Effect of Serum Deprivation Stress on Signal Induction Regulatory Protein-Alpha (SIRP-Alpha)-Mediated Erythrophagocytosis by Macrophages

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Background: Hemophagocytic lymphohistiocytosis (HLH) is a rare syndrome that involves loss of macrophages’ self-cells recognition resulting in auto-phagocytosis of erythrocytes, leukocytes, and platelets and leading to multi-system effects. The pathogenesis of HLH is unclear but can be explained by malfunction of the physiologic inhibitory pathway through interaction between macrophage SIRP-α and erythrocyte CD 47. The goal of the present study was to evaluate if erythrocytes phagocytosis occurs as a result of altered macrophage SIRP-α expression and its during inflammatory/stressful conditions as seen in HLH.

Material/Methods: RAW264.7 macrophages were cultured in serum-free media (SFM) and complete media (CM) to simulate stressful and physiologic conditions, respectively. CD47+ mouse erythrocytes were used to test interactions with macrophages at different stages. SIRP-α expressions and phagocytosis assays were measured and analyzed at different steps. The study was in vitro and used murine cells to simulate in vivo human interactions.

Results: SIRP-α expressions and phagocytosis rates were higher in SFM compared to CM. Interestingly, after adding SIRP-α blocking antibodies (Ab), phagocytosis rates significantly decreased.

Conclusions: Serum deprivation and LPS/IFN-Gamma induction resulted in increased SIRP-α expression and erythrophagocytosis. Using SIRP-α Ab during this condition decreased the rate of erythrophagocytosis, which indicates that SIRP-α receptor can have pro-phagocytic activity.

MeSH Keywords: Lymphohistiocytosis, Hemophagocytic • Macrophages

Abbreviations: Ab – antibody; BSA – bovine serum albumin; CO₂ – carbon dioxide; CD47 – cluster differentiation 47; cDNA – complementary DNA; CM – complete media; CTL – cytotoxic lymphocytes; EDTA – ethylenediaminetetraacetic acid; HLH – hemophagocytic lymphohistiocytosis; LPS/IFN-γ – lipopolysaccharide/Interferon-γ; μg – microgram; μl – microliter; nM – nanomolar; NK – natural killer, PMA – phorbol myristate acetate; PBS – phosphate-buffered saline; qRT-PCR – quantitative reverse transcriptase polymerase chain reaction; RBCs – red blood cells; RNA – ribonucleic acid; SFM – serum-free media; SHP-1 – Src homology region 2 domain-containing phosphatase-1; SIRP-alpha – signal induction regulatory protein-alpha; WBCs – white blood cells

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Background

Macrophages are powerful phagocytic cells that are involved in inflammation, innate immune response, and repair of damaged tissues [1]. They also assist in erythrocyte synthesis and maturation [2]. Macrophages avoid phagocytosing self-cells through surface receptor interactions. One of these receptors is the signal induction regulatory protein alpha (SIRP-α) [3], which is expressed on the surface of macrophages and dendritic cells, and primarily acts to inhibit the phagocytosis of Cluster of Differentiation 47 (CD47)-expressing cells, such as erythrocytes [4]. CD47-SIRP-α interaction functions as a signal for self-cell recognition, hence foreign cells that lack CD47 are more readily phagocytosed than those expressing it. The expression of CD47 decreases in erythrocytes as they age, resulting in higher rates of phagocytosis [5]. In rare circumstances, abnormal macrophage behavior results in failure of self-cell recognition, leading to phagocytosis of erythrocytes, leukocytes, and platelets, a condition known as hemophagocytic lymphohistiocytosis (HLH) [6].

HLH can be primary (familial) when genetic mutations are involved, or can be secondary to infections, autoimmune conditions, or malignancies [7]. During inflammatory process, macrophages interact with different cells, including natural killer (NK) cells and cytotoxic T lymphocytes (CTL), which play an important role in regulating macrophage activity. Impaired NK and CTL functions during inflammation can cause loss of inhibition of macrophage activity and continued cytokine stimulation, which accounts for the pathophysiology of HLH [6,8].

Macrophage phagocytic function can also be influenced by other cytokines and cell signals. X aus et al. found that lipopolysaccharides (LPS) can impede macrophage phagocytosis by inhibiting the major histocompatibility complex II (MHC II) expression [9]. Moreover, Bian et al. showed that LPS can increase phagocytosis towards self-cells by downregulation of SIRP-α [10]. On the other hand, X aus et al. demonstrated that initial activation by interferon-gamma (INF-γ) rendered macrophages unresponsive to apoptotic signals by the induction of P21WAF1. Additionally, Martinet et al. showed that macrophage phagocytic activity against heat inactivated gram-positive and gram-negative bacteria was increased by nutrient deprivation, thus demonstrating the crucial role of macrophage environment in regulating phagocytic activity [11]. Our hypothesis is that erythropagocytosis can occur as a result of failure of macrophage self-recognition of erythrocytes. In this study, we explored the possible correlation between SIRP-α expression and rate of phagocytosis.

Material and Methods

In our experiments, we used red blood cells (RBCs) that express CD47 on their surface in combination with murine macrophages that express SIRP-α with the final goal of better understanding the role of SIRP-α/CD47 interaction in the induction of RBCs phagocytosis under different treatments that resemble inflammatory/stressful conditions. In all experiments, RAW 264.7 cells were treated with Phorbol 12-myristate 13-acetate (PMA) to ensure their differentiation into M1 macrophages, as demonstrated by Martinez et al. [12]. Cells were cultured in complete media (CM) and serum-free media (SFM) to resemble physiologic and stressful conditions, respectively. IFN-γ and LPS were then added to both media.

Cell culture and treatments

RAW264.7 macrophage cells (ATCC, Manassas, VA) were seeded in 6-well plates in CM, composed of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (GE Healthcare Life Sciences, Logan, UT). For macrophages differentiation into M1, cells were incubated in a 5% CO₂ incubator at 37°C for 24 h, and 50 nM of PMA was then added. After 48 h, the medium was replaced with either CM+/- PMA or SFM+/- PMA, and cells were incubated at 37°C for an additional 24 h. IFN-γ (Sigma-Aldrich, St. Louis, MO) and LPS (Sigma-Aldrich, St. Louis, MO; 10 µM each) were added to both media and incubated for 24 h. The experiment was repeated 3 times for consistency.

CD47 staining

RBCs with CD 47⁺/- (tested for CD 47 positivity by fluorescence-activated cell sorter)were bought from Astarte Biologics (Bothell, WA). They were kept at 4°C for up to 1 month. For each experiment, 1 ml of RBCs from the main stock vial was centrifugated at 960 rpm for 8 min at a temperature of 4°C. Supernatant was aspirated and the cell pellet was gently resuspended in 1 ml of phosphate-buffered solution (PBS) containing 5% of bovine serum albumin (BSA). We then added 100 ng/µl of the CD47 antibody (Ab; Clone miap301; BD Pharmingen, San Jose, CA). The cell suspension was gently mixed and incubated at 37°C in a 5% CO₂ incubator for 1 h. Cells were then washed and re-suspended in 1 ml of PBS solution containing 5% of BSA. A 10-µl cell aliquot was placed onto the center of a glass slide, a cover-slip was placed over the top, and the CD47 staining was assessed using a Zeiss AxioVert 200M microscope (63X oil objective). Percentages of CD47-positive cells were calculated per field of view in more than 20 randomly selected fields (Figure 1A). The experiment was repeated 3 times.

RNA extraction and quantitative Reverse Transcriptase PCR (qRT-PCR):

Total RNAs were isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was then synthesized from 1 µg of RNA...
using the Verso cDNA kit (Thermo Scientific, Pittsburgh, PA). To measure SIRP-α cDNA, PCR was performed using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) on a MyIQ real-time PCR system (BioRad, Hercules, CA) using SIRP-α-specific primers (Cat# PPM05351E-200, Qiagen, Valencia, CA), and following the defined program: 1× heating for 10 min at 95°C; 40× denaturing for 15 s at 95°C, followed by annealing/extension 1 minute at 60°C/60× extension for 20 s at 60°C. Transcript levels were normalized to the S15 housekeeping gene (Forward: 5'-CAACGGCAAGACCTTCAAC-3’/ Reverse: 5'-GGCTTGTAGGTGATGGAGAAC-3’). The CT values were determined by automated threshold analysis with MyIQ version 1.0 software. SIRP-α fold change was then calculated using the $$\Delta\Delta Ct$$ method. Each treatment sample was tested in triplicate. The size of the amplicon was then verified on a 2% agarose gel. The experiment was repeated 3 times for consistency.

**RBCs phagocytosis in vitro assay**

The present protocol was optimized based on our previously published work [13]. Briefly, 2.2×10^5 RAW 246.7 cells were seeded in complete media onto 6-well plates and incubated at 37°C for 24 h, and 50 nM of PMA was then added. After 48 h, the medium was replaced with either CM+/− PMA or SFM+/− PMA. After 24 h, the media was replaced with the following treatments: 1. CM; 2. CM+PMA; 3. SFM+PMA; 4. CM+PMA+IFN-γ/LPS; 5. SFM+PMA+IFN-γ/LPS+anti-SIRP-α Ab (5 µl of a 0.03 µg/5µl Ab solution); 6. CM+PMA+IFN-γ/LPS+anti-SIRP-α Ab. The cells were incubated at 37°C for another 24 h. RBCs previously dyed (Cell Tracker Red CMTPX dye-Life Technologies) as recommended by the manufacturer (Figure 1B) were then added to the macrophages at a ratio 1: 10 (macrophages: RBCs) for 24 h. Confocal images were obtained using the Nikon T1-E microscope with A1 confocal and STORM super-resolution with a 60× objective (N.A. 1.4; oil; Z-stack). After imaging, Z-stacks were merged (Figure 2). Percent phagocytosis was determined by counting the number of macrophages containing red fluorescent over the total number of macrophages. Staining with Trypan blue (0.4% in PBS) was done to assess the viability of the RBCs before co-culturing with the macrophages, and 24 h after to verify that RBCs death was not due to the different treatments. The experiment was repeated 3 times for consistency.

**Figure 1.** Two representative pictures showing RBCs stained with anti-CD47 antibody (Green) in A or with the Cell Tracker Red CMTPX dye (Red) before phagocytosis in B.
Statistical methods

Data were analyzed using IBM SPSS Statistics for Windows version 23 (IBM Corp., Armonk, NY). Seven groups of different mixtures were identified and phagocytosis incidents were counted in each group. All data were summarized into frequencies and percentages. The chi-square test ($\chi^2$) was used to examine differences between groups of categorical data. All $p$-values were two-tailed. A $p$-value of $<0.05$ was considered significant. As for SIRP-α expression assay, the Mann-Whitney U test was employed.

Results

SIRP-α mRNA expression in relation to phagocytic activity in macrophages in vitro

We measured SIRP-α mRNA expression under various conditions. SIRP-α expression was higher in SFM compared to CM conditions, and was also higher after adding IFN-γ/LPS (we used them because they are commonly produced in inflammation) to both CM and SFM (Figure 3, Table 1). The results of the phagocytosis assay showed that the rate of the phagocytosis was also higher in SFM conditions and IFN-γ/LPS combination conditions when compared to CM and conditions without IFN-γ/LPS, respectively (Tables 2, 3).

Figure 2. Role of SIRP-α in the phagocytosis of RBCs by macrophages in vitro. Red fluorescent RBCs were co-cultured with RAW264.7 macrophages (Ratio 10:1) under the different indicated treatments. After 24 h, phagocytosis was analyzed using the Nikon T1-E microscope with A1 confocal and STORM super-resolution with a 60× objective (N.A. 1.4; oil; Z-stack). After imaging, the number of macrophages containing red fluorescence in the cytoplasm and the total number of macrophages (>150 per treatment condition) were counted. Representative pictures (overlay between red fluorescence and Nomarski picture) of RBCs (Red) – macrophages (unstained) co-cultures under the different treatments. Black arrows: intracellular vesicles located within the macrophage’s cytoplasm which contain Red fluorescent RBCs.

Figure 3. SIRP-α mRNA expression in RAW264.7 macrophages in vitro. Data points represent mean ± SD of triplicate samples from 3 independent experiments (* $p<0.05$). Comparison between SIRP-α mRNA expression among different groups was performed using the Mann-Whitney U test.

SIRP-α blockage significantly inhibits the phagocytosis of RBC in vitro

To determine the potential effect of SIRP-α in RBCs phagocytosis, we next used specific SIRP-α blocking antibodies and we compared its effect in stressed and unstressed conditions. The results showed...
that after adding anti-SIRP-α Ab to the SFM+PMA+Combo (representing stressed macrophages in serum free media), the phagocytosis rate significantly decreased by 28.4% (Table 2). However, adding those antibodies to the CM+PMA+Combo (representing stressed macrophages in complete media) did not affect the phagocytosis rate. A summary graph represents the relation between phagocytosis rate and SIRP-α mRNA expression in Figure 4.

**Discussion**

After reviewing literature, we found few studies that explored the relationship between erythrophagocytosis and SIRP-α expression. A study de Almeida et al. showed that erythrophagocytosis by monocytes was decreased when stimulated by either IFN-β or IFN-γ/TNF-α, independent of SIRP-α expression [14].

**Table 1.** Statistical analyzes of the SIRP-α mRNA expression. (*) p-value >0.05.

| Comparison          | Median | Mean rank | P-value |
|---------------------|--------|-----------|---------|
| CM versus SFM       | 136.60 | 7.18      | 0.006   |
| PMA versus Combo    | 125.55 | 6.13      | 0.016   |

**Table 2.** Descriptive table of different treatment conditions and the rate of phagocytosis in each condition.

| Treatment condition code | Treatment condition description | Percentage (%) of phagocytosis |
|--------------------------|---------------------------------|-------------------------------|
| 1                        | Raw+RBC                         | 5.6                           |
| 2                        | CM+Raw+PMA+RBC                  | 21.7                          |
| 3                        | SFM+Raw+PMA+RBC                 | 40.3                          |
| 4                        | CM+Raw+PMA+RBC+INF-γ/LPS        | 54.8                          |
| 5                        | SFM+Raw+PMA+RBC+INF-γ/LPS       | 67.7                          |
| 6                        | CM+Raw+PMA+INF-γ/LPS+anti-SIRP-α| 59.6                          |
| 7                        | SFM+Raw+PMA+INF-γ/LPS+anti-SIRP-α| 39.3                         |

**Table 3.** Comparisons between phagocytosis percentages among different treatment conditions and their significance levels. (*) represents comparisons that were statistically insignificant (p-value >0.05) except those marked with.

| Comparison between treatment conditions (TC) | Phagocytosis % in 1st treatment condition (TC) | Phagocytosis % in 2nd treatment condition (TC) | X²** | P-value |
|---------------------------------------------|-----------------------------------------------|-----------------------------------------------|------|---------|
| TC (2) versus (3)                           | 21.7                                          | 40.3                                          | 6.185| 0.013   |
| TC (4) versus (5)                           | 54.8                                          | 67.7                                          | 1.638| 0.201*  |
| TC (6) versus (7)                           | 59.6                                          | 39.3                                          | 4.211| 0.049   |
| TC (3) versus (5)                           | 40.3                                          | 67.7                                          | 6.216| 0.013   |
| TC (3) versus (7)                           | 40.3                                          | 39.3                                          | 0.013| 0.909*  |
| TC (5) versus (7)                           | 67.7                                          | 39.3                                          | 6.464| 0.011   |
| TC (2) versus (4)                           | 21.7                                          | 54.8                                          | 22.381| <0.001 |
| TC (2) versus (6)                           | 21.7                                          | 59.6                                          | 19.698| <0.001 |
| TC (4) versus (6)                           | 54.8%                                         | 59.6%                                         | 0.299| 0.585*  |

**Figure 4.** Representation of phagocytosis rate in relation with SIRP-α mRNA copies expression in treatment conditions from 1 to 6. 1. CM+PMA; 2. SFM+PMA; 3. CM+PMA+INF-γ/LPS; 4. SFM+PMA+INF-γ/LPS; 5. CM+PMA+INF-γ/LPS+anti-SIRP-α Ab; 6. SFM+PMA+INF-γ/LPS+anti-SIRP-α Ab.
Although it the methods used by de Almeida et al. are, to some extent, similar to ours, we used different stimulators and inducers in stressed and non-stressed media for comparison purposes, and to clearly bring out the effect of SIRP-α expression. Using anti-SIRP-α antibodies on stressed (in complete media) macrophages resulted in non-significant change in phagocytosis rate, which is mostly compatible with the results of de Almeida et al. However, using the antibodies on stressed macrophages (nutrient deprived in serum-free media) led to significant reduction in phagocytosis rate, suggesting that erythrophagocytosis is also dependent on SIRP-α expression.

Another study, also by de Almeida et al., on erythrophagocytosis in vitro showed that SIRP-α expression on monocytes in autoimmune hemolytic anemia (AIHA) was higher than in a healthy population. When monocytes from AIHA were treated with dexamethasone, SIRP-α expression was reduced without affecting erythrophagocytic activity. Although this study also indicates that erythrophagocytosis is independent on SIRP-α expression, the that monocytes in their study were only treated with steroids and there were no stimulants or inducers used, in contrast to our study. Moreover, the possibility of pro-phagocytic activity of SIRP-α was not ruled out by using anti-SIRP-α antibodies.

Takuro Kuriyama et al. hypothesized that HLH occurs as a result of SIRP-α/CD 47 interaction, which is in accordance with our hypothesis. However, they found that hematopoietic stem cell (HSC) CD 47 expression was decreased in HLH in correlation with cytokine surge. They also concluded that SIRP-α expression or mutation is irrelevant to HLH. It should be noted that their study focused mainly on granulocytes, megakaryocytes, and erythrocyte progenitors to prove their theory. However, the mature forms of these cells are also phagocytosed, which means that HLH pathogenesis can include other pathways. Furthermore, the SIRP-α expression and mutations assay were analyzed using blood samples from 15 patients with HLH, but the authors did not mention whether those patients were receiving treatment, which makes a huge difference in data interpretation. In our study, we used mature erythrocytes from CD 47+/− mice to exclude the possibility of phagocytosis being carried out due to immaturity. Also, as mentioned previously, the fact that SIRP-α expression and the treatment anti-SIRP-α antibody in non-stressed macrophages did not alter significantly the phagocytosis rate is compatible with these above-mentioned studies.

Anti-SIRP-α antibody was used as potential treatment against melanoma and renal cell carcinoma cells in a study done by Yanagita et al. [17]. This study also proves that SIRP-α has anti-phagocytic function, but since our results showed that inhibition of SIRP-α in stressed macrophages led to decreased phagocytosis, this may indicate that SIRP-α receptors go through morphological changes during phagocytosis and become pro-phagocytic. SIRP-α phagocytic activity is supported by a study by Burger et al., which demonstrated that CD 47 receptors on aged RBCs go through conformational changes that eventually act as a target for phagocytosis by pulp macrophages in the spleen after binding to SIRP-α [18]. Another study, by Tada et al., revealed that apoptotic cells are engulfed by macrophages via CD 47/SIRP-α interaction, which is mediated by phosphatidylinerine [19]. This study indicates that SIRP-α can function as a phagocytic promotor, depending on the stimulator on the target cell.

**Study limitations**

1. We used mouse cells instead of human cells since we plan to continue the experiment in mice, and RAW 264.7 cells were specifically used because our investigators are experienced with their culturing, cell cycle analysis, and phagocytosis assay. The phagocytosis model and the expected macrophages/RAW 264.7 behavior in our experiment were based on 2 separate studies [12,13] that showed similar results as ours. Future experiments will be performed using primary macrophages to confirm our preliminary data.

2. Although PMA is an artificial activator of monocytes, the only purpose of using it was to transform and differentiate monocytes to macrophages, and thus, the effect of LPS and INF-γ would be clearly appreciated.

**Conclusions**

Self-recognition receptors like SIRP-α, when interacting with CD47, can normally inhibit phagocytosis during non-inflammatory conditions. However, in the presence of macrophage stressors (i.e., culturing in serum deprivation and induction by LPS/INF-gamma), SIRP-α expression increases along with phagocytosis rate. Moreover, blocking the SIRP-α receptor, under the effect of stressors (mainly serum deprivation), unexpectedly led to decreased erythrophagocytosis, which indicates that the SIRP-α receptor functions as pro-phagocytic mediator.

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**Conflicts of interest**

None.
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