Extracellular vesicles induced by intravenously administered syngeneic red blood cells modulate macrophage phagocytic activity in mouse humoral immunity*

Zewnątrzkomórkowe pęcherzyki błonowe indukowane podaniem dożylnym syngenicznych erytrocytów modulują aktywność fagocytarną makrofagów w odporności humoralnej u myszy

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Summary
Phagocytosing macrophages are involved in the induction of humoral immunity to corpuscular antigens. Recently, we demonstrated that B cell response to haptenated sheep red blood cells (SRBC) could be suppressed by extracellular vesicles (EVs) produced by suppressor T cells activated through intravenous administration of a high dose of syngeneic mouse red blood cells (sMRBC). However, the mechanism underlying the inhibitory effect of sMRBC-induced EVs on macrophages involved in activation of humoral immunity remained unclear. Thus, the current studies aimed at investigating the phagocytic and antigen-presenting activity of macrophages treated with sMRBC-induced EVs.

Material/Methods: Mouse thioglycollate-induced peritoneal macrophages were treated with sMRBC-induced EVs and then pulsed with either native or fluorescein isothiocyanate-conjugated SRBC. Afterwards, macrophages were, respectively, administered intraperitoneally into naive recipients or subjected to flow cytometric analysis. The elicited humoral immune response was evaluated in plaque forming and haemagglutination assays.

Results: Decreased number of B cells secreting SRBC-specific antibodies was shown in spleens of mouse recipients of SRBC-pulsed macrophages pretreated with sMRBC-induced EVs along with an increased ratio of IgM to IgG serum antibodies. Furthermore, pretreatment of macrophages with sMRBC-induced EVs reduced their phagocytic activity and expression of costimulatory molecules involved in antigen phagocytosis and presentation.

Conclusions: Current research findings demonstrated the impaired ability of macrophages to activate B cells due to the action of sMRBC-induced EVs, which may play a role in suppressing self-reactive B cells. Thus, our results seem to have translational potential in development of therapeutic strategies to prevent the macrophage-induced humoral immunity against nonpathogenic antigens.

Keywords: extracellular vesicles • humoral immunity • immune tolerance • macrophages • phagocytosis

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INTRODUCTION

Due to their ability to phagocytose and intracellularly process antigen to finally present its determinants complexed with major histocompatibility (MHC) class II molecules, macrophages are considered as cells cross linking innate and adaptive immunity. Depending on the type of activating stimuli, macrophages can be classified as classically activated M1 pro-inflammatory phenotype or alternatively activated M2 anti-inflammatory phenotype. However, due to the high plasticity of phenotype switching regulated by current tissue conditions, macrophages usually express the intermediary activation status [21]. Interestingly, recent studies revealed that phagocytic activity of macrophages not only results from but also imprints the heterogeneity of tissue-resident macrophage populations, expressing a great impact on cellular homeostasis of the organism [1]. Consequently, phagocytosing macrophages contribute to both anti-microbial responses activating host immune defense and clearance of cellular debris, allowing immunization to self-cells to be avoided [21]. However, still little is known about phagocytic functions of macrophages involved in the activation of immune tolerance to non-self-antigens.

Our current research findings in mouse model of contact (CHS) and delayed-type hypersensitivity (DTH) reactions [4, 5, 22, 26] demonstrated the crucial role of macrophages in the activation of immune tolerance by CD8\(^+\) suppressor T (Ts) cells [25], activated through intravenous administration of a high dose of syngeneic mouse red blood cells (sMRBC) conjugated with hapten or protein antigen [4]. These cells then release regulatory exosomes carrying miRNA-150 and further coated with antibody light chains [20], enabling the delivery of exosomes to antigen-presenting cells [22]. This finally results in antigen-specific suppression of CHS and DTH reactions in mice [4, 5, 20, 22, 25, 26]. Antigen-presenting macrophages altered with miRNA-150-carrying exosomes, when pulsed with hapten-conjugated sheep red blood cells (SRBC), expressed impaired ability to induce antigen-specific antibody production by B cells [25]. Surprisingly, a similar inhibitory effect was observed in the case of macrophages pretreated with extracellular vesicles (EVs) released by Ts cells activated through intravenous injection of a high dose of sMRBC alone [25]. However, the mechanism underlying the inhibitory effect of sMRBC-induced EVs on macrophages involved in the activation of humoral immunity remained unclear. Thus, we have undertaken the current studies to investigate the phagocytic and antigen-presenting activity of macrophages treated with sMRBC-induced EVs prior to pulsing with SRBC to induce B cell response.

MATERIALS AND METHODS

Mice

Ten to twelve week-old CBA mice from the Breeding Unit of Faculty of Medicine of the Jagiellonian University Medical College (Krakow, Poland) were standardly fed with autoclaved food and water. All experiments were conducted according to the guidelines of Jagiellonian University and Local Ethics Committee (approvals No 39/2011 and 154/2013). Each experiment was repeated at least twice, and the representative results are shown in the figures, and, additionally, the general scheme of experiments is depicted in Fig. 1.

Generation of sMRBC-induced EVs

Freshly collected and deprived of buffy coat syngeneic mouse red blood cells (sMRBC) were intravenously injected in 0.2ml of 10% DPBS suspension on days 0 and 4 into naive mice. On day 11 spleens and lymph nodes of tolerized mice, containing activated Ts cells, were collected and single cell suspensions were cultured in protein-free Mishell-Dutton medium at a concentration of 2x10\(^7\) cells per ml for 48 hours [4, 5, 25]. The resulting supernatant was then centrifuged at 300g and 3,000g for 10 minutes, filtered through 0.45 µm and 0.22 µm molecular filters, which was followed by double ultracentrifugation at 100,000g for 70 minutes at 4\(^\circ\)C [4, 25]. Obtained pellet was resuspended in DPBS and used as sMRBC-induced EVs [4, 25].

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Abbreviations: CHS – contact hypersensitivity; DTH – delayed-type hypersensitivity; EVs – extracellular vesicles; FITC – fluorescein isothiocyanate; FITC-SRBC – fluorescein isothiocyanate-conjugated sheep red blood cells; PFC – plaque forming cells; ROIs – reactive oxygen intermediates; sMRBC – syngeneic mouse red blood cells; SRBC – sheep red blood cells; Ts cells – suppressor T cells.
Macrophage harvest

Peritoneal exudate macrophages were induced by intraperitoneal injection of 2 ml of thioglycolate medium (Gibco Life Technologies, Grand Island, NY) [10] and harvested 5 days later by washing the peritoneal cavity with ice-cold DPBS containing heparin (5U/ml, Polfa, Warszawa, Poland). Then, the percentage of macrophages in isolated exudates was evaluated with non-specific esterase assay [7] and in each case exceeded 96%.

Plaque Forming (PFA) and Haemagglutination (HA) Assays

Macrophages were treated or not in vitro with sMRBC-induced EVs resuspended in DPBS in a dose of around 4×10⁹ EVs (as estimated by Nanoparticle Tracking Analysis [4]) per 1×10⁶ cells for 30 minutes at 37°C waterbath. Then, macrophages were pulsed with sheep red blood cells (SRBC, Graso Biotech, Starogard Gdanski, Poland) by incubation for additional 30 minutes at 37°C in a ratio of 10 SRBC per single macrophage. This was followed by the removal of non-phagocytosed SRBC by osmotic shock. Finally, SRBC-pulsed macrophages were injected intraperitoneally (4×10⁶ cells per recipient) into naive mice. A week later blood sera and spleens of EV-pretreated, SRBC-pulsed macrophage recipients were individually collected.

Single cell suspensions of each spleen in RPMI1640 were incubated with 20% SRBC in the presence of guinea pig complement (Biomed, Lublin, Poland) for 90 minutes at 37°C. Then, the number of hemolytic plaque forming cells (PFC) formed in a PFA performed by a slide technique was enumerated for each spleen in triplicates [8, 10, 23, 25]. The averaged results were then expressed as the number of PFC per spleen.

Titers of anti-SRBC antibodies (IgM and IgG) in whole sera along with titers of IgG antibodies in sera preincubated with 0.15 M 2-mercaptoethanol (Sigma, St. Louis, MO, USA) to eliminate IgM activity were measured in direct HA, as described previously [10, 23, 25]. The results were then expressed as a log2 of titers. Titers of SRBC-specific IgM antibodies were estimated by subtraction of IgG titers from total antibody titers.

The data was analyzed with GraphPad Prism and Excel software with the assessment of statistical significance of differences between groups in two-tailed Student’s T test, and p<0.05 was considered statistically significant.
Phagocytosis assay

Macrophages were treated in vitro with sMRBC-induced EVs as above. Phagocytosis assay was then performed, as recently described by us [9]. Briefly, SRBC, after labelling with fluorescein isothiocyanate (FITC, Sigma, St. Louis, MO, USA), were added to macrophages in a ratio of 10 FITC-SRBC per single macrophage. Cells were incubated for 20 minutes at 37°C, which was followed by removal of non-phagocytosed FITC-SRBC by osmotic shock. To assess the efficacy of FITC-SRBC phagocytosis by EV-pre-treated macrophages, part of the cells, after addition of viability dye, namely 7-amino-actinomycin D (7-AAD, BD Biosciences, San Diego, CA, USA), was stained with allophycocyanin (APC)-conjugated anti-mouse F4/80 monoclonal antibody (eBioscience Inc., San Diego, CA, USA) and then immediately subjected to flow cytometry analysis on FACS Calibur (BD Biosciences, San Jose, CA, USA).

The acquired data were analyzed with BD CellQuest Pro, GraphPad Prism 8 and Excel software. The remaining FITC-SRBC-pulsed macrophages, after osmotic shock, were incubated for additional 120 minutes in 37°C water-bath to allow for eventual antigen processing. Then, the efficacy of phagocytosis was similarly analyzed by flow cytometry after staining with APC-conjugated anti-mouse F4/80 monoclonal antibody and addition of 7-AAD viability dye. The gating strategy and representative results are shown in Fig. 2.

Assessment of macrophage surface markers’ expression

Macrophages incubated or not with sMRBC-induced EVs were pulsed with FITC-SRBC as described above and, after osmotic shock, were incubated for additional 120 minutes in 37°C water-bath to stimulate the processing
and eventual presentation of antigen. Afterwards, cells were stained with APC-conjugated anti-mouse F4/80 monoclonal antibody and with phycoerythrin (PE)-conjugated monoclonal antibodies against one of the following mouse markers: I-A<sup>k</sup>, CD80, CD86, CD40, CD11b, Mac-3 from (BD Biosciences, San Diego, CA, USA), and CD68 or CD206 from (eBioscience Inc., San Diego, CA, USA). Just before cytometric analysis on FACS Calibur (BD Biosciences, San Jose, CA, USA), propidium iodide (PI, BD Pharmingen, San Diego, CA, USA) was added to each sample. The acquired data was then initially analyzed with BD CellQuest Pro software and statistically analyzed as above with GraphPad Prism 5 and Excel software. The gating strategy and representative results are shown in Fig. 2.

**RESULTS**

**Activation of B cell response by macrophages is impaired under the influence of sMRBC-induced EVs**

Our prior studies revealed that both hapten-specific, miRNA-150-carrying exosomes and sMRBC-induced EVs reduce macrophage ability to induce humoral immune response to hapten-conjugated SRBC [25]. However, it has remained unclear whether the suppressive action of EVs on macrophages involved in the activation of B cells could be achieved either due to hapten binding to SRBC or is a general phenomenon, and thus can be observed in the case of humoral immunity to native antigen. To solve these doubts, we have pretreated macrophages with sMRBC-induced EVs prior to pulsing with native SRBC and then transferred them into naive recipients, which was followed by assessment of humoral immune response activation a week later. Consequently, we found the significantly decreased number of B cells releasing SRBC-specific antibodies in spleens of recipients in which untreated macrophages were transferred (Fig. 3A). Furthermore, we detected an increased ratio of SRBC-specific IgM to IgG antibodies in sera of mice administered with EVs-pretreated macrophages (Fig. 3B). These results demonstrated that sMRBC-induced EVs impair macrophage ability to activate antigen-specific B cells and their maturation. Thus, our data confirms that sMRBC-induced EVs affect macrophage functions not only in cell-mediated immune response [25], but also in a humoral immunity.

**sMRBC-induced EVs affect macrophage phagocytic activity**

The above results showed that sMRBC-induced EVs impair macrophage-mediated activation of B cells. However, the mechanism underlying this phenomenon has been yet undefined. We assumed that the impairment of humoral immunity may result from the reduction of antigen phagocytosis or presentation by macrophages, or from altered interaction of macrophages with B cells and T helper lymphocytes. To test these assumptions, we had incubated EV-pretreated macrophages with FITC-coupled SRBC, and then cytometrically analyzed cell viability and the efficacy of phagocytosis. Assessment of macrophage viability after phagocytosis of FITC-SRBC showed no differences between untreated and EV-treated macrophages (Fig. 4A). However, the significantly decreased percentage of viable macrophages that phagocytosed FITC-SRBC was demonstrated in the case of cells pretreated with sMRBC-induced EVs, both when assessed immediately or 2 hours after incubation with antigen (Fig. 4B). Furthermore, after treatment of peritoneal exudate cells with sMRBC-induced EVs, the increased percentage of F4/80<sup>pos</sup> macrophages was detected (Fig. 5A) along with the slightly enhanced expression of this marker on EV-treated macrophages (Fig. 5B). Finally, treatment with sMRBC-induced EVs resulted in significantly reduced percentage of F4/80<sup>pos</sup> macrophages that phagocytosed FITC-SRBC (Fig. 5C). These findings confirmed that sMRBC-induced EVs alter the phagocytic activity of mouse peritoneal macrophages.

**sMRBC-induced EVs influence macrophage activation status**

We assumed that alteration of antigen phagocytosis by F4/80<sup>pos</sup> macrophages [28] can further affect its processing and eventual presentation. To test this hypothesis, we have stained EV-pretreated and FITC-SRBC-pulsed macrophages with fluorochrome-conjugated antibodies against surface
markers of macrophage activation and antigen presentation. Accordingly, we have found no significant differences in expression of MHC class II and CD40 molecules between untreated and EV-pretreated F4/80<sup>pos</sup> macrophages. However, we have observed the decreased percentage of F4/80<sup>pos</sup>CD80<sup>pos</sup> cells among EV-pretreated macrophages along with lower intensity of PE fluorescence emitted by these cells and F4/80<sup>pos</sup>CD86<sup>pos</sup> macrophages (Table 1). These observations suggest that treatment of macrophages with sMRBC-induced EVs slightly influences the expression of costimulatory molecules.

Further, we have analyzed the expression of molecules recognized as markers of macrophage phenotype and activation status, including CD206, i.e. mannose receptor, characteristic for M2 polarized macrophages [21], and Mac3 considered as a marker of activated macrophages [10, 14]. While cytometric analysis demonstrated the similar percentage of cells expressing CD11b and CD68 markers among F4/80<sup>pos</sup> population, the increased percentage of cells expressing Mac3 marker and decreased percentage of cells displaying CD206 receptor was found in the case of F4/80<sup>pos</sup> macrophages pretreated with sMRBC-induced EVs, with

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**Fig. 4.** Assessment of (A) viability and (B) phagocytic activity of peritoneal macrophages pretreated with extracellular vesicles (EVs) induced by syngeneic mouse red blood cell injection prior to pulsing with fluorescein isothiocyanate-conjugated sheep red blood cells (FITC-SRBC), expressed as the percentage of cells not stained with 7-AAD viability dye and expressing FITC fluorescence, respectively. Graphs depict mean ± standard deviation (SD), *p <0.05; **p <0.01.

**Fig. 5.** Assessment of (A) viability, (B) intensity of F4/80 marker expression and (C) phagocytic activity of F4/80<sup>pos</sup> peritoneal macrophages pretreated with extracellular vesicles (EVs) induced by syngeneic mouse red blood cell injection prior to pulsing with fluorescein isothiocyanate-conjugated sheep red blood cells (FITC-SRBC), expressed as the percentage of F4/80<sup>pos</sup> cells not stained with 7-AAD viability dye, as geometric mean of fluorescence intensity (MFI) and the percentage of F4/80<sup>pos</sup> cells expressing FITC fluorescence, respectively. Graphs depict mean ± standard deviation (SD), *p <0.05; ***p <0.005.
corresponding changes in fluorescence intensity (Table 1). Taking into account the similar expression of MHC class II, but lower expression of CD80, one can assume that some of the antigen-primed macrophages influenced with sMRBC-induced EVs seem to undergo the cytotoxic activation, which is in line with the previously reported enhancement of generation of reactive oxygen intermediates (ROIs) by mouse macrophages treated with miRNA-150-carrying EVs induced by intravenous administration of haptenated sMRBC [24].

**DISCUSSION**

Our previous reports implied that mere intravenous administration of a high dose of sMRBC may induce the generation of immune suppressive EVs that target antigen-primed macrophages to eventually down-regulate humoral response against haptenated SRBC [25]. However, the mechanism underlying the observed effect remained unknown. Current research findings revealed that macrophages pretreated with sMRBC-induced EVs express the reduced ability to activate B cell response against native SRBC (Fig. 3) to a similar extent as in the case of haptenated SRBC [25]. Furthermore, an increased ratio of SRBC-specific IgM to IgG antibodies was also observed, suggesting the impairment of immunoglobulin class switching during maturation of antigen-specific B cells. These observations suggest that targeting of macrophages with sMRBC-induced EVs prior to pulsing with an antigen generally affects macrophage ability to activate antigen-specific B cells. sMRBC-induced EVs are suspected to contain immune regulatory miRNA-150 (Nazimek et al., manuscript in preparation) [25], which was formerly shown to down-regulate B lymphocyte maturation [29, 31] and inflammatory functions [30]. However, the effects of miRNA-150 carried in sMRBC-induced EVs on phagocytic macrophages involved in induction of B cell function have not yet been elucidated. Apart from ability to emit fluorescence after excitation, FITC molecules express haptenic properties [6], similarly to trinitrophenol previously used for conjugation with SRBC [25]. Thus, we selected FITC-coupled SRBC to test macrophage phagocytic activity, which showed the decreased percentage of peritoneal exudate macrophages that phagocytosed FITC-SRBC when pretreated with sMRBC-induced EVs (Fig. 4). This result was especially clearly seen in the case of F4/80pos macrophages that tended to slightly dominate the population of viable peritoneal cells after treatment with sMRBC-induced EVs (Fig. 5). However, the difference in the percentage of F4/80pos macrophages that phagocytosed FITC-SRBC between untreated and EV-treated cells was less pronounced after 2-hour incubation. This may result from lower cell death ratio in the case of EV-pretreated F4/80pos macrophages (Fig. 5A). On the other hand, some of the F4/80pos macrophages pulsed with sMRBC-induced EVs and then with antigen seem to be characterized by an intermediary pattern of activation associated with an increased Mac3 and decreased CD206 markers’ expression along with reduced expression of costimulatory molecules (Table 1). It was previously suggested that CD80 and CD86 costimulatory molecules could overlap each other’s function during immunoglobulin class switch towards IgG in a response to antigen administered with an adjuvant [3]. In addition, CD86 may additionally costimulate the activation of Th1 cells in the case of emulsified antigens [2]. This seems to be in line with our observations that a slight decrease in CD80 and CD86 caused by sMRBC-induced EVs may impair the production of IgG in a response to haptenated antigens. Besides, elevated Mac3 expression, in some circumstances observed in classically activated macrophages [11], accompanied with lower expression of CD206 considered as a marker of an alternative macrophage activation [21] suggests that some of the antigen-primed F4/80pos macrophages underwent cytotoxic activation after pretreatment with sMRBC-induced EVs. This is in line with a formerly observed enhancement of generation of ROIs by peritoneal macrophages harvested from mice intravenously administered with a high dose of sMRBC [24] as another pattern of cytotoxic activation, which may be directed against effector T cells [27].

**Table 1.** Expression of surface markers of antigen phagocytosis and presentation, and cell activation by macrophages treated with sMRBC-induced EVs.

| marker (bound by PE-conjugated monoclonal antibodies) | F4/80pos mouse macrophages |
|--------------------------------------------------------|-----------------------------|
|                                                        | untreated (control)         | treated with sMRBC-induced EVs |
|                                                        | % of positive cells | geomean of PE fluorescence | % of positive cells | geomean of PE fluorescence |
|---------------------------------|------------------|----------------------|------------------|----------------------|
| CD11b                           | 97.3 ± 0.00      | 453.8 ± 0.00         | 97.1 ± 0.35      | 462.2 ± 47.3         |
| Mac3                            | 34.6 ± 0.92      | 159.8 ± 8.6          | 41.2 ± 2.76      | 187.5 ± 20.7         |
| I-A^k (MHC II)                  | 26.7 ± 2.33      | 232.1 ± 21.40        | 28.1 ± 0.21      | 230.5 ± 5.30         |
| CD80                            | 64.4 ± 3.25      | 106.1 ± 5.80         | 58.3 ± 1.48      | 98.3 ± 0.99          |
| CD86                            | 78.4 ± 0.78      | 149.9 ± 3.39         | 77.6 ± 1.77      | 142.8 ± 2.76         |
| CD40                            | 10.5 ± 1.63      | 70.8 ± 1.70          | 12.5 ± 0.42      | 71.9 ± 0.85          |
| CD86                            | 92.8 ± 1.48      | 220.7 ± 25.74        | 92.7 ± 0.35      | 223.9 ± 4.03         |
| CD206                           | 50.9 ± 0.92      | 146.2 ± 1.41         | 46.2 ± 0.28      | 137.1 ± 1.91         |
Although the direct macrophage cytotrophic action on B cells remains a matter of speculation, extracellularly-derived ROIs were suggested to induce the B cell apoptosis, which could be reversed by IL-6 [19]. Interestingly, mice lacking the ability to generate ROIs were shown to be more prone to develop autoimmune responses with enhanced IgG and Th1 cell-mediated responses against self-antigen [15]. This observation brings another piece of evidence for the ability of cytotoxically-activated macrophages to down-regulate humoral immunity. Interestingly, miRNA-150 was also suggested to up-regulate the cellular oxidation process, which led to cell senescence [18]. Nonetheless, the involvement of other macrophage-derive secretory factors, like cytokines and EVs especially [13], cannot be excluded. It is worth noting that macrophages treated with miRNA-150-carrying EVs induced in mice by intravenous administration of haptenated sMRBC were suggested to release their own EVs that in turn suppress Th1 lymphocytes [22]. Thus, a similar EV-mediated mechanism could also be responsible for the suppression of B cell response observed under the influence of macrophages treated with sMRBC-induced EVs.

Phenotypic analysis of cells subjected to current assays revealed that they resemble the population of infiltrating macrophages, characteristic for thioglycollate-induced peritoneal exudate [12]. Furthermore, our results imply that sMRBC-induced EVs to some extent may enhance the persistence of F4/80pos macrophages (Fig. 5A). F4/80

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**CONCLUSION**

Our current research findings demonstrated subtle differences in macrophage activation status caused by pre-treatment with sMRBC-induced EVs prior to pulsing with haptenated SRBC. The resulting reduced phagocytic activity and expression of costimulatory molecules likely accompanied by cytotoxic activation, together seem to contribute to impaired ability of macrophages to activate B cells in humoral immune response. Simultaneously, other mechanisms, including EV-mediated intracellular signaling, could preserve this suppressive effect. Furthermore, such an inhibitory effect of sMRBC-induced EVs may play a role in suppressing of self-reactive B cells. Altogether, the impact of sMRBC-induced EVs on macrophage phagocytic and antigen-presenting activity in humoral immune response seems to have an important translational potential in the development of therapeutic strategies to prevent the macrophage-induced humoral immunity against nonpathogenic and self-antigens.
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