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Experimental analysis of apoptotic thymocyte engulfment by macrophages

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TITLE:

Experimental Analysis of Apoptotic Thymocyte Engulfment by Macrophages

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KEYWORDS:

macrophage; apoptotic cells; efferocytosis; immunofluorescent microscopy; flow cytometry; TAM receptor tyrosine kinases

SUMMARY:

Here, we present a protocol to prepare apoptotic thymocytes and peritoneal macrophages and analyze the efficiency of efferocytosis and the specific inhibitor-mediated blocking of apoptotic thymocytes engulfment. This protocol has a broad application in cell-mediated clearance of other particles including artificial beads and bacteria.

ABSTRACT:

Cell apoptosis is a natural process and plays a critical role in embryonic development, homeostatic regulation, immune tolerance induction, and resolution of inflammation. Accumulation of apoptotic debris in the body may trigger chronic inflammatory responses that lead to systemic autoimmune diseases over time. Impaired apoptotic cell clearance has been implicated in a variety of autoimmune diseases. Apoptotic clearance is a complex process rarely detected under physiological conditions. It involves abundant surface receptors and signaling molecules. Studying the process of apoptotic cell clearance provides insightful molecular mechanisms and subsequent biological responses, which may lead to the development of new therapeutics. Here, we describe protocols for the induction of apoptotic thymocytes, the preparation of peritoneal macrophages, and the analysis of apoptotic cell clearance by flow cytometry and microscopy. All cells will undergo apoptosis at a certain stage, and many residential and circulating cells can uptake apoptotic debris. Therefore, the protocol described here can be used in many applications to characterize apoptotic cell binding and ingestion by many other cell types.

INTRODUCTION:

Our body generates 1-10 billion apoptotic cells on a daily basis. Such a large number of apoptotic cells must be cleared in a way that the immune responses remain quiescent. To ensure the clearance of apoptotic cells in a timely manner, numerous types of tissue resident cells and circulating cells develop mechanisms to engulf apoptotic cells[1]. Dysfunctional regulation of apoptosis has been implicated in the onset and progression of various inflammatory disease and autoimmunity[2]. Apoptosis also plays a critical role in the pathogenesis of cancer development and its subsequent resistance to conventional treatments[3,4]. Removal of apoptotic cells generally promotes an anti-inflammatory response, which may be linked to immunological tolerance[5]. Disturbance of apoptotic cell clearance drives self-immunization and contributes to the development of systemic autoimmune diseases in both humans and mice[6].

When cells undergo apoptosis, they expose the phosphatidylserine (PtdSer) from the inner leaflet to the outer leaflet of the membrane. PtdSer will then be recognized by phagocytes through surface receptors. Over a dozen receptors have been identified to recognize and/or facilitate the engulfment of apoptotic cells. In general, there are at least three types of surface receptors involved in the apoptotic cell clearance: tethering receptors, recognize apoptotic cells; tickling receptors, initiate engulfment; chaperoning receptors, facilitate the whole process[7]. TAM receptor tyrosine kinases (TAM RTKs) consist of Tyro-3, Axl, and Mer and are primarily expressed by myeloid cells of the immune system[8]. The primary function of TAM RTKs is to serve as tethering receptors, facilitating the phagocytic removal of apoptotic cells and debris. Our group has studied TAM mediated apoptotic cell clearance in the setting of autoimmunity for many years. The vitamin K-dependent protein growth arrest specific protein 6 (Gas6) and protein S (ProS) binds to and activates TAM receptors[9,10]. Gas6 is produced in the heart, kidneys, and lungs. ProS is mainly produced in the liver[11]. TAM recognizes of apoptotic cells in such a way that the N-terminal of Gas6/ProS binds to the PtdSer on an apoptotic cell and the C-terminal of Gas6/ProS binds to TAM receptors that anchored on the surface of phagocytes. Together with the other receptors, engulfment of apoptotic cells occurs[12]. Though Mer can bind to both the ligands ProS and Gas6, we found that Gas6 appears to be the sole ligand for Mer-mediated macrophage phagocytosis of apoptotic cells, which can be blocked by anti-Mer antibody[13]. Macrophages are professional phagocytes. Rapid clearance of apoptotic cells by macrophages is important for the inhibition of inflammation and autoimmune responses against intracellular antigens. Mer receptor tyrosine kinase is critical for the macrophage engulfment and efficient clearance of apoptotic cells[14]. In mouse spleen, Mer mainly expresses on the marginal zone and tangible body macrophages[13].

The protocol presented here describes a basic method to induce cell apoptosis and demonstrate ways to measure the process and the efficiency of efferocytosis. These protocols can be readily adapted to study efferocytosis by other cell types in engulfment of apoptotic cells of different origins.

PROTOCOL:

Experimental mice were bred and maintained in our mice colony. All animal work was conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati.

1. Preparation of CFSE labeled apoptotic thymocytes

1.1. Euthanize two naïve C57/B6 mice by CO_2 inhalation for 10 min and dissect to open the chest cavity, remove (pull out) the thymus with curved fine-tip forceps into tissue culture petri dish containing 10 mL of RPMI1640 medium.

1.2. Obtain single cell suspension by grinding the whole thymus against two frosted ends of the microscope slides and then filter the suspension through 100 μm cell strainer.

1.3. Collect the 10 mL thymus suspension into a 50 mL tube and centrifuge at 300 *x g* for 5 min.

1.4. Remove the supernatant, resuspend in 40 mL of 1x PBS, and count cell numbers with a hemocytometer.

1.5. Centrifuge at 300 *x g* for 5 min and remove the supernatant.

1.6. Resuspend in 20 mL of 1x PBS in a 50 mL tube as a single cell suspension with up to 2 $\times$ 10^8 cells (if more than 2 $\times$ 10^8 cells, resuspend the rest of the cells in another 20 mL of 1 $\times$ PBS in a different 50 mL tube).

1.7. Make 5 μM of CFSE in equal volume (20 mL) of 1x PBS in a separate 50 mL tube by pipetting 40 μL of CFSE stock solution (2.5 μM) into 20 mL of 1x PBS and mix well by inverting the tube 2 - 3 times.

1.8. Add the 20 mL of CFSE from step 1.7 into the 20 mL of cell suspension from step 1.6 (the final concentration of CFSE is now 2.5 μM).

NOTE: For every 20 mL cell suspension, a 20 mL of CFSE tube is needed.

1.9. Invert the cell and CFSE mixture tube 2 - 3 times and incubate the mixture in the dark at room temperature for a maximum of 2 min, then stop the reaction by adding 10 mL of heat-inactivated horse serum.

1.10. Centrifuge the 50 mL tube mixture at 300 *x g* for 5 min at room temperature.

NOTE: If the CFSE labeling is successful, cell pellet will become light yellow in color.

1.11. Remove the supernatant and resuspend the cell pellet in 40 mL of 1x PBS and count cell numbers with a hemocytometer.

1.12. Centrifuge the cell suspension at 300 *x g* for 5 min and discard the supernatant.

1.13. Wash the cell pellet again with 40 mL of RPMI1640 medium.

1.14. Remove the supernatant and resuspend cells with RPMI1640 tissue culture medium (RPMI1640, 20 mM HEPES, 10% FBS (heat inactivated), 20 mM glutamine, and 1x Pen/Strep) at a concentration of 7×10^6 cells/mL in a 100 mm tissue culture dish.

NOTE: If more cells are obtained, a separate 100 mm tissue culture dish will be needed.

1.15. Add staurosporine into the cell suspension culture at a final concentration of 1 μM and culture for 4 h at 37 °C in a tissue culture incubator supplied with 5% CO_2.

2. Preparation of peritoneal macrophages

2.1. Inject two C57B6 mice (or any gene-manipulated mice in the lab) intraperitoneally with 1 mL of 3% aged thioglycollate at day 0.

2.2. Euthanize the mice at day 5 as in step 1.1, cut and peel open the abdominal skin but leave the peritoneum intact. Flush the peritoneal cavity by quickly pushing 10 mL of wash buffer (RPMI1640, 2% FBS, 0.04% EDTA) into the peritoneal cavity using a 10 mL syringe attached with a 16 G needle.

2.3. Retrieve the wash buffer slowly with the same needle/syringe, and collect the wash buffer into a 50 mL tube (details refer to Janssen lab article[15]).

2.4. Wash the peritoneal gavage twice with 1x PBS, resuspend the peritoneal macrophages in RPMI1640 tissue culture medium at a density of 2×10^6 cells/mL and aliquot 500 μL into each well of the 24-well plate. Leave the plate in a tissue culture incubator for 2 h.

2.5. Remove the floating cells by aspirating and replacing with 500 μL of fresh culture medium, twice.

2.6. Optionally, add TAM receptor tyrosine inhibitor, RXDX-106, at concentrations indicated in the figure legends into each well of the macrophage culture and incubate for another 2 h.

3. Co-culture of peritoneal macrophages with apoptotic thymocytes

3.1. Collect apoptotic thymocytes from step 1 and wash three times with RPMI1640 medium. The efficiency of apoptotic induction can be measured at this stage with the Annexin V/7-AAD kit.

3.2. Distribute 0 - 12 $\times$ 10^6 cells (in 500 μL medium) into each well of the macrophage cultures from protocol #2, according to the experimental arrangement (e.g., see **Figure 1**). This makes the whole culture volume of 1 mL in each well of the 24-well plate. Add the blocking antibody into the culture immediately before the addition of apoptotic thymocytes.

3.3. Culture the cell mixture at 37 °C for 4 h in a tissue culture incubator supplied with 5% CO_2.

3.4. Wash each well of the culture with 1x PBS (containing 500 μM EDTA) twice to remove the free-floating apoptotic cells.

3.5. Stain the plate-bound macrophages with CD11b-PE at this stage.

3.5.1. Wash the plate-bound macrophages with staining buffer (1x PBS, 1% BSA) once.

3.5.2. Add 200 μL of staining buffer containing 2 μL CD11b-PE into each well.

3.5.3. Incubate the plate at 4 °C for 20 min.

3.5.4. Wash each well of plate three times with the staining buffer.

3.5.5. Add 200 μL of staining buffer and proceed the plate for image analysis under the fluorescent microscope.

3.6. Alternatively, detach the plate-bound macrophages by adding 1 mL of 1% lidocaine in PBS and incubating for 10 min at 37 °C.

3.7. Detach plate-bound macrophages with repeat pipetting.

3.8. Transfer macrophage suspension into an individual 5 mL round-bottom FACS tube from each well of the 24-well plate.

3.9. Centrifuge at 300 *x g* for 5 min.

3.10. Remove the supernatant and add 200 μL of staining buffer containing 2 μL of CD11b-PE (1:200 dilution in staining buffer).

3.9. Incubate the cell suspension in staining buffer at 4 °C for 20 min.

3.10. Wash the cell suspension twice with staining buffer.

3.11. Add 200 μL of staining buffer and proceed for FACS analysis with a flow cytometer and analyze for the percentage of CFSE positivity macrophages.

REPRESENTATIVE RESULTS:

Analysis of peritoneal macrophage-mediated engulfment of apoptotic thymocytes. Peritoneal macrophages and apoptotic cells were prepared and co-cultured as described in the protocol. Macrophages were detached and stained with PE conjugated anti-CD11b antibody for 30 min on ice. Macrophages were then washed and processed in a flow cytometer. As seen, there is no CFSE positive macrophage in the bottom right quadrant when no apoptotic cells were added into the culture (**Figure 1A** and **Figure 2**, first panel). Thioglycollate-stimulated peritoneal macrophages have the variable capacity to engulf apoptotic cells. Up to 30% of macrophages showed positive in the CFSE channel, indicating they have ingested CFSE-labeled apoptotic cells in this experiment (**Figure 1**). It is worth to note that CFSE positive macrophages spread out in the bottom right quadrant due to different intensities, indicating that the number of apoptotic cells within macrophages is different. Therefore, microscopic observation of macrophage engulfment of apoptotic cells is essential to investigate the capacity of macrophages to ingest apoptotic cells (**Figure 2**). The higher ratio of apoptotic cells to macrophages not only increases the number of macrophages ingesting apoptotic cells but also enhances the ability of macrophages to ingest more apoptotic cells (**Figure 2**).

Dose-dependent inhibition of efferocytosis by Mer blockage.

In another set of experiments, we found that about 15% of the macrophages became CFSE positive when CFSE-labeled apoptotic thymocytes (ration of 6:1) were added into the macrophage culture for 4 h, indicating they are the phagocytic macrophages (**Figure 3B**). One function of Mer on macrophages is to recognize and mediate phagocytosis of apoptotic cells through the bridging molecule, Gas6. To test the percentage of efferocytosis attributed by Mer inhibition, we added anti-Mer antibodies into the culture to block Mer-mediated efferocytosis. Mer antibody blocks macrophage efferocytosis in a dose-dependent manner (**Figure 3C**, **Figure 3D**) and the overall blockage may account for about 30% of the efferocytosis efficiency in the current setting (**Figure 3**), This data was also confirmed in our previous study with Mer knockout macrophages[13]. We then tested the efficiency of inhibition with the newly FDA approved TAM receptor inhibitor, RXDX-106, which inhibits all TAM receptors with different affinities (Axl>>Tyro3>Mer). RXDX-106 was added into the macrophage culture 2 hours before co-incubation with apoptotic thymocytes. As shown in **Figure 4**, RXDX-106 inhibited macrophage efferocytosis in a dose dependent manner. The saturated inhibition concentration was about 100 nM (**Figure 4**, solid line), a concentration that appears to be more effective than Mer-deficiency (**Figure 4** dotted line) or Mer antibody (**Figure 3**, panel D) alone. Since macrophage expresses all three TAM receptors on the surface[16], it is expected to see that higher concentrations of RXDX-106 (over 100 nM) will block all three receptors, and therefore, will be more effective in blocking efferocytosis than targeting a single TAM receptor, Mer.

FIGURES LEGENDS:

Figure 1. Percentage of phagocytosis of apoptotic thymocytes by peritoneal macrophages. Apoptotic thymocytes were induced by incubating with 1 μM of staurosporine for 4 h and added into macrophage culture at a ratio as indicated in the figure panels: (**A**) 1:0; (**B**) 1:1; (**C**) 1:2; (**D**) 1:4; (**E**) 1:6; (**F**) 1:8; (**G**) 1:10; (**H**) 1:12. Data were acquired with a flow cytometer. Percentage of phagocytic macrophages was analyzed using the software associated with the flow cytometer.

Figure 2. Microscopic analysis of efferocytosis. Efferocytosis was prepared as in **Figure 1**. Phagocytic macrophages were stained in situ on the plate with CD11b-PE, fixed with paraformaldehyde, and evaluated. Images were acquired using the fluorescent microscope and analyzed with the software associated with the microscope. Representative insertions were digitally enlarged and shown below in each image.

Figure 3. Inhibition of peritoneal macrophage efferocytosis by an anti-Mer antibody. Apoptotic cells were prepared and co-cultured with macrophages for 4 h as described in the protocol. Anti-Mer Ab was added into the macrophage culture immediately before the co-culture with apoptotic cells. Phagocytic macrophages were then detached and stained with anti-mouse CD11b-PE antibody on ice for 30 min. Data were acquired and analyzed as in **Figure 1**.

Figure 4. RXDX-106 mediated inhibition of macrophage efferocytosis. Efferocytosis was set up as described in **Figure 3**. RXDX-106 was added two hrs before the co-culture with apoptotic thymocytes. Mer-deficient peritoneal macrophages were prepared similarly and served as the control group. Data were acquired and the percentage of phagocytic macrophages were gated on CD11b positive cells and analyzed using the flow cytometer software.

DISCUSSION:

Apoptosis is a highly conserved cell death process that involves many signal cascades and induces protein expression, secretion, and transportation. Apoptosis is often associated with cellular morphology changes[17]. Apoptotic cells actively release cytokines and chemokines that attract phagocytes to migrate to the site and initiate the process of engulfment, an extremely complex pathway under tight control[18]. On the other hand, necrotic cell death releases danger signals that trigger inflammatory responses[1]. Defective or prolonged clearance of apoptotic cells will lead to secondary necrosis of these cells[19]. Therefore, timing in apoptosis induction is very critical in the experiment. There are numerous ways to induce cell apoptosis[20]. However, the duration and strength of induction are cell type dependent. We recommend setting up a titration experiment to determine the optimal condition of maximum (90-95%) apoptosis production. The Annexin-V/7-AAD Apoptotic Detection Kit was used and the instructions were followed in our lab to evaluate the apoptotic efficiency. Our laboratory has tried two different ways

to induce thymocytes apoptosis in the past. Gamma-radiation seems to be a better method, as it has no chemical residuals in the culture and induces few necrotic cell deaths. We expose thymocytes to 500 rad of γ-radiation followed by 4-h culture in RPMI1640 medium. We observed about 95% apoptotic thymocytes[13]. When a radiator is not available, we induced thymocytes to undergo apoptosis with 1 μM of staurosporine in the culture for 4 h (Step 1.15). Primary cells are generally more sensitive to apoptosis induction. Extensive washing steps are also required to remove the chemicals from culture before co-culture with phagocytes. There are many ways to label apoptotic cells. Though toxicity has been associated with high concentration, CFSE is very efficiently retained within the cytoplasm when carefully optimized[21]. pHrodo is an acid-sensitive dye, which increases in fluorescence as the pH of the environment decreases. Due to the low pH of the phagolysosome, phagocytized apoptotic cells can be easily distinguished from physically attached but not engulfed apoptotic cells in the assay[22].

Numerous surface receptors (TAM receptors, mannose receptor, integrins (CD11b/CD18), scavenger receptors, Fc receptors, et al) allow phagocytes to distinguish self from pathogens and discriminate the subsequent responses[23]. Different receptors work together to finish a rather complex process. Blocking receptor(s) likely results in a partial inhibition of phagocytosis. Primary macrophages can be prepared from different resources with various methods (bone marrow derived macrophages, peritoneal macrophages, spleen macrophages, et al.). The advantage of thioglycollate-induced macrophages is the skewed phagocytic phenotype of the macrophages; the disadvantage is that thioglycollate needs to be aged in the dark for at least 3 months. However, thioglycollate solution has a shelf life of 2 years. A constant stock of the solution should overcome this disadvantage. In the preparation of peritoneal macrophages, the trace amount of red blood cells in the peritoneal macrophage collection should not affect the experiment, since they are going to be washed away along with the floating cells after 2 h of macrophage culture. A large amount of red blood cells (visible pellet) may require treatment with ACK lysing buffer. Macrophages tend to adhere tightly to the culture surface. Different methods have been cited in the literature to detach adhesion cells from the surface[24]. Enzyme-based detachment yields the highest levels of cell recovery but may damage surface receptors, impairing cell function and associated analyses. Alteration of surface receptors may also affect receptor-based flow cytometry analysis. Lidocaine causes cell morphology to change to a more spherical conformation due to the blockade of calcium ion channels[25]. It is the best way to detach macrophages from the surface with no noticeable damage in our experiment.

Macrophages containing apoptotic cells can be analyzed by flow cytometry-based or microscope-based assays. FACS can be applied to analyze a large number of cells in a short time and can further identify cell subtypes by differential staining with antibodies against the specific surface or cytoplasmic proteins. An optimal concentration (ratio) of apoptotic cell numbers in the co-culture system can also be decided by the FACS analysis. Microscopic analysis has limitations regarding cell numbers and differential staining as compared to the FACS analysis. However, fluorescent microscopy provides in-depth

information. Microscopic analysis of macrophage engulfment of apoptotic cells is essential to investigate the capacity and the kinetics of macrophages to ingest apoptotic cells. A time-lapse of the whole ingestion progression may provide detailed information regarding how long it takes to engulf one apoptotic cell, and how many apoptotic cells can a single macrophage ingest simultaneously. Phagocytic macrophages can also be analyzed by Western blot to investigate signaling molecules regulated by the engulfment process. Gene expression profiles associated with this process can be evaluated by real time PCR.

Finally, this protocol provides a basic platform in the experimental analysis of efferocytosis. Other cell types (dendritic cells, mesangial cells, epithelial cells) can also function to uptake apoptotic cells at their residential sites. Those cells have preferences in utilizing different receptors to recognize and initiate the process of apoptotic cell clearance[2]. The overall phagocytic efficiency may be influenced by several factors, including experimental and cell type specific factors. Variable results reported in the literature are probably due to 1) duration of co-culture; 2) resource and preparation of phagocytes and apoptotic cells; 3) methods to dissociate the apoptotic cells from phagocytes. The protocol described here may apply to the phagocytic potential of other cells. Optimization may be required to maximize the phagocytic potential of target cells. We have analyzed renal mesangial cell phagocytosis of apoptotic thymocytes generated in the same way as described in this protocol. Neutrophils play a key role in the innate immune system through elimination of pathogens. Neutrophil phagocytosis of labeled bacteria or particles can be analyzed by flow cytometry[26]. However, neutrophils have a very short half-life (6-8 h) and neutrophil phagocytosis of bacteria or fungi occurs within seconds to minutes[27,28].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

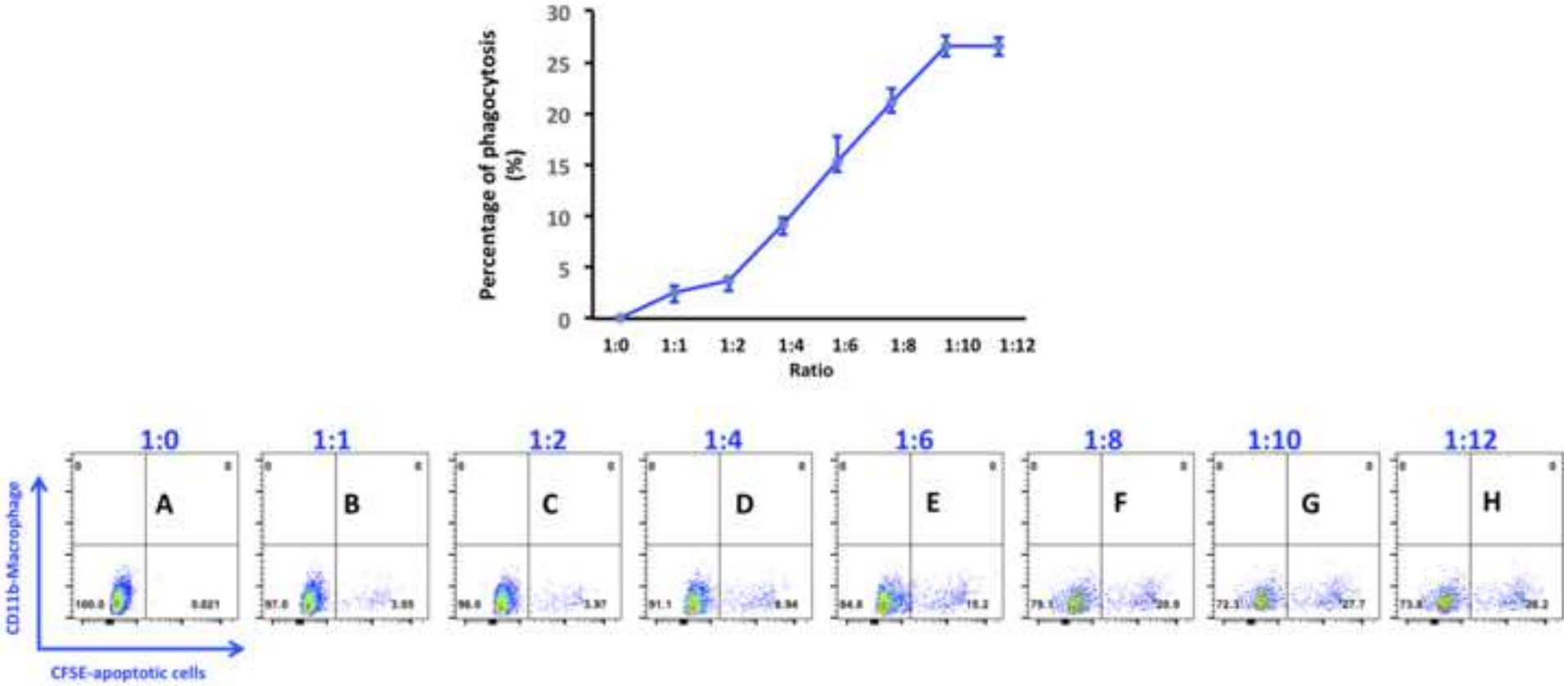

Figure 2

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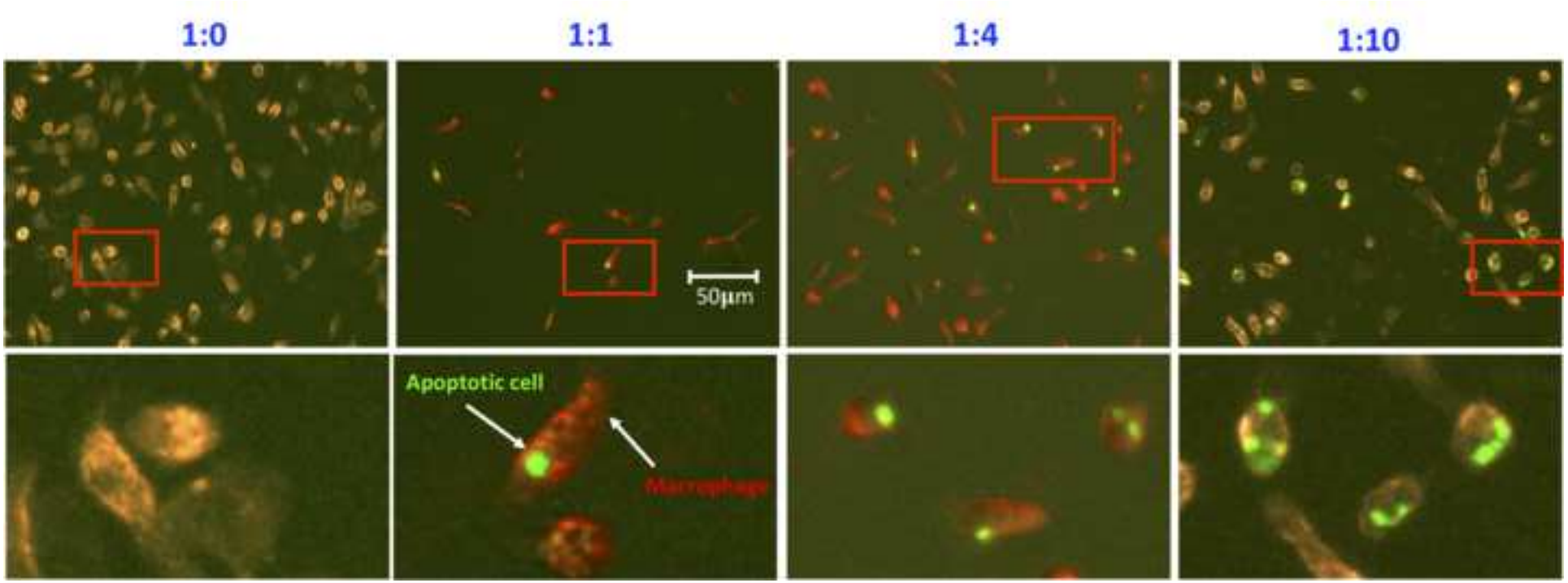

Figure 3

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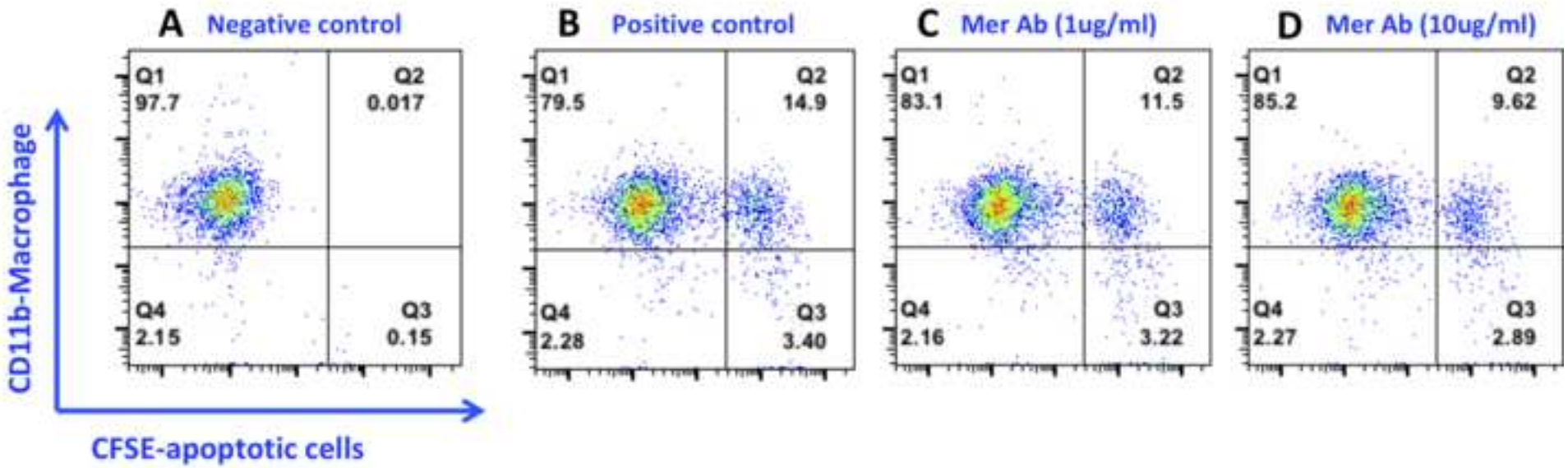
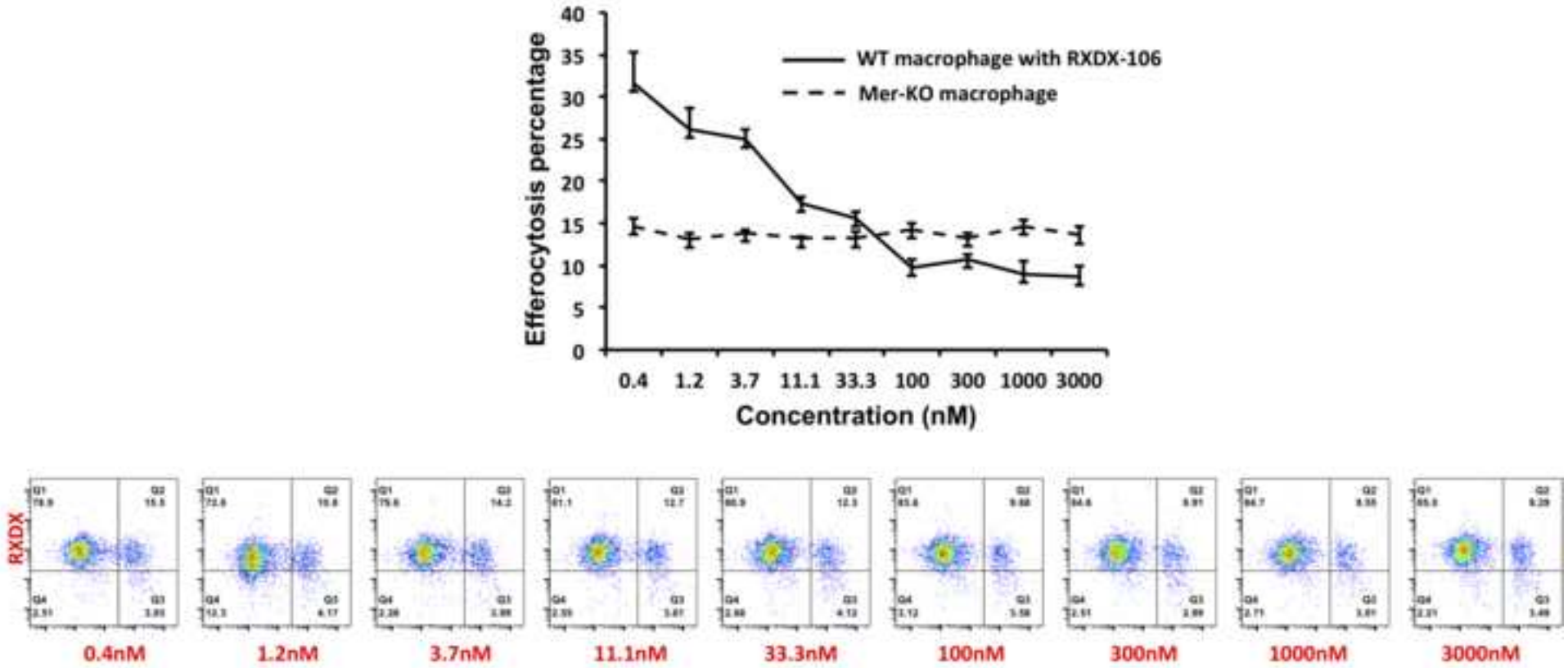


Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Ack lysing buffer	GIBCO	A10492	
Annexin V/7-AAD	BD Pharmingen	559763	
Anti-Mer antibody	R&D Systems	BAF591	
CD11b-PE (clone M1/70)	BD Pharmingen	553311	
CFSE	Invitrogen	C1157	
DMSO	Sigma-Aldrich	D-2650	
EDTA (0.5 mM)	GIBCO	15575-020	
FACS tubes	BD Biosciences	352017	
Frosted slides	Fisher Scientific	12-552-343	
Horse Serum (Heat-inactivated)	Invitrogen	26050088	
Lidocaine	Sigma-Aldrich	L-5647	Prepare 1% buffer in 1x PBS
PBS, 1x	Corning	21040CV	
RPMI-1640	Corning	10040CV	
RXDX-106	Selleck Chemicals	CEP-40783	
Staurosprine (100**m**g)	Fisher Scientific	BP2541-100	Add 214.3 ml of DMSO into 100mg to make 1mM stocking solution
Thioglycolate Medium Brewer Modified	BD Biosciences	243010	Prepare 3% thioglycolate buffer in 1´PBS, autoclaved, and store in the dark for 3 months.

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Dear Editor,

We are grateful to the editorial panel and the scientific reviewers for their critical reading of the manuscript and their valuable comments. We have carefully followed the editorial comments and revised the manuscript thoroughly. We also included a point-to-point responses to concerns raised by the reviewers. All the revision was highlighted in blue. We also highlighted the protocol parts in yellow for the video. Meanwhile, we had an English speaking scientist proof read the manuscript.
We hope this manuscript is now acceptable for publication.

Sincerely,

Wenhai Shao, PhD
For the authors

Point-to-point response:

Reviewer #1:

- Title: the article only analyses/illustrates the phagocytic clearance of apoptotic thymocytes by peritoneal macrophages. I suggest to make this more clear from the title. eg consider to change "Experimental analysis of apoptotic cell engulfment" to "Experimental analysis of apoptotic thymocyte engulfment by macrophages" or similar. Alternatively the authors should try to show that the protocol presented here can be readily adapted to study efferocytosis by other cell types/ engulfment of apoptotic bodies of different origins.

We thank the reviewer's suggestion of title change. We have changed the title to "Experimental analysis of apoptotic thymocyte engulfment by macrophages". We described the broad application/adaptation of the protocol in the introduction.

- Introduction: I feel the introduction is only introducing apoptosis but does not sufficiently introduce the protocols and the applications/potential uses of this protocol. I'd advise to include another short paragraph on this to help the reader follow more easily the protocols described below.

We thank the reviewer's suggestion. We included the short paragraph at the end of the introduction.

- Discussion: currently the discussion is concluded almost unexpectedly by listing a series of assays that can be done to further analyse phagocytic macrophages. I suggest instead to include some concluding remark summarising the impact and the relevance of this protocol. Also, could the authors discuss a bit if/how variants to this protocol could be made to fit other research lines? e.g. is it possible with the same treatments to obtain apoptotic cells from other tissues/cell types such as for example neurons? Are apoptotic thymocytes a suitable substrate also to mimic/quantify phagocytic clearance by neutrophils, microglial cells etc? Discussing limits and potentials seems

appropriate, especially to emphasise the breath of applications for this protocol.

We are grateful for the reviewer's comments. We now discussed the impact and relevance of this protocol. However, this manuscript provides a basic protocol, the extend analysis to other cell lines is possible but requires careful preliminary study, which is out the scope of the current manuscript.

Minor Concerns:
- Abstract: The abstract is well written but I feel the authors list and describe unnecessarily the three different types of apoptosis receptors (which could be useful in the intro but maybe too distracting from the goal of the article when presented in the abstract), while they could spend few more words instead emphasising more the possible applications of this methods to study pathological and physiological processes.

We thank the review for the thoughtful input. We now diminished the TAM receptor information in the abstract. Instead, described the possible applications of the methods.

- Line 99: "Therefore, contribute to the resistance of cancer therapy". --> sentence requires some grammar fixes.

We deleted this sentence and rearranged the introduction.

- Inconsistent spacing between numbers and units e.g. L.133: 40ml versus L. 135: 40 ml.

We corrected it in the text, consistently as "40 ml" with a space.

- Line 138: 100m tissue culture dish. Is it 100 mm?

This should be "100 mm". We corrected it in the text.

- Line 195: "It's worth to note" Line 301: "It's the best way" Avoid contracted forms in written scientific English.

We rewrote the sentences in the long form now.

- Line 228: "Phagocytic cure of peritoneal macrophage engulfment of apoptotic thymocytes" I suggest rephrasing/simplifying this title e.g. "Percentage of phagocytosis of apoptotic thymocytes by peritoneal macrophages"

We changed the title of Figure 1 as suggested, "Percentage of phagocytosis of apoptotic thymocytes by peritoneal macrophages".

- Line 230: "1: to 0, 1, 2, 4, 6, 8, 10, 12" Remove ":"

We removed the colon punctuation from the text.

- Figure 2:please include a scale bar and a label that indicates what the colours are

We now included the scale bar and labeled the color in figure 2.

Reviewer #2:
1. The thioglycolate induced macrophages have been shown to be very effective in clearing out apoptotic cells. There are few publications showing about 60 -70% efferocytosis following co-culture of macrophages and apoptotic cells for 1hr. Authors have seen only 26% of efferocytosis after 4 hr. co-incubation. Can authors provide explanation for such a slow rate and inefficient efferocytosis?

The differences of phagocytic efficiency is probably due to several reasons: 1. Duration of co-culture; 2. Resource and preparation of phagocytes and apoptotic cells. 3. Methods to detach the apoptotic cells from phagocytes. 1 hr incubation may result in higher number of apoptotic cells being ingested. However, our data are supported by the publication here (Mol Cell Biol. 2014 Apr; 34(8): 1512-1520). We have included all these in our discussion part of the text.

2. One of the publication showing above mentioned phenotype, "Mol Cell Biol. 2014 Apr; 34(8): 1512-1520." However there are few more publications showing similar phenotypes.

We thank the reviewer mention this publication. This article showed similar percentage of phagocytosis to our data in the current manuscript.

3. Is CFSE labeling of apoptotic cells is efficient? Authors can use other well used methods for staining apoptotic cells, like pHrodo dye. This might increase the detection of macrophages which have engulfed the apoptotic cells by both the methods.

4. Authors have mentioned in discussion that they can generate apoptotic cell by 2 methods, γ-radiation and staurosporine treatment. Does authors have analyzed rate of efferocytosis when apoptotic cells generated by either methods side by side? Is there any difference in rate of efferocytosis was observed?

We would like to compare the 2 methods in apoptosis induction. Unfortunately, get a license of using radioactive methods is extremely hard and require FBI background check, which may take over 1 year. We favor the γ-radiation due to the reason discussed in the article, but have no choice at this stage.

5. Its appreciable that, authors have used RxDX-106 (pan-TAM inhibitor) and Mertk KO macrophages as a positive control to depict Mertk dependent efferocytosis, however in both cases we do observe only 35-40% decrease in efferocytosis. How significant is this?

Data presented here are for demonstration only. However, statistical analysis was also not presented in the article "Mol Cell Biol. 2014 Apr; 34(8): 1512-1520." (point #2, provided by this reviewer). As mentioned in the discussion, there are redundant receptors fulfilling this important task. 30% reduction of phagocytosis is dramatic enough to cause lupus-like autoimmune disease in the Mer-/- mouse (Cohen PL, J. Exp. Med. 2002).

6. Authors have also mentioned in discussion that they analyzed PS exposure by Annexin-V/7-AAd apoptosis detection kits. Can authors provide the data?

We did the assay in the past at the previous institute. We would like to repeat the assay if required in the manuscript. However, we need longer time (one month at least) to prepare the experiment and present the data.

7. Authors have mentioned few times about TAM receptor ligand, Gas6, throughout the text. Does authors have used Gas6 during the experiment. If so, can authors provide information about dose and source of Gas6.

There are several commercial resources of Gas6 available. However, the activities of those compounds remain to be identified. Lemke's group studied different compound and found out that truncated Gas6 can physically bind to the receptor but doesn't induce any signals. Similarly, those compounds do not have the ability to mediate apoptotic cell engulfment. Gas6 are abundant in the serum (Shao WH, CI, 2010). Addition of Gas6 into the culture medium doesn't improve the phagocytosis in the assay.

8. The text needs to be edited one more time. There are several typo errors throughout the text as well it needs significant work on the language.

We thank the review. We now have an English-speaking scientist edited the language.

Reviewer #3:
The paper shows a very basic protocol for CSFE labeling of apoptotic thymocytes by peritoneal macrophages. It is not clear why at several places in the text the importance of TAM RTK's are mentioned, it is less clear why these have been singled out, also since the authors mention themselves that over a dozen receptors are known to be involved in engulfment of apoptotic cells. Also the data in Figs 3 and 4 show that blocking the TAM RTK pathways either with antibodies or small molecules has only a partial effect (even going up to 3000 nM of the inhibitor, see Fig 4).

We mentioned the TAM RTKs, because we are one of the groups studying TAM associated apoptotic cells and systemic lupus disease. All the data presented in this manuscript are related to the TAM RTK. We think it's reasonable to introduce the proper background to make the data easy to understand. Regarding the partial inhibition, please refer to Reveiwer #2, question #7.

Other points:
-It is unclear how the presented protocols would be able to distinguish between the different receptor types mentioned in the abstract;

This is a basic protocol rather for phagocytosis analysis, but not to distinguish the different receptor types. However, we present data here to use specific antibodies and/or inhibitors to identify the receptor-specific contribution to the phagocytosis. Such idea may apply to other receptors.

-The physiological relevance of generating apoptotic thymocytes through staurosporin addition and their subsequent phagocytosis by peritoneal macrophages is unclear. These cells are at distinct sites in the body; also, the thymus already consist of a large number of apoptotic cells, and this could even be used to make the uptake assay more selective;

Again, here we present a basic protocol. In general, apoptotic cells flip phosphatidylserine (PtdSer) to the out membrane of the cell, serving as an "eat-me" signal to attract phagocytes to recognize them. There are many eat-me signals released by apoptotic cells. PtdSer is the most important and not cell type specific. Therefore, apoptotic cells will be engulfed through a general pathway.

-The introduction describes the TAM RTK's as well as Gas6, but it is unclear what Gas6 actually is doing and on what cells this is expressed

We included the background information in the introduction section.

-The last paragraph of the introduction discusses Gas6, Mer, Mc2 macrophages that appears outside of the scope of this paper; in fact, a clear link to the presented protocol is lacking;

We rewrote the introduction regarding the Mer/Gas6 on macrophages. Please refer to the first major point raised by this reviewer #3.

-The authors use Lidocaine that is an ion channel modulator—does this not influence the assay?

We tried several different ways to detach macrophages after culture. Lidocaine seems to be the most efficient method. We discussed this in the article.

-Much of what is shown is rather obvious; for example, see the explanation of the data shown in Fig 2 (the statement beginning on line 199 'higher ratio of apoptotic cell to macrophage …apoptotic cells').

We thank the reviewer's comment.

-The Figure 2 legend mentions a magnification of 100x, but the upper and lower row show different magnifications! Size bars would have been appropriate here;

We now included a scale bar in the figure.

-The manuscript would benefit from proofreading.

As mentioned above, we now have an English-speaking scientist edited the language.