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ENHANCED GENERATION OF REACTIVE OXYGEN INTERMEDIATES BY SUPPRESSOR T CELL-DERIVED EXOSOME-TREATED MACROPHAGES

Abstract: Macrophages (Mφ) as efficient phagocytes able to present the antigen and playing an effector role induce and orchestrate the immune response also through the release of soluble factors. Recently described T CD8+ cell-derived suppressive exosomes carrying miRNA-150, that act antigen-specifically, seem to inhibit murine contact sensitivity reaction indirectly by affecting antigen presenting cells, especially Mφ. Present studies investigated the influence of suppressive exosomes on secretory activity of Mφ assessed as their ability to generate reactive oxygen intermediates (ROIs), nitric oxide, cytokines as well as their viability and expression of antigen phagocytosis and presentation markers. Interestingly, *in vivo* and *in vitro* treatment of Mφ with assayed hapten-specific exosomes affected only ROIs generation, significantly enhancing their production. Current results suggest that ROIs may participate in antigen-specific tolerance mechanism mediated by suppressive T lymphocyte-derived exosome-influenced Mφ, by inhibition of effector T cell proliferation and induction of T regulatory lymphocytes.

Key words: macrophages, reactive oxygen intermediates, exosomes, T suppressor lymphocytes, immune suppression, immune tolerance, cytokines, nitric oxide.

INTRODUCTION

Macrophages (Mφ) are the efficient phagocytes present in all body tissues that are responsible for anti-microbial response and clearance of cellular debris. Furthermore, Mφ as the antigen presenting and effector cells induce, orchestrate as well as regulate the adaptive immune response. The immune activity of these cells is *inter alia* mediated by soluble factors released after Mφ activation. The composition of secreted factors mainly is conditioned by the actual phenotype of Mφ, which is activated by various cell-influencing signals [1].

T suppressor factor (TsF) described to be able to inhibit hapten-induced contact sensitivity response in mice was shown to impair Mφ antigen-presenting capacity [2]. Furthermore, previous studies suggested that Mφ after binding of

TsF may release a soluble factor termed macrophage suppressor factor (MSF) inhibiting CS effector cells in an antigen non-specific manner [3–5]. Recent studies defined TsF as miRNA-150 molecule carried by exosomes coated with hapten-specific antibody light chains [6–9]. The TsF exosomes were shown to bind to hapten, enabling their specific purification by antigen affinity column chromatography, and to act antigen-specifically mediating the immune tolerance. Noteworthy, tolerogenesis procedure leading to induction of suppressor T(Ts) cell release of hapten-specific TsF exosomes includes the intravenous administration of hapten-conjugated syngeneic erythrocytes followed by contact immunization with the same hapten. The latter procedure activates the release of B1 cell-derived specific light chains that coat the suppressive, hapten-specific exosomes. However, it was also shown that intravenous administration of high dose of unlabelled syngeneic erythrocytes also induces T suppressor lymphocytes to release exosomes containing miRNA-150 that are not coated with antibody light chains but express similar suppressive activity in antigen non-specific immune assays [9]. These non-specific suppressive exosomes were termed *Sham* factor (SHAM-F).

Since TsF exosomes were previously suggested to affect the various immune functions of Mφ [10], the present studies were aimed to investigate the influence of TsF exosomes on secretory activity of murine peritoneal Mφ assayed as their ability to release cytokines, nitric oxide and reactive oxygen intermediates as well as to determine if exosome treatment affects the Mφ viability along with the expression of markers of antigen phagocytosis and presentation.

MATERIALS AND METHODS

Ten-week-old CBA/J mice were from breeding unit of Department of Immunology, Jagiellonian University Medical College and were treated according to the guidelines of Jagiellonian University Ethics Committee (approval number 39/2011). Each experiment was repeated at least twice and representative results were statistically analyzed and interpreted.

Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Mac-3 mAb, phycoerythrin (PE)-conjugated rat anti-mouse $H2^k$, anti-mouse CD80, anti-mouse CD86, anti-mouse CD11b, anti-mouse CD14 and anti-mouse CD16/32 mAb, FITC-conjugated annexin V, propidium iodide (BD Pharmingen, San Diego, CA), oxazolone (Aldrich Chemical Company, Milwaukee, WI), picryl chloride (PCL, trinitrophenol chloride, TNP-Cl) (Chemtronix, Swannanoa, NC), trinitrobenzene sulphonic acid (TNBSA) (Eastman Chemicals, Rochester, NY) were used.

Mishell-Dutton Medium (MDM), RPMI1640, minimal essential medium with amino acids, HEPES, cacodylic buffer, TRIS buffer, 2-mercaptoethanol, luminol, lucigenin, DMSO, mineral oil heavy fraction, zymosan (Sigma, St. Louis, MO),

fetal calf serum (FCS), Dulbecco's phosphate buffered saline (DPBS), Pen-Strep, sodium pyruvate, L-glutamine (Gibco Life Technologies, Grand Island, NY), acetone, ethanol, glucose, reagents for Griess reaction (P.O.Ch., Gliwice, Poland), heparin (Polfa, Warszawa, Poland), ethylenediaminetetraacetic acid (EDTA) (BDH, Pool, England) and lipopolysaccharide (LPS) (BIO-Whittaker, Walkersville, MD) were used.

Mouse TNF alpha ELISA Ready-SET-Go kit (sensitivity 8 pg/ml), Mouse IL-6 ELISA Ready-SET-Go kit (sensitivity 4 pg/ml), Mouse IL-10 Platinum ELISA Test (sensitivity 5 pg/ml), Mouse TGF-beta1 Platinum ELISA Test (sensitivity 8 pg/ml) (eBioscience, San Diego, CA), Mouse IL-12p40 BD OptEIA Set (sensitivity 15.6 pg/ml) (BD Bioscience, San Diego, CA) were used to measure cytokine concentration in cell culture supernatants.

Ts lymphocytes were induced by double *i.v.* injections of 0.2 ml of 10% suspension of hapten-labelled syngeneic erythrocytes on days 0 and 4 followed by contact immunization on shaved abdomen skin with 0.15 ml of hapten solution on day 9. Then, on day 11 spleens and lymph nodes were harvested to isolate Ts cells for 48-hour culture in MDM at concentration 2×10^7 cells/ml [9]. The released exosomes were isolated from culture supernatant (SN) by double ultracentrifugation at 100000 g for 70 minutes at 4°C after centrifugation at 300 g and 10000 g and filtration through 0.45 μm, 0.22 μm and 0.1 μm molecular filters. Pellet was then resuspended in DPBS [9] and used as hapten-specific suppressive exosomes. SHAM-F exosomes [9] were obtained as above from SN of culture of Ts cells induced by injection of unlabelled MRBC treated as for hapten conjugation and skin application of vehicle without hapten.

Mφ were isolated as mineral oil-induced peritoneal exudate cells from either naive, hapten-contact immunized (Mφ were then treated with exosomes *in vitro* for 30 minutes in 37°C water-bath followed by washing at 300 g) or tolerized mice (Mφ treated with exosomes *in vivo*). In some instances exosome-treated Mφ were labelled with TNP hapten by 10-minute incubation in darkness with TNBSA solution (2 mg/ml) in ratio 0.2 mg TNBSA per 10^7 cells.

Naive or tolerized donor-derived Mφ treated with suppressive exosomes were assayed by flow cytofluorometric analysis (FACSCalibur, BD Biosciences, San Jose, CA, USA) for expression of $H2^k$ (MHC class II), CD80, CD86, CD11b, CD14, CD16/32 surface markers by staining with appropriate monoclonal antibodies as well as for their viability after staining with FITC-conjugated annexin V (for evaluation of apoptosis) and propidium iodide (for evaluation of necrosis).

After similar treatment with exosomes Mφ unstimulated or stimulated with LPS (20 μl of 10 μg/ml solution per well) were cultured in standard conditions (2×10^6 cells per 2 ml per well) and resulting supernatants were collected 24 and 48 hours later for measurement of concentration of nitric oxide in method based on Griess reaction [11] and selected cytokines in ELISA performed according to manufacturer procedures.

The generation of ROIs by Mφ *in vivo* and *in vitro* treated with exosomes was assessed in luminol- and lucigenin-dependent chemiluminescence after stimulation of Mφ oxidative burst with zymosan [12, 13].

RESULTS

THE RELEASE OF CYTOKINES AND NITRIC OXIDE BY CULTURED EXOSOME-TREATED MΦ

Treatment of Mφ with suppressive exosomes did not significantly influence their unstimulated (Fig. 1a) or LPS-stimulated (Fig. 1b) ability to release nitric oxide

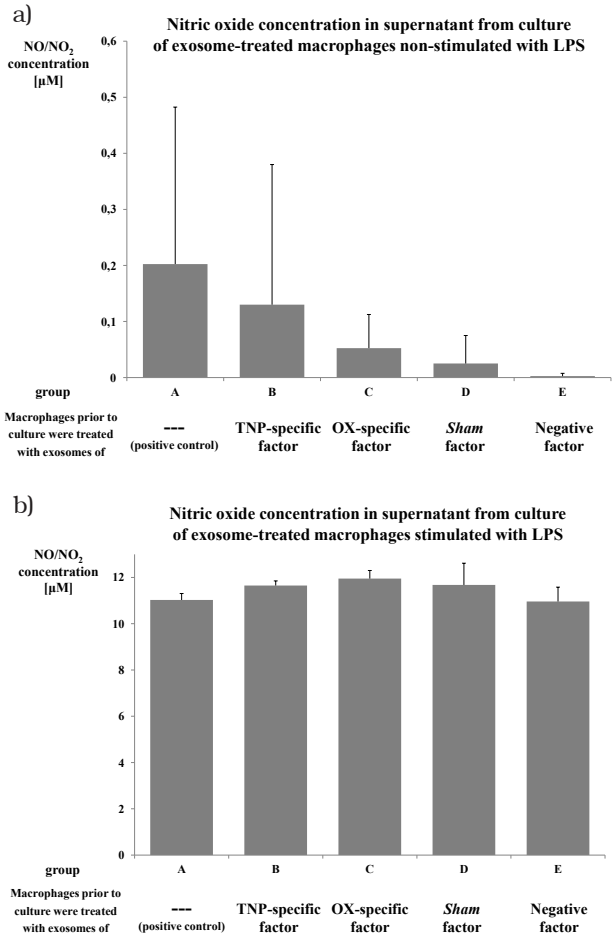

Fig. 1. Macrophages were harvested from naive mice and then treated in vitro with proper exosomes prior to cell culture of macrophages (at concentration 1×10^6 cells per ml) in indicated groups stimulated with LPS (100 ng per 10^6 cells). Concentration of nitric oxide was measured in supernatant collected after 24 hours of cell culture and is expressed in μmoles.

Table 1

Release of pro- and anti-inflammatory cytokines by macrophages treated *in vivo* or *in vitro* with suppressive exosomes.

Cytokine / Macrophages	IL-6	TNFα	IL-12p40	IL-10	TGF-β1
Mφ [control]	56	8	39	19	893±67.0
Mφ [control]+LPS	28006	1860	1110±311.4	378±20.2	642±76.2
Mφ [TNP-TsF]	31	<4	34	<5	706±163.8
Mφ [TNP-TsF]+LPS	25644	2755	1053±162.6	508±24.7	653±206.4
Mφ [OX-TsF]	33	4	36	<5	728±177.1
Mφ [OX-TsF]+LPS	17481	2572±455.0	1147±218.3	525±35.4	559±158.1
Mφ [SHAM-F]	115	11	62	<5	714±145.2
Mφ [SHAM-F]+LPS	24625	2750	1124±175.2	355±7.1	494±48.0
TNP-Mφ control	366±45.1	25±3.9	29±7.1	55±1.3	1136±68.2
TNP-Mφ control+LPS	33809±4238.2	332±12.0	346±40.1	365±40.0	1356±220.8
TNP-Mφ+TNP-TsF	653±3.5	33±1.8	25±2.8	56±6.8	1063±104.2
TNP-Mφ+TNP-TsF+LPS	25741±587.8	348±7.1	379±22.5	331±25.9	1419±217.4
TNP-Mφ+NF	1106±275.9	36±2.1	28±1.5	55±2.8	1652±205.1
TNP-Mφ+NF+LPS	27622±1082.8	296±24.8	379±48.4	333±33.8	1781±412.6
Mφ control	165±30.1	5±0.9	8±1.4	15±0.2	1289±131.3
Mφ control+LPS	33331±13302.4	324±43.8	150±1.4	293±20.8	995±132.3
Mφ+TNP-TsF	163±5.9	5±1.3	7±1.4	18±1.4	1498±85.9
Mφ+TNP-TsF+LPS	30013±9970.2	223±10.0	126±7.1	245±57.7	1097±108.4
Mφ+OX-TsF	200±51.2	<4	12±2.9	12±0.2	1412±101.7
Mφ+OX-TsF+LPS	29341±1533.5	<4	164±11.3	201±27.5	1012±91.1
Mφ+SHAM-F	156±55.1	5±0.64	10±2.8	13±0.2	1220±319.1
Mφ+SHAM-F+LPS	24050±7663.3	286±10.6	145±2.8	208±14.7	1155±136.3
Mφ+NF	235±14.2	7±1.4	14±0.8	12±0.2	1499±148.0
Mφ+NF+LPS	26634±359.5	305±49.5	143±9.9	224±19.8	1043±129.3

First experiment: macrophages were harvested from either naive mice (Mφ [control]) or from donors of exosomes of trinitrophenol-specific T suppressor factor (Mφ [TNP-TsF]), oxazolone-specific TsF (Mφ [OX-TsF]) or *Sham* factor (Mφ [SHAM-F]). Second experiment: macrophages were harvested from naive donors and *in vitro* treated with exosomes of TNP-specific TsF (TNP-Mφ + TNP-TsF) or negative factor (TNP-Mφ + NF) followed by conjugation with TNP hapten. Third experiment: macrophages were harvested from naive mice and then treated *in vitro* with exosomes of TNP-specific TsF (Mφ + TNP-TsF), OX-specific TsF (Mφ + OX-TsF), *Sham* factor (Mφ + SHAM-F) or negative factor (Mφ + NF). Concentrations of tested cytokines are expressed in pg/ml of supernatant collected after 24 hours (IL-6, TNFα, IL-12p40) or 48 hours (IL-10, TGF-β1) of culture of macrophages (at concentration 1×10^6 cells per ml) in indicated groups stimulated with LPS (100 ng per 10^6 cells).

into cell culture supernatant. Further, the unstimulated as well as LPS-stimulated Mφ secretion of pro-inflammatory TNFα, IL-6, IL-12p40 and anti-inflammatory IL-10, TGFβ1 cytokines was not significantly affected by *in vivo* and *in vitro* suppressive exosome-treatment (Table 1). However, the inhibition of TNFα release by unstimulated and LPS-stimulated Mφ treated with oxazolone-specific TsF exosomes was demonstrated.

THE VIABILITY AND EXPRESSION OF SURFACE MARKERS OF MΦ TREATED WITH EXOSOMES

In vivo and *in vitro* treatment of Mφ with assayed suppressive exosomes did not significantly alter the cytofluorometrically analyzed expression of markers of antigen phagocytosis (CD11b, CD14, CD16/32) and presentation (MHC class II, CD80, CD86) (Table 2a). However, apart from CD11b-expressing cells, the slight decrease in the number of Mac-3+ cells with the expression of analyzed markers was assessed. Further, despite the lack of significant differences in viability, the increased ratio of apoptotic to necrotic cells was observed in case of suppressive exosome-treated Mφ with the strongest effect caused by SHAM-F exosomes (Table 2b).

Table 2a

Percentage of macrophages expressing specific surface markers of antigen phagocytosis (CD11b, CD14, CD16/32) and presentation (MHC class II, CD80, CD86).

Marker Macrophages	CD11b	CD14	CD16/32	MHC II	CD80	CD86
Mφ total	96.4	25.8	61.2	45.3	84.9	57.1
Mφ Mac-3$^+$	62.4	14.6	22.1	22.8	13.0	16.6
Mφ total + TNP-TsF	97.0	28.6	62.5	47.4	84.7	56.3
Mφ Mac-3$^+$ + TNP-TsF	43.3	8.7	12.3	11.5	5.9	6.3
Mφ total [TNP-TsF]	92.9	35.6	83.9	81.2	87.8	83.3
Mφ Mac-3$^+$ [TNP-TsF]	48.2	22.6	24.9	35.0	25.5	28.1
Mφ total [SHAM-F]	93.3	40.0	77.4	73.1	89.8	76.6
Mφ Mac-3$^+$ [SHAM-F]	45.2	25.3	27.6	34.2	24.2	26.2
PCL-Mφ total	87.8	45.4	81.5	93.6	85.7	85.2
PCL-Mφ Mac-3$^+$	10.5	37.2	35.9	40.9	34.0	34.3

First experiment: macrophages were harvested from naive mice (Mφ) and treated *in vitro* with exosomes of TNP-specific suppressor factor (Mφ + TNP-TsF). Second experiment: macrophages were harvested either from mice contact sensitized with picryl chloride (PCL-Mφ) or from donors of exosomes of TNP-specific TsF (Mφ [TNP-TsF]) or *Sham* factor (Mφ [SHAM-F]). Macrophages were analyzed as total population of harvested viable cells (Mφ total), that did not bind propidium iodide, or as cells expressing Mac-3 marker (Mφ Mac-3$^+$).

Table 2b

Assessment of viability of exosome-treated macrophages as percentage of cells binding annexin V ($AnnV^+$) and/or propidium iodide (PI^+).

	Mφ	Mφ + TNP-TsF	Mφ [TNP-TsF]	Mφ [SHAM]	PCL-Mφ
PI^+ (necrosis)	18.6	12.9	6.7	10.8	3.9
$AnnV^+$ (early apoptosis)	6.4	9.2	22.2	16.7	29.2
PI^+AnnV^+ (late apoptosis)	8.8	12.0	15.1	20.3	9.0
Total %	33.4	34.1	44	47.8	42.1

First experiment: macrophages were harvested from naive mice (Mφ) and treated *in vitro* with exosomes of TNP-specific suppressor factor (Mφ + TNP-TsF). Second experiment: macrophages were harvested either from mice contact sensitized with picryl chloride (PCL-Mφ) or from donors of exosomes of TNP-specific TsF (Mφ [TNP-TsF]) or *Sham* factor (Mφ [SHAM-F]).

GENERATION OF ROIS BY EXOSOME-TREATED MΦ

The significant enhancement of zymosan-activated generation of ROIs by Mj collected from tolerized donors was observed in luminol- and lucigenin-dependent chemiluminescence assays, in which oxidative burst was activated either just after (Fig. 2a and 2b) or 24 hours after collection of Mj (Fig. 2c and 2d). Similarly,

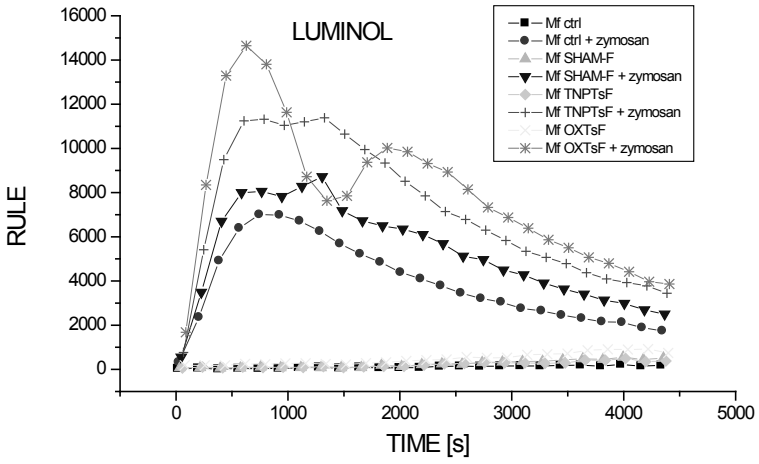

Fig. 2a. Luminol-dependent chemiluminescence measurement of reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from naive mice (Mf ctrl) or donors of exosomes of either Sham factor (Mf SHAM-F), trinitrophenol-specific T suppressor factor (Mf TNPTsF) or oxazolone-specific TsF (Mf OXTsF). ROIs production was stimulated just after harvest of macrophages with zymosan (50 µl). Results are expressed in relative units of luminescence emission (RULE).

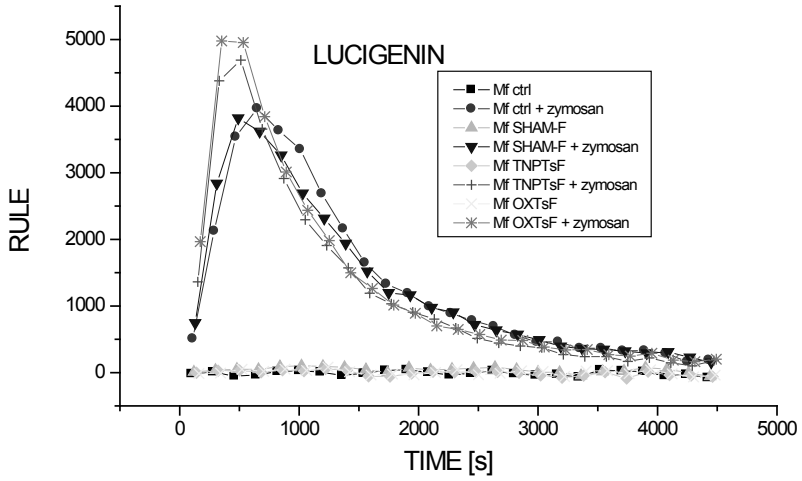

Fig. 2b. Lucigenin-dependent chemiluminescence measurement of reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from naive mice (Mf ctrl) or donors of exosomes of either *Sham* factor (Mf SHAM-F), trinitrophenol-specific T suppressor factor (Mf TNPTsF) or oxazolone-specific TsF (Mf OXTsF). ROIs production was stimulated just after harvest of macrophages with zymosan (50 µl). Results are expressed in relative units of luminescence emission (RULE).

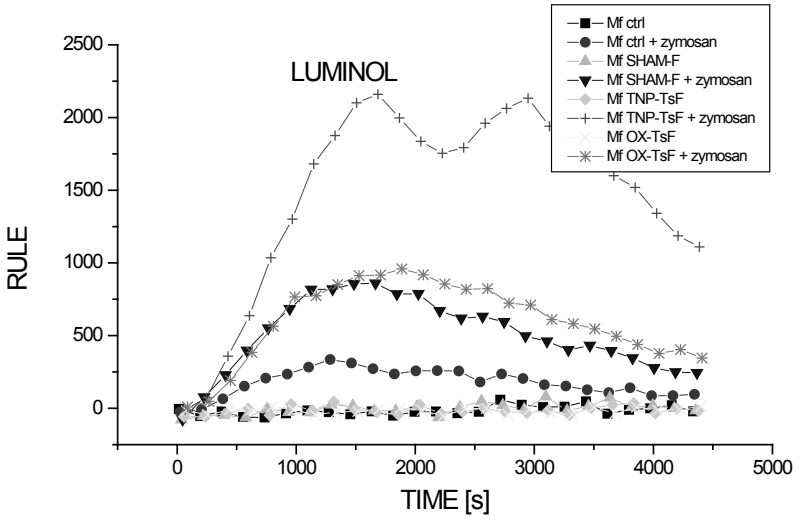

Fig. 2c. Luminol-dependent chemiluminescence measurement of reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from naive mice (Mf ctrl) or donors of exosomes of either *Sham* factor (Mf SHAM-F), trinitrophenol-specific T suppressor factor (Mf TNPTsF) or oxazolone-specific TsF (Mf OXTsF). ROIs production was stimulated after 24 hour culture of macrophages with zymosan (50 µl). Results are expressed in relative units of luminescence emission (RULE).

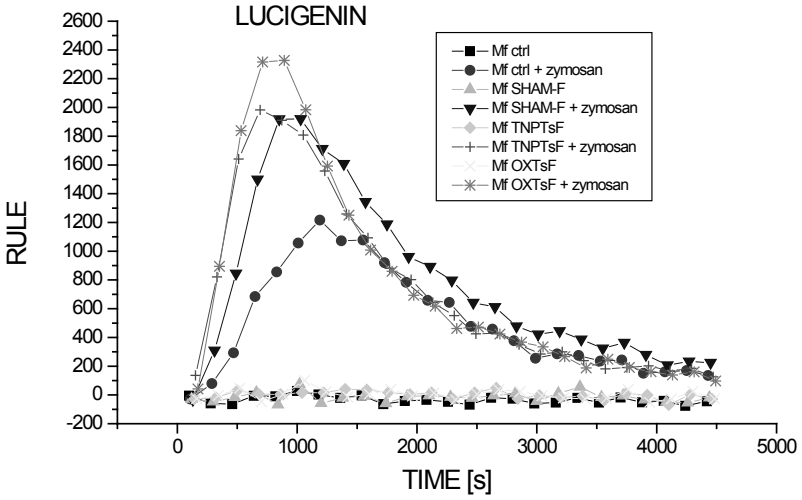

Fig. 2d. Lucigenin-dependent chemiluminescence measurement of reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from naive mice (Mf ctrl) or donors of exosomes of either *Sham* factor (Mf SHAM-F), trinitrophenol-specific T suppressor factor (Mf TNPTsF) or oxazolone-specific TsF (Mf OXTsF). ROIs production was stimulated after 24 hour culture of macrophages with zymosan (50 µl). Results are expressed in relative units of luminescence emission (RULE).

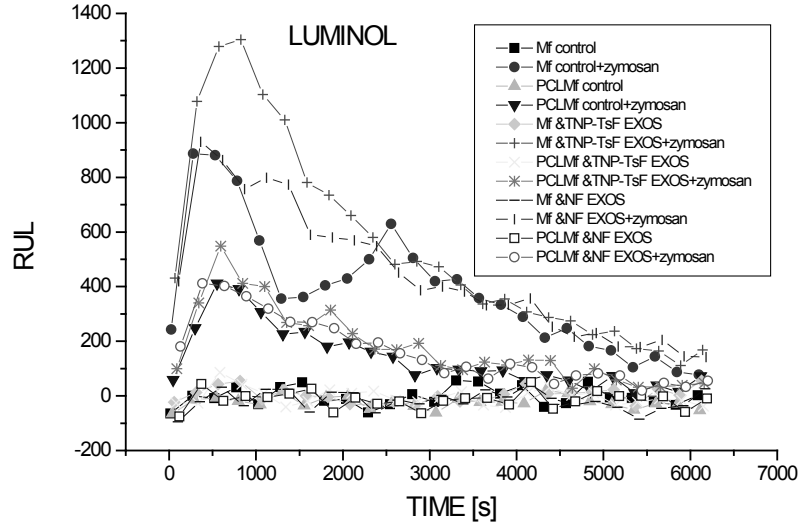

Fig. 3a. Luminol-dependent chemiluminescence measurement of zymosan-stimulated reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from either naive mice (Mf control) or mice contact sensitized with picryl chloride (PCLMf control) that were then *in vitro* treated with exosomes of trinitrophenol-specific T suppressor factor (Mf & TNP-TsF EXOS; PCLMf & TNP-TsF EXOS) or negative factor (Mf & NF EXOS; PCLMf & NF EXOS). Results are expressed in relative units of luminescence (RUL).

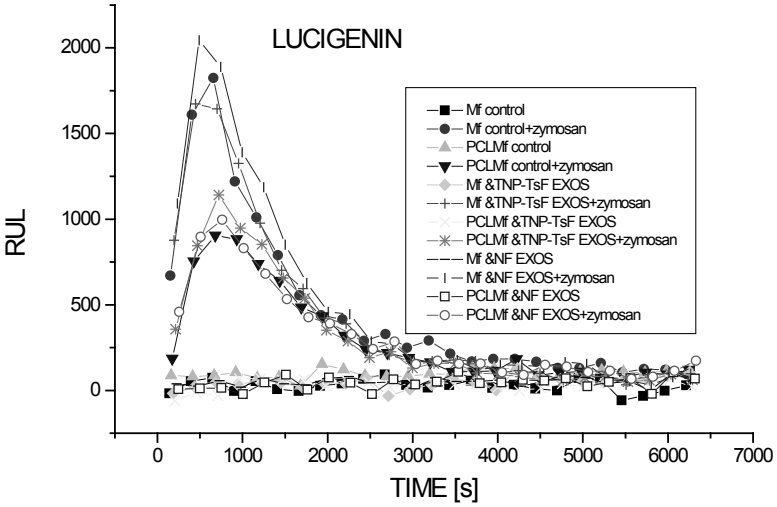

Fig. 3b. Lucigenin-dependent chemiluminescence measurement of zymosan-stimulated reactive oxygen intermediates generation by macrophages (1×10^6 cells per well) harvested from either naive mice (Mf control) or mice contact sensitized with picryl chloride (PCLMf control) that were then *in vitro* treated with exosomes of trinitrophenol-specific T suppressor factor (Mf & TNP-TsF EXOS; PCLMf & TNP-TsF EXOS) or negative factor (Mf & NF EXOS; PCLMf & NF EXOS). Results expressed in relative units of luminescence (RUL).

treatment of oil-induced peritoneal Mj harvested from either naive or PCL hapten contact immunized donors with suppressive TsF and SHAM-F exosomes also resulted in increased generation of ROIs (Fig. 3a and 3b). Interestingly, the chemiluminescence signal was higher for Mj from naive mice in comparison to immunized mice. The results suggest that ROIs as the only one of tested factors released by Mj may participate in investigated antigen-specific suppression mechanism.

DISCUSSION

Results of Ptak *et al.* [2, 4, 5, 10] suggested that TsF released by T CD8+ lymphocytes inhibits murine contact sensitivity reaction through the action on antigen presenting Mφ that then transmit regulatory signal to effector cells of immune response. Moreover, Mφ were shown to be able to bind TsF, which activated their release of secondary suppressor factor termed as macrophage suppressor factor (MSF) [3, 5, 14, 15]. Recently, our research group identified TsF as miRNA-150 secreted by Ts lymphocytes in exosomes coated with B1 cell-derived hapten specific antibody light chains [6–9]. This allowed to re-investigate previously described mechanism of TsF activity. The ability of Mφ to induce adaptive immune response also depends on expression of markers involved in antigen engulfment and presentation as well as on soluble factors released by Mφ, including cytokines,

nitric oxide and ROIs. Therefore, the present studies were aimed to determine if treatment of Mφ with TsF exosomes influence their secretory activity, expression of specific surface markers and viability.

Firstly, the unstimulated and stimulated with pathogen associated molecular patterns (PAMPs) secretory activity of Mφ was assessed after either *in vivo* or *in vitro* treatment of tested cells with suppressive TsF or SHAM-F exosomes. The zymosan-stimulated generation of ROIs by Mφ harvested from naive or hapten contact immunized donors as well as from tolerized mice that were induced for Ts cell release of TsF was measured in the luminol and lucigenin dependent chemiluminescence assay. Noteworthy, luminol may cross cellular membrane, which allows to measure both, extra- and intracellular pools of ROIs, while lucigenin is oxidized only by extracellular ROIs [12, 13, 16, 17].

The significant enhancement of intra- and extracellular ROIs generation was observed under the influence of hapten (either trinitrophenol or oxazolone) specific TsF and SHAM-F exosomes when peritoneal Mφ were obtained from tolerized donors (Fig. 2a and 2b). Interestingly, the same effect was observed even when Mφ oxidative burst was stimulated with zymosan after 24 hour culture of Mφ (Fig. 2c and 2d). However, after cell culture the chemiluminescence signal was reduced, which suggested the diminished metabolic activity and viability of Mφ. Similarly, the increased generation of ROIs was observed as a result of *in vitro* treatment of Mφ from naive or PCL contact sensitized mice with TNP-specific TsF exosomes (Fig. 3a and 3b). Furthermore, the chemiluminescence signal was reduced in case of Mφ collected from sensitized donors in comparison to naive mice, which suggests that previous contact with hapten may influence the Mφ inflammatory response to stimulation with PAMPs.

Current observations suggest that TsF exosomes do not affect phagocytic activity of Mφ. However, Blackstock *et al.* [18] determined the reduced phagocytosis of bacterial antigens by Mφ incubated with supernatant containing crude TsF, which may be the result of unspecific inhibition of phagocytosis during the engulfment of TsF by Mφ. Nevertheless, the presently observed enhancement of ROIs generation by suppressive exosome-treated Mφ could increase their cytotoxic activity. In delayed-type hypersensitivity (DTH), including CS, Mφ apart from antigen-presenting capability, express effector cytotoxic activity, which during the sterile, non-related to infection, inflammatory response is directed against effector lymphocytes leading to the inhibition of their proliferation and differentiation. This in turn suppresses the effector immune response enabling alleviation of allergic clinical symptoms. Tripathi and Hildeman [19] described one of the naturally occurring mechanisms of the limitation of the active immune reaction involving induction of T effector cell apoptosis by ROIs generation, which results in inhibition of T cell expansion and in survival of only the memory T lymphocytes. Worth to note is that ROIs express very short biological half-life and their activity is limited to the cells in the nearest neighborhood of Mφ [20]. Thus, it may be directed aga-

inst T lymphocyte recognizing hapten presented by Mφ [20, 21] to mediate the suppressive effect of TsF exosomes. Additionally, ROIs generated by Mφ may also affect their intracellular signalization pathways [22]. However, antigen presenting cell-derived ROIs along with cytokines were also shown as activators of effector response in immunological synapse [23]. On the other hand, knock-out mice that are unable to generate ROIs after stimulation express increased susceptibility to develop autoimmune response [24]. Moreover, generation of ROIs by Mφ was shown to express protective effect associated with the activation of T regulatory lymphocytes in a course of rheumatoid arthritis [25, 26]. This may suggest that presently observed enhancement of ROIs generation by Mφ treated with suppressive TsF exosomes may also lead to development of T regulatory cells mediating the specific suppression of immune response.

Apart from ROIs, also nitric oxide is an efficient mediator of the cytotoxic response of Mφ. However, *in vitro* treatment of Mφ with hapten-specific TsF or SHAM-F exosomes did not significantly affect their ability to secrete nitric oxide (Fig. 1a and 1b). Macrophage-derived nitric oxide mediates intracellular pathogen -induced DTH reaction, which seems to confirm that TsF exosomes do not impair phagocytic and anti-microbial activity of Mφ. Further, nitric oxide was identified as a potent immunoregulatory factor involved in the suppression of T cell-dependent immunity [27–30], including CS [31]. Therefore, TsF exosomes possibly do not influence nitric oxide-dependent mechanisms of immunoregulation.

Cytokines are the further assayed secretory factors that mediate and orchestrate Mφ antigen non-specific immune activity. Present results showed that *in vivo* and *in vitro* treatment of Mφ with suppressive TsF and SHAM-F exosomes does not significantly influence their unstimulated or LPS-stimulated release of pro-inflammatory IL-6, TNFα, IL-12p40 as well as anti-inflammatory IL-10 and TGF-β_1 cytokines into culture supernatant (Table 1). The observed inhibition of macrophage-activating TNFα release by Mφ treated with oxazolone-specific TsF exosomes may result from difference in mechanism of CS induced by this hapten, which is mediated by T CD8+ cytotoxic lymphocytes rather than Th1 cells and Mφ [32]. Since anti-inflammatory cytokine activity suppresses the response in an antigen non-specific manner, the observed results seem to confirm antigen-specific mechanism of action of TsF exosomes through Mφ, Moreover, it was shown recently that treatment of patients with recombinant IL-10 leads to development of antigen non-specific immune suppression due to the inhibition of expression of co-stimulatory molecules on the surface of antigen presenting cells [33].

To test the potential involvement of alteration of Mφ surface markers expression mediating antigen phagocytosis and presentation in presently studied tolerance mechanism, we have analyzed cytofluorometrically the expression of CD11b, CD14, CD16/32, CD80, CD86 and MHC class II on the surface of Mφ treated either *in vivo* or *in vitro* with TsF or SHAM-F exosomes. Interestingly, the analysis failed to show significant differences in the expression of tested markers on the

surface of total population of assayed cells as well as of the Mac-3+ population of pro-inflammatory Mφ [12] under the influence of exosomes (Table 2a), which confirms aforementioned high specificity of investigated TsF exosome-dependent suppression mechanism. This further allows to avoid the bystander antigen effect often observed in immune suppression induced by non-specific blocking of antigen presentation. Recently, Mi *et al.* described the impairment of antigen presenting capacity of Langerhans cells in miRNA-150 knock-out mice. However, the expression of markers of antigen phagocytosis and presentation was comparable to these assessed in wild type mouse Langerhans cells [34]. Present and latter results suggests that miRNA-150 does not affect the phenotype of Mφ defined as the expression of specific functional markers.

Further, the viability of Mφ treated *in vivo* and *in vitro* with suppressive exosomes was assessed as the percentage of cells binding FITC-conjugated annexin V and/or propidium iodide [35, 36], wherein the cells that bound only either the annexin V or propidium iodide were considered as, subsequently, early apoptotic or necrotic and cells that bound both dyes as late apoptotic.

Although treatment of Mφ with TsF and SHAM-F exosomes did not influence their viability, we observed the increase of the late apoptotic cell percentage in the total analyzed population of Mφ (Table 2b). This suggests that suppressive exosomes may promote the apoptotic cell death rather than necrosis to then prevent the development of inflammation and autoimmunization as well.

To summarize, our results indicate that TsF exosome-mediated immune suppression could be mediated by macrophage-derived ROIs, suggesting their involvement in antigen-specific immune tolerance mechanism. Mφ releasing ROIs may therefore inhibit the proliferation of effector T cells as well as induce the regulatory phenotype of naive T lymphocytes, leading to alleviation of clinical symptoms of allergic cellular immune response observed in mice under the influence of T CD8+ cell-derived suppressive exosomes. However, our preliminary data suggest the release of MSF-remaining factor by Mφ treated with TsF exosomes [37], which, apart from or together with ROIs, may mediate the studied suppression mechanism.

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DISCLOSURES

K.N. planned and performed all the experiments and wrote the manuscript; B.N. performed flow cytofluorometric measurements and was supervised by J.M. who

also revised the manuscript; M.P. supported the preparation of cell cultures; W.P. consulted the experimental protocols and revised the manuscript; K.B. consulted and assisted in planning and performance of experiments, revised manuscript and supervised K.N.

ABBREVIATIONS

Mφ — macrophages
MSF — macrophage suppressor factor
NF — negative factor
OX-TsF — oxazolone-specific T suppressor factor
SHAM-F — control *Sham* factor
TNP-TsF — trinitrophenol-specific T suppressor factor
TsF — T suppressor factor

REFERENCES

1. *Nazimek K., Bryniarski K.*: The activity of macrophages in health and disease. Postepy Hig Med Dosw. 2012; 66: 507–520. **— 2.** *Ptak W., Gershon R.K.*: Immunological agnosis: a state that derives from T suppressor cell inhibition of antigen presenting cells. Proc Natl Acad Sci USA. 1982; 79: 2645–2648. **— 3.** *Marcinkiewicz J., Ptak W.*: Macrophage suppressor factor in contact sensitivity. Mechanisms of its release and action. Immunology. 1980; 41: 211–216. **— 4.** *Ptak W., Zembala M., Gershon R.K.*: Intermediary role of macrophages in the passage of suppressor signals between T-cell subsets. J Exp Med. 1978; 148: 424–434. **— 5.** *Ptak W., Zembala M., Hanczakowski-Rewicka M., Asherson G.L.*: Nonspecific macrophage suppressor factor: its role in the inhibition of contact sensitivity to picryl chloride by specific T suppressor factor. Eur J Immunol. 1978; 8: 645–649. **— 6.** *Bryniarski K., Nazimek K., Martin E., Ptak M., Askenase P.W., Ptak W.*: T CD8+ cell-derived exosomal miRNA-150 suppresses induction and effector phases of murine contact sensitivity as well as symptoms of active allergy. Centr Eur J Immunol. 2014; 39 (Suppl. 1): 20. **— 7.** *Bryniarski K., Nazimek K., Sikora E., et al.*: miRNA-150 inhibits contact sensitivity response via antigen-specific exosomes produced by T suppressor cells. Immunology. 2012; 137 (Suppl. 1): 91. **— 8.** *Bryniarski K., Nazimek K., Sikora E., Ptak M., Askenase P.W., Ptak W.*: TCD8+ suppressor cells produce antigen-specific exosomes carrying miRNA-150 to inhibit contact sensitivity response. Front Immunol. 2013. doi: 10.3389/conf.fimmu.2013.02.00077. **— 9.** *Bryniarski K., Ptak W., Jayakumar A., et al.*: Antigen specific, antibody coated, exosome-like nanovesicles deliver suppressor T cell miRNA-150 to effector T cells in contact sensitivity. J Allergy Clin Immunol. 2013; 132: 170–181.e9. **— 10.** *Ptak W., Marcinkiewicz J., Rewicka M., Różycka D.*: The requirement for T lymphocyte-specific suppressor factors and corresponding antigen in the production of nonspecific suppressor monokines by macrophages. J Reticuloendothel Soc. 1980; 27: 575–584.

11. *Marzinzig M., Nussler A.K., Stadler J., et al.*: Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate, and S-nitrosothiols. Nitric Oxide. 1997; 1: 177–189. **— 12.** *Filipczak-Bryniarska I., Nowak B., Sikora E., et al.*: The influence of opioids on the humoral and cell-mediated immune responses in mice. The role of macrophages. Pharmacol Rep. 2012; 64: 1200–1215. **— 13.** *Nazimek K., Nowak B., Ptak W., Bryniarski K.*: Exosomal T cell suppressor factor inhibits the generation of reactive oxygen intermediates in murine peritoneal macrophages. Immunology. 2012; 137 (Suppl. 1): 693. **— 14.** *Asherson G.L., Zembala M.*: T cell suppression of contact sensitivity in the mouse. III. The role of macrophages and the specific triggering of nonspecific sup-

pression. Eur J Immunol. 1974; 4: 804–807. — **15.** *Zembala M., Asherson G.L.*: T cell suppression of contact sensitivity in the mouse. II. The role of soluble suppressor factor and its interaction with macrophages. Eur J Immunol. 1974; 4: 799–804. — **16.** *Caldefie-Chezet F., Walrand S., Moinard C., Tridon A., Chassagne J., Vasson M.P.*: Is the neutrophil reactive oxygen species production measured by luminol and lucigenin chemiluminescence intra or extracellular? Comparison with DCFH-DA flow cytometry and cytochrome c reduction. Clin Chim Acta. 2002; 319: 9–17. — **17.** *Kopprasch S., Pietzsch J., Graessler J.*: Validation of different chemilumigenic substrates for detecting extracellular generation of reactive oxygen species by phagocytes and endothelial cells. Luminescence. 2003; 18: 268–273. — **18.** *Blackstock R., Zembala M., Asherson G.L.*: Functional equivalence of cryptococcal and haptene-specific T suppressor factor (TsF). I. Picryl and oxazolone-specific TsF, which inhibit transfer of contact sensitivity, also inhibit phagocytosis by a subset of macrophages. Cell Immunol. 1991; 136: 435–447. — **19.** *Tripathi P., Hilderman D.*: Sensitization of T cells to apoptosis — a role for ROS? Apoptosis. 2004; 9: 515–523. — **20.** *Kusmartsev S., Nefedova Y., Yoder D., Gabrilovich D.I.*: Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol. 2004; 172: 989–999.

21. *Hultqvist M., Olsson L.M., Gelderman K.A., Holmdahl R.*: The protective role of ROS in autoimmune disease. Trends Immunol. 2009; 30: 201–208. — **22.** *Forman H.J., Torres M.*: Signaling by the respiratory burst in macrophages. IUBMB Life. 2001; 51: 365–371. — **23.** *Tse H.M., Milton M.J., Schreiner S., Profozich J.L., Trucco M., Piganelli J.D.*: Disruption of innate-mediated proinflammatory cytokine and reactive oxygen species third signal leads to antigen-specific hyporesponsiveness. J Immunol. 2007; 178: 908–917. — **24.** *Hultqvist M., Olofsson P., Holmberg J., Backstrom B.T., Tordsson J., Holmdahl R.*: Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to mutation in the Ncf1 gene. Proc Natl Acad Sci USA. 2004; 101: 12646–12651. — **25.** *Gelderman K.A., Hultqvist M., Pizzolla A., et al.*: Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species. J Clin Invest. 2007; 117: 3020–3028. — **26.** *Kraaij M.D., Savage N.D., van der Kooij S.W., et al.*: Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species. Proc Natl Acad Sci USA. 2010; 107: 17686–17691. — **27.** *Albina J.E., Abate J.A., Henry W.L. Jr.*: Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-γ in the induction of the nitric oxide-synthesizing pathway. J Immunol. 1991; 147: 144–148. — **28.** *Bingisser R.M., Tilbrook P.A., Holt P.G., Kees U.R.*: Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol. 1998; 160: 5729–5734. — **29.** *Silberman D., Bucknum A., Bartlett T., et al.*: CD28 ligation increases macrophage suppression of T cell proliferation. Cell Mol Immunol. 2012; 9: 341–349. — **30.** *Silberman D., Bucknum A., Kozlowski M., Matlack R., Riggs J.*: Cytokine treatment of macrophage suppression of T cell activation. Immunobiology. 2010; 215: 70–80.

31. *Ross R., Reske-Kunz A.B.*: The role of NO in contact sensitivity. Int Immunopharmacol. 2001; 1: 1469–1478. — **32.** *Xu H., Banerjee A., Dilulio N.A., Fairchild R.L.*: Development of effector CD8+ T cells in contact hypersensitivity occurs independently of CD4+ T cells. J Immunol. 1997; 158: 4721–4728. — **33.** *Sky Ng T.H., Britton G.J., Hill E.V., Verhagen J., Burton B.R., Wraith D.C.*: Regulation of adaptive immunity; the role of interleukin-10. Front Immunol. 2013. doi: 10.3389/fimmu.2013.00129. — **34.** *Mi Q.S., Xu Y.P., Qi R.Q., Shi Y.L., Zhou L.*: Lack of miRNA miR-150 reduces the capacity of epidermal Langerhans cell cross-presentation. Exp Dermatol. 2012; 21: 876–877. — **35.** *Lecoeur H., Prevost M.C., Gougeon M.L.*: Oncosis is associated with exposure of phosphatidylserine residues on the outside layer of the plasma membrane: a reconsideration of the specificity of the annexin V/propidium iodide assay. Cytometry. 2001; 44: 65–72. — **36.** *Vermes I., Haanen C., Steffens-Nakken H., Reutelingsperger C.*: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995; 17: 39–51. — **37.** *Nazimek K., Nowak B., Ptak M., Ptak W., Bryniarski K.*: Essential role of macrophages in antigen-specific suppression of immune response mediated by T CD8+ lymphocyte-derived regulatory exosomes. Centr Eur J Immunol. 2014; 39 (Suppl. 1): 10.

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