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Identification of TLT2 as an engulfment receptor for apoptotic cells

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Abstract

Clearance of apoptotic cells (efferocytosis) is critical to the homeostasis of immune system by restraining inflammation and autoimmune response to intracellular antigens released from dying cells. Toll-like receptors (TLRs) mediated innate immunity plays important role in pathogen clearance and in regulation of adaptive immune response. However, the regulation of efferocytosis by activation of TLRs has not been well characterized. In this study, we found that activation of TLR3 or TLR9, but not of TLR2, enhances engulfment of apoptotic cells by macrophages. We found that the activation of TLR3 upregulates the expression of Triggering receptor expressed on myeloid cells (TREM)-like protein 2 (TLT2), a member of TREM receptor family, on the surface of macrophages. Blocking TLT2 on macrophage surface by either specific anti-TLT2 antibody or soluble TLT2 extracellular domain attenuated the enhanced ability of macrophages with TLR3 activation to engulf apoptotic cells. To the contrary, overexpression of TLT2 increased the phagocytosis of apoptotic cells. We found that TLT2 specifically binds to phosphatidylserine (PS), a major “eat me” signal that is exposed on the surface of apoptotic cells. Furthermore, we found that TLT2 mediates phagocytosis of apoptotic cells *in vivo*. Thus, our studies identified TLT2 as an engulfment receptor for apoptotic cells. Our data also suggest a novel mechanism by which TREM receptors regulate inflammation and autoimmune response.

Keywords

efferocytosis; Triggering receptor expressed on myeloid cells (TREM)-like protein 2; Toll like receptor; inflammation

Introduction

Clearance of apoptotic cells, a process known as efferocytosis, is critical to the homeostasis of the immune system by preventing inflammation and autoimmune response to intracellular antigens released from dying cells [1]. Redistribution of phosphatidylserine (PS) to the outer

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leaflet of the plasma membrane at early stages of apoptosis is a key signal for recognition of apoptotic cells by phagocytes [1]. Several receptors have been recently identified to recognize PS, and thereby mediating efferocytosis. Deficiencies in these receptors lead to aggravation of inflammation and autoimmune diseases [2,3,4,5].

Innate immunity is critical to pathogen clearance and is also involved in the activation and regulation of adaptive immune response [6]. Sensing pathogens and endogenous danger signals is achieved through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) [6]. TLRs recognize microbial, such as lipopolysaccharide (LPS), lipopeptides, and nucleic acids, and therefore initiate specific signaling pathways that lead to distinct immune responses [6].

In the present study, we examined the effects of TLR activation on efferocytosis. Our data demonstrated that activation of macrophages with Poly I:C (TLR3 ligand) or ODN (TLR9 ligand), but not with PAM (TLR2 ligand), enhances ingestion of apoptotic cells. Using gene microarray assays, we found that Triggering receptor expressed on myeloid cells-like protein 2 (TLT2 or TREML2) is upregulated after TLR3 activation. TLT2 is a member of TREM receptor family that regulates innate immune response [7,8]. Our study demonstrated that TLT2 is directly involved in the engulfment of apoptotic cells through binding to PS. Our results reveal a novel function for TLT2 in mediating clearance of apoptotic cells and suggest additional mechanisms by which TREM receptors regulate inflammation and autoimmune responses.

Material and Methods

Mice

Female C57BL/6 mice (WT) were purchased from the National Cancer Institute (NCI)-Frederick. Female TLR3 deficient mice, B6129SF2 (WT control for TLR3 deficient mice) and TRIF deficient mice (TRIF KO) were purchased from Jackson Laboratory. All animal protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (UAB). Experiments were performed using 8- to 10-week-old mice.

Reagents

Penicillin-streptomycin, Brewer thioglycollate, cytochalasin D and PKH26 red fluorescent cell linker kit were from Sigma-Aldrich. GM-CSF was from PeproTech. CCL-1 conditioned medium was from American Type Culture Collection (ATCC). PE conjugated anti-mouse TLT2 Antibody and PE conjugated isotype IgG control were from Biolegend. Goat anti-human TLT2 Abs were from Santa Cruz. Recombinant chimeric human TLT2-Fc and human IgG1-Fc were from R&D Systems. The antibody against the extracellular portion of TLT2, clone Ab1H4, was a gift from Dr. Louis Justement at UAB. Red fluorescent carboxylate-modified microspheres and pHrodo succinimidyl ester were from Invitrogen. Anti-CD16/CD32 antibodies, FITC-conjugated anti-CD11b and allophycocyanin (APC)-conjugated anti-CD90.2 antibodies were from BD Pharmingen. PS, PC and PE were from Avanti Polar Lipids. Annexin V was from BD biosciences. Pam(3)CysSK(4) (PAM), Lipopolysaccharide (LPS), CpG oligodeoxynucleotides (ODN) and polyinosinic-polycytidylic acid (Poly I:C) were from InvivoGen. Chromeo-488 antibody labeling kit was from Active Motif.

Culture of mouse peritoneal macrophages

Peritoneal macrophages were elicited from WT and TRIF KO mice by i.p. injection of 1 ml sterile 4% Brewer thioglycollate. Cells were harvested 4 days later by peritoneal lavage.

Macrophages were plated on 24-well plates at a concentration of 0.5×10^6 cells/well in RPMI 1640 medium containing 10% FBS at 37°C. After 1 hour at 37°C, non-adherent cells were removed by washing with medium.

Culture of bone marrow derived macrophages (BMDMs)

BMDMs were induced as previously described [9,10] with modifications. In brief, bone marrow cells devoid of red blood cells were cultured in RPMI 1640 containing 10% CCL-1 conditioned media and 10 ng/ml GM-CSF overnight. Non-adherent cells were collected and plated at 1×10^7 cells/150-mm plate. 5 days after the induction, BMDMs were collected by scraping and plated in GM-CSF-free RPMI complete media for 24h before phagocytosis assays.

Induction of apoptosis in thymocytes

Mouse thymocytes were labeled with PKH26 red fluorescent dye according to the manufacture's instructions. To induce apoptosis, thymocytes were resuspended in RPMI 1640 medium containing 10% FBS and 1 μM dexamethasone at a concentration of 6×10^6 cells/ml and incubated at 37°C in 5% CO_2 overnight. At this time point, >90% of thymocytes were apoptotic [4].

Solid-phase ELISA

Solid-phase ELISA to determine the binding between recombinant chimeric TLT2-Fc and phospholipids was performed as previously described [11]. Phospholipid solutions of PS, PC or PE at a concentration of 5 μg/ ml in methanol were added to 96-well plates and air-dried at room temperature. The plates were then blocked with 10 mg/ml BSA in PBS. Recombinant chimeric TLT2-Fc was added to the wells and incubated at room temperature for 2 hours. Bound TLT2-Fc was quantified by ELISA with anti-TLT2 Abs (sc-109096) and peroxidase-conjugated secondary Ab.

In vitro efferocytosis assay

Phagocytosis of PKH26-labeled apoptotic thymocytes by macrophages (efferocytosis) was evaluated by addition of 0.5×10^6 apoptotic thymocytes suspended in 500 μl of RPMI 1640 medium containing 10% FBS to each well of a 24-well plate containing macrophages, followed by incubation at 37°C for 30 min. Macrophages were then washed 5 times with cold PBS, resuspended in PBS containing 1% albumin, FITC-conjugated anti-CD11b (macrophage marker) antibody, and APC-conjugated anti-CD90.2 antibody (thymocyte marker used to differentiate thymocytes engulfed by macrophages from thymocytes simply attached to macrophages), and analyzed by flow cytometry. The phagocytic index was calculated as the ratio of FITC+PKH26+APC− cells to all cells gated. Engulfed thymocytes are not accessible to the APC-conjugated anti-CD90.2 antibody. Therefore, FITC +PKH26+APC− cells are macrophages that have engulfed PKH-labeled thymocytes, whereas the APC+PKH+FITC+ cells were macrophages to which thymocytes are adherent but not engulfed. Phagocytosis of red fluorescent carboxylate-modified microspheres (beads) was determined by addition of 1 μl of beads suspended in 500 μl of RPMI 1640 medium containing 10% FBS, followed by incubation at 37°C for 30 min. After incubation, 500 μl of such solutions were added to each well of a 24-well plate containing macrophages, followed by incubation at 37°C for 30 min. Samples were washed 5 times with cold PBS and resuspended in PBS containing 1% albumin and FITC-conjugated anti-CD11b (macrophage marker) antibody. Phagocytosis was determined by flow cytometry.

In vivo efferocytosis assay

The in vivo efferocytosis assay was performed as previously described [12]. To determine the effect of TLT2 on phagocytosis of apoptotic cells, PKH26-labeled apoptotic thymocytes (10×10^6) were incubated with 4 μg/ml recombinant IgG1-Fc or 4 μg/ml recombinant TLT2-Fc for 2 hours at room temperature with rotation and then injected i.p to mice. Mice were sacrificed 2h after the injections, and peritoneal lavage was performed. Cells from the lavage fluids were resuspended in PBS containing 1% albumin, FITC-conjugated anti-CD11b (macrophage marker) antibody, and APC-conjugated anti-CD90.2 (thymocyte marker) antibody. Phagocytosis was evaluated by flow cytometry. To evaluate the role of TLR3 in efferocytosis in vivo, B6129SF2 or TLR3−/− mice were injected i.p. 200 μg Poly I:C. 24h after the injection, PKH26-labeled apoptotic thymocytes (10×10^6) were administered i.p. to mice. Mice were sacrificed 2 hours after the injection and peritoneal lavages performed. Phagocytosis was evaluated by flow cytometry.

Plasmids

Mouse TLT2 cDNA was obtained by PCR amplification and inserted in-frame into multicloning sites in pGEFP-C3 vector (Clontech).

Real-time PCR

The assay was performed as previously described [13,14]. Primer sequences were: Mouse GAPDH: sense, CGACTTCAACAGCAACTCCCACTCTTCC; antisense, TGGGTGGTCCAGGGTTTCTTACTCCTT. Mouse TLT2: sense, TGGTGGTGGTGTTGACATTTCTTCC; antisense, ATCCAGGGTTTAGCATAGTTGCTGC.

Flow cytometry

To determine levels of TLT2 on the surface of macrophages, cells were pretreated with anti-CD16/CD32 antibodies (2 μg/ml) for 30 min to block FcR. The cells were then washed three times with serum-free RPMI 1640 medium and incubated with PE conjugated isotype IgG (Control-PE) or PE conjugated isotype anti-TLT2 antibody (TLT2-PE) for 30 minutes, followed by washing and analyzed by flow cytometry.

Fluorescent labeling of BSA and TLT2-ECD

BSA and TLT2-ECD were labeled by Chromeo-488 green fluorescent dye (Active Motif) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SD for each experimental group. One-way ANOVA followed by Bonferroni test was performed for comparisons among multiple groups, and Student *t* test was used for comparisons between two groups. A p value < 0.05 was considered statistically significant.

Results

Activation of TLRs differentially regulates the ability of macrophages to engulf apoptotic cells

Initial studies were carried out to determine the effects of TLR activation on efferocytosis. For this purpose, peritoneal macrophages were pretreated overnight with TLR3 ligand (Poly I:C), TLR2 ligand (PAM) or TLR4 ligand (LPS) and the uptake of PKH26 labeled apoptotic thymocytes were measured. As shown in Fig. 1A, Poly I:C treated macrophages

demonstrated significantly enhanced ability to engulf apoptotic thymocytes, as compared to the untreated macrophage controls. To the contrary, activation of TLR2 slightly reduced the ability of macrophages to ingest apoptotic cells. Activation of TLR4 also increased the efferocytosis, but to a lesser extent than TLR3 activation did. These data suggest that TLRs activation differentially regulates efferocytosis.

To determine whether the effect of TLRs activation on the uptake of apoptotic cells is specific to peritoneal macrophages or a general phenomenon to other macrophage populations, we performed the same treatments described above in bone marrow-derived macrophages (BMDMs). As shown in Supplementary Fig. 1 and similar to those observed in peritoneal macrophages, activation of TLR3 and TLR4, but not TLR2, enhanced the ability of BMDMs to engulf apoptotic thymocytes.

Efferocytosis is a multi-step process that starts with recognition of apoptotic cells by specific receptors on the surface of phagocytes [1]. In addition, a number of soluble proteins, such as milk fat globule-EGF factor 8 protein (MFG-E8) and growth arrest-specific 6 (GAS6), can serve as “bridging molecules” to facilitate the recognition of apoptotic cells by phagocytes [15,16]. To delineate the mechanism by which TLR3 activation enhances efferocytosis, we first investigated if the increased phagocytic activity involves soluble “bridging molecules” secreted by Poly I:C treated macrophages or is caused by cellular alterations with the Poly I:C treated cells. To do this, macrophages were incubated overnight with Poly I:C. The culture supernatants were then removed and the treated macrophages were washed 3 times. Apoptotic thymocytes were added to the macrophages and phagocytosis assays were performed. As shown in Fig. 1B, Poly I:C treated macrophages demonstrated elevated efferocytosis, even when the original supernatants were removed from the cell culture. In the other set of experiments, efferocytosis assays were performed in the presence of the supernatants collected from Poly I:C treated macrophages. As shown in Fig. 1C, efferocytosis by untreated macrophages demonstrated minimum changes in the presence of supernatants from Poly I:C treated macrophages, as compared to that in the presence of fresh media (Control supernatant). These data suggest that the increased efferocytosis after TLR3 activation is caused by cellular alterations of, but not by secreted molecules from, the Poly I:C treated macrophages.

To determine if the enhanced phagocytic activity after TLR3 activation is an effect specific to apoptotic cells, or represents a general phenomenon while ingesting other foreign targets, we performed phagocytosis assays using carboxylate-modified beads. We found that TLR3 stimulation did not enhance uptake of carboxylate-modified beads by macrophages (Fig. 1D), a process that occurs through mechanisms distinct from those involved in apoptotic cell clearance [17]. However, pretreatment of macrophages with LPS or PAM enormously increased the uptake of beads (Fig. 1D), consistent with previous reports [18]. Taken together, these data suggest that TLR3 activation specifically enhances efferocytosis.

Enhanced efferocytosis after TLR3 activation requires adaptor TRIF

Engagement of TLR3 by Poly I:C recruits the adaptor protein, TIR-domain-containing adapter-inducing interferon-β (TRIF), to the receptor. This event subsequently leads to the activation of interferon regulatory factor 3 (IFR3) and type I interferon response [6,19]. Sensing viral dsRNAs by TLR3 and the resulted type I interferon response are primary host defenses to viral infection [6]. To elucidate the mechanism by which TLR3 activation specifically enhances efferocytosis, wild type (WT) and TRIF knockout (TRIF KO) macrophages were stimulated with medium or Poly I:C and the uptake of apoptotic thymocytes was evaluated. As shown in Fig. 2A, although Poly I:C treatment increased the activity of WT macrophages to ingest apoptotic cells, it had no effect on TRIF KO

macrophages. These data suggest that efferocytosis enhanced by TLR3 activation requires an intact, TRIF-mediated signaling event.

Another IRF family member, IRF-7, regulates a number of common target genes with IRF3. IRF7 is activated upon TLR9 engagement [6,19]. To determine if enhanced efferocytosis also occurs in macrophages with TLR9 activation, we pretreated macrophages with CpG oligodeoxynucleotides (ODN), a specific synthetic ligand of TLR9. As shown in Fig. 2B–2C and similar to those observed with Poly I:C treated cells, ODN pretreatment significantly enhanced the ability of macrophages to ingest apoptotic cells, but not beads. These data suggest that the enhanced efferocytosis is a common phenomenon shared by IRF3 and IRF7 activations.

TLR3 activation upregulates TLT2 expression

To identify molecules that mediate the elevated efferocytosis after TLR3 activation, we compared the expression profile of untreated, Poly I:C treated, and PAM treated macrophages by microarray assays. Our hypothesis was that specific genes that could mediate efferocytosis would be upregulated by Poly I:C stimulation, whereas downregulated or not altered by PAM stimulation. As shown in Supplementary Table, a number of genes were increased in Poly I:C treated, but decreased in PAM treated macrophages. The complete gene array data were deposited in to GEO database and can be accessed through http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36891 with accession number GSE36891. Genes of particular interest to us were cellular receptors, including TLT2 that belongs to the TREM receptor family involved in inflammatory regulation [20].

To validate the microarray findings, we performed realtime PCR assays and found that TLT2 is significantly upregulated in Poly I:C treated, whereas slightly decreased in PAM treated macrophages (Fig. 3A), concordant with the findings that Poly I:C, but not PAM pretreatment enhances efferocytosis (Fig. 1A). Of note, TLT2 expression was also increased in ODN and LPS treated macrophages, but to a lesser extent (Fig. 3B). To further characterize TLT2 expression on macrophages with TLR3 activation, we performed flow cytometry assays using specific anti-TLT2 antibody and found that TLT2 expression on the surface of Poly I:C treated macrophages are markedly increased (Fig. 3C). These data suggest that the elevated levels of TLT2 on the surface of macrophages could be responsible for the enhanced efferocytosis found with Poly I:C treated cells.

Enhanced efferocytosis after TLR3 activation is dependent on TLT2

To determine if the enhanced efferocytosis after TLR3 activation is dependent on TLT2, we pretreated Poly I:C stimulated macrophages with specific anti-TLT2 antibodies. As shown in Fig. 4A, pretreatment with anti-TLT2 antibodies significantly attenuated the enhanced ability of Poly I:C stimulated macrophages to uptake apoptotic macrophages. These data suggest that the enhanced efferocytosis found in Poly I:C treated macrophages requires TLT2. However, we found that pretreatment with anti-TLT2 antibodies has no effect on macrophages to ingest beads, consistent with the findings that Poly I:C stimulated cells do not have enhanced ability to uptake beads (Fig. 4B). These data suggest that TLT2 specifically mediates ingestion of apoptotic cells.

The above results have thus far implicated TLT2 as a potential receptor for recognition of apoptotic cells. Next, we asked if a soluble TLT2 extracellular domain (TLT2-ECD) could compete with TLT2 on the surface of macrophages to recognize apoptotic cells, and thus block efferocytosis. To test this hypothesis, we examined the ability of Poly I:C treated macrophages to ingest apoptotic cells in the presence of soluble TLT2 extracellular fragments. As shown in Fig. 5A, TLT2 extracellular fragments significantly attenuated the

enhanced ability of Poly I:C treated macrophages to ingest apoptotic macrophages. However, TLT2 extracellular fragments had no effect on ingestion of beads by macrophages (Fig. 5B). These data reinforce the conclusion that TLT2 specifically mediate efferocytosis.

Preincubation of soluble TLT2 extracellular fragments with apoptotic cells, but not with macrophages, attenuates efferocytosis

Our results showed that the enhanced efferocytosis found with Poly I:C treated macrophages is diminished in the presence of soluble TLT2 extracellular fragments. However, it is not known what is necessary for such an inhibitory effect, the interactions between the soluble TLT2 extracellular fragments and macrophages or apoptotic cells? To delineate this, we preincubated macrophages or apoptotic cells with soluble TLT2 extracellular fragments before performing efferocytosis assays. As shown in Supplementary Fig. 2A, the enhanced efferocytosis found with Poly I:C treated macrophages was diminished in the presence of soluble TLT2 extracellular fragments, as expected. Furthermore, we found that preincubation of apoptotic cells, but not macrophages, with soluble TLT2 extracellular fragments, diminished the ability of macrophages to ingest apoptotic cells (Supplementary Fig. 2B–2C). These data suggest that TLT2 extracellular fragments compete with TLT2 receptor on the surface of macrophages to recognize and bind to apoptotic cells.

TLT2 mediates efferocytosis

We have shown that blocking TLT2 with either specific anti-TLT2 antibody or soluble TLT2 extracellular fragments attenuates enhanced efferocytosis found with Poly I:C stimulated macrophages, we next determined if increasing TLT2 levels promotes efferocytosis. We expressed GFP fused TLT2 (GFP-TLT2) in NIH3T3 cells and found that cells expressing GFP-TLT2 demonstrated much higher ability to ingest apoptotic cells, as compared to cells expressing GFP alone (Fig. 6A). However, GFP negative cell populations in the cells that were transfected with constructs expressing GFP or GFP-TLT2 had only comparably low basal levels of efferocytosis (Fig. 6A). Cells expressing GFP-TLT2 did not show enhanced activity to ingest beads (Fig. 6B), consistent with the findings that blocking TLT2 did not affect macrophages to uptake beads. Furthermore, we found that soluble TLT2 extracellular fragments diminished the enhanced efferocytosis found in cells expressing GFP-TLT2 (Fig. 6C). Of note, TLT2 extracellular fragments had no effect on GFP negative cells (Fig. 6C). Together, these data suggest that TLT2 specifically and directly mediates ingestion of apoptotic cells.

TLT2 binds to phosphatidylserine (PS)

Phagocytes directly recognize PS through receptors such as Brain-specific angiogenesis inhibitor 1 (BAI1) and receptor for advanced glycation endproducts (RAGE), or indirectly through soluble bridging molecules that bind to both PS and specific phagocyte receptors [2,3,4,21]. Given that TLT2 mediates efferocytosis, we next asked if TLT2 does so through recognizing and binding to PS.

Using solid-phase ELISA assays, we found that TLT2 extracellular fragments specifically bind to PS, but not to PC or PE (Fig. 7A–7C), two major lipids on the outer leaflet of plasma membrane of both viable and apoptotic cells. We also found that preincubation with annexin V, which specifically binds to PS [22], diminished the binding of TLT2 to PS (Fig. 7D). Furthermore, we found that the binding between TLT2 to PS was inhibited by specific anti-TLT2 antibody (Fig. 7E). To determine if TLT2 extracellular fragments specifically bind to apoptotic cell, we incubated live and apoptotic thymocytes with Chromeo-488 labeled TLT2-ECD and found that TLT2-ECD demonstrates much greater binding to apoptotic cells than to live cells (Fig. 7F). Using con-focal microscopy, we found that TLT2-ECD specifically binds to the surface of apoptotic, but not live thymocytes (Fig. 7G). Of note,

BSA had no binding to either live or apoptotic cells (Fig. 7F–G). Taken together, these data suggest that TLT2 mediates engulfment of apoptotic cells by specifically recognizing and binding to PS, a process that can be blocked by anti-TLT2 antibody. We next investigated if TLT2 is involved in binding of apoptotic cells to macrophages. We pretreated macrophages with cytochalasin D that blocks internalization by inhibiting actin polymerization. We found that Poly I:C augments the binding of apoptotic thymocytes to cytochalasin D treated macrophages. Poly I:C enhanced binding was diminished by TLT2-ECD (Fig. 7H). These data suggest that TLT2 increases efferocytosis by enhancing macrophage binding to apoptotic cells.

We have shown that TLT2 mediates binding of apoptotic cells to macrophages. We next asked if TLT2 is involved in the internalization of apoptotic cells and phagosome formation. We labeled the apoptotic cells with pHrodo dye that lacks fluorescent signal in the neutral extracellular environment, but dramatically increases in red fluorescence as the pH of its surroundings becomes more acidic, like those found in phagosomes/lysosomes. We found that internalized apoptotic cells are wrapped by TLT2-GFP (Supplementary Fig. 3), suggesting that cell membrane bound TLT2-GFP is internalized together with the apoptotic cells and becomes components of phagosomes.

TLT-2 mediates efferocytosis *in vivo*

Next, we evaluated whether TLT2 mediates efferocytosis *in vivo*. To do this, PKH26-labeled apoptotic thymocytes were preincubated with or without soluble TLT2 extracellular fragments, as performed for *in vitro* efferocytosis assays. The PKH-labeled apoptotic thymocytes were then administered i.p. to mice. 2h later, peritoneal lavage fluids were harvested and phagocytic indexes determined. As shown in Fig. 8A, phagocytosis by resident peritoneal macrophages of apoptotic thymocytes pretreated with soluble TLT2 extracellular fragments was significantly diminished, as compared to that found with apoptotic thymocytes incubated with control fragments. These data further support a role for TLT2 in mediating engulfment of apoptotic cells. To determine if TLR3 deficiency affects clearance of apoptotic cells in vivo, we treated TLR3−/− mice with poly I:C before phagocytosis. We found that Poly I:C treated TLR3 deficient mice demonstrated less phagocytosis of apoptotic cells, as compared to Poly I:C treated WT mice (Fig. 8B). These data suggest that an increase in efferocytosis in response to TLR3 activation, such as those observed during viral infection, is a self-protective mechanism to contain viral infection by promoting elimination of the virally infected, apoptotic cells.

Discussion

TLRs are critical for hosts to recognize invading organisms and to initiate proper inflammatory response to combat infections [6]. In the process of inflammation, immune cells are activated to produce cytokines and chemokines and are destined to undergo apoptotic cell death. Rapid removal of apoptotic cells is one of the major mechanisms for constraining excessive inflammation since this process prevents release of irritating intracellular contents from broken apoptotic cells [23]. In case of non-infected inflammation, clearance of apoptotic cells prevents auto-immune response to nucleic acids and other cellular components from apoptotic cells [24,25,26]. Deficiency in clearance of apoptotic cells have been well documented in various inflammatory conditions [25]. However, there is no systemic characterization of how TLR activation regulates this process. In this study, we thoroughly investigated the effects of TLR activation on clearance of apoptotic cells and found that activation of TLRs differentially regulates this process. We found that activation of TLR3 or TLR9, but not activation of TLR2 enhances the ability of macrophages to engulf apoptotic cells.

TLR3 and TLR9 are two major PRRs that recognize dsRNA and DNA from RNA virus and other intracellular pathogens [6]. Virus infection and intracellular invasion of pathogens cause apoptotic cell death. This phenomenon is a well known representation of mechanisms by which hosts interrupt the life cycle of these pathogens [27]. Here, we found that macrophages with TLR3 or TLR9 activation have enhanced ability to engulf apoptotic cells. Therefore, such an enhanced ability to engulf apoptotic cells could be an evolutionally conserved self-protected mechanism by which hosts are able to constrain the spreading of infections.

We found that TLT2 expression is upregulated by activation of TLR3, and to a lesser extent, activation of TLR9. It is known that two IRF transcriptional factors, IRF3 and IRF7, are activated upon stimulation of TLR3 and TLR9, respectively [6]. Additionally, IRF3 and IRF7 share a number of common target genes [28]. After searching the promoter region within the TLT2 gene, we found that there are two IRF responsive elements at 3.5 and 2.5 kb upstream of the transcriptional starting site (data not shown). There are also several responsive elements for transcriptional factor STAT family, which shares binding sites with the IRF family members, within the proximal promoter region of the TLT2 gene. Therefore, enhanced expression of TLT2 appears to be a conserved response to the activation of IRF transcriptional factors.

Although the identities of ligands for all TREM family receptors have been elusive [8], we found that TLT2 binds to PS. TLT2 does not bind to PC or PE, two lipids that express on the outer leaflet of plasma membrane of both live and apoptotic cells. Thus, TLT2 joins a small family of PS receptors, such as BAI-1, RAGE, stabilin-1, and Tim-4, all of which have been shown to directly mediate clearance of apoptotic cells [2,3,4,21]. Identifying PS receptors is of great importance for understanding the process of apoptotic cell clearance and for addressing the mechanisms involved in the resolution of inflammation and autoimmune responses. We found that the binding between TLT2 extracellular domain and PS is diminished, but not abolished by annexin V, which indicates a partially overlapped binding site between TLT2 and annexin V. It is also wroth pointing out that the binding between TLT2 extracellular domain and PS is attenuated by specific anti-TLT2 antibody. These data are consistent with the finding that anti-TLT2 antibody diminishes the ability of macrophages to engulf apoptotic cells. Furthermore, TLT2 extracellular domain demonstrates much greater binding to the surface of apoptotic cells than to that of live cells, concordant with redistribution of the “eat me” signal, PS, to the outer leaflet of apoptotic cells [29]. These data suggest that TLT2 mediates efferocytosis directly through binding to PS. The modest binding of TLT2 to live thymocytes that we found indicates potential interactions of TLT2 with other molecules on the cell surface. This hypothesis is likely true because a recent study found that TLT2 binds to the B7 family member B7-H3, which is expressed on T cells [30]. The fact that live cells are normally not engulfed by phagocytes indicates no involvement of B7-H3 in efferocytosis. It is currently unknown if levels of B7-H3 are increased when T cells are becoming apoptotic. However, it is still interesting to know if B7-H3 is involved in the TLT2 mediated, PS dependent engulfment of apoptotic cells.

We found that the soluble TLT2 extracellular domain inhibits ingestion of apoptotic cells, likely through competing with TLT2 receptors on the surface of macrophages to bind to apoptotic cells. There is currently no evidence that TLT2 on cell surface sheds its extracellular domain to surrounding environment. However, the other three TREM family members, TREM1, TREM2, and TLT1, can all release their extracellular domains under inflammatory conditions, such as experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD), and sepsis [31,32,33]. The soluble forms of TREM family receptors play important roles in regulating the functions of TREMs [7,8]. Given that

all TREM family receptors share similar structures, it is plausible to predict that TLT2 could also shed its extracellular domain under certain inflammatory conditions, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). SLE and RA are autoimmune diseases that involve a deficiency in clearance of apoptotic cells [24,34]. Therefore, the presence of soluble TLT2 extracellular domains in the sera of SLE and RA patients may represent a novel mechanism that contributes to the pathogenesis of these diseases.

Although specific anti-TLT2 antibody or TLT2 extracellular domain diminishes the enhanced ability of macrophages with TLR3 activation to engulf apoptotic cells, neither can abolish such an increased activity. It is likely that TLR3 activation enhances expression of some additional receptors that can also mediate phagocytosis of apoptotic cells, in the absence of TLT2. Identification and characterization of such receptors are currently being undertaken in our laboratories.

We found that TLR9 activation enhanced phagocytosis of apoptotic cells. However, unlike those found with TLR3 activation induced phagocytosis, soluble TLT2-ECD did not significantly attenuate TLR9 enhanced phagocytosis (data not shown). These data suggest a less important role of TLT2 in TLR9 activation induced engulfment of apoptotic cells. These data seem consistent with a lesser induction of TLT2 in the TLR9 activated macrophages than in the TLR3 activated cells (Fig. 3B). These data also imply that TLR9 activation enhanced phagocytosis is mediated by other receptors and/or intracellular pathways.

Our studies uncover a novel role for TLT2 in mediating phagocytosis of apoptotic cells and suggest additional mechanisms by which TREM receptors regulate inflammation and autoimmune responses

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. The Journal of cell biology. 2010; 189:1059–1070. [PubMed: 20584912]
2. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature. 2007; 450:430–434. [PubMed: 17960134]
3. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, et al. Identification of Tim4 as a phosphatidylserine receptor. Nature. 2007; 450:435–439. [PubMed: 17960135]
4. Friggeri A, Banerjee S, Biswas S, de Freitas A, Liu G, et al. Participation of the receptor for advanced glycation end products in efferocytosis. J Immunol. 2011; 186:6191–6198. [PubMed: 21502377]
5. Park SY, Jung MY, Lee SJ, Kang KB, Gratchev A, et al. Stabilin-1 mediates phosphatidylserine-dependent clearance of cell corpses in alternatively activated macrophages. Journal of cell science. 2009; 122:3365–3373. [PubMed: 19726632]
6. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010; 11:373–384. [PubMed: 20404851]

7. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. Nat Immunol. 2006; 7:1266–1273. [PubMed: 17110943]
8. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. Curr Opin Immunol. 2009; 21:38–46. [PubMed: 19230638]
9. Westcott MM, Henry CJ, Cook AS, Grant KW, Hiltbold EM. Differential susceptibility of bone marrow-derived dendritic cells and macrophages to productive infection with Listeria monocytogenes. Cell Microbiol. 2007; 9:1397–1411. [PubMed: 17250592]
10. Wan M, Shi X, Feng X, Cao X. Transcriptional mechanisms of bone morphogenetic protein-induced osteoprotegrin gene expression. J Biol Chem. 2001; 276:10119–10125. [PubMed: 11139569]
11. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, et al. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002; 417:182–187. [PubMed: 12000961]
12. Rodriguez-Manzanet R, Sanjuan MA, Wu HY, Quintana FJ, Xiao S, et al. T and B cell hyperactivity and autoimmunity associated with niche-specific defects in apoptotic body clearance in TIM-4-deficient mice. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:8706–8711. [PubMed: 20368430]
13. Liu G, Friggeri A, Yang Y, Park YJ, Tsuruta Y, et al. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. Proc Natl Acad Sci U S A. 2009; 106:15819–15824. [PubMed: 19721002]
14. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, et al. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. The Journal of experimental medicine. 2010; 207:1589–1597. [PubMed: 20643828]
15. Hanayama R, Miyasaka K, Nakaya M, Nagata S. MFG-E8-dependent clearance of apoptotic cells, and autoimmunity caused by its failure. Curr Dir Autoimmun. 2006; 9:162–172. [PubMed: 16394660]
16. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends Cell Biol. 2006; 16:189–197. [PubMed: 16529932]
17. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005; 123:321–334. [PubMed: 16239148]
18. Yoon HJ, Jeon SB, Kim IH, Park EJ. Regulation of TLR2 expression by prostaglandins in brain glia. J Immunol. 2008; 180:8400–8409. [PubMed: 18523308]
19. Perry AK, Chen G, Zheng D, Tang H, Cheng G. The host type I interferon response to viral and bacterial infections. Cell Res. 2005; 15:407–422. [PubMed: 15987599]
20. King RG, Herrin BR, Justement LB. Trem-like transcript 2 is expressed on cells of the myeloid/granuloid and B lymphoid lineage and is up-regulated in response to inflammation. J Immunol. 2006; 176:6012–6021. [PubMed: 16670310]
21. Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, et al. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. Cell Death Differ. 2008; 15:192–201. [PubMed: 17962816]
22. Munoz LE, Frey B, Pausch F, Baum W, Mueller RB, et al. The role of annexin A5 in the modulation of the immune response against dying and dead cells. Current medicinal chemistry. 2007; 14:271–277. [PubMed: 17305532]
23. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol. 2007; 7:964–974. [PubMed: 18037898]
24. Baumann I, Kolowos W, Voll RE, Manger B, Gaipl U, et al. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis Rheum. 2002; 46:191–201. [PubMed: 11817590]
25. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science. 2004; 304:1147–1150. [PubMed: 15155946]
26. Tas SW, Quartier P, Botto M, Fossati-Jimack L. Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells. Ann Rheum Dis. 2006; 65:216–221. [PubMed: 16014673]

27. Fujimoto I, Pan J, Takizawa T, Nakanishi Y. Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages. J Virol. 2000; 74:3399–3403. [PubMed: 10708457]

28. Hiscott J. Triggering the innate antiviral response through IRF-3 activation. J Biol Chem. 2007; 282:15325–15329. [PubMed: 17395583]

29. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med. 1999; 160:S5–11. [PubMed: 10556161]

30. Hashiguchi M, Kobori H, Ritprajak P, Kamimura Y, Kozono H, et al. Triggering receptor expressed on myeloid cell-like transcript 2 (TLT-2) is a counter-receptor for B7-H3 and enhances T cell responses. Proc Natl Acad Sci U S A. 2008; 105:10495–10500. [PubMed: 18650384]

31. Gibot S, Kolopp-Sarda MN, Bene MC, Bollaert PE, Lozniewski A, et al. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. The Journal of experimental medicine. 2004; 200:1419–1426. [PubMed: 15557347]

32. Piccio L, Buonsanti C, Cella M, Tassi I, Schmidt RE, et al. Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. Brain. 2008; 131:3081–3091. [PubMed: 18790823]

33. Jung YS, Kim SW, Yoon JY, Lee JH, Jeon SM, et al. Expression of a soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) correlates with clinical disease activity in intestinal Behcet's disease. Inflamm Bowel Dis. 2011; 17:2130–2137. [PubMed: 21910175]

34. Notley CA, Brown MA, Wright GP, Ehrenstein MR. Natural IgM is required for suppression of inflammatory arthritis by apoptotic cells. J Immunol. 2011; 186:4967–4972. [PubMed: 21383247]

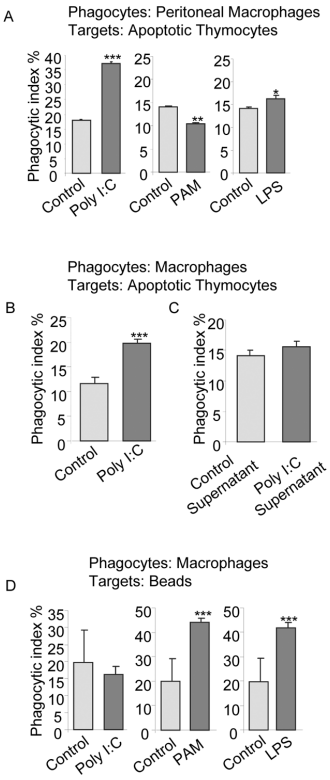

Fig. 1. Activation of TLRs differentially regulates the ability of macrophages to engulf apoptotic cells

(A) Peritoneal macrophages were stimulated overnight with medium (Control), 2μg/ml Poly I:C, 1μg/ml PAM or 10 ng/ml LPS. Culture supernatants were then removed and cells incubated with PKH26 labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed as described in Materials and Methods. *p<0.05; **p<0.01 and ***p<0.001 compared to the control group. Representative results of at least three independent experiments are shown. (B) Macrophages were stimulated overnight with medium (Control) or 2μg/ml Poly I:C. Culture supernatants were then removed. Macrophages were incubated with PKH26 labeled apoptotic thymocytes for 30 min and phagocytosis assays performed. ***p< 0.001 compared to the control group. Representative results of at least three independent experiments are shown. (C) Macrophages were incubated with PKH26 labeled apoptotic thymocytes for 30 min, in the presence of culture supernatants collected from macrophages treated overnight with medium or Poly I:C. Representative results of at least three independent experiments are shown. (D) Macrophages were stimulated overnight with medium (Control), 2μg/ml Poly I:C, 1μg/ml PAM or 10 ng/ml LPS. Supernatants were then removed and cells incubated with carboxylate-modified beads for 30 min. Phagocytosis assays were performed. ***p<0.001 compared to the control group. Representative results of two independent experiments are shown.

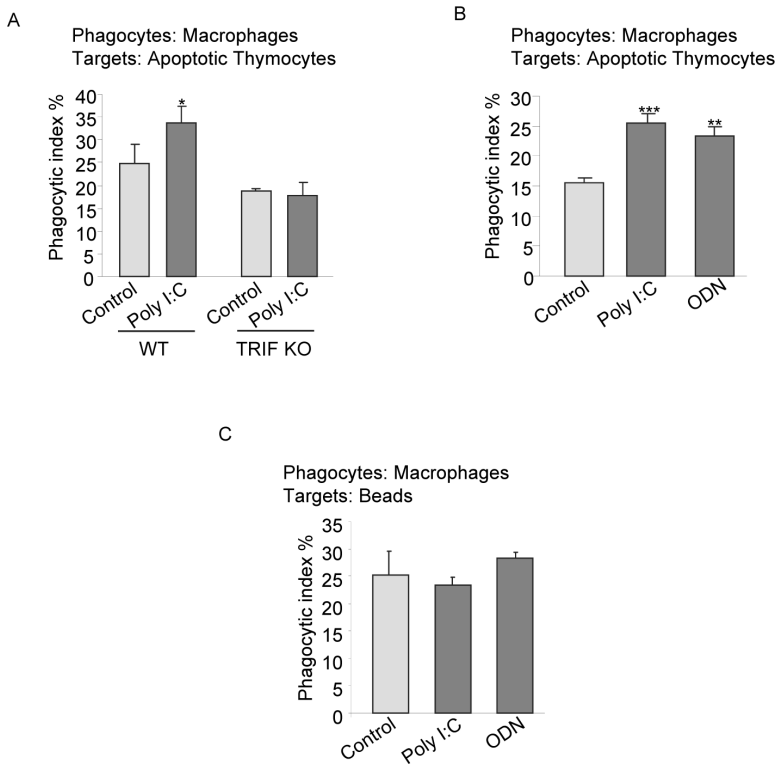

Fig. 2. Enhanced efferocytosis after TLR3 activation requires adaptor TRIF

(A) Macrophages isolated from WT mice and TRIF KO mice were stimulated for 6 h with medium (Control) or 2μg/ml Poly I:C. After stimulations, the supernatants were removed, macrophages incubated with PKH26 labeled apoptotic thymocytes for 30 min, and phagocytosis assays performed. *p< 0.01 compared to WT macrophages in the control group. Representative experiments are shown. Two additional independent experiments provided similar results. (B) Macrophages isolated from WT mice were stimulated overnight with medium (Control), 2μg/ml Poly I:C or 2μM ODN. After the indicated treatments, the supernatants were removed and cells incubated with PKH26 labeled apoptotic thymocytes for 30 min. The uptake of apoptotic cells was evaluated. **p<0.01 and ***p<0.001 compared to the control group. Representative results of at least three independent experiments are shown. (C) Macrophages were stimulated overnight with medium (Control), 2μg/ml Poly I:C, or 2μM ODN. After the treatments, the supernatants were removed, cells incubated with carboxylate-modified beads for 30 min and phagocytosis assays performed. Representative results of at least three independent experiments are shown.

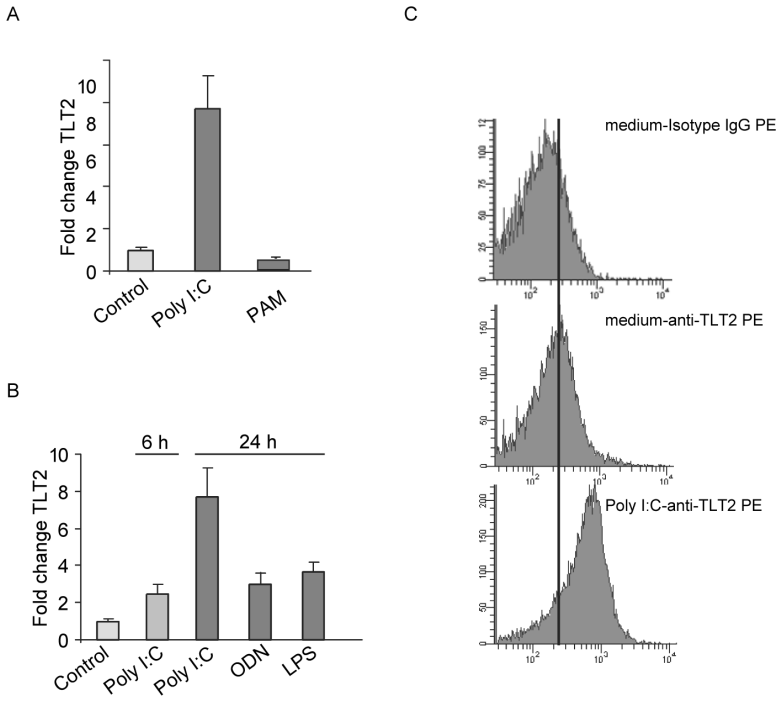

Fig. 3. TLR3 activation upregulates TLT2 expression

(A–B) Peritoneal macrophages were stimulated with medium (Control), 1μg/ml PAM, 10 ng/ml LPS, 2μM ODN or 2μg/ml Poly I:C for 6h or 24h. Levels of TLT2 were determined by real-time PCR assays. Representative experiments are shown. Two additional independent experiments provided similar results. (C) Macrophages were treated overnight with medium (Control) or 2μg/ml Poly I:C. Cells were then incubated with anti-CD16/ CD32 antibodies (2 μg/ml) for 30 min to block FcR on the cell surface, followed by incubation with PE conjugated isotype IgG (Isotype PE) or PE conjugated anti-TLT2 antibody (TLT2 PE). Flow cytometry assays were performed.

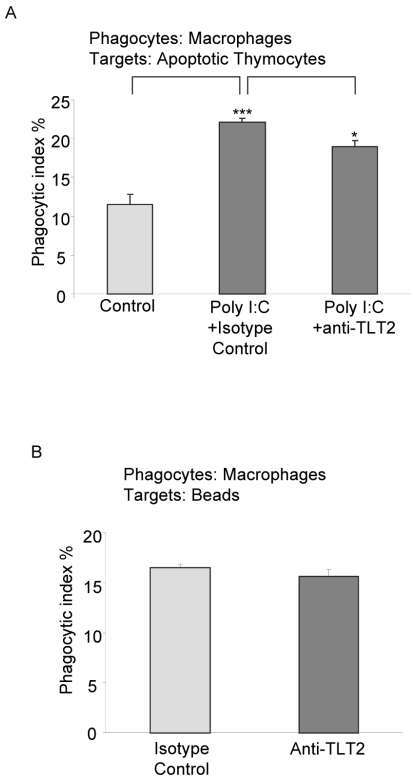

Fig. 4. Anti-TLT2 antibody diminishes the enhanced efferocytosis after TLR3 activation

(A) Macrophages were stimulated overnight with medium (Control) or 2 μg/ml Poly I:C. Culture supernatants were then removed and macrophages treated with anti-CD16/CD32 Abs (2 μg/ml) for 30 min to block FcR on the cell surface. The cells were then washed three times with serum-free medium and pre-incubated with medium (control), 20 μg/ml isotype control or specific anti-TLT2 antibody (clone Ab1H4) for 5 min, followed by incubation with PKH26 labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed. *p<0.05 compared to Poly I:C+BSA group, ***p< 0.001 compared to the control group. Representative experiments are shown. Two additional independent experiments provided similar results. (B) The experiments were performed as in "A", except that the apoptotic thymocytes were replaced with carboxylate-modified beads.

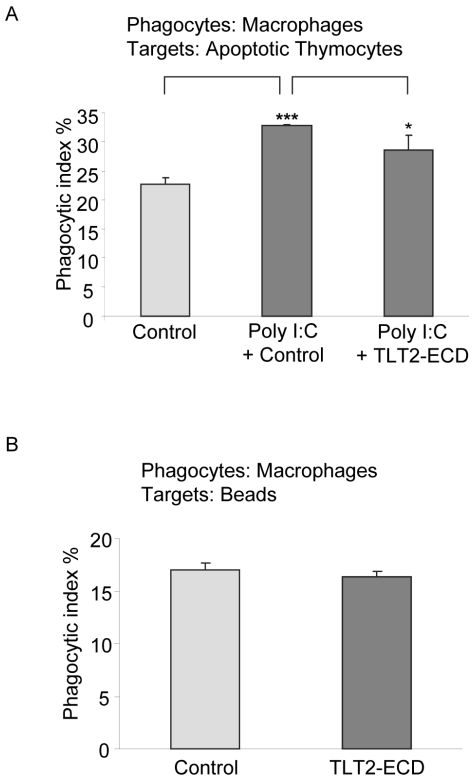

Fig. 5. Soluble TLT2 extracellular domain diminishes the enhanced efferocytosis after TLR3 activation

(A) Macrophages were stimulated overnight with medium (Control) or 2 μg/ml Poly I:C. Culture supernatants were removed and macrophages treated with anti-CD16/CD32 Abs (2 μg/ml) for 30 min to block FcR on the cell surface. The cells were then washed three times with serum-free medium and pre-incubated with medium (control), 4 μg/ml BSA or 4 μg/ml soluble TLT2 extracellular domain (TLT2-ECD) for 5 min, followed by incubation with PKH26 labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed. *p<0.05 compared to the group with macrophages stimulated with Poly I:C and incubated with control fragments (IgG-Fc fragments), ***p< 0.001 compared to the control group. Representative experiments are shown. Two additional independent experiments provided similar results. (B) The experiments were performed as in “A”, except that the apoptotic thymocytes were replaced with carboxylate-modified beads.

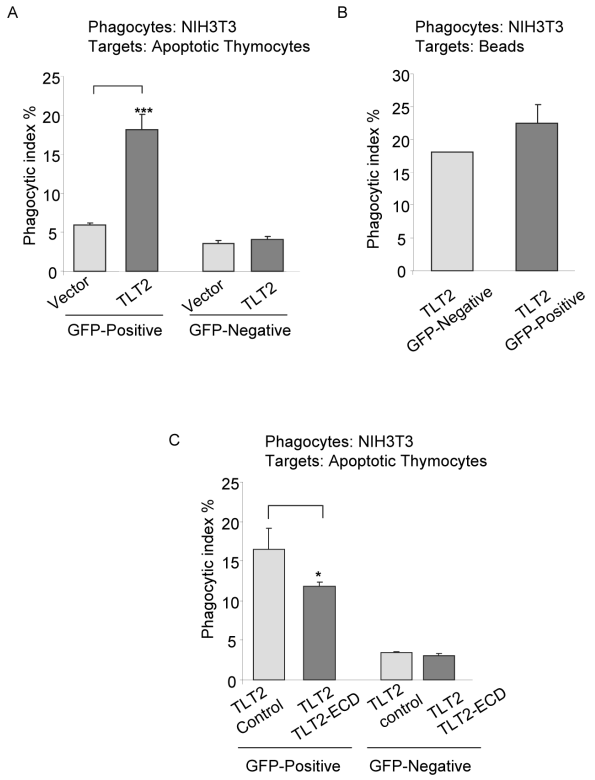

Fig. 6. TLT2 mediates efferocytosis

(A) NIH3T3 cells were transfected with constructs that express GFP alone or GFP-TLT2. 48h after the transfection, apoptotic thymoytes were added to the cells and phagocytosis assays performed. The results are presented as phagocytic indexes for cells with expression of GFP or GFP-TLT2 (GFP positive) and cells with transfection, but without either GFP or GFP-TLT2 expression (GFP negative). ***$p<0.001$ compared to the group with cells expressing GFP alone. Representative experiments are shown. Two additional independent experiments provided similar results (B) The experiments were performed as in "A", except that apoptotic thymocytes were replaced with carboxylate-modified beads. (C) NIH3T3 cells were transfected as in A. Cells were then washed and preincubated with 4 μg/ml control fragments or 4 μg/ml soluble TLT2 extracellular domain (TLT2-ECD) for 5 min, followed by incubation with PKH26 labeled apoptotic thymocytes for 120 min. Phagocytosis assays were performed. *$p<0.05$ compared to the group with cells expressing GFP-TLT2 that were preincubated with control fragments.

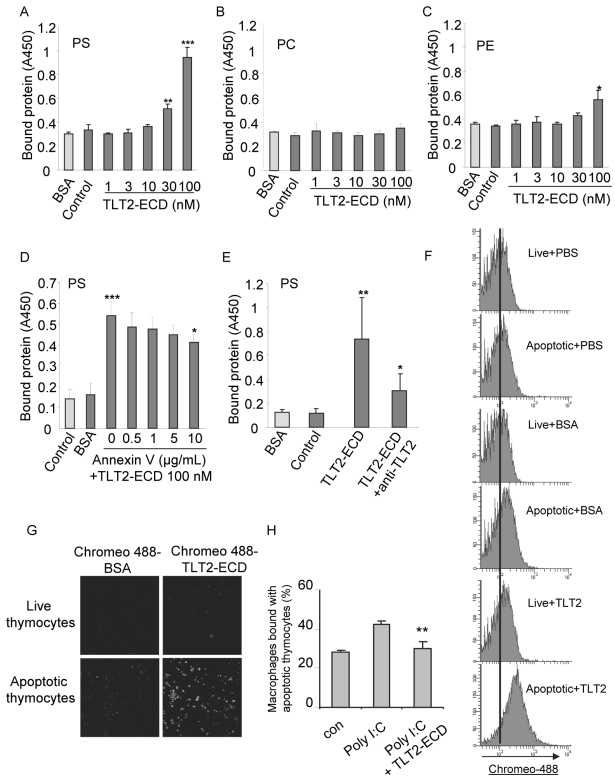

Fig. 7. TLT2 binds to phosphatidylserine

(A–C) 96-well plates were incubated with PS (A), PC (B) or PE (C). The plates were then washed three times with PBS, followed by blocking with PBS containing 1% BSA for 1 h. After removing the blocking solution, the plates were incubated with 100 nM BSA, 100 nM recombinant IgG1-Fc (control) or increasing concentrations of recombinant TLT2-ECD-Fc dissolved in PBS (1, 3,10, 30 or 100 nM) for 60 min. Recombinant proteins not bound to the plates were removed by washing and bound proteins quantified by ELISA as described in Materials and Methods. $^{**}p< 0.01$, $^{***}p<0.001$ compared to the Control group. A450, absorbance at 450 nm. Representative experiments are shown. Two additional independent experiments provided similar results. (D) PS coated plates were incubated with increasing doses of annexin V (0, 0.5, 1, 5, or 10 μg/ml) for 60 min. The plates were then washed three times with PBS and then incubated with 100 nM BSA, 100 nM recombinant IgG1-Fc (control) or 100 nM recombinant TLT2-ECD-Fc for 60 min. Unbound proteins were removed and bound proteins quantified by ELISA. $^{***}p<0.001$ compared to the IgG1-Fc group; $^{*}p<0.05$ compared to the group pretreated without annexin V. Representative experiments are shown. Another independent experiment provided similar results. (E) PS coated plates were blocked with BSA and then incubated for 1h with 100 nM BSA, 100 nM recombinant IgG1-Fc (control), 100 nM recombinant TLT2-ECD-Fc, or 100 nM recombinant TLT2-ECD-Fc that was preincubated with specific antibody against the extracellular domain of TLT2 (clone Ab1H4, 20 μg/ml) for 2 hours. Bound proteins were quantified by ELISA. $^{**}p< 0.01$ compared to the IgG1-Fc group; $^{*}p< 0.05$ compared to the TLT2-Fc group without preincubation with anti-TLT2 antibody. Representative experiments are shown. Another independent experiment provided similar results. (F) Live and apoptotic thymocytes were incubated with PBS, 5 μg/ml Chromeo-488 labeled BSA or 5 μg/ml Chromeo-488 labeled TLT2-ECD for 1h. Cells were then washed extensively with PBS and flow cytometry performed. Original color images were converted into grayscale, with the intensity of brightness representing the intensity of original green fluorescence. Representative experiments are shown. Another independent experiment provided similar

results. (G) Live and apoptotic thymocytes were incubated with 5 μg/ml Chromeo-488 labeled BSA or 5 μg/ml Chromeo-488 labeled TLT2-ECD for 1h. Cells were then washed extensively with PBS. Cytospin slides were prepared and con-focal microscopy performed. Representative experiments are shown. Another independent experiment provided similar results. (H) TLT2 inhibits the binding of apoptotic thymocytes to macrophages. Macrophages were treated without or with 2 μg/ml poly I:C overnight. Cells were then washed with PBS and pretreated with vehicle or 1 μg/ml cytochalasin D for 1h. PKH26-labeled apoptotic thymocytes resuspended in medium containing 4 μg/ml BSA or TLT2-ECD were added into the macrophages. 30 minutes after the addition of the thymocytes, the media were removed and macrophages were collected for flow cytometry analysis.

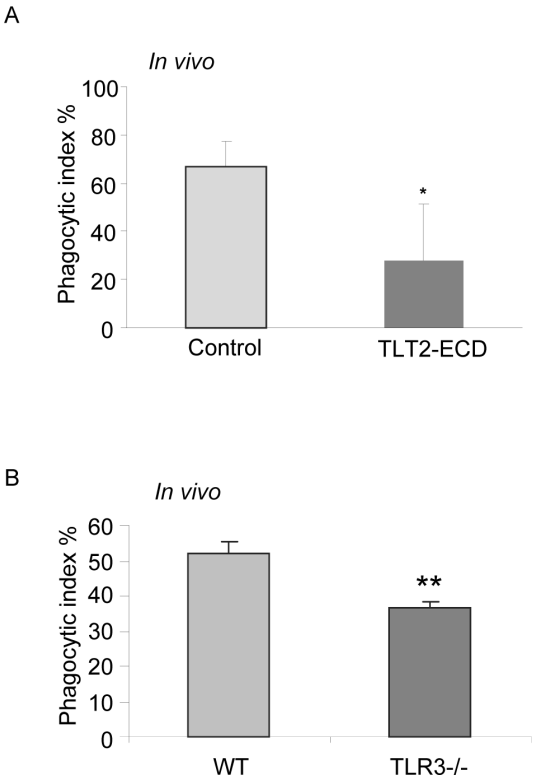

Fig. 8. TLT-2 mediates efferocytosis *in vivo*

(A) PKH26-labeled apoptotic thymocytes (1×10^7) were incubated with 4 μg/ml recombinant IgG1-Fc (Control) or 4 μg/ml recombinant TLT2-ECD-Fc for 2 hours. The apoptotic thymocytes were then administered i.p. to mice. Mice were sacrificed 2 hours after the injection and peritoneal lavages performed. Phagocytic indexes were determined as described in Materials and Methods. * $p<0.05$ compared to the group with mice injected with apoptotic thymocytes that were preincubated with recombinant IgG1-Fc. Data are representative of two independent experiments. (B) B6129SF2 (WT control) or TLR3−/− mice were injected peritoneally 200 μg Poly I:C. 24h after the injection, PKH26-labeled apoptotic thymocytes (10×10^6) were administered i.p. to mice. Mice were sacrificed 2 hours after the injection and peritoneal lavages performed. Phagocytic indexes were determined. ** $p<0.01$ compared to the WT group.