the surface of phagocytes.

Peptide screen to examine interaction of P2X 7 with apoptotic targets
To explore the initial interaction between P2X 7 and apoptotic epitopes, we measured the binding of a series of peptides (12–16 residues mimicking regions of P2X 7-ED) to the surface of three different apoptotic cell types. Four peptide sequences showed binding to the surface of apoptotic cells (peptides 5, 7, 8, and 22), but only peptide 22 showed binding to cell membrane lipids including PS. Moreover, peptide 22 also showed binding to a wide variety of nonopsonized particles (latex beads, live and dead bacteria, and apoptotic cells), which are recognized targets for SR. Peptide 22 has a high positive charge (pI 11.5), and modeling suggests that it is in part on the surface of the P2X 7 molecule, thus allowing interactions of an electrostatic nature with adjoining surfaces. The binding assay also suggests that P2X 7 may recognize different particles via more than one motif in the extracellular domain, and the region around residues 306–320 seems critical for recognition of particles of many types. Moreover, deletion of the peptide 22 region from a wild-type P2X 7 construct greatly impaired phagocytosis of apoptotic HPB cells by transfected HEK-293 cells, confirming the importance of this extracellular region of P2X 7 for apoptotic recognition.

Three other peptides (5, 7, and 8) also showed binding to apoptotic cells but not to viable cells, suggesting these sequences contain a second interacting region between P2X 7 and the apoptotic cell. Modeling based on the crystal structure of zP2X 7 (42) shows these three peptides are located on an exposed nodule of the molecule. However, none of these three peptides showed binding to the membrane lipid strip, suggesting that this region (115–162) does not recognize PS on the apoptotic cell surface. Purified Annexin V, which does bind to exposed PS, did not affect the phagocytosis of apoptotic lymphocytes by autologous macrophages (data not shown), suggesting this second interacting region has a major role in the initial interaction between apoptotic cell and phagocyte. This result supports the concept that P2X 7 may interact with a second region on apoptotic cell surface, which is different to the newly exposed PS. We also found that one or more disulfide bonds in the P2X 7-ED played an important role in attaching the phagocyte to the target cell surface because breaking disulfide bonds by DTT, GSH, or N-acetylcysteine abolished the binding of peptides 5, 7, and 8 to the apoptotic cell surface. However, not all the cysteine-containing peptides bound to the apoptotic cell surface, suggesting other nearby residues confer specificity to the recognition. Recent studies of platelet biology have revealed that thiol-disulfide isomerization involving protein disulfide isomerase is an essential step in the firm attachment of activated platelets to endothelial cells during thrombus formation (43). The potential role of this enzyme or a similar thioredoxin molecule will be the subject of further studies exploring the interaction of P2X 7 with apoptotic cells.

A variety of SR have been shown to participate in the phagocytosis of apoptotic cells, and this redundancy may reflect the importance of this process. We have previously demonstrated that P2X 7 regulates phagocytosis of nonopsonized beads and bacteria by monocytes in the absence of ATP. Our present data demonstrate that P2X 7 on the phagocytic cell as well as P2X 7-transfected HEK-293 cells directly participates in the recognition and engulfment of a range of apoptotic target cells, both in vitro and in vivo. When compared with other phagocytic pathways, this P2X 7-mediated phagocytosis is unique in several aspects: first, the P2X 7 membrane complex can mediate the phagocytosis of a broad range of nonopsonized particles, from beads and live and dead bacteria to apoptotic cells, suggesting P2X 7 has an important role in innate immunity. Second, P2X 7 is a regulatory target of extracellular ATP, a ubiquitous signaling molecule, with autocrine and paracrine effects to promote inflammation and inhibit phagocytosis. Finally, our data suggest that certain conserved disulfide bonds in the P2X 7-ED are critical for the initial firm attachment of apoptotic cells to phagocytes. Removal of apoptotic cells during tissue remodeling is a continual process that in most organs and tissues occurs in the absence of serum. Our data suggest that P2X 7 is an important addition to the small number of receptors that participate in this process in serum-poor settings.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


## Supplemental Information

**Table S1. The sequences of P2X7 extracellular domain mimicking peptides**

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Figure S1: P2X7 extracellular motifs involved in phagocytosis of YG beads. HEK-293 cells were transfected with DsRed (mock), DsRed-tagged wild type P2X7 or deleted P2X7 constructs. a.&b. Typical uptake curves showing phagocytosis of YG beads by gated HEK-293 cells with medium DsRed intensity (a.) or with high DsRed intensity (b.). All the deletion constructs showed similar phagocytosis ability in YG bead uptake when gated on cells expressing high DsRed fluorescence (the rest YG bead uptake curves are not shown). c. The composite values for phagocytosis of YG beads by transfected HEK-293 cells with medium DsRed intensity (n=4).
Figure S2. The binding profile of peptides mimicking P2X7 extracellular domain sequences. 24 biotin-tagged peptides were incubated with YG beads (3 µm, 5 µL), Alexa 488 conjugated S.aureus or E.coli (2 mg/mL, 5μL), live S.aureus or E.coli (OD690 = 0.9, 10 µL each) for 30 min at a concentration of 10 µg/mL, followed by washing and incubation with HRP-labeled streptavidin for 30 min.
Figure S3. Typical flow cytometry histogram showing phagocytosis of apoptotic SH-SY5Y cells by macrophages. SH-SY5Y cells were labeled with CFSE and induced to apoptosis with 0.2 µM staurosporine overnight in RPMI1640 medium with 20% FCS. Macrophages were labeled with CMTMR and pre-treated with 1 mM ATP for 15 min, 0.3 mM OxATP for 30 min, 20 µM CytD for 30 min or fixed with 2% paraformaldehyde prior to the addition of apoptotic SH-SY5Y cells. Mixed cells were incubated for 3 hours before fixed and assayed by flow cytometry on gated CMTMR⁺ macrophages.