MACROPHAGE-DERIVED EXTRACELLULAR RIBONUCLEIC ACID INFLUENCES INFLAMMATORY PROFILE OF MACROPHAGES IN DOSE-DEPENDENT MANNER

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Abstract

Ribonucleic Acid (RNA) released from damaged cells is shown to modify proinflammatory activities of innate immune cells. Macrophages are the first responders during inflammation, where extracellular (ex)RNA derives from damaged cells (self-exRNA). There are controversies about the mechanisms of self-exRNA's influence on the profile of activated macrophages. We aimed to study the responses of activated cultured macrophages to self (macrophage-derived) exRNA to identify the proinflammatory characteristics that undergo changes upon such stimulation. We analyzed expression of the major histocompatibility complex (MHC)II, expression of nuclear factor-kappa B1 (NFkB1) along with production of tumor necrosis factor alpha (TNFα) and interleukin (IL)6. We concluded that stimulation of macrophages with 1 μg/mL of self-exRNA resulted in upregulation of MHCII and NFkB1 expression by activated macrophages, and increased production of TNFα and IL6. Stimulation of macrophages with 5 μg/mL of self-exRNA resulted in significant downregulation of MHCII, and reduced TNFα and IL6 production. At the same time, transcription of NFkB1 was found upregulated in both settings suggesting active inflammatory responses. Understanding the influence of self-exRNA, the product of cellular damage, on proinflammatory phenotype of innate immune cells will enable us to better understand the mechanisms of healing, immune tolerance, and autoimmunity.

Introduction

During inflammatory responses RNA is released from damaged cells. It is mostly micro-RNA (mi)RNA and ribosomal RNA (r)RNA [1, 4, and 15]. The role of self-exRNA in inflammatory processes has been a topic of research interest for some time aimed to find a better understanding of the mechanisms of tissue healing, immune tolerance, and autoimmunity. The self-exRNA is shown to modify inflammatory responses of activated innate immune cells and specifically macrophages [1, 3, 4, and 10]. Fisher, S., et al. have shown upregulation of proinflammatory responses of macrophages stimulated with exRNA [3]. Other authors showed downregulation of macrophages' proinflammatory profile upon co-stimulation with self-exRNA [2, 4, and 13]. In addition, there are variable data describing influences of self versus non-self-exRNA on activated macrophages. Milillo, M. A., et al. [13] have shown that bacterial exRNA downregulates MHCII expression by macrophages activated with bacterial lipoprotein L-Omp19. T. Takuya Uehata, et al. [1] described RNA-mediated inflammatory responses as a posttranscriptional mechanism of gene expression's regulation that can be different for self vs non-self-exRNA. The authors also linked responses of innate immune cells to exRNA to activation of CD4+ and CD8+ T cells through MHCII or MHCI upregulation.

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The described differences and controversies of exRNA influence on inflammatory profile of innate immune cells have been linked to the involvement of different toll-like receptors (TLRs) in the initial activation of macrophages [3, 4]. Moreover, K.Preisner, et al. suggested the role for self-exRNA in development of autoimmune responses [4]. Addressing the differences in the influences of self vs. non-self-exRNA resulting in upregulation or downregulation of inflammatory responses can be an additional step in understanding of immune pathways for autoimmunity and immune tolerance. In addition, studying the influence of self-exRNA that comes from cells damaged during inflammation on the profile of innate immune cells can provide a better understanding of the immune mechanisms of tissue healing.

To study responses of activated macrophages to self-exRNA we used RNA extracted from cultured bone-marrow derived RAW264.7 macrophages. To activate macrophages, we used TLR4 agonist, LPS, with or without addition of self-exRNA. Moreover, we compared effects of two doses of self-exRNA on activated by LPS macrophages.

As it has been stated earlier, RNA released from destroyed cells is one of the main alarmins that can be sensed by pattern recognition receptors (PRR) on macrophages [3, 5], and therefore can alter proinflammatory activities of macrophages. It has been shown that exRNA can alter TLRs signaling pathways, leading to increased vascular permeability and thrombosis [3, 5], which in turn can enhance cellular damage leading to chronic inflammation and autoimmunity.

The present study was designed to investigate the alterations in proinflammatory profile of macrophages activated by TLR4 agonist and stimulated by two different doses of self-exRNA. We showed that stimulation with self-exRNA of activated through TLR4 signaling pathway macrophages results in the dose-dependent changes of MHCII expression by CD11b+ cells and production of TNFα and IL6. At the same time, expression of NFkB1 mRNA remains similar to that of macrophages activated by LPS alone, which is consistent with upregulation of proinflammatory signaling pathway upon co-stimulation of macrophages with self-exRNA. The next step would be to study the influence of self-exRNA on proinflammatory profile of macrophages activated by different TLRs agonists. This will enable us to define a TLR-dependent mechanism involved in self-exRNA induced alterations of proinflammatory features of activated macrophages.

Material and Methods

Cell Culture

Mouse Monocyte/Macrophage cells (ATCC, #CCL-107, RAW264.7) were cultured in complete (c)RPMI (ThermoFisher Scientific, #61870127) and differentiated using granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, #315-03) at 20ng/mL [6, 7, 8, 10, and 12]. Cells were plated in 10mL of media supplemented with GM-CSF in 75cm^2 flasks and incubated at 37°C with 5% CO_2 for three days. The media has been refreshed on day three using cRPMI supplemented with 20ng/mL GM-CSF [7 and 10]. Non-adherent cells were collected, and RNA was extracted to be later used for self-exRNA stimulation. On day six, an equal volume of cRPMI with 20ng/mL GM-CSF was added to the flask. The cells and supernatants were collected on day seven by removing the media and washing the cells with PBS as described elsewhere [9]. The cells were then incubated in 5mL of prewarmed trypsin/EDTA (Sigma Aldrich, #93595) for detachment. Cells were plated in a 24-well plate at 0.25x10^6 per well in 0.5 mL of cRPMI supplemented with 20ng/mL of GM-CSF [8, 9, and 12].

Stimulation of Macrophages

Stimuli were prepared as followed and preincubated for 1 hour at 37°C: LPS (Thermo Fisher Scientific, #2270741) 20 ng/mL, macrophage-derived RNA (1 μg/mL and 5 μg/mL) [5]. Double concentration of each stimulus in 0.5mL of cRPMI was added to each well as shown in Fig. 1. 24 hours later, the supernatants from each well were collected and placed into a sterile 24-well plate in order corresponding to the order of the original plate (see Fig.1). The supernatants were used for TNFα and IL6 Enzyme Linked Immunosorbent Assay (ELISA).

The wells of the original plate were washed with sterile Dulbecco's phosphate-buffered saline (dPBS) (Sigma Aldrich, #D8537). Cells were detached by trypsinization as described above.

Once detached, the cells were collected in sterile aliquot tubes labeled in correspondence to the wells of the original plate. Collected cells have been used for FACS and RNA extraction followed by NFkB1 RT-PCR.

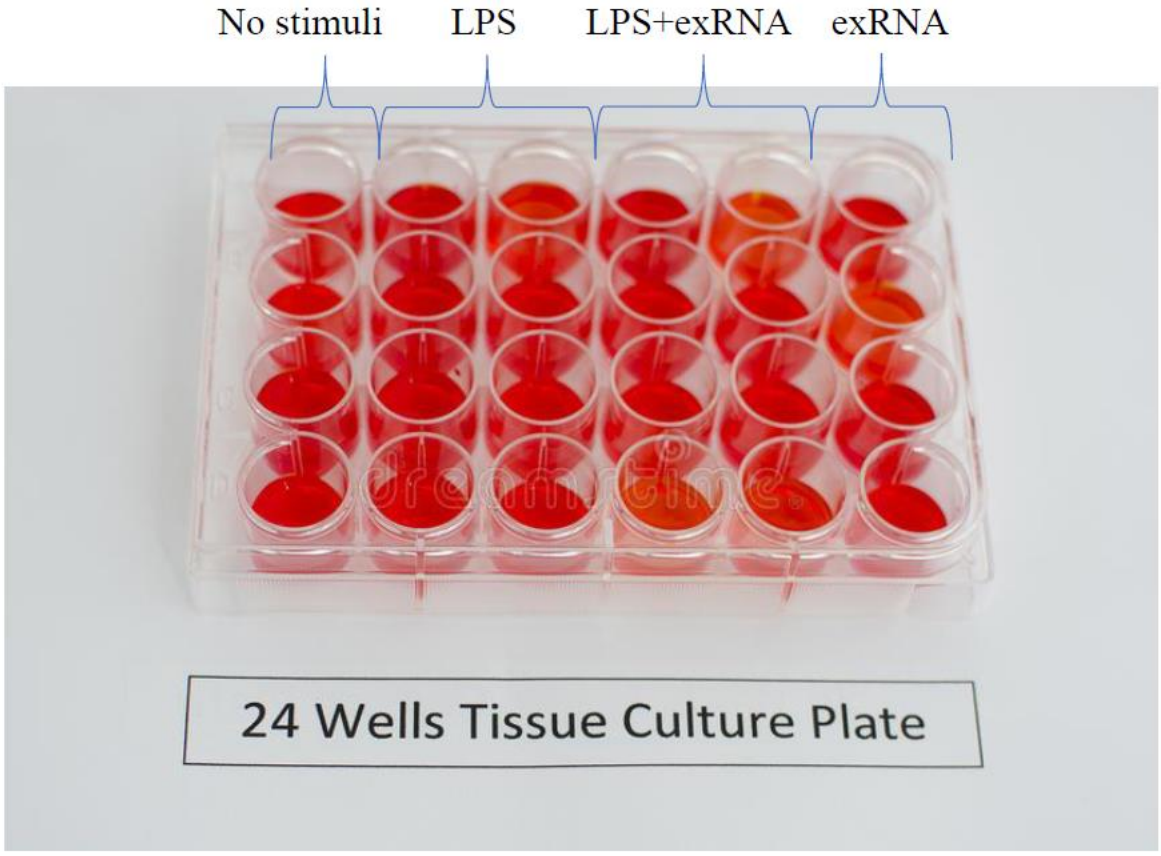


Figure 1. Stimulation of activated by LPS macrophages with self-exRNA. Cultured in a presence of 20 ng/mL GM-CSF murine bone-marrow-derived RAW264.7 macrophages have been activated by 20 ng/mL of LPS alone, LPS and 1 μg/mL or 5 μg/mL of self-exRNA, or by self-exRNA alone using non-activated and non-RNA stimulated cells as a control (No stimuli).

Flow Cytometry (FACS) analysis

24 hours after stimulation, half of the cells collected by trypsinization from each experimental group have been assessed by fluorescently activated cell sorting (FACS) using BD AcuriC6 counter to determine expression by CD11b+ cells MCHII and TLR4 co-receptor CD14 using PerCp-Cy5.5-anti-

CD11b, FITC-anti-MHCII (I-A/I-E), and FITC-anti-CD14 antibodies (Biolegend, #101227, #107605, #123307, 0.1-10 μg/mL respectively). Standard flow cytometry protocols were followed. Data was processed using FlowJo™ v10.8 Software (BD Life Sciences).

RNA extraction and RT-PCR

RNA used for stimulation was extracted from collected, non-adherent cultured cells from day three and from some adherent cells after trypsinization using RNA extraction kit (QIAGEN, #74804) following the protocol presented by Tsatas and Ghasemlou [20]. Cells were first treated with 1 mL of lysis buffer (Qiagen #79306) then transferred to a 2 mL Eppendorf tube. The mixture was vortexed then mixed on a rocker for 5 minutes at room temperature. 200 μL chloroform (Sigma Aldrich #319988) was added and the tube was shaken for 15 seconds, then the tube was left undisturbed at room temperature for 3 minutes. The resulting mixture was centrifuged for 15 minutes at 4°C. The top aqueous layer was transferred to a new 2 mL Eppendorf tube, then treated with 600 μL of 70% ethanol. The solution was vortexed then transferred in 700 μL aliquots to a Qiagen mini spin column and centrifuged at 9000 g for 15 s and the flow-through was discarded. Upon complete transfer to the mini spin column, 700 μL Buffer RW1 was added to the mini spin column then centrifuged at 9000 g for 15 seconds. The flow through was discarded then the column was charged with 500 μL of buffer RPE and centrifuged at 9000 g for 15 seconds. The flow through was discarded then the column was charged with a second 500 μL aliquot of buffer RPE and centrifuged at 9000 g for 2 minutes. The flow through and collection tube were discarded. The mini spin column was placed in a new 1.5 mL collection tube and charged with 50 μL of gently warmed RNase-free water and centrifuged at 9000 g for 1 minute. A second 50 μL aliquot of RNase-free water was added to the mini spin column and centrifuged at 9000 g for 1 min. The flow-through was collected and quantified for RNA content. The RNA was quantified using SpectraMax® i3x Multi-Mode Microplate spectrophotometer. To perform RT-PCR after stimulation, half of the cells from each experimental group have been collected to extract RNA for NFkB1 RT-PCR (BioRad, cDNA Synthesis kit #1708890 and Cyber-Green kit BioRad, #1725270). We used GAPDH as housekeeping gene (Origene, #SR193830) and NFkB1 primers (BioRad, #qMmuCED0047222). DNA amplification and RT-PCR were performed using a thermal cycler and BioRad RT-PCR cycler according to manufacturer protocol.

TNFα and IL6 ELISA

24 hours after stimulation, supernatants have been collected for TNFα and IL6 ELISA (Biolegend, #430901 and #431301, respectfully). The necessary reagents and assay were performed according to manufacturer's protocol. The results have been acquired using SpectraMax® i3x Multi-Mode Microplate Reader and corresponding software SoftMax Pro.

Statistical Analysis

Each dose of exRNA has been tested in two independent experiments with evaluation of 2 or 4 samples in each group. For statistical analysis, the data from each group was analyzed by a one-way ANOVA test. All analysis was performed using GraphPad Prism version 9.0.0 for macOS (GraphPad Software, San Diego, California USA).

Results

Stimulation of activated macrophages with self-exRNA has no influence on CD14 co-receptor, however, it influences MHCII expression by activated macrophages in dose-dependent manner.

First, we tested the effect of 1 μg/mL of self-exRNA on MHCII expression by CD11b+ macrophages activated with 20 ng/mL of LPS (Fig. 2). We found upregulation of MHCII in cells stimulated by LPS alone and in macrophages co-stimulated with LPS and 1 μg/mL of self-exRNA. However, stimulation of macrophages with 1 μg/mL self-exRNA alone resulted in downregulation of MHCII (Fig. 2c), which corresponds to results of Milillo et al. [13] that showed downregulation of MHCII expression by macrophages stimulated with bacterial RNA. Next, we increased the dose of self-exRNA to 5 μg/mL (Fig. 3). We found no influence of exRNA on expression of macrophages' co-receptor CD14 (Fig. 3d). Our results showed that the expression of CD14 was comparable in all experimental groups. All groups of macrophages had over 5% CD14+ cells. On the other hand, addition of 5 μg/mL of self-exRNA to macrophages activated with 20 ng/mL LPS resulted in significant downregulation of MHCII (Fig. 3c). MHCII, the main antigen presenting molecule in adaptive immune responses was upregulated by TLR4 agonist, LPS, alone; however, it was significantly reduced upon co-stimulation of activated by LPS macrophages with 5 μg/mL of self-exRNA or with 5 μg/mL of self-exRNA alone (Fig. 3c).

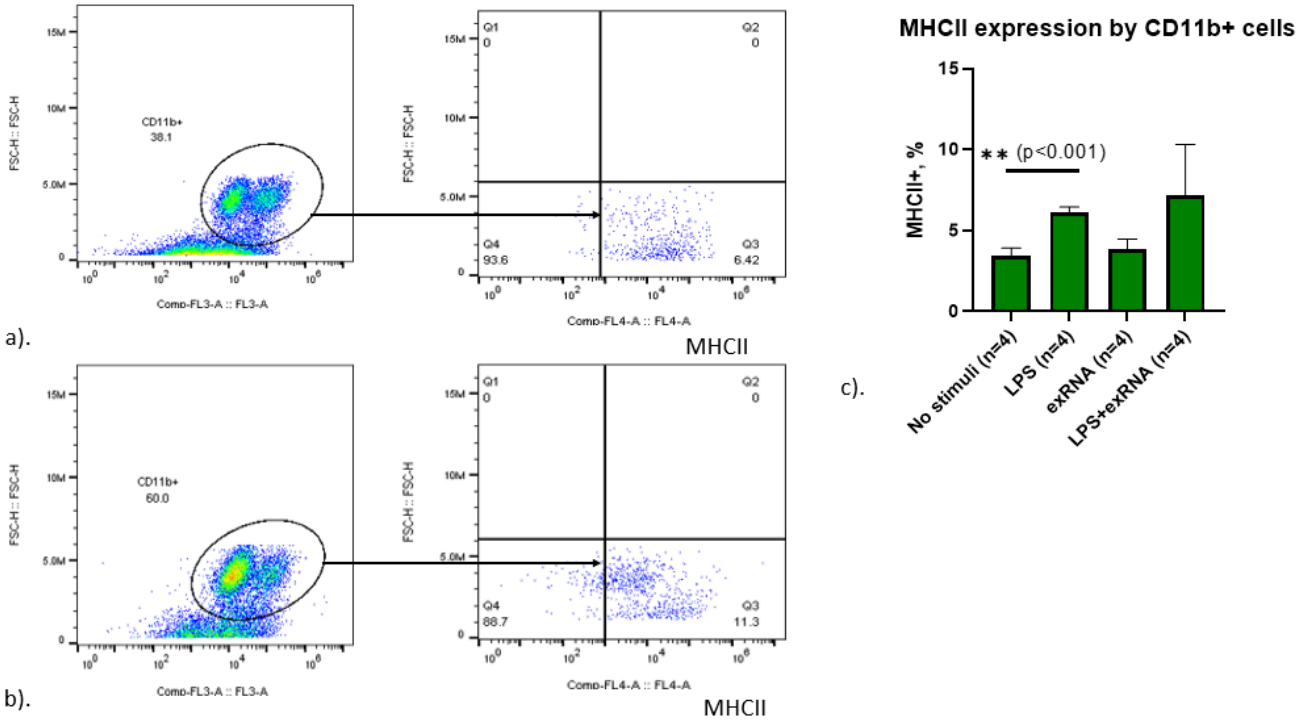


Figure 2. Influence of 1 μg/mL of self-exRNA on pro-inflammatory phenotype of macrophage activated by TLR4 agonist. Cells that did not receive LPS and exRNA have been used as no stimuli control. a). CD11b and MHCII expression by CD11b+ RAW264.7 cells in response to LPS alone (representative FACS images); b). CD11b and MHCII expression by CD11b+ RAW264.7 cells in response to activation with LPS followed by self-exRNA 1 μg/mL stimulation (representative FACS images); c). MHCII expression by CD11b+ RAW264.7 macrophages stimulated with LPS alone, co-stimulated with LPS and 1 μg/mL of self-exRNA, or 1 μg/mL of self-exRNA alone.

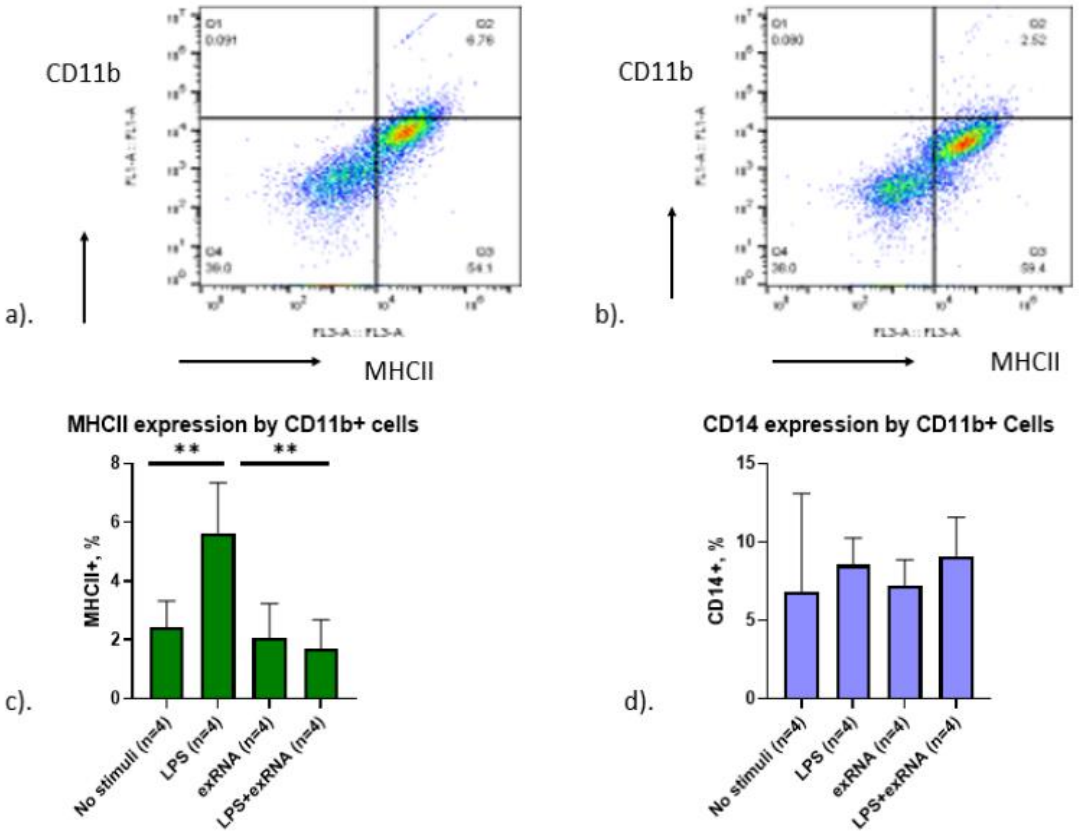


Figure 3. Influence of 5 μg/mL of self-exRNA on pro-inflammatory phenotype of macrophage activated by TLR4 agonist. Cells that did not receive LPS and exRNA have been used as no stimuli control; a). CD11b and MHCII expression by CD11b+ RAW264.7 cells in response to LPS alone (representative FACS images); b). CD11b and MHCII expression by CD11b+ RAW264.7 cells in response to activation with LPS followed by self-exRNA 5 μg/mL stimulation (representative FACS images); c). MHCII expression by CD11b+ RAW264.7 macrophages stimulated with LPS alone, co-stimulated with LPS and 5 μg/mL of self-exRNA, or 5 μg/mL of self-exRNA alone; d). CD14 expression by CD11b+ macrophages stimulated with LPS alone, co-stimulated with LPS and 5 μg/mL of self-exRNA, or 5 μg/mL of self-exRNA alone.

Stimulation of activated macrophages with self-exRNA alters TNFα and IL6 production in dose-dependent manner. Next, we tested the influence of self-exRNA on production of two major proinflammatory cytokines by activated macrophages. Our results showed that co-stimulation of macrophages with 20 ng/mL LPS and 1μg/mL of self-exRNA did not reduce IL6 or TNFα production by macrophages compared to cells stimulated with LPS alone (Figs. 4a and 4b). On the other hand, co-stimulation of activated macrophages with 20 ng/mL LPS and 5 μg/mL of self-exRNA resulted in significant reduction of both TNFα and IL6 compared to cells stimulated with LPS alone (Figs. 4c and 4d). Those results correspond to changes in MHCII expression by macrophages co-stimulated with self-exRNA (Fig. 3c).

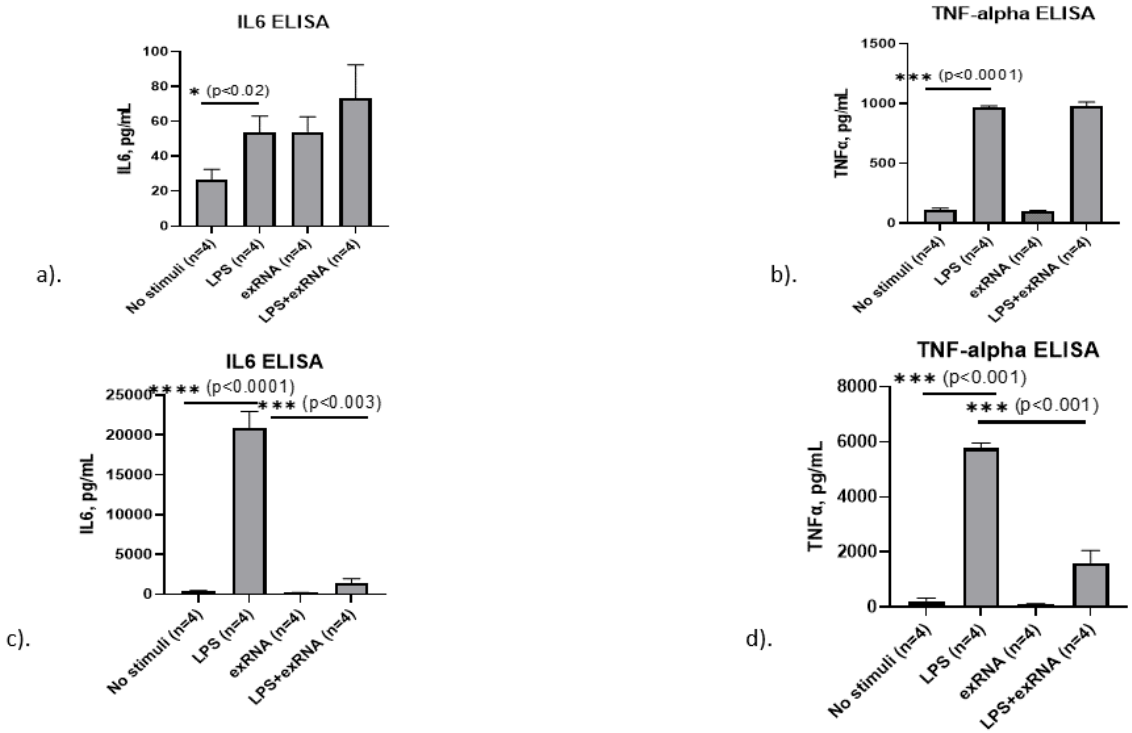
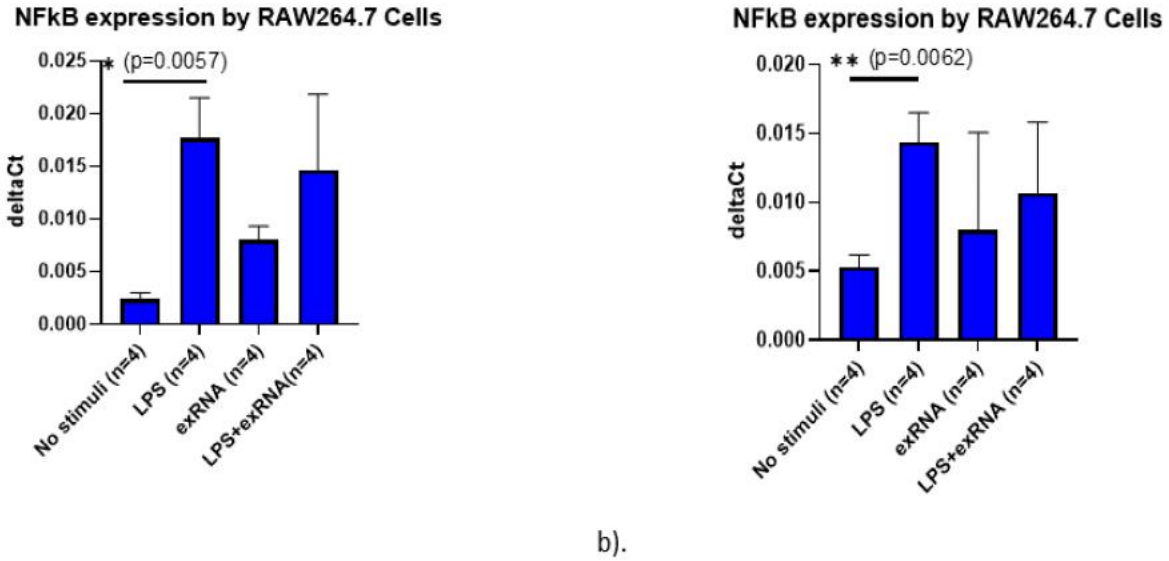


Figure 4. Dose-dependent alteration of proinflammatory cytokines IL6 and TNFα production upon co-stimulation of macrophages with TLR4 agonist and self-exRNA. Cells that did not receive LPS and exRNA have been used as no stimuli control. a). IL6 produced by macrophages stimulated with either LPS alone, with LPS and 1 μg/mL exRNA, or with 1 μg/mL exRNA alone; b). TNFα produced by macrophages stimulated with either LPS alone, with LPS and 1 μg/mL exRNA, or with 1 μg/mL exRNA alone; c). IL6 produced by macrophages stimulated with LPS alone, with LPS and 5 μg/mL exRNA, or with 5 μg/mL exRNA alone; d). TNFα produced by macrophages stimulated with LPS alone, with LPS and 5 μg/mL exRNA, or with 5 μg/mL exRNA alone.

Stimulation of activated by LPS macrophages with self-exRNA does not result in alteration of NFkB1 expression. One of the parameters for evaluation of cellular proinflammatory response is expression of transcription factor NFkB1. In our experiments, adding self-exRNA to the activated by LPS macrophages at either 1 μg/mL or 5 μg/mL did not change expression of NFkB1 mRNA by activated via TLR4 signaling pathway macrophages compared to the responses of macrophages stimulated with LPS alone (Figs. 4a and 4b). Expression of NFkB1 mRNA was significantly higher in cells activated with LPS alone, or co-stimulated with LPS and 1 μg/mL of self-exRNA indicating upregulated proinflammatory responses to both stimuli (Fig.4a). Moreover, the expression of NFkB1 mRNA by macrophages co-stimulated with LPS and 5 μg/mL self-exRNA macrophages also was not changed compared to macrophages activated with LPS alone (Fig. 4b).



a). b).

Figure 5. Expression of NFkB1 mRNA by LPS activated and co-stimulated by two different doses of self-exRNA macrophages. a). expression of NFkB1 mRNA by cultured RAW264.7 macrophages in response to no stimulation, stimulation with 20 ng/mL of LPS alone, with 1 μg/mL of self-exRNA alone, or with 20 ng/mL of LPS and 1 μg/mL of self-exRNA. b). expression of NFkB1 mRNA by cultured RAW264.7 macrophages in response to stimulation with 20 ng/mL of LPS alone, with 5 μg/mL of self-exRNA alone, or with 20 ng/mL of LPS and 5 μg/mL of self-exRNA.

Discussion

RNAs released from damaged cells is considered a danger associated molecules acting as an alarmin to induce a proinflammatory response when combined with TLRs agonists [3, 4, and 5]. Lately it was also described as immune regulatory molecules [15] that include micro (mi)RNA along with ribosomal (r)RNA and long non-coding (lnc)RNA [15 and 16]. It has been previously shown that macrophages stimulated with 10 μg/mL of exRNA extracted from *B. abortus* for 48 hours in presence of bacterial lipoprotein (Omp19) and interferon (IFN)γ showed significant reduction of MHCII expression [13]. In addition, co-stimulation of astrocytes with exRNA and TLR2 agonist Pam2CSK4 resulted in both increased proinflammatory cytokine production and NFkB activity compared to stimulation with exRNA alone [3].

The present study confirmed that self-exRNA causes alterations in the proinflammatory profile of cultured macrophages activated via TLR4 signaling pathway. Our results showed that stimulation of macrophages with LPS alone or with LPS plus 1 μg/mL of self-exRNA results in upregulation of MHCII by CD11b+ macrophages compared to unstimulated cells, whereas stimulation of macrophages with 1 μg/mL of self-exRNA alone significantly reduced expression of MHCII by CD11b+ cells.

On the other hand, increased to 5 μg/mL dose of self-exRNA leads to significantly reduced expression of antigen-presenting molecule MHCII and production of both proinflammatory cytokines, IL6 and TNFα, by activated macrophages, whereas expression of key proinflammatory transcription factor NFkB1 remains upregulated even upon stimulation of macrophages with 5 μg/mL of self-exRNA alone.

This dose-dependent response of activated through TLR4 signaling macrophages to self-exRNA might suggest one of the possible scenarios:

1). Reduction of MHCII expression along with significantly decreased IL6 and TNFα production by macrophages co-stimulated with self-exRNA leads to downregulation of inflammation, prevention of autoimmunity and setting the stage for healing. This scenario is supported by reports linking self-exRNA to autoimmunity [4]. Paola de Candia et al. [15] note that there are increasing numbers of studies demonstrating a direct role for one of the extracellular RNAs' component, miRNA in regulation of immune responses, including its role in development of regulatory T cells and immune tolerance. In support to P. deCadua et al. [15], Shanshan Liu and co-authors [16] described the role for exRNAs in pathway of systemic lupus erythematosus, specifically pointing out influence of exRNA on T cells function by modulating initial stages of immune responses.

2). Downregulation of MHCII expression induced by co-stimulation of macrophages with higher dose of self-exRNA could possibly result in upregulation of MHCI expression, which would increase population of cytotoxic T cells and enhance tissue damage. As shown in Fig. 5, one of the possible mechanisms would be an enhanced upregulation of Toll-like Receptor Adaptor Molecule (TRAM) – Interferon Regulatory Factor (IRF)3 pathway by engaging TLR4 signaling along with endogenous TLR signaling activated by self-exRNA ligation. Involvement of TLR4 signaling in TRAM-IRF3/9-dependent pathway of interferon (IFN)β production has been known for a long time [17 and 18]. This might lead to upregulation of MHCI expression possibly through type I IFN-dependent pathway (Fig. 6). It has been shown by Ying Chen and co-authors [19] that type I IFN receptors' signaling pathway leads to upregulation of MHCI expression. To show this, authors used type I IFN receptor knockout mice in viral infection's model. The authors' results correspond to the explanation proposed here that activation of TRAM-IRFs pathway though endogenous TLRs ligation by self-exRNA results in upregulation of type I IFNs production leading to MHCI upregulation. M. Tatematsu and co-authors [11] also outline upregulation of IFNs type I production via TRAM-IRFs pathway activated by exRNA. We speculated that this could result in downregulation of MHCII expression and reduced TNFα and IL6 production (Fig. 6). We did not find data showing reciprocal relationships between expression of MHCI and MHCII molecules, or link between upregulation of MHCI and reduced production of TNFα and IL6 by macrophages co-stimulated with self-exRNA, which leaves this possibility open for future research.

Macrophages and astrocytes stimulated with exRNA in combination with TLR2 agonist (Pam2CSK4) have been shown to increase expression of proinflammatory cytokines, specifically TNFα, IL1β and IL6 [3 and 5]. Our data showed that co-stimulation of activated by TLR4 agonist macrophages with 5μg/mL of self-exRNA resulted in the downregulation of both IL6 and TNFα. These results, however, do not rule out ongoing inflammatory responses. There could be increased production of other proinflammatory cytokines (e.g., IL1β). Involvement of IL10 as anti-inflammatory cytokine in this pathway also must be considered as an anti-inflammatory mechanism in this scenario. D. Mosser and Z. Zhang [14] highlighted functional plasticity of macrophages in response to different stimuli, some of which can promote wound healing and anti-inflammatory functions of macrophages.

Thus, we have shown that self-exRNA in dose-dependent manner can influence TLR4 signaling pathway and change the proinflammatory profile of cultured macrophages compared to stimulation of macrophages with TLR4 agonist alone.

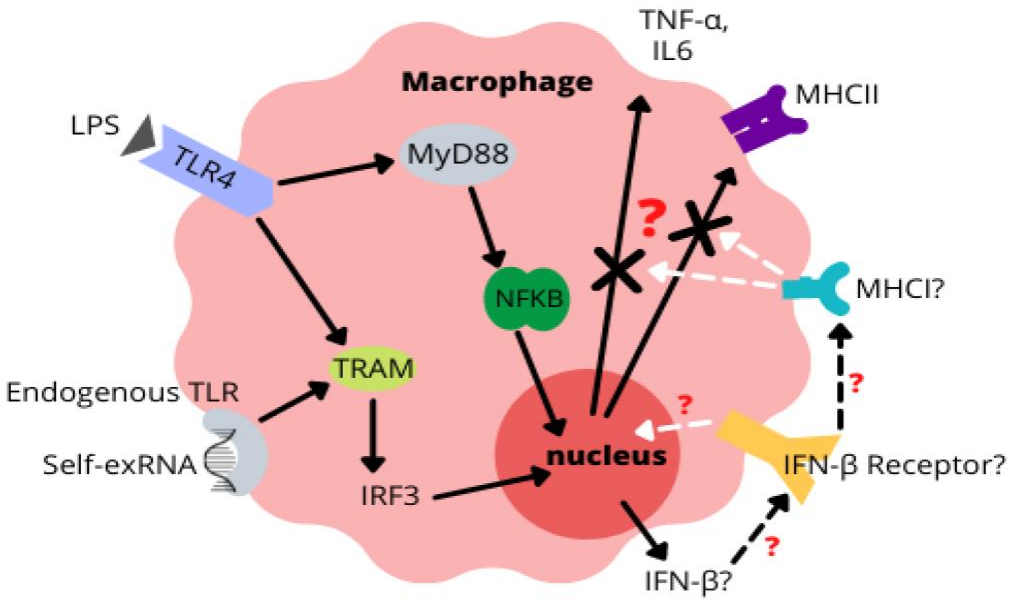


Figure 6. Possible pathway, by which co-stimulation of TLR4 agonist with self-exRNA reduces expression of MHCII and production of TNFα and IL6 in RAW264.7 mouse bone-marrow derived cultured macrophages. Ligation of TLR4 without co-stimulation with self-exRNA leads to activation of Myeloid differentiation primary response 88 (MyD88) adaptor protein, which leads to activation of transcription factor NFkB1 and results in upregulation of MHCII expression, and increased production of TNFα and IL6. Co-stimulation of activated by TLR4 agonist macrophages with self-exRNA leads to downregulation of MHCII expression and reduced production of TNFα and IL6. One of the possible mechanisms would be an enhanced upregulation of TRAM–IRF3 pathway by engaging TLR4 along with endogenous TLR activated by self-exRNA ligation. This might lead to upregulation of MHCI expression (through IFN-dependent pathway), which might result in downregulation of MHCII expression and reduced TNFα and IL6 production.

To further investigate the role for self-exRNA in proinflammatory responses of monocyte-derived cells, we intend to evaluate responses of macrophages activated by different TLRs agonists and explore the involvement of TRAM-IRF signaling pathway that leads to downregulation of MHCII and IL6 and TNFα production.

Acknowledgement

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‡ **TATIANA JONES**, M.D., Ph.D. earned her M.D. degree from Pavlov's State Medical University in St. Petersburg, Russia. From 1987 to 1999 she practiced Rheumatology and completed postdoctoral training along with defending her Ph.D. Thesis. After moving to the USA in 2000, she worked in immunology research at Harvard Medical School. This extremely interesting work resulted in multiple peer-reviewed publications. However, she always had passion for teaching, and since 2013 she has been working as a College Professor and conducting immunology research with undergraduate students. In her free time, she hikes, works on conservation projects, and does some archery. She loves reading a good book and listening to good music.