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Abraham and Gang Liu

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Identification of TLT2 as an Engulfment Receptor for Apoptotic Cells

Andressa de Freitas,* Sami Banerjee,* Na Xie,* Huachun Cui,* Kasey I. Davis,[†] Arnaud Friggeri,^{‡,§} Mingui Fu,[¶] Edward Abraham,^{||} and Gang Liu*

Clearance of apoptotic cells (efferocytosis) is critical to the homeostasis of the immune system by restraining inflammation and autoimmune response to intracellular Ags released from dying cells. TLRs-mediated innate immunity plays an important role in pathogen clearance and in regulation of the 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 the TREM receptor family, on the surface of macrophages. Blocking TLT2 on the macrophage surface by either specific anti-TLT2 Ab 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, 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. *The Journal of Immunology*, 2012, 188: 6381–6388.

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 Ags released from dying cells (1). Redistribution of phosphatidylserine (PS) to the outer 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 recently been identified to recognize PS, and thereby mediating efferocytosis. Deficiencies in these receptors led to aggravation of inflammation and autoimmune diseases (2–5).

Innate immunity is critical to pathogen clearance and is also involved in the activation and regulation of the adaptive immune

response (6). Sensing of pathogens and endogenous danger signals is achieved through pattern recognition receptors, such as TLRs (6). TLRs recognize microbial components, such as LPS, lipopeptides, and nucleic acids, and therefore initiate specific signaling pathways that lead to distinct immune responses (6).

In the current study, we examined the effects of TLR activation on efferocytosis. Our data demonstrated that activation of macrophages with polyinosinic-polycytidylic acid (poly I:C; TLR3 ligand) or CpG oligodeoxynucleotides (ODN; TLR9 ligand), but not with Pam3CysSK4 (PAM; TLR2 ligand), enhances ingestion of apoptotic cells. Using gene microarray assays, we found that triggering receptor expressed on myeloid cells (TREM)-like protein 2 (TLT2, or TREML2) is upregulated after TLR3 activation. TLT2 is a member of the 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.

Materials and Methods

Mice

Female C57BL/6 mice (wild type; WT) were purchased from the National Cancer Institute-Frederick. Female TLR3-deficient mice, B6129SF2 (WT control for TLR3-deficient mice), and TIR domain-containing adapter inducing IFN- β (TRIF)-deficient mice (TRIF knockout) were purchased from The Jackson Laboratory. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Experiments were performed using 8- to 10-wk-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. PE-conjugated anti-mouse TLT2 Ab 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

*Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294; [†]Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294; [‡]Pole Anesthésie Réanimation, Centre Hospitalier Universitaire, Amiens 80054, France; [§]INSERM U-1088, Amiens 80054, France; [¶]University of Missouri-Kansas City, Kansas City, MO 64108; and ^{||}Wake Forest School of Medicine, Winston-Salem, NC 27157

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Address correspondence and reprint requests to Dr. Gang Liu or Dr. Edward Abraham, Department of Medicine, University of Alabama at Birmingham, 901 19th Street So., BMR II 233, Birmingham, AL 35294 (G.L.) or Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157 (E.A.). E-mail addresses: gliu@uab.edu (G.L.) and eabraham@wakehealth.edu (E.A.)

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; IRF, IFN regulatory factor; KO, knockout; ODN, oligodeoxynucleotide; PAM, Pam3-CysSK4; PC, phosphatidylcholine; poly I:C, polyinosinic-polycytidylic acid; PS, phosphatidylserine; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TLT2, TREM-like protein 2; TLT2-ECD, TLT2 extracellular domain; TREM, triggering receptor expressed on myeloid cells; TRIF, TIR domain-containing adapter inducing IFN- β ; WT, wild type.

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were from R&D Systems. The Ab against the extracellular portion of TLT2, clone Ab1H4, was a gift from Dr. Louis Justement (University of Alabama at Birmingham). Red fluorescent carboxylate-modified microspheres and pHrodo succinimidyl ester were from Invitrogen. Anti-CD16/CD32 Abs, FITC-conjugated anti-CD11b, and allophycocyanin-conjugated anti-CD90.2 Abs were from BD Pharmingen. PS, phosphatidylcholine (PC), and PE were from Avanti Polar Lipids. Annexin V was from BD Biosciences. PAM, LPS, CpG ODN, and poly I:C were from Invivogen. Chromeo-488 Ab labeling kit was from Active Motif.

Culture of mouse peritoneal macrophages

Peritoneal macrophages were elicited from WT and TRIF knockout (KO) mice by i.p. injection of 1 ml sterile 4% Brewer thioglycollate. Cells were harvested 4 d 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 h at 37°C, nonadherent cells were removed by washing with medium.

Culture of bone marrow-derived macrophages

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

Induction of apoptosis in thymocytes

Mouse thymocytes were labeled with PKH26 red fluorescent dye according to the manufacturer's instructions. To induce apoptosis, thymocytes were resuspended in RPMI 1640 medium containing 10% FBS and 1 μ M dexamethasone at a concentration of 6×10^6 cells/ml and incubated at 37°C in 5% CO₂ 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 μ g/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 h. 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 μ l 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 five times with cold PBS, resuspended in PBS containing 1% albumin, FITC-conjugated anti-CD11b (macrophage marker) Ab, and allophycocyanin-conjugated anti-CD90.2 Ab (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⁺allophycocyanin⁺ cells to all cells gated. Engulfed thymocytes are not accessible to the allophycocyanin-conjugated anti-CD90.2 Ab. Therefore, FITC⁺PKH26⁺allophycocyanin⁺ cells are macrophages that have engulfed PKH-labeled thymocytes, whereas the allophycocyanin⁺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 μ l beads suspended in 500 μ l RPMI 1640 medium containing 10% FBS, followed by incubation at 37°C for 30 min. After incubation, 500 μ l 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 five times with cold PBS and resuspended in PBS containing 1% albumin and FITC-conjugated anti-CD11b (macrophage marker) Ab. 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 μ g/ml recombinant IgG1-Fc or 4 μ g/ml recombinant TLT2-Fc for 2 h at

room temperature with rotation and then injected i.p. to mice. Mice were sacrificed 2 h 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) Ab, and allophycocyanin-conjugated anti-CD90.2 (thymocyte marker) Ab. Phagocytosis was evaluated by flow cytometry. To evaluate the role of TLR3 in efferocytosis in vivo, B6129SF2 or TLR3^{-/-} mice were injected i.p. with 200 μ g poly I:C. Twenty-four hours after the injection, PKH26-labeled apoptotic thymocytes (10×10^6) were administered i.p. to mice. Mice were sacrificed 2 h 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 pGFP-C3 vector (Clontech).

Real-time PCR

The assay was performed as previously described (13, 14). Primer sequences were as follows: mouse GAPDH: sense 5'-CGACTTCAACAGCAACTCC-CACTCTTCC-3', antisense 5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3'; mouse TLT2: sense 5'-TGGTGGTGGTGTGACATTCTTCC-3', antisense 5'-ATCCAGGGTTTAGCATAGTGTCTGC-3'.

Flow cytometry

To determine levels of TLT2 on the surface of macrophages, cells were pretreated with anti-CD16/CD32 Abs (2 μ g/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 Ab (TLT2-PE) for 30 min, followed by washing and analysis by flow cytometry.

Fluorescent labeling of BSA and TLT2 extracellular domain

BSA and TLT2 extracellular domain (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 \pm 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 was measured. As shown in Fig. 1A, poly I:C-treated macrophages demonstrated significantly enhanced ability to engulf apoptotic thymocytes compared with 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 that by TLR3 activation. 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 of other macrophage populations, we performed in BMDMs the same treatments described above. As shown in Supplemental Fig. 1 and similar to effects observed in peritoneal macrophages, activation of TLR3 and TLR4, but not TLR2, enhanced the ability of BMDMs to engulf apoptotic thymocytes.

Efferocytosis is a multistep 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 MFG-E8 and GAS6, can serve as "bridging molecules" to facilitate the

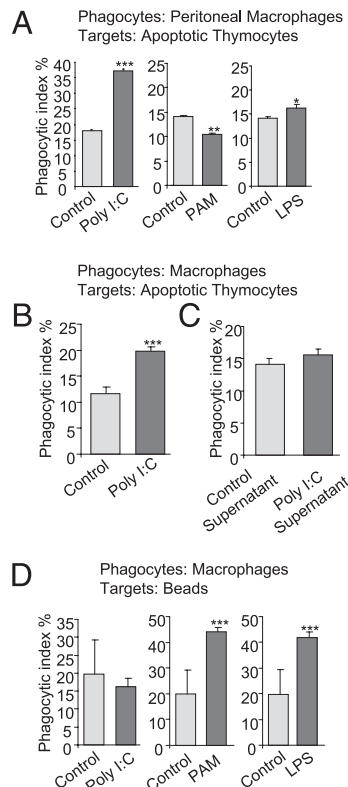


FIGURE 1. Activation of TLRs differentially regulates the ability of macrophages to engulf apoptotic cells. **(A)** Peritoneal macrophages were stimulated overnight with medium (control), 2 $\mu\text{g}/\text{ml}$ poly I:C, 1 $\mu\text{g}/\text{ml}$ PAM, or 10 ng/ml LPS. Culture supernatants were then removed and cells incubated with PKH26-labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed as described in *Materials and Methods*. Representative results of at least three independent experiments are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (compared with the control group). **(B)** Macrophages were stimulated overnight with medium (control) or 2 $\mu\text{g}/\text{ml}$ poly I:C. Culture supernatants were then removed. Macrophages were incubated with PKH26-labeled apoptotic thymocytes for 30 min and phagocytosis assays performed. Representative results of at least three independent experiments are shown. $***p < 0.001$ (compared with the control group). **(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), 2 $\mu\text{g}/\text{ml}$ poly I:C, 1 $\mu\text{g}/\text{ml}$ PAM, or 10 ng/ml LPS. Supernatants were then removed and cells incubated with carboxylate-modified beads for 30 min. Phagocytosis assays were performed. Representative results of two independent experiments are shown. $***p < 0.001$ (compared with the control group).

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 three 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 compared with 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 adapter TRIF

Engagement of TLR3 by poly I:C recruits the adapter protein TRIF to the receptor. This event subsequently leads to the activation of IFN regulatory factor (IRF)3 and type I IFN response (6, 19).

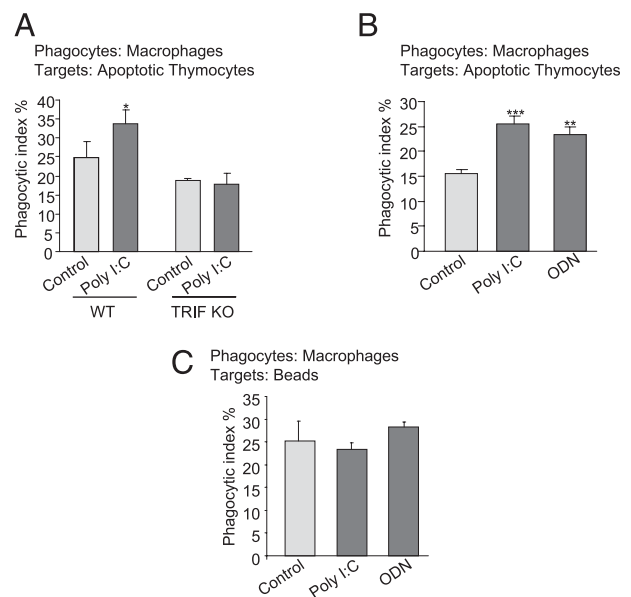


FIGURE 2. Enhanced efferocytosis after TLR3 activation requires adapter TRIF. **(A)** Macrophages isolated from WT mice and TRIF KO mice were stimulated for 6 h with medium (control) or 2 $\mu\text{g}/\text{ml}$ poly I:C. After stimulations, the supernatants were removed, macrophages incubated with PKH26-labeled apoptotic thymocytes for 30 min, and phagocytosis assays performed. Representative experiments are shown. Two additional independent experiments provided similar results. $*p < 0.01$ (compared with WT macrophages in the control group). **(B)** Macrophages isolated from WT mice were stimulated overnight with medium (control), 2 $\mu\text{g}/\text{ml}$ poly I:C, or 2 μM 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. Representative results of at least three independent experiments are shown. $**p < 0.01$, $***p < 0.001$ (compared with the control group). **(C)** Macrophages were stimulated overnight with medium (control), 2 $\mu\text{g}/\text{ml}$ poly I:C, or 2 μM 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.

Sensing of viral dsRNAs by TLR3 and the resulting type I IFN response are primary host defenses to viral infection (6). To elucidate the mechanism by which TLR3 activation specifically enhances efferocytosis, WT and 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, IRF7, 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 ODN, a specific synthetic ligand of TLR9. As shown in Fig. 2B and 2C and similar to effects 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 but downregulated or not altered by PAM stimulation. As shown in Supplemental Table I, a number of genes were increased in poly I:C-treated macrophages but decreased in PAM-treated macrophages. The complete gene array data have been deposited in the Gene Expression Omnibus database (accession number GSE36891; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36891>). Genes of particular interest to us were cellular receptors, including

TLT2, which belongs to the TREM receptor family involved in inflammatory regulation (20).

To validate the microarray findings, we performed real-time PCR assays and found that TLT2 is significantly upregulated in poly I:C-treated macrophages but 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 characterize further TLT2 expression on macrophages with TLR3 activation, we performed flow cytometry assays using specific anti-TLT2 Ab and found that TLT2 expression on the surface of poly I:C-treated macrophages is 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 Abs. As shown in Fig. 4A, pretreatment with anti-TLT2 Abs 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 Abs has no effect on macrophages to ingest beads, consistent with the

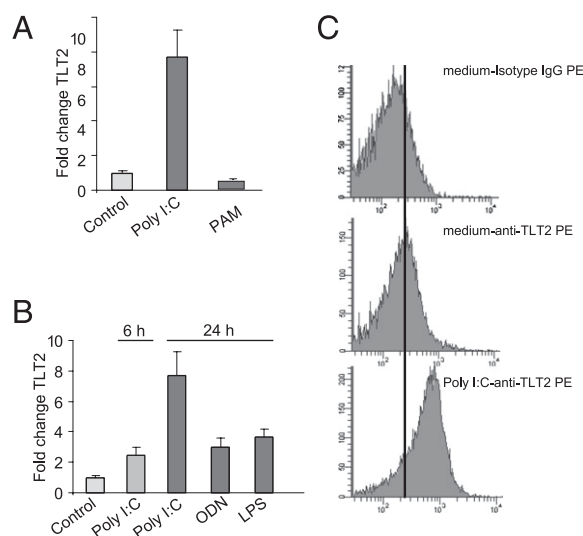


FIGURE 3. TLR3 activation upregulates TLT2 expression. **(A and B)** Peritoneal macrophages were stimulated with medium (control), 1 μ g/ml PAM, 10 ng/ml LPS, 2 μ M ODN, or 2 μ g/ml poly I:C for 6 or 24 h. Levels of TLT2 were determined by real-time PCR assays. Representative experiments are shown. Two additional independent experiments provided similar results. **(C)** Macrophages were treated overnight with medium (control) or 2 μ g/ml poly I:C. Cells were then incubated with anti-CD16/CD32 Abs (2 μ g/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 Ab (TLT2 PE). Flow cytometry assays were performed.

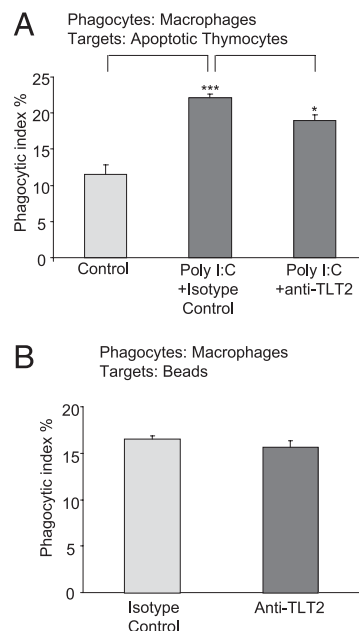


FIGURE 4. Anti-TLT2 Ab diminishes the enhanced efferocytosis after TLR3 activation. **(A)** Macrophages were stimulated overnight with medium (control) or 2 μ g/ml poly I:C. Culture supernatants were then removed and macrophages treated with anti-CD16/CD32 Abs (2 μ g/ml) for 30 min to block FcR on the cell surface. The cells were then washed three times with serum-free medium and preincubated with medium (control), 20 μ g/ml isotype control, or specific anti-TLT2 Ab (clone Ab1H4) for 5 min, followed by incubation with PKH26-labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed. Representative experiments are shown. Two additional independent experiments provided similar results. * p < 0.05 (compared with poly I:C + isotype control group), *** p < 0.001 (compared with the control group). **(B)** The experiments were performed as in (A), except that the apoptotic thymocytes were replaced with carboxylate-modified beads.

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-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 mediates 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 Supplemental 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 (Supplemental 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 showed that blocking of TLT2 with either specific anti-TLT2 Ab or soluble TLT2 extracellular fragments attenuates enhanced efferocytosis found with poly I:C-stimulated macrophages, and 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 compared with 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 of 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

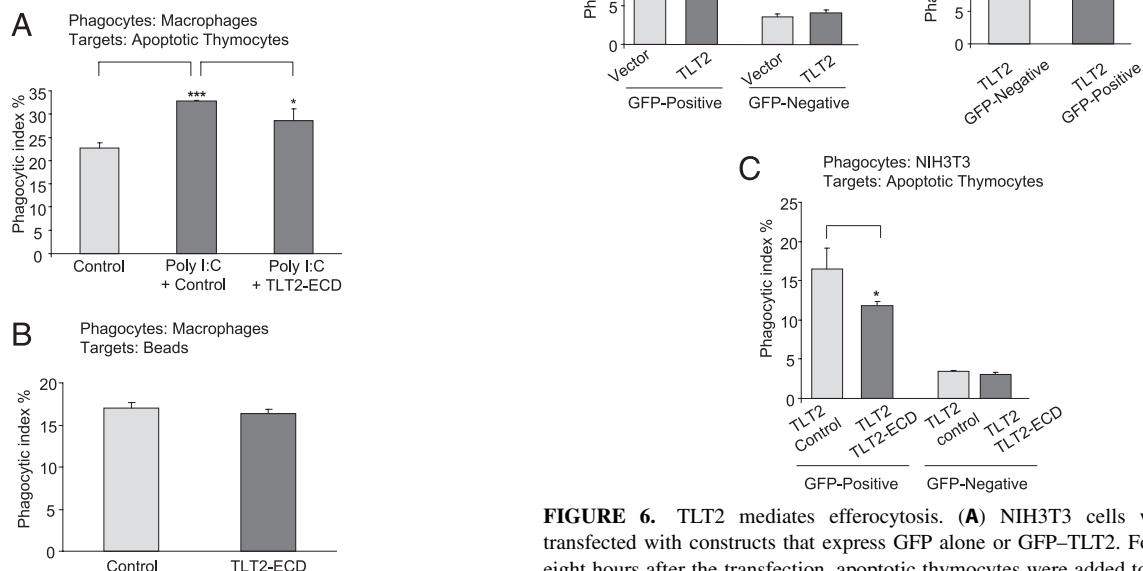


FIGURE 5. Soluble TLT2-ECD diminishes the enhanced efferocytosis after TLR3 activation. **(A)** Macrophages were stimulated overnight with medium (control) or 2 μ g/ml poly I:C. Culture supernatants were removed and macrophages treated with anti-CD16/CD32 Abs (2 μ g/ml) for 30 min to block FcR on the cell surface. The cells were then washed three times with serum-free medium and preincubated with medium (control), 4 μ g/ml BSA, or 4 μ g/ml soluble TLT2-ECD for 5 min, followed by incubation with PKH26-labeled apoptotic thymocytes for 30 min. Phagocytosis assays were performed. Representative experiments are shown. Two additional independent experiments provided similar results. * $p < 0.05$ (compared with the group with macrophages stimulated with poly I:C and incubated with BSA), *** $p < 0.001$ (compared with the control group). **(B)** The experiments were performed as in (A), except that the apoptotic thymocytes were replaced with carboxylate-modified beads.

FIGURE 6. TLT2 mediates efferocytosis. **(A)** NIH3T3 cells were transfected with constructs that express GFP alone or GFP-TLT2. Forty-eight hours after the transfection, apoptotic thymocytes 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). Representative experiments are shown. Two additional independent experiments provided similar results. *** $p < 0.001$ (compared with the group with cells expressing GFP alone). **(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 μ g/ml control fragments or 4 μ g/ml soluble TLT2-ECD for 5 min, followed by incubation with PKH26-labeled apoptotic thymocytes for 120 min. Phagocytosis assays were performed. * $p < 0.05$ (compared with the group with cells expressing GFP-TLT2 that were preincubated with control fragments).

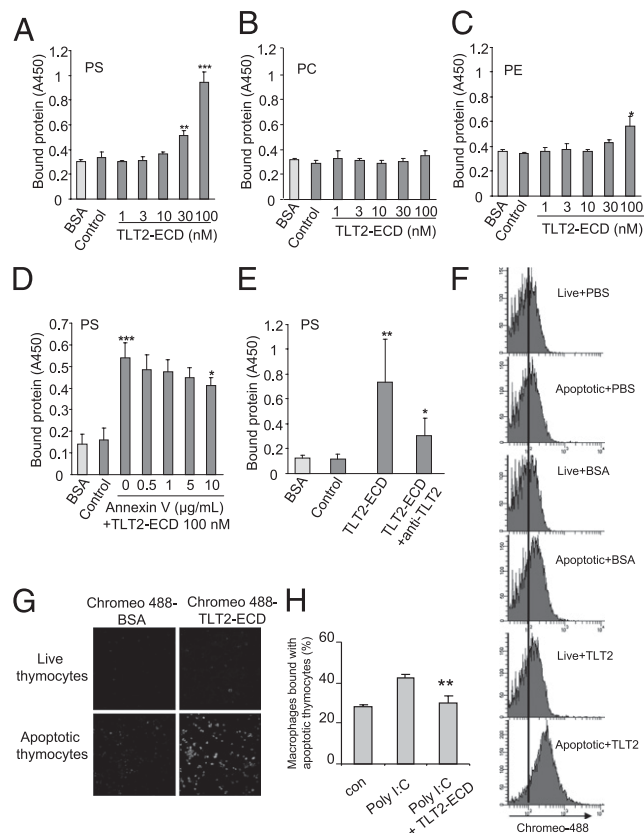


FIGURE 7. TLT2 binds to PS. **(A–C)** Ninety-six–well plates were incubated with PS (A), PC (B), or PE (C). The plates were then washed three times with PBS, followed by blocking with PBS containing 1% BSA for 1 h. After removing the blocking solution, the plates were incubated with 100 nM BSA, 100 nM 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*. Representative experiments are shown. Two additional independent experiments provided similar results. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (compared with the control group). A450, absorbance at 450 nm. **(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. Representative experiments are shown. Another independent experiment provided similar results. $*p < 0.05$ (compared with the group pretreated without annexin V), $***p < 0.001$ (compared with the IgG1-Fc group). **(E)** PS-coated plates were blocked with BSA and then incubated for 1 h 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 Ab against the extracellular domain of TLT2 (clone Ab1H4, 20 μg/ml) for 2 h. Bound proteins were quantified by ELISA. Representative experiments are shown. Another independent experiment provided similar results. $*p < 0.05$ (compared with the TLT2-Fc group without preincubation with anti-TLT2 Ab), $**p < 0.01$ (compared with the IgG1-Fc group). **(F)** Live and apoptotic thymocytes were incubated with PBS, 5 μg/ml Chromo-488–labeled BSA, or 5 μg/ml Chromo-488–labeled TLT2-ECD for 1 h. 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 Chromo-488–labeled BSA or 5 μg/ml Chromo-488–labeled TLT2-ECD for 1 h. Cells were then washed extensively with PBS. Cytospin slides were prepared and confocal micros-

copy 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 1 h. PKH26-labeled apoptotic thymocytes resuspended in medium containing 4 μg/ml BSA or TLT2-ECD were added into the macrophages. Thirty minutes after the addition of the thymocytes, the media were removed, and macrophages were collected for flow cytometry analysis. $**p < 0.01$ compared to poly I:C group.

TLT2 binds to PS

Phagocytes directly recognize PS through receptors such as BAI1 and RAGE or indirectly through soluble bridging molecules that bind to both PS and specific phagocyte receptors (2–4, 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–C), 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 and PS was inhibited by specific anti-TLT2 Ab (Fig. 7E). To determine if TLT2 extracellular fragments specifically bind to apoptotic cell, we incubated live and apoptotic thymocytes with Chromo-488–labeled TLT2-ECD and found that TLT2-ECD demonstrates much greater binding to apoptotic cells than to live cells (Fig. 7F). Using confocal 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, 7G). 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 Ab. We next investigated if TLT2 is involved in binding of apoptotic cells to macrophages. We pretreated macrophages with cytochalasin D, which 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, which 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 (Supplemental Fig. 3), suggesting that cell membrane-bound TLT2–GFP is internalized together with the apoptotic cells and becomes a component of phagosomes.

TLT2 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

copy 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 1 h. PKH26-labeled apoptotic thymocytes resuspended in medium containing 4 μg/ml BSA or TLT2-ECD were added into the macrophages. Thirty minutes after the addition of the thymocytes, the media were removed, and macrophages were collected for flow cytometry analysis. $**p < 0.01$ compared to poly I:C group.

performed for *in vitro* efferocytosis assays. The PKH-labeled apoptotic thymocytes were then administered *i.p.* to mice. Two hours 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 compared with 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 compared with that of poly I:C-treated WT mice (Fig. 8B). These data suggest that an increase in efferocytosis in response to TLR3 activation, such as that 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, as this process prevents release of irritating intracellular contents from broken apoptotic cells (23). In case of noninfected inflammation, clearance of apoptotic cells prevents autoimmune response to nucleic acids and other cellular components from apoptotic cells (24–26). Deficiency in clearance of apoptotic cells has 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 pattern recognition receptors 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). In this study, 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 evolutionarily conserved self-protection 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 the 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 the plasma membrane of both live and apoptotic cells. Thus, TLT2 joins a small family of PS receptors, such as BAI1, RAGE, stabilin-1, and Tim-4, all of which have been shown directly to mediate clearance of apoptotic cells (2–4, 21). Identification of 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-ECD 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 worth pointing out that the binding between TLT2-ECD and PS is attenuated by specific anti-TLT2 Ab. These data are consistent with the finding that anti-TLT2 Ab diminishes the ability of macrophages to engulf apoptotic cells. Furthermore, TLT2-ECD 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 whether B7-H3 is involved in the TLT2-mediated, PS-dependent engulfment of apoptotic cells.

We found that the soluble TLT2-ECD inhibits ingestion of apoptotic cells, likely through competing with TLT2 receptors on the surface of macrophages to bind to apoptotic cells. There is

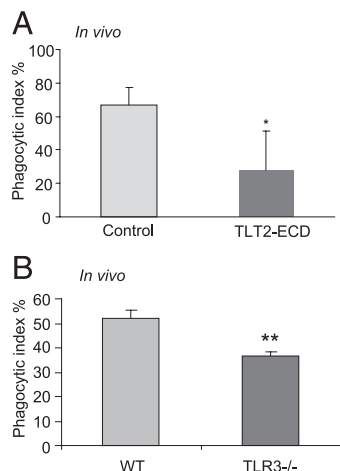


FIGURE 8. TLT2 mediates efferocytosis *in vivo*. **(A)** PKH26-labeled apoptotic thymocytes (1×10^7) were incubated with 4 $\mu\text{g/ml}$ recombinant IgG1-Fc (control) or 4 $\mu\text{g/ml}$ recombinant TLT2-ECD-Fc for 2 h. The apoptotic thymocytes were then administered *i.p.* to mice. Mice were sacrificed 2 h after the injection and peritoneal lavages performed. Phagocytic indexes were determined as described in *Materials and Methods*. Data are representative of two independent experiments. * $p < 0.05$ (compared with the group with mice injected with apoptotic thymocytes that were preincubated with recombinant IgG1-Fc). **(B)** B6129SF2 (WT control) or TLR3^{-/-} mice were injected peritoneally with 200 μg poly I:C. Twenty-four hours after the injection, PKH26-labeled apoptotic thymocytes (10×10^6) were administered *i.p.* to mice. Mice were sacrificed 2 h after the injection and peritoneal lavages performed. Phagocytic indexes were determined. ** $p < 0.01$ (compared with the WT group).

currently no evidence that TLT2 on the cell surface sheds its extracellular domain to the 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, inflammatory bowel disease, and sepsis (31–33). 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-ECDs 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 Ab or TLT2-ECD 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

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Disclosures

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