

ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Syngeneic red blood cell-induced extracellular vesicles suppress delayed-type hypersensitivity to self-antigens in mice

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Abstract

Background: At present, the role of autologous cells as antigen carriers inducing immune tolerance is appreciated. Accordingly, intravenous administration of haptenated syngeneic mouse red blood cells (sMRBC) leads to hapten-specific suppression of contact hypersensitivity (CHS) in mice, mediated by light chain-coated extracellular vesicles (EVs). Subsequent studies suggested that mice intravenously administered with sMRBC alone may also generate regulatory EVs, revealing the possible self-tolerogenic potential of autologous erythrocytes.

Objectives: The current study investigated the immune effects induced by mere intravenous administration of a high dose of sMRBC in mice.

Methods: The self-tolerogenic potential of EVs was determined in a newly developed mouse model of delayed-type hypersensitivity (DTH) to sMRBC. The effects of EV's action on DTH effector cells were evaluated cytometrically. The suppressive activity of EVs, after coating with anti-hapten antibody light chains, was assessed in hapten-induced CHS in wild-type or miRNA-150^{-/-} mice.

Results: Intravenous administration of sMRBC led to the generation of CD9 + CD81+ EVs that suppressed sMRBC-induced DTH in a miRNA-150-dependent manner. Furthermore, the treatment of DTH effector cells with sMRBC-induced EVs decreased the activation of T cells but enhanced their apoptosis. Finally, EVs coated with antibody light chains inhibited hapten-induced CHS.

Conclusions and Clinical Relevance: The current study describes a newly discovered mechanism of self-tolerance induced by the intravenous delivery of a high dose of sMRBC that is mediated by EVs in a miRNA-150-dependent manner. This mechanism implies the concept of naturally occurring immune tolerance, presumably activated by overloading of the organism with altered self-antigens.

KEYWORDS

delayed-type hypersensitivity to self-antigen, extracellular vesicles, immune suppression, miRNA-150, mouse model of delayed-type hypersensitivity to self-antigen, self-tolerance

1 | INTRODUCTION

Peripheral immune tolerance to self-antigens is conferred by immune cells expressing regulatory phenotypes, including different T cell subsets.^{1,2} However, regulatory T (Treg) cell-mediated tolerance can be broken quite easily.³ This promotes the development of autoimmunity, drawing attention to the clinical importance of newly elaborated methods that enable the preservation or restoration of immune regulatory cell activity. Some of the currently investigated approaches are based on the observation that antigen coupled to autologous cells induces tolerance rather than effector responses.⁴ Accordingly, autologous erythrocytes were proposed to constitute good carriers of antigens to induce specific Treg cells.⁵

Along these lines, recent findings revealed that intravenous administration of a high dose of syngeneic mouse red blood cells (sMRBC) coupled with hapten or protein antigen induces CD8⁺ suppressor T (Ts) cells in mice.^{6,7} These regulatory cells inhibit contact (CHS) or delayed-type hypersensitivity (DTH) reactions by releasing miRNA-150-carrying extracellular vesicles (EVs), further coated with B1 cell-derived, antigen-specific antibody free light chains (LCs).⁸⁻¹⁵ It is worth noting that surface expression of LCs confers antigen specificity of tolerance mediated by EV-carried miRNA-150.¹¹ Simultaneously, mice merely injected with a high dose of sMRBC alone generate EVs capable of inhibiting the function of antigen-presenting macrophages,¹⁴ which implies their tolerogenic potential and possible self-protecting activity. Therefore, our current study aimed to investigate the immune effects induced by EVs generated in response to mere intravenous administration of a high dose of sMRBC

in mice. We also assessed the self-protecting potential of EVs by establishing a new model of DTH to sMRBC.

2 | METHODS

2.1 | Mice

Ten- to 12-week-old male mice (C57BL/6, miRNA-150^{-/-} and CBA inbred strains) were purchased from Jackson Laboratories, and some of the CBA mice were obtained from the Breeding Unit of the Jagiellonian University Medical College, Faculty of Medicine. Mice were fed autoclaved food and water ad libitum. This study was carried out in accordance with the guidelines of the European Union Directive 2010/63/EU and the National Institutes of Health guide for the care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee of Yale University, New Haven, CT (Permit Number 07381) and 1st Local Ethics Committee of the Jagiellonian University, Krakow, Poland (Permit Number 39/2011, 40/2011, 106/2012 and 124/2018). All procedures were performed under ether or isoflurane anaesthesia, and all efforts were made to minimize suffering. The experimental design is depicted in Figure 1.

2.2 | Induction and elicitation of active DTH to self-antigens of sMRBC

Freshly collected sMRBC in 50% DPBS suspension were coupled with ovalbumin (OVA, Sigma) by incubation with 1% OVA solution

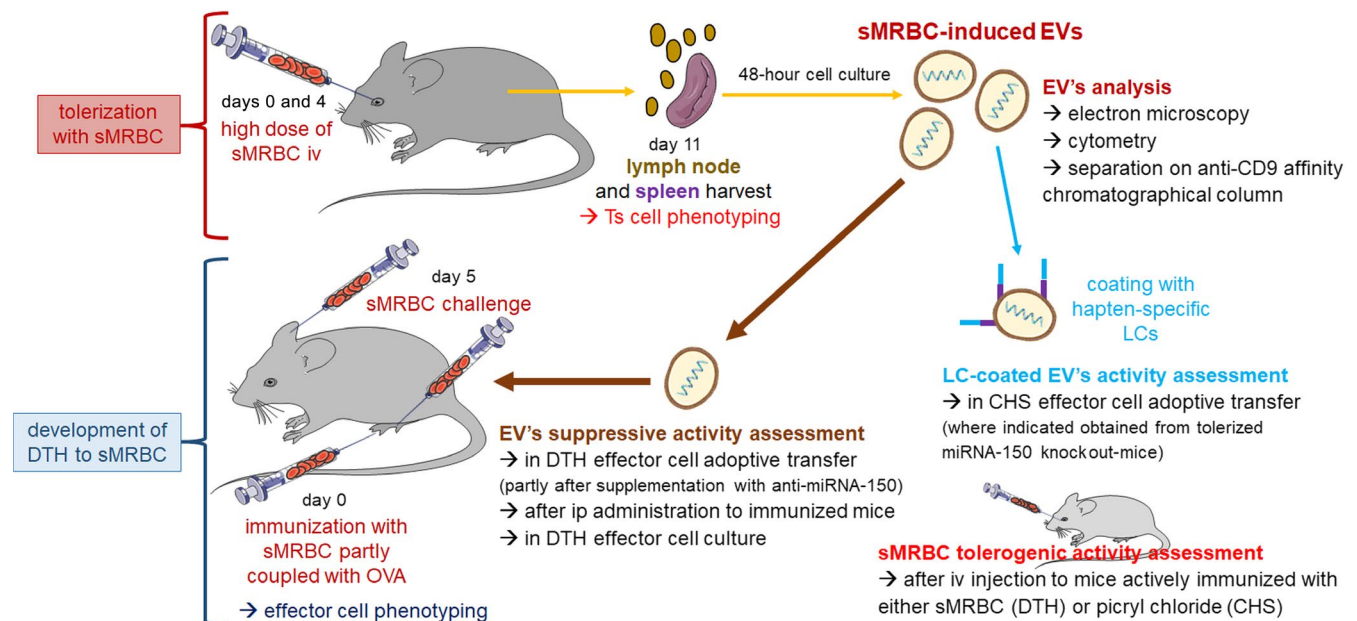


FIGURE 1 Experimental design. To induce immune tolerance, mice were intravenously (iv) injected with a high dose of syngeneic red blood cells (sMRBC) at days 0 and 4, and 7 d later lymph nodes and spleens were collected for phenotyping and culturing of suppressor T (Ts) cells that release sMRBC-induced extracellular vesicles (EVs). Otherwise, mice were intradermally injected into footpads with sMRBC mixed with ovalbumin (OVA)-coupled sMRBC, and 5 d later were intradermally injected into footpads and ears with sMRBC alone to elicit delayed-type hypersensitivity (DTH) reaction, with phenotyping of effector cells. Further, sMRBC-induced EVs were analysed for their biological properties and immune function in DTH and, after coating with hapten-specific antibody light chains (LCs), in contact hypersensitivity (CHS)

in DPBS (in a ratio 1:5 v/v) for 60 minutes at room temperature on a haematological roller in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Pierce, Thermo Fisher Scientific).¹¹

Naive or sMRBC-tolerized mice were actively immunized with sMRBC by intradermal injection of 50 μ L of 10% suspension of the 1:1 mixture of sMRBC and OVA-coupled sMRBC in 0.9% NaCl into each hind footpad. Five days later, immunized mice were challenged by intradermal injections with 10 μ L of 20% sMRBC in 0.9% NaCl per ear and with 15 μ L of 20% sMRBC in 0.9% NaCl per footpad into both ears or both hindlimbs, respectively, to elicit DTH. The resulting ear and footpad swelling was measured between 24 and 120 hours after challenge with an engineer's micrometre by a blinded observer, as described below. Where indicated, mice immunized with the sMRBC/OVA-sMRBC mixture were challenged with 20% sheep red-blood-cell (SRBC) suspension in 10 μ L or 15 μ L of 0.9% NaCl injected into the ear or footpad, respectively, to assess the antigen specificity of the developed DTH swelling response. Additionally, just after measuring the 24-hour DTH swelling response, some mice were intraperitoneally administered with sMRBC-induced EVs. Where indicated, blood serum was collected from sMRBC-immunized mice 48 hours after challenge.

2.3 | Adoptive transfer of sMRBC-induced DTH effector cells

Five days after immunization with sMRBC/OVA-sMRBC, mice were killed, and lymph nodes and spleens were collected to enrich for DTH effector cells. Where indicated, DTH effector cells were depleted using monoclonal antibodies against CD4 (clone GK1.5) or CD8 (clone 53-6.7 TIB-105) with added rabbit complement for 1 hour in a 37°C water bath. All hybridoma cells were from American Type Culture Collection (ATCC), and antibodies were obtained and chromatographically purified at the Department of Immunology, Jagiellonian University Medical College, Krakow, Poland. Otherwise, cells were positively selected with CD4 (L3T4) MicroBeads separated on a MiniMACS MS column according to the manufacturer's procedure (Miltenyi Biotec). The efficacy of magnetic-activated cell sorting (MACS) was then assessed with flow cytometry and reached 95%. DTH effector cells were treated either with miRNA-150 alone or with sMRBC-induced EVs, in some cases supplemented with anti-miR-150, for 30 minutes in a 37°C water bath.⁹ After washing, DTH effector cells were transferred intravenously (7×10^7 cells per mouse) into naive recipients; 24 hours later, the recipients were challenged with sMRBC to elicit a DTH response. This newly elaborated methodology was partly modelled on the DTH response to SRBC.¹⁶ In parallel, lymph node DTH effector cells were cultured for 24 hours in the presence of anti-CD3 monoclonal antibodies (clone 145-2C11 CRL-1975, ATCC, antibodies were produced and chromatographically purified at the Department of Immunology, Jagiellonian University Medical College) and/or sMRBC-induced EVs to produce culture supernatant. Along with blood sera, the supernatant was further used to

measure IFN- γ concentrations by ELISA (according to the manufacturer's instructions with the use of Mouse IFN γ ELISA Ready-SET-Go kit, eBioscience Inc). Blood sera were also subjected to direct haemagglutination to determine the potential presence of antibodies against sMRBC.¹⁴

2.4 | Intravenous administration of sMRBC (tolerization) and harvesting of EVs

On days 0 and 4, CBA or miRNA-150^{-/-} mice were intravenously injected with 0.2 mL of 10% DPBS suspension of sMRBC.¹⁴ On day 11, lymph nodes and spleens were collected, and single-cell suspension (2×10^7 cells per mL) was cultured in protein-free Mishell-Dutton medium for 48 hours at 37°C and 5% CO₂ after enriching for CD8⁺ cells by MACS or depleting CD3⁺ cells with specific antibodies and rabbit complement, where indicated, with efficacy reaching 92% and 100%, respectively.^{9,10,13,14} The resulting culture supernatant was subsequently centrifuged at 300 g and 3 000 g to remove cellular debris, filtered through 0.45- μ m and 0.22- μ m molecular filters and then ultracentrifuged twice at 100 000 g for 70 minutes at 4°C. Pelleted EVs were resuspended in DPBS and, in some instances, incubated overnight on ice with anti-hapten (trinitrophenol, TNP, or oxazolone, OX) monoclonal antibody LCs,^{9,11,14} produced as formerly described.¹⁷ This was followed by ultracentrifugation to remove unbound LCs. Further, EVs from miRNA-150^{-/-} mice were additionally incubated overnight on ice with synthetic miRNA-150 (mmu-miR-150-5p, GE Dharmacon), followed by ultracentrifugation to remove excessive miRNA molecules. Otherwise, sMRBC-induced EVs of wild-type mice were supplemented with anti-miR-150 (mmu-miR-150-5p hairpin inhibitor, GE Dharmacon) with a 1-hour incubation at 37°C.⁹ Finally, sMRBC-induced EVs were visualized by transmission electron microscopy or were separated by affinity chromatography on columns filled with CNBr-activated Sepharose (GE Healthcare) conjugated to anti-CD9 monoclonal antibodies (BD Biosciences).⁹

2.5 | Cytometric analysis of sMRBC-induced EVs

DPBS-resuspended EVs were coated onto aldehyde/sulphate latex beads (4% w/v, 4 μ m; Life Technologies, Thermo Fisher Scientific, Carlsbad, CA) for 2 hours at room temperature with gentle agitation. Then, any remaining binding sites were blocked with 1 mL of 100 mmol/L glycine solution by a 30-minute incubation at room temperature with gentle agitation. After extensive washing in DPBS with 0.1% bovine serum albumin (BSA) at 600 g, bead-coated EVs were stained with phycoerythrin (PE)-conjugated monoclonal antibodies against mouse CD9 (clone KMC8), CD63 (clone NVG-2) or CD81 (clone Eat2) molecules and with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against mouse κ light chains (clone 187.1, all from BD Biosciences) for 40 minutes at room temperature in the dark. Cells were then washed with 0.1%

BSA and acquired by a BD FACSCalibur, with data analysis using BD CellQuest Pro software (Figure S1A).

2.6 | DTH effector cell culture

Thioglycollate-induced peritoneal macrophages were pulsed with sMRBC by a 20-minute incubation at 37°C in water bath, followed by osmotic shock to remove non-phagocytosed sMRBC. DTH effector cells (1×10^6 cells per well) from the draining lymph nodes of mice immunized with sMRBC were stimulated with either anti-CD3 monoclonal antibodies (2.4 µg/well) and IL-2 (3 U/well) or with sMRBC-pulsed macrophages (1×10^5 cells per well). They were then partly treated with sMRBC-induced EVs and cultured for either 3 hours to assess apoptosis; 18 hours to assess CD69 expression; or 24 hours to analyse expression of CD25, CD62L and CD44 markers on CD4 + cells by flow cytometry with the use of annexin-V and propidium iodide or fluorescent monoclonal antibodies (all from BD Biosciences, Figure S1B).

2.7 | Induction and elicitation of active or adoptively transferred CHS reaction

Naive or sMRBC-tolerized mice were actively contact sensitized and 5 days later challenged with picryl chloride (PCL, Chemtronix) as described elsewhere.^{9,10,12,14} After 24 hours, ear swelling was measured with an engineer's micrometre (Mitutoyo) by a blinded observer.¹⁸ Background non-specific increases in ear thickness in non-sensitized, but similarly challenged, littermates were subtracted from experimental groups to yield a net swelling value expressed as $\Delta \pm$ standard error (SE) [$U \times 10^{-2}$ mm]. OX hapten (Sigma) in 3% solution was selected to efficiently sensitize miRNA-150^{-/-} and C57BL/6 mice.⁹⁻¹¹ CHS effector cells were collected 5 days after sensitization with PCL or OX and then treated with various vesicle preparations for 30 minutes at 37°C in water bath.^{9,10} These cells were then intravenously transferred (7×10^7 cells per mouse) into naive recipients that were immediately challenged to elicit CHS ear swelling, as measured as above.

2.8 | Statistical analysis

Each experiment was carried out at least 2×, and the results of representative experiments are shown in the figures. Experimental and control groups consisted of 4-6 mice. Average values of non-specific increases of ear thickness due to chemical irritation by vehicle and hapten in challenged, but not sensitized mouse littermates, were subtracted from average values in experimental groups to obtain a net swelling value (Δ). In the case of in vitro tests, the results of repeated experiments were pooled for statistical analysis. Statistical significance of the data was estimated (after control of meeting of test assumptions) by one-way or two-way analysis of variance (ANOVA) with post hoc RIR Tukey test or two-tailed Student's *t* test, and $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | DTH to self-antigens of red blood cells is mediated by CD4 + T cells and macrophages

Measurable swelling of ear and footpad skin, peaking 48-72 hours after challenge, was detected in mice immunized with the mixture of sMRBC and OVA-sMRBC and challenged with sMRBC. Importantly, it was significantly greater than the background, non-specific swelling of ear and footpad skin caused by the administration of sMRBC to non-immunized littermates (Figure 2A). To assess whether the developed immune reaction was antigen-specific and directed against sMRBC antigens, we challenged OVA-MRBC/sMRBC-immunized mice with SRBC. This resulted in significantly lower net swelling of ear and footpad skin when compared to the response elicited by sMRBC (Figure 2B). Adoptive transfer of lymph node cells and splenocytes of immunized mice allowed elicited skin swelling in recipient mice challenged with sMRBC (Figure 3). However, depletion of CD4 + T cells greatly reduced the elicited skin swelling response mediated by the adoptively transferred DTH effector cells, similar to the removal of nylon-adherent cells, while depletion of CD8 + T cells slightly enhanced the swelling response (Figure 3A). Additionally, adoptive transfer of positively selected CD4 + effector T cells induced a stronger DTH reaction in recipient mice than unseparated DTH effector cells (Figure 3B). In parallel, sMRBC-agglutinating antibodies were not detected in the sera of mice actively immunized with sMRBC (data not shown). Altogether, the above results indicate that the immune response to sMRBC is mediated by CD4 + helper T lymphocytes and macrophages, which is typical for the effector phase of a classical DTH reaction.¹⁹

3.2 | DTH to self-red blood cells is suppressed by sMRBC-induced EVs in a miRNA-150-dependent manner

Subsequently, we investigated whether intravenous administration of sMRBC and induced EVs suppress DTH to self-red blood cells. Accordingly, sMRBC-tolerized mice developed an inhibited DTH reaction, similar to mice that received an intraperitoneal injection of sMRBC-induced EVs 24 hours after challenge (Figure 4A). Further, treatment of DTH effector cells with sMRBC-induced EVs led to significant suppression of the skin swelling elicited in adoptively transferred recipients, while supplementation of sMRBC-induced EVs with anti-miR-150 completely blocked their suppressive activity (Figure 4B).

However, while we did not detect IFN- γ in the blood sera of mice in each treatment group shown in Figure 4A (data not shown), lymph node effector cells from mice immunized with a mixture of sMRBC/OVA-sMRBC released vast amounts of IFN- γ into the culture supernatant after stimulation with anti-CD3, and the addition of sMRBC-induced EVs failed to significantly modulate this effect (Figure 4C).

This supports our hypothesis on the DTH nature of the immune response to sMRBC.

3.3 | sMRBC-induced EVs are produced by CD3 + CD8 + Ts cells and express CD9 and CD81 tetraspanins

To determine the phenotype of EV-releasing cells, we enriched CD8 + T cells or depleted CD3 + lymphocytes from sMRBC-tolerized mouse lymph nodes and spleens prior to culture. Accordingly, EVs produced by enriched CD8 + T cells suppressed adoptively transferred DTH effector cells, while EVs released by populations deprived of CD8 + or CD3 + T cells failed to inhibit the transferred DTH response (Figure 5A).

Further, electron microscopy revealed the presence of EVs with a bilamellar membrane, resembling exosome-like nanovesicles, in the culture supernatant of lymph node and spleen cells of

sMRBC-tolerized mice (Figure 5B). By means of flow cytometry, sMRBC-induced EVs were shown to express CD9 and CD81 but not CD63 tetraspanins (Figure 5C). Subsequent fractionation of the sMRBC-induced EVs on an anti-CD9 affinity column showed that CD9 + EVs present in the column eluate suppress the DTH reaction and that this effect is prevented by supplementation of eluted EVs with anti-miR-150 (Figure 5D). Additionally, incubation of effector cells with miRNA-150 alone suppressed the transferred DTH reaction (Figure 5D).

3.4 | In vitro treatment of DTH effector cells with sMRBC-induced EVs enhances apoptosis and impairs CD4 + effector T cell activation

To determine the possible mechanism underlying the suppressive activity of EVs, we treated DTH effector cells with sMRBC-induced EVs and then cultured them with either IL-2 and anti-CD3 antibodies

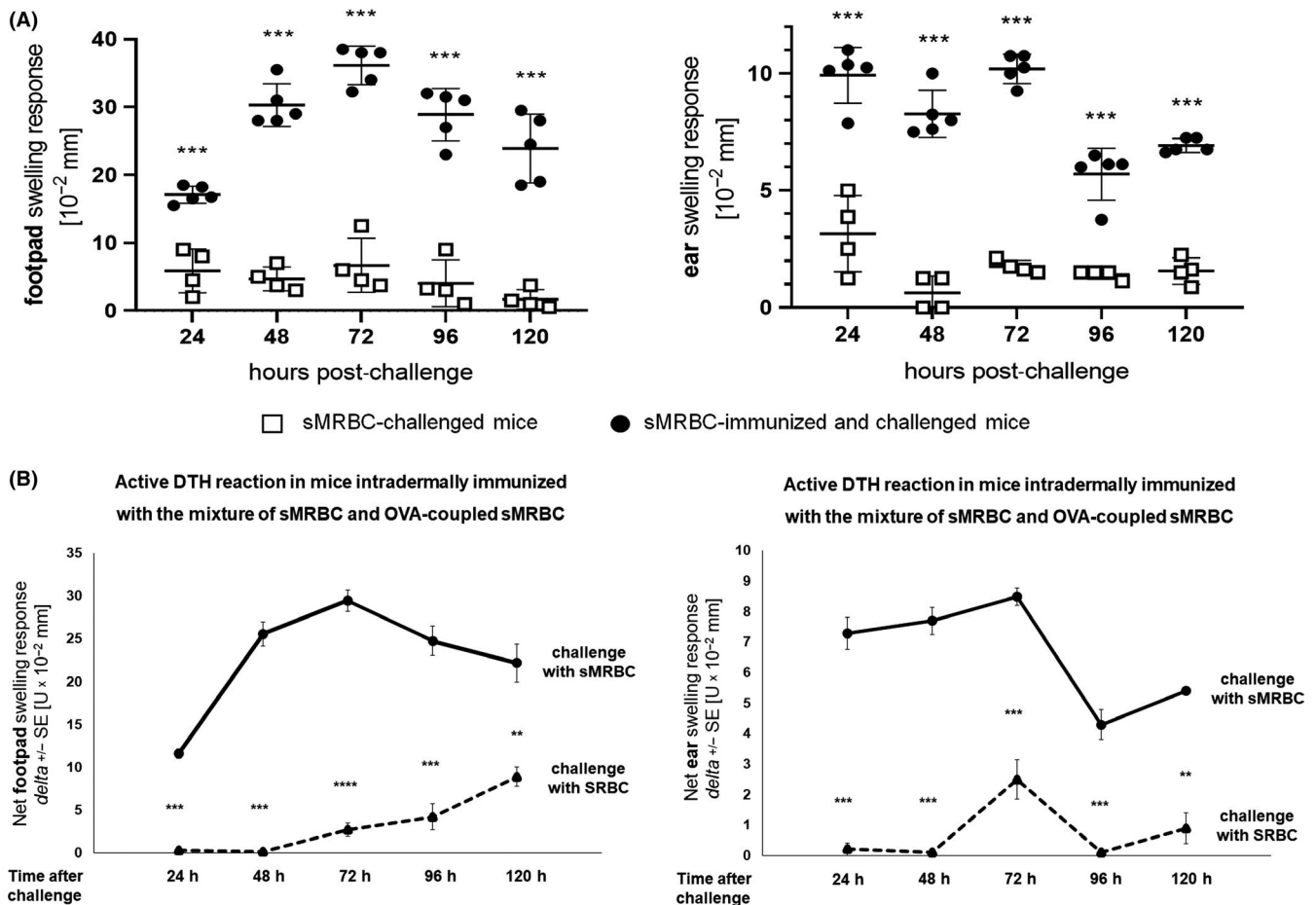


FIGURE 2 CBA mice can be antigen-specifically immunized with syngeneic mouse red blood cells (sMRBC) to elicit delayed-type hypersensitivity (DTH) reaction. A, Footpad (left graph) or ear (right graph) swelling response of mice that were either only challenged by intradermal injection of sMRBC (open squares) or firstly immunized by intradermal injection of sMRBC mixed with ovalbumin-coupled sMRBC into footpad skin and then similarly challenged by intradermal injection of sMRBC into footpad or ear skin (black dots), measured up to 120 h after challenge. Bars represent the mean and standard deviation. B, Footpad (left graph) or ear (right graph) swelling response of mice that were immunized by intradermal injection of sMRBC mixed with ovalbumin-coupled sMRBC into footpad skin and then challenged by intradermal injection of either sMRBC (solid line) or sheep red blood cells (SRBC, dotted line) into footpad or ear skin, measured up to 120 h after challenge. Two-tailed Student's t test ($n = 5$). ** $P < .01$, *** $P < .005$

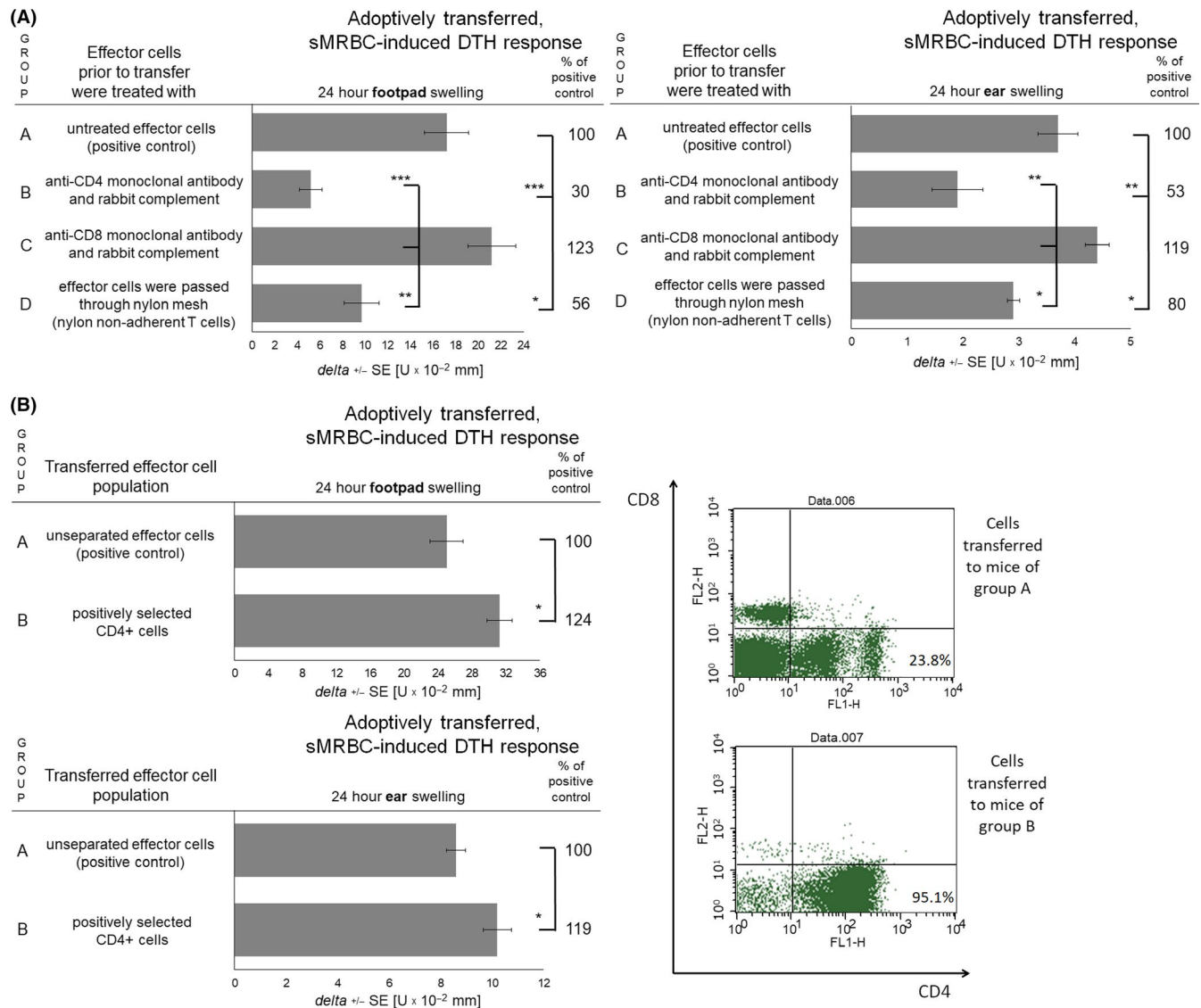


FIGURE 3 Delayed-type hypersensitivity (DTH) reaction to syngeneic mouse red blood cells (sMRBC) is mediated by lymph node and spleen CD4⁺ T cells. A, Footpad (left graph) or ear (right graph) swelling response of mouse recipients of either total population of lymph node and spleen DTH effector cells induced by intradermal injection of sMRBC mixed with ovalbumin-coupled sMRBC in group A or negatively selected DTH effector cells, that is depleted of CD4⁺ cells in group B, depleted of CD8⁺ cells in group C or deprived of nylon-adherent cells in group D. B, Footpad (upper graph) or ear (lower graph) swelling response of mouse recipients of either total population of lymph node and spleen DTH effector cells induced by intradermal injection of sMRBC mixed with ovalbumin-coupled sMRBC in group A or positively selected CD4⁺ DTH effector cells in group B, with cytometric control of selection efficacy (right panel). One-way ANOVA with RIR Tukey test or two-tailed Student's *t* test (*n* = 5). **P* < .05, ***P* < .01, ****P* < .005

or with sMRBC-pulsed macrophages. Afterwards, the viability and activation of CD4⁺ T cells were assessed. In the case of unstimulated DTH effector T cells, an increased percentage of early apoptotic cells was detected (Figure 6A) along with a decreased percentage of CD69-expressing T lymphocytes (Figure 6B). A similar trend was observed when analysing CD25 expression (Figure 6B). Re-stimulation of EV-treated DTH effector T cells with IL-2 and anti-CD3 antibodies resulted in a decreased percentage of necrotic cells (Figure 6A) and intensity of CD44 expression by activated T cells, simultaneously increasing the ratio of CD62L^{low} to CD62L^{high} cells (Figure 6C).²⁰ In the case of EV-treated DTH effector T cells cultured

with sMRBC-pulsed macrophages, an increased percentage of early apoptotic cells (Figure 6A), decreased percentage of CD4⁺ CD69⁺ cells and reduced intensity of CD69 expression were observed (Figure 6B). Analogously, a decreased ratio of CD62L^{low} to CD62L^{high} cells was determined (Figure 6C). However, these cells tended to increase their expression of CD25 after culture with sMRBC-pulsed macrophages (Figure 6B).

Similar tendencies were observed in the case of tolerized mice and naive human T cells treated with sMRBC-induced EVs in the presence of antigen-presenting cells (APCs, Figures S2 and S3). Additionally, our preliminary data suggest that miRNA-150

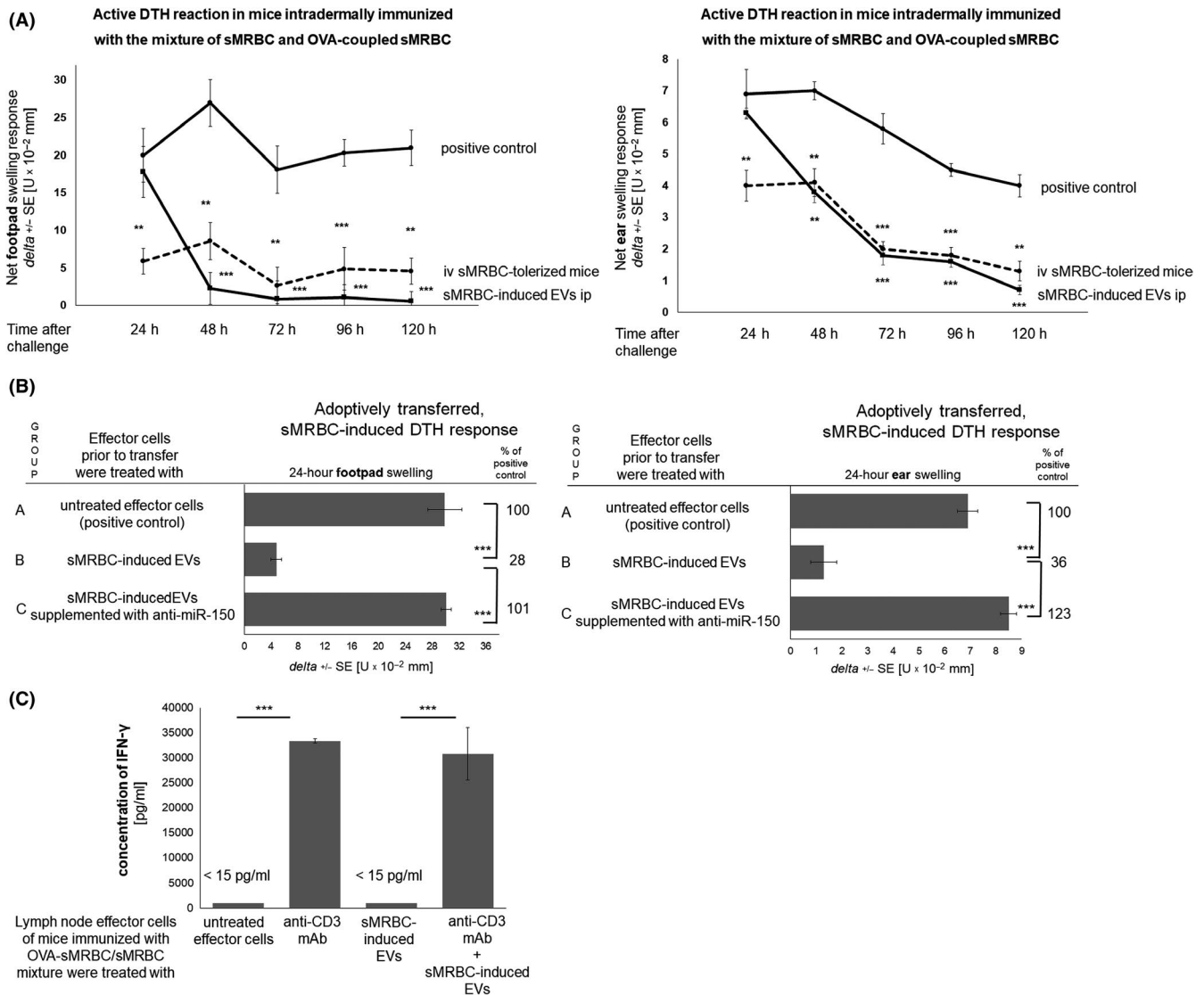


FIGURE 4 Delayed-type hypersensitivity (DTH) reaction to syngeneic mouse red blood cells (sMRBC) can be suppressed by intravenous (iv) administration of sMRBC and the resulting extracellular vesicles (EVs) acting in a miRNA-150-dependent manner. A, Footpad (left graph) or ear (right graph) swelling response either of mice immunized by intradermal injection of sMRBC mixed with ovalbumin-coupled sMRBC (OVA-sMRBC) into footpad skin and then challenged by intradermal injection of sMRBC (solid line with circles), or of mice that had been intravenously administered with sMRBC prior to immunization (dotted line with circles), or of mice intraperitoneally (ip) administered with sMRBC-induced EVs 24 h after challenge (solid line with squares), measured up to 120 h after challenge. B, Footpad (left graph) or ear (right graph) swelling response of mouse recipients of either untreated DTH effector cells induced by intradermal injection of sMRBC mixed with OVA-sMRBC in group A, or DTH effector cells treated prior to transfer with sMRBC-induced EVs in group B, or DTH effector cells treated prior to transfer with sMRBC-induced EVs supplemented with anti-miR-150 (miRNA-150 hairpin inhibitor) in group C. C, The concentration of interferon γ (IFN- γ) released to 24-hour culture supernatant by lymph node DTH effector cells induced by intradermal injection of sMRBC mixed with OVA-sMRBC and cultured alone or in the presence of anti-CD3 monoclonal antibodies (mAb) and/or sMRBC-induced EVs. One-way ANOVA with RIR Tukey test ($n = 5$). $**P < .01$, $***P < .005$

may down-regulate the IL-2-dependent viability of effector cells (Figure S4).

3.5 | sMRBC-induced EVs suppress hapten-induced CHS after coating with hapten-specific antibody LCs

Next, we attempted to investigate whether the tolerogenic activity of sMRBC is limited solely to DTH against self-antigens. Thus,

we tolerized mice with sMRBC prior to skin sensitization with hapten, which inhibit the elicited ear swelling response (Figure 7A). This effect may have resulted from the resulting surface coating of sMRBC-induced EVs with hapten-specific LCs provided by hapten-activated B1 cells.¹¹ To test this hypothesis, we cytometrically analysed the expression of mouse κ LCs on sMRBC-induced EVs originating from CD8 + Ts cells, cultured alone or with hapten-primed B1 lymphocytes, showing that sMRBC-induced EVs acquire

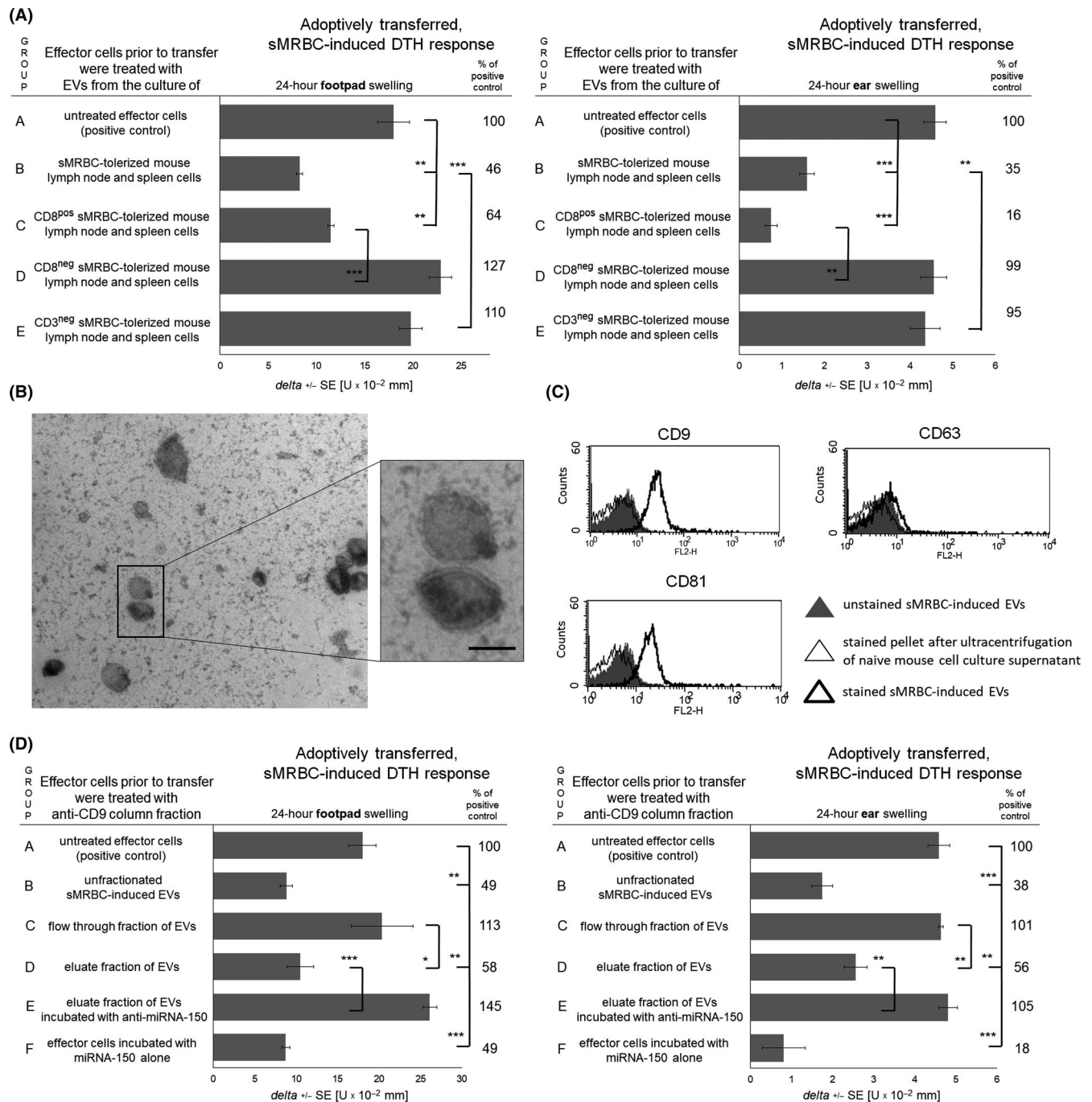


FIGURE 5 Syngeneic mouse red blood cell (sMRBC)-induced extracellular vesicles (EVs) are generated by CD3 + CD8⁺ T cells and express CD9 and CD81 tetraspanins. A, Footpad (left graph) or ear (right graph) swelling response of mouse recipients of unaffected delayed-type hypersensitivity (DTH) effector cells in group A, or DTH effector cells pretreated with sMRBC-induced EVs produced by tolerized mouse lymph node and spleen cells cultured either as total population in group B, or after enrichment of CD8⁺ cells in group C, or after depleting of CD8⁺ or CD3⁺ cells in groups D and E, respectively. B, Electron microscopy analysis of sMRBC-induced EVs (bar express 100 nm). C, Cytometric analysis of sMRBC-induced EVs with staining against CD9, CD63 or CD81 vesicle markers. D, Footpad (left graph) or ear (right graph) swelling response of mouse recipients of untreated DTH effector cells in group A, or DTH effector cells treated prior to transfer with either unfractionated sMRBC-induced EVs in group B, or EVs passing through anti-CD9 affinity column in group C, or EVs eluted from anti-CD9 column alone in group D or supplemented with anti-miRNA-150 (miRNA-150 hairpin inhibitor) in group E, or of recipients of DTH effector cells pretreated with miRNA-150 alone. One-way ANOVA with RIR Tukey test ($n = 4-5$). * $P < .05$, ** $P < .01$, *** $P < .005$

LC-coating in the presence of B1 cells (Figure 7B). Thus, we pre-incubated sMRBC-induced EVs with TNP-specific LCs prior to treatment of adoptively transferred CHS effector cells, which rendered

them fully suppressive, in contrast to coating with specific antibody heavy chains (Figure 7C). This observation was reproduced in the dose-response experiment (Figure S5). Simultaneously, further

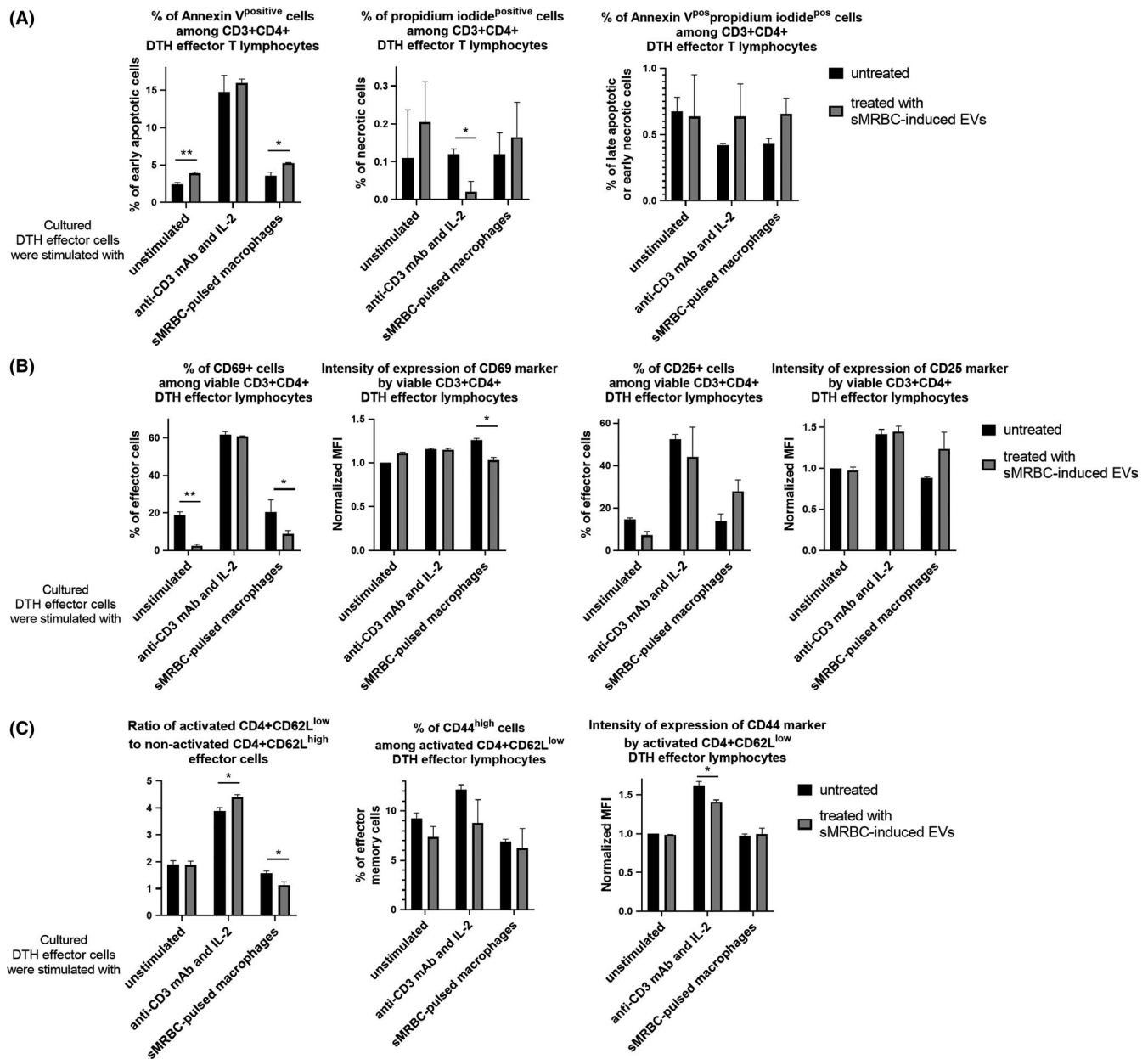


FIGURE 6 Syngeneic mouse red blood cell (sMRBC)-induced extracellular vesicles (EVs) increase the apoptosis and decrease the activation of delayed-type hypersensitivity (DTH) effector cells. A, Cytometrically assessed percentage of CD3 + CD4 + DTH effector cells that are positive in staining with annexin-V and/or propidium iodide, after 3-hour culture in the presence (grey bars) or absence (black bars) of sMRBC-induced EVs. B, Percentage of viable CD3 + CD4 + DTH effector cells that express either CD69 or CD25 markers and geometric mean of fluorescence intensity (MFI) of fluorescent dyes conjugated to monoclonal antibodies (mAb) binding to either CD69 or CD25 markers, cytometrically evaluated after 18- or 24-hour culture in the presence (grey bars) or absence (black bars) of sMRBC-induced EVs. C, Ratio of activated to non-activated DTH effector cells calculated as the percentage of CD62L^{low} cells divided by the percentage of CD62L^{high} cells, among CD3 + CD4 + T lymphocytes, or percentage of CD4 + CD62L^{low} DTH effector cells that highly express CD44 marker with geometric mean of fluorescence intensity (MFI) of fluorescent dye conjugated to mAb against CD44, cytometrically evaluated after 24-hour culture in the presence (grey bars) or absence (black bars) of sMRBC-induced EVs. MFI has been normalized to the value obtained while analysing control group of unstimulated and EV-untreated cells. Graph bars depict mean $\pm$ standard deviation (SD). Two-way ANOVA with RIR Tukey test ($n = 4$). * $P < .05$, ** $P < .01$

results implied that this effect is mediated by the buoyant fraction of EVs (Figure S6A) induced by intravenous administration of sMRBC, but not of allogeneic or xenogeneic erythrocytes (Figure S6B), and depends on the RNA cargo of the EVs (Figure S7). This suggested that LC-coated, sMRBC-induced EVs suppress CHS in a

miRNA-150-dependent manner. To verify this assumption, we tolerated miRNA-150^{-/-} mice with sMRBC, but their derived EVs failed to suppress adoptively transferred CHS effector cells, even when pre-incubated with either hapten-specific LCs or synthetic miRNA-150 (Figure 7D). However, pre-incubation of sMRBC-induced EVs

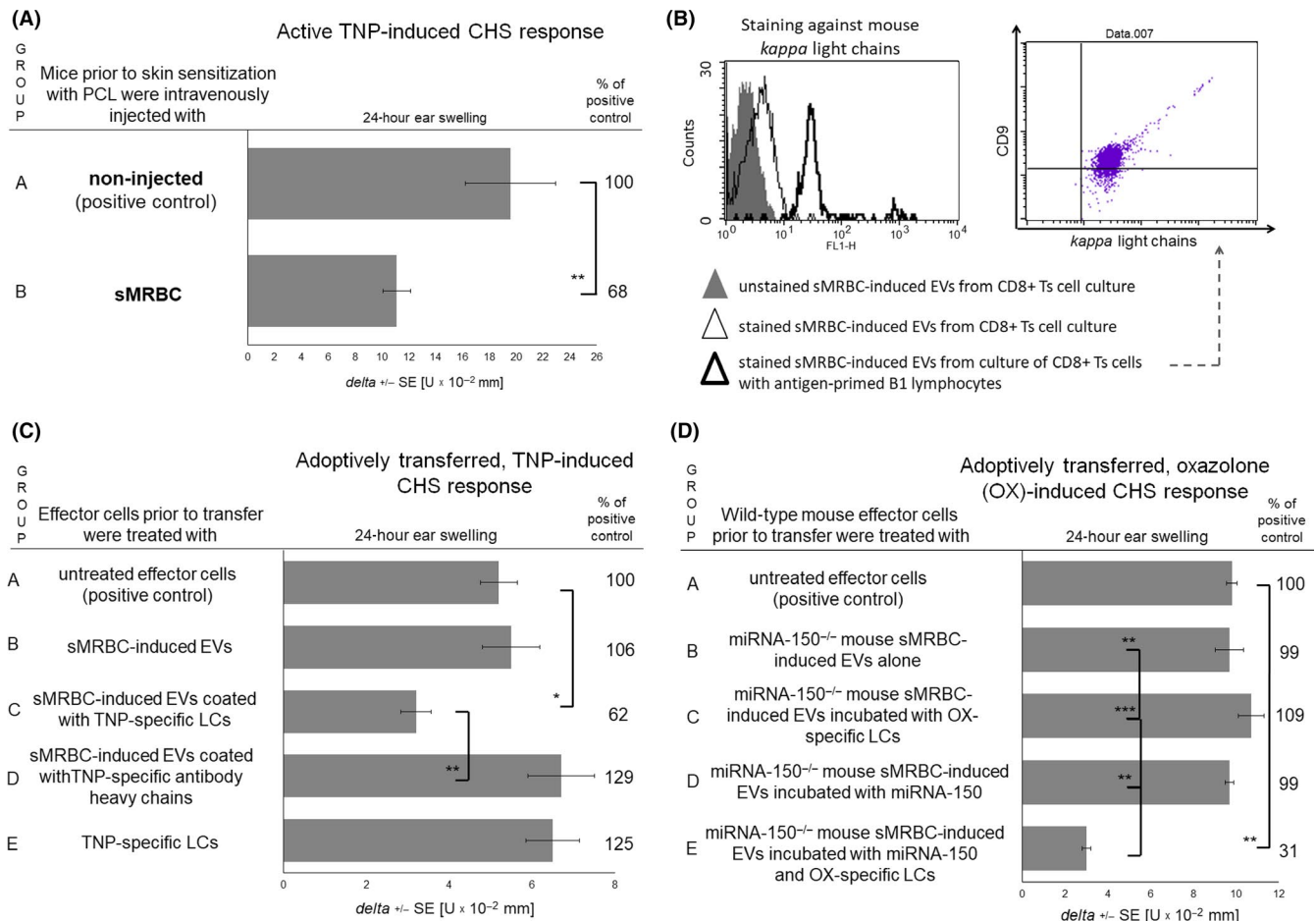


FIGURE 7 Syngeneic red blood cell (sMRBC)-induced extracellular vesicles (EVs) gain full suppressive activity in contact hypersensitivity (CHS) to trinitrophenol (TNP) or oxazolone (OX) hapten after coating with hapten-specific antibody light chains (LCs). A, Ear swelling response of mice actively sensitized with picryl chloride (PCL) in group A or intravenously injected with sMRBC prior to active sensitization in group B. B, Cytometric analysis of sMRBC-induced EVs, that had been produced by either enriched CD8 + suppressor T (Ts) cells cultured alone or with antigen-primed B1 lymphocytes, and stained against mouse antibody kappa light chains and CD9 tetraspanin. C, Ear swelling response of mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with either non-supplemented sMRBC-induced EVs in group B or EVs supplemented with antibody light or heavy chains in groups C and D, or of CHS effector cells incubated with LCs alone in group E. D, Ear swelling response of mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with miRNA-150^{-/-} mouse sMRBC-induced EVs alone in group B, or supplemented with synthetic miRNA-150 mimic and/or OX-specific LCs in groups C-E. One-way ANOVA with RIR Tukey test or two-tailed Student's *t* test (*n* = 5). **P* < .05, ***P* < .01, ****P* < .005

from miRNA-150^{-/-} mice with hapten-specific LCs and synthetic miRNA-150 together rendered them fully suppressive (Figure 7D).

4 | DISCUSSION

Administration of OVA-altered sMRBC via the intradermal route, which is considered immunogenic,¹⁵ results in the breaking of immune tolerance and subsequent development of immune responses against self-erythrocytes. Positive and negative selection assays have revealed that the immune response to altered sMRBC is mediated by CD4 + T cells and macrophages. Furthermore, it peaks at 48-72 hours post-challenge and is also measurable at a site distant (ear skin) to the area of initial antigen deposition (footpad skin).²¹

Upon stimulation, lymph node cells of mice immunized with sMRBC released IFN-γ, while immunized mouse blood sera did not contain antibodies agglutinating sMRBC. Thus, these overall characteristics confirm that the immune response to sMRBC is a classical DTH reaction mediated by CD4 + helper T cells activating the cytotoxic function of macrophages. This further implies that IFN-γ-producing CD4 + Th1 cells dominate the effector cell population.²²

Some self-reactive T cells escape negative selection in the thymus,³ and their responsiveness seems to positively correlate with the dose of self-antigen.²³ In such conditions, immune tolerance is conferred by Treg cells but is less durable and can be quite easily broken by challenges with antigen.³ Furthermore, in the current circumstances, breaking of tolerance was additionally facilitated by delivering self-antigen together with a foreign, immunogenic

protein. Thus, after extravascular delivery, OVA-altered sMRBC undergo phagocytosis by infiltrating macrophages and skin resident APCs that then present the resulting immunogenic determinants to naive CD4 + T cells, beginning the induction phase of the DTH reaction.^{15,19} Consequently, proliferating effector T cells are reactive to sMRBC self-antigens, allowing initiation of the effector phase of DTH by subsequent intradermal injection of intact sMRBC.

On the other hand, intravenous administration of sMRBC causes sudden alterations in blood parameters, including viscosity, due to the elevated blood volume and increased number of circulating erythrocytes. Consequently, this drives compensatory mechanisms to restore physiological conditions, including removal of excessive, altered or senescent blood cells by hepatic and splenic macrophages,^{24,25} which have recently been proposed to activate Treg cells.²⁶ However, in the currently studied conditions, delivery of sMRBC in a huge excess likely led to the overloading of phagocytosing macrophages, which forced them to ensure the protection of self-antigens against eventual recognition by self-reactive cells. This resulted in the generation of EVs by Ts cells. Our previous data, consistent with this prediction, demonstrated that depletion of clodronate-sensitive cells, mostly macrophages, prior to tolerization significantly impairs the generation of suppressive EVs.¹⁴ Thus, circulating sMRBC-induced EVs seem to confer protection against possible autoimmunization, likely by delivering regulatory miRNA-150. Instead, when these self-protecting EVs gain surface coating with antigen-specific LCs, they become redirected towards the suppression of immune responses against the corresponding antigen. Since the excessive inflammatory reaction to non-pathogenic antigens could be self-damaging, in this regard, LC-coated EVs still preserve their self-protecting function.

Furthermore, haptentation of self-proteins leads to the development of allergies and hypersensitivities in susceptible individuals.²⁷ Thus, hapten-specific LC-coated, sMRBC-induced EVs, redirected towards the suppression of sensitized effector cells, would greatly support the activation of immune tolerance to the hapten. This opens up the possibility to therapeutically induce immune tolerance to a particular allergen but also suggests that LC-coated EVs may mediate the naturally occurring, possibly miRNA-150-dependent, immune tolerance to haptens in non-allergic individuals, which requires further investigation.

Along these lines, one can speculate that, in some circumstances, DTH-inducing allergens, such as haptens, may bind to host erythrocytes while passing to the circulation. These in turn would be sensed by splenic and hepatic macrophages, ultimately activating Ts cell release of EVs. Hence, as mentioned before, sMRBC-induced EVs presumably may be considered as mediators of the possible, naturally occurring mechanism of immune tolerance to DTH-inducing allergens. Validity of this speculation may be supported by the fact that not all individuals exposed to contact allergens suffer from contact allergy.

Our research findings demonstrate that sMRBC alone or conjugated with contact allergens induce immune tolerance after systemic injection of a high dose.^{6,9,10,14} This is in line with studies by

Cremel et al highlighting the role of erythrocytes as carriers for immunogenic antigens that make them tolerogenic after intravenous administration.²⁸ Interestingly, it was reported that intravenous administration of MRBC coupled with OVA and then treated with [bis(sulfosuccinimidyl)] suberate (BS3), a chemical promoting liver targeting, prior to immunization with OVA mixed with an adjuvant down-regulated the response of OVA-specific CD8 + T cells and B lymphocytes and increased the number of splenic Treg cells. Instead, in those conditions, administration of MRBC treated with BS3 alone failed to significantly modulate the immune response triggered by immunization with OVA mixed with an adjuvant.²⁸ However, compared to our current experimental settings, crucial differences include the immunization protocols and the means of MRBC treatment prior to injections. In the discussed study, stored sMRBC were treated with a liver targeting promoter and then partly reconstituted with mouse plasma. In contrast, in our conditions, freshly collected sMRBC deprived of buffy coat were used as a plain DPBS suspension. Furthermore, while boosting of mice with OVA in the presence of various adjuvants triggers cytotoxic T cell responses and the production of antibodies,²⁸ epicutaneous sensitization with hapten alone or intradermal immunization with OVA without an adjuvant induces CHS or DTH reactions, respectively.^{9,12,29} Finally, our current observations demonstrate that intradermal administration of sMRBC mixed with OVA-coupled sMRBC also induces a DTH reaction and that both hapten-induced CHS and DTH to self-erythrocytes could be antigen-specifically suppressed by intravenous administration of a high dose of intact sMRBC. Thus, the herein presented research findings contribute to our understanding of erythrocyte-induced immune tolerance by characterizing the underlying biological mechanism involving EVs that target APCs to ultimately suppress effector T cells.¹⁴

Accordingly, attempts to determine the mechanisms underlying the suppressive effects of sMRBC-induced EVs revealed that they diminish T cell activation by sMRBC-pulsed macrophages but not by anti-CD3 monoclonal antibodies and IL-2.³⁰⁻³² This seems to prove the involvement of APCs in the regulatory action of EVs, which is in line with the previously described crucial role of antigen-primed macrophages in the suppression of CHS effector T cells by miRNA-150-carrying EVs.¹⁴ Furthermore, we demonstrate the proapoptotic effect of sMRBC-induced EVs exerted on DTH effector T cells, which may result from miRNA-150 activity, as reported in the case of CD8 + memory T cell differentiation.³³ In addition, sMRBC-induced EVs express anti-proliferative potential and influence the IL-2-dependent viability of HT-2 cells.¹⁴ These effects also seem to be regulated by miRNA-150 and are likely responsible for the eventual inhibition of the sMRBC-induced DTH reaction.³⁴⁻³⁶

It is worth noting that miRNA-150 is well known to regulate haematopoiesis, including erythropoiesis.^{37,38} Overexpression of miRNA-150 was recently shown to inhibit erythroid proliferation and terminal differentiation.³⁹ Accordingly, miRNA-150-mediated inhibition of the release of matured erythrocytes from the bone marrow to the circulation would greatly support the process of restoration of homeostatic conditions after infusion of excessive sMRBC. To

the best of our knowledge, this is the first report that immune cells, namely, CD8 + Ts lymphocytes, contribute to the downregulation of erythropoiesis by releasing miRNA-150 in EVs after a significant systemic increase in red-blood-cell number.¹⁴

From another point of view, the transfusion-related immunomodulation (TRIM) effect is observed in critically ill patients transfused with allogeneic blood or packed red blood cells,⁴⁰⁻⁴² supposedly leading to life-threatening complications.^{43,44} Accordingly, replacement of allogeneic blood transfusions by the administration of autologous (syngeneic) blood or its products was suggested for clinical use to prevent adverse TRIM effects in severely injured individuals.⁴⁵ Furthermore, our current data obtained in a mouse model indicate that, in particular circumstances, sMRBC infusion could induce immune tolerance to self- and non-pathogenic antigens. This in turn could exert beneficial effects by suppressing excessive or unwanted inflammatory responses in the prevention or alleviation of immune-related symptoms of allergies, hypersensitivities and autoimmune disorders, highlighting the clinical importance and translational potential of the herein described tolerance mechanism.

Additionally, DTH reactions to non-pathogenic antigens underlie the pathogenesis of various immune-related disorders, including contact dermatitis as well as coeliac and Crohn's diseases.^{19,46} In such conditions, therapeutic strategies to precisely induce immune tolerance to particular antigens, simultaneously preserving immune responsiveness to other antigens, would bring the most beneficial effects. In this regard, the currently described mechanism of DTH suppression expands our current understanding of the process of immune regulation via EVs and miRNAs and the possible means of its induction.

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CONFLICT OF INTEREST

The Authors declare that they have no relevant conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPLEMENTARY INFORMATION

to a research article

Syngeneic red blood cell-induced extracellular vesicles suppress delayed-type hypersensitivity to self-antigens in mice

by *Katarzyna Nazimek, Eugenio Bustos-Morán, Noelia Blas-Rus, Bernadeta Nowak, Włodzimierz Ptak, Philip W. Askenase, Francisco Sánchez-Madrid and Krzysztof Bryniarski**

submitted to Clinical and Experimental Allergy

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Supplementary Methods

Characterization of syngeneic mouse red blood cell (sMRBC)-induced extracellular vesicles (EVs)

sMRBC-induced EVs pelleted by ultracentrifugation were further subjected to sucrose gradient ultracentrifugation overnight at 100,000 g and the resulting buoyant fraction was used to treat contact hypersensitivity (CHS) effector cells prior to their adoptive transfer. Where indicated, aMRBC-induced EVs were coated with decreasing doses of trinitrophenol (TNP)-specific antibody light chains (LCs) by overnight incubation at 4°C, followed by ultracentrifugation to remove unbound LCs. Otherwise, culture supernatants containing sMRBC-induced EVs were extracted with phenol-chloroform by modified Chomczynski method to obtain DNA/RNA mixtures. Then phenol-chloroform extracts were used to supplement EVs derived from B1 cells of mice actively sensitized with picryl chloride (PCL, Chemtronix, Swannanoa, NC). These B1 cell-derived EVs, representing a good carrier for miRNA cargo, are known to be non-suppressive and surface coated with antigen-specific antibodies. B1-cell EVs supplemented with phenol-chloroform extracted DNA/RNA of sMRBC-induced EVs were incubated with CHS effector cells prior to their intravenous transfer into naive mice.

In some cases, CBA mice were intravenously injected with 0.2 ml of 10% DPBS suspension of either BALB/c allogeneic red blood cells or sheep red blood cells (SRBC, Graso Biotech, Starogard Gdanski, Poland), or solely with 0.2 ml of DPBS prior to collection of lymph nodes and spleens for the culture of single cell suspensions. The resulting supernatants were processed to obtain EVs, then used to treat adoptively transferred CHS effector cells.

Assessment of sMRBC and EV's tolerogenic activity in CHS reaction

Naive or sMRBC-tolerized mice were actively contact sensitized with PCL by application of 0.15 ml of 5% PCL solution in an ethanol : acetone mixture (3:1 v/v) on shaved abdominal skin. Five days later mice were challenged to elicit CHS by topical application of 10 µl of 0.4% PCL in acetone : olive oil mixture (1:1 v/v) on each side of both ears. After 24 hours ear swelling was measured with an engineer's micrometer (Mitutoyo, Japan) by a blinded observer. Just after measurement of 24 hour CHS mice were sacrificed for

collection of draining lymph nodes and spleens, from which CD3⁺CD4⁺ T cells were individually analyzed by flow cytometry on a FACS Calibur with BD CellQuest Pro software (BD Bioscience, San Jose, CA, USA) for the expression of CD69 and CD25 activation markers with the use of fluoresceinated monoclonal antibodies (all from BD Pharmingen, San Diego, CA, USA). Otherwise, five days after sensitization with PCL, mice were sacrificed and lymph nodes and spleens were collected to enrich CHS effector cells that were then treated with various vesicle preparations for 30 minutes in 37°C water-bath. After washing, CHS effector cells were transferred intravenously (7×10^7 cells per mouse) into naive recipients that were immediately challenged with PCL to elicit CHS response, measured 24 hours later.

Cell lines

The human Jurkat T cell line (E6.1 clone) and the lymphoblastoid Raji B cell line (Burkitt lymphoma, both purchased from the DSMZ Organization) were cultured in RPMI 1640 medium supplemented with GlutaMAX, HEPES (Gibco Life Technologies, Grand Island, NY) and 10% fetal bovine serum (FBS, Hyclone, ThermoFisher, Waltham, MA, USA). At least 2 hours prior to experimental culturing, cells were moved to medium supplemented with 10% FBS previously ultracentrifuged overnight to remove its own vesicles.

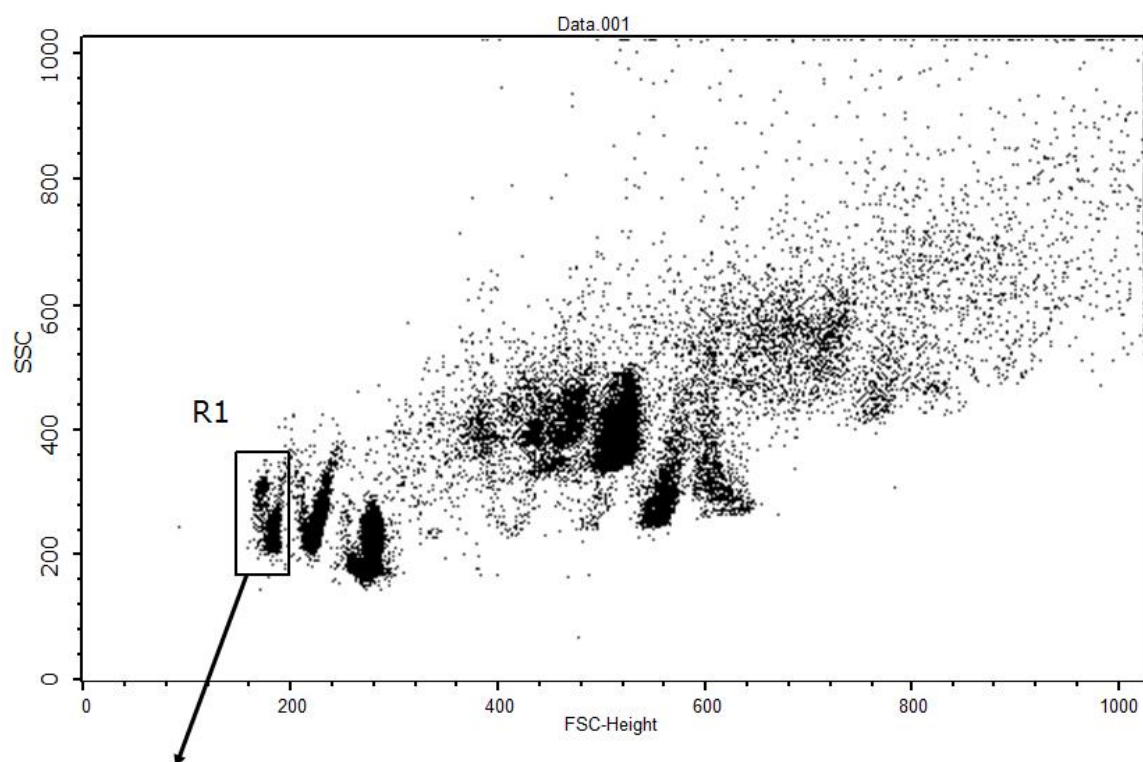
For conjugate formation, Raji B cells were pre-pulsed with SEE superantigen (0.5 µg/ml) for 30 minutes at 37°C. For T cell activation assays, Jurkat T cells cultured either on plates coated with anti-CD3 (5 µg/µl) and anti-CD28 (3 µg/µl) monoclonal antibodies or in the presence of SEE-pulsed Raji B cells in a ratio 1:2 (T cells to APCs) for 16 hours (for evaluation of CD69 expression) in the presence of sMRBC-induced EVs or negative control nanovesicles not carrying miRNA-150. Then, expression of CD69 T cell activation marker was analyzed cytometrically.

For flow cytometric analysis, cell surface Fc receptors were blocked in a buffer containing 1% bovine serum albumin (BSA), 1% FBS and human γ-globulin (100 µg/ml), and then stained with fluoresceinated antibodies against human CD19 (TONBO Biosciences, San Diego, CA, USA; also allowing to distinguish CD19^{neg} T cells), and against human CD69 (Immunotools, Friesoythe, Germany). A viability staining was

also performed (Ghost Dye V510, TONBO Biosciences, San Diego, CA, USA). Cells were analyzed in a FACS Canto II (BD Bioscience, San Jose, CA, USA) and then FCS files were analyzed in FlowJo software.

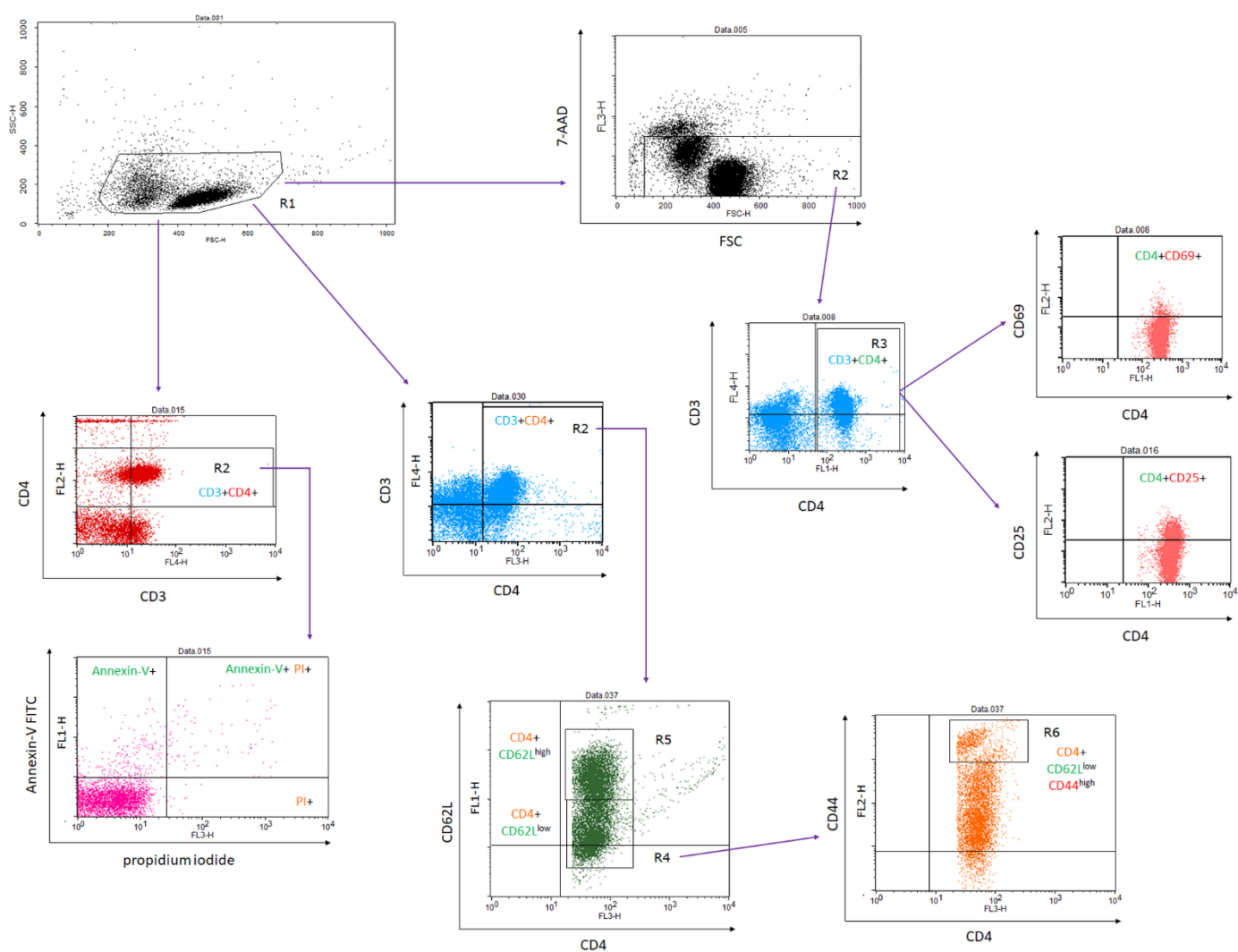
The mouse IL-2-dependent HT-2 T cell line (American Type Culture Collection, Manassas, VA) was cultured in high-glucose Dulbecco modified Eagle medium (DMEM, Gibco Life Technologies, Grand Island, NY) supplemented with 5% FBS (ultracentrifuged at 100,000g to remove vesicles) and 100 U/mL hrIL-2. At least 2 hours before the test, IL-2 was removed from cell culture by extensive washing. Then 10^4 cells per microwell in DMEM containing 3 U/mL hrIL-2 were plated and various doses of sMRBC-induced EVs or control EVs from naive mice were added to the wells. The starting vesicle concentration was estimated by using NTA before serial dilutions. Plates were incubated at 37°C in 5% CO₂, and after 18 hours, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After next 3 hours, 120 µL of acidic isopropanol with 0.04 mol/L HCl was added, and the mixture was incubated overnight in darkness at 25°C. The absorbance of developed dark blue dye was measured at 570 nm, and results were compared with the standard curve. In the standard curve, viability of HT-2 cells cultured in a presence of 3 U/mL hrIL-2 represented 100%, whereas viability of HT-2 cells cultured without hrIL-2 represented 0% (no viability). All cell lines were routinely tested for mycoplasmas.

Supplementary Figures

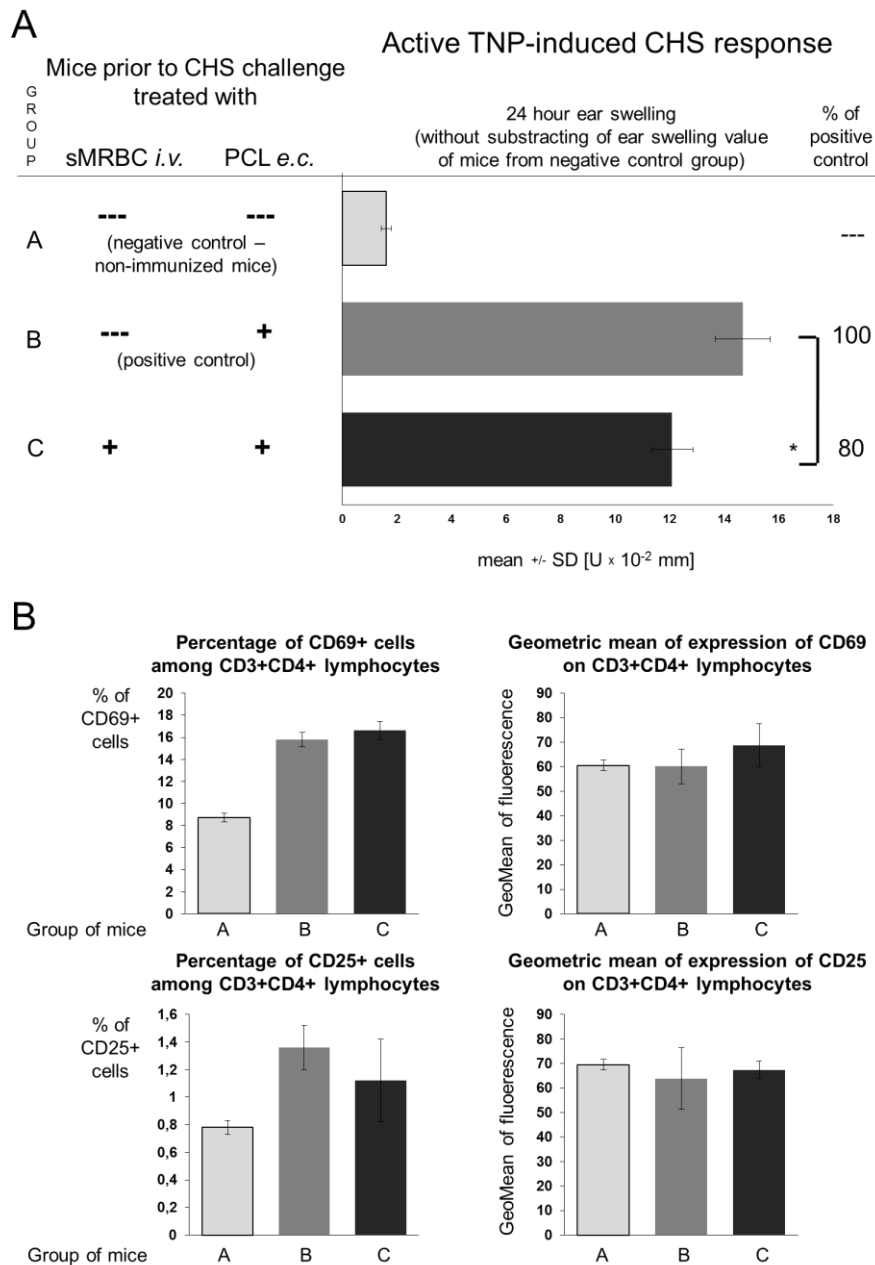


considered as singlets; i.e. single beads individually coated with EVs;
and thus subjected to further analysis

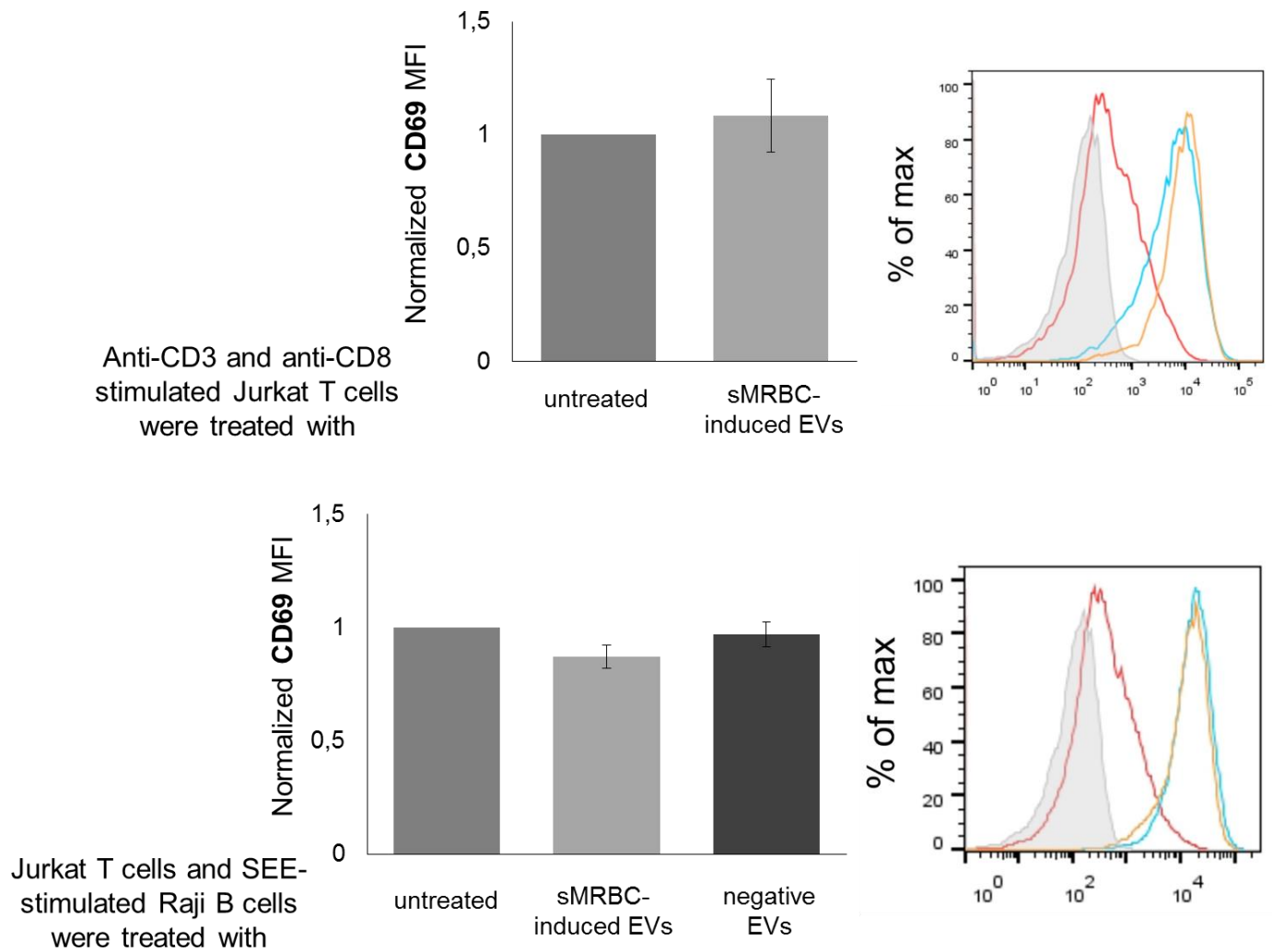
Suppl. Fig. 1A. Gating of beads coated with syngeneic red blood cell-induced extracellular vesicles (EVs) for further cytometric analysis of tetraspanin and light chain expression.



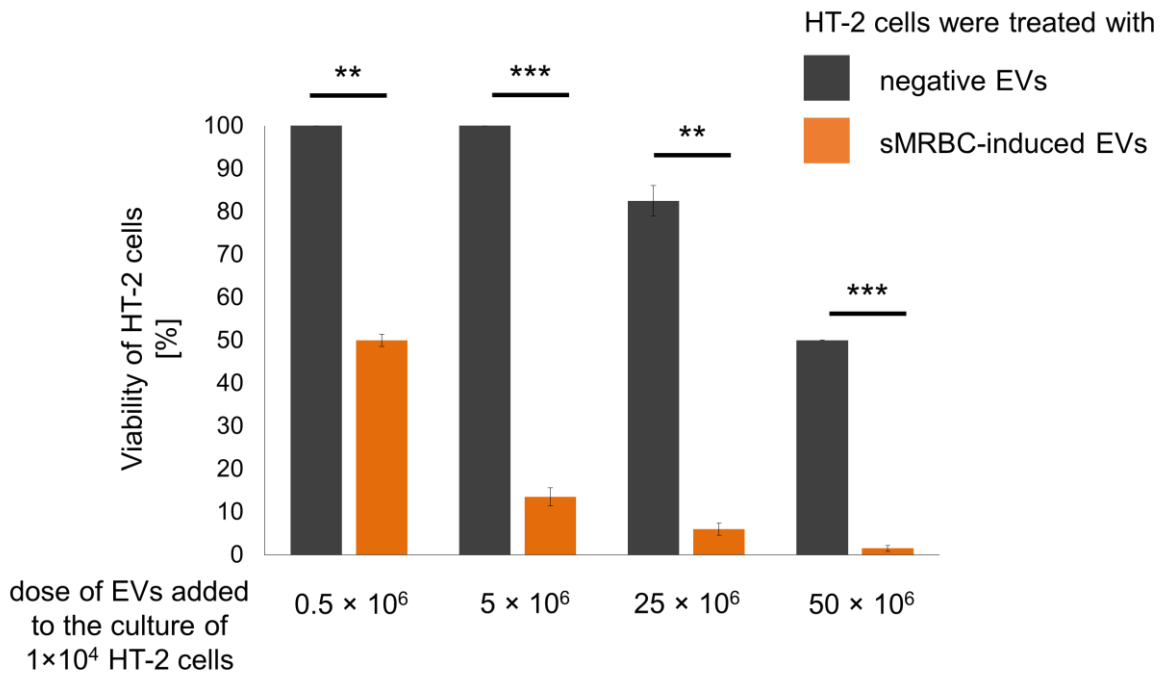
Suppl. Fig. 1B. Gating strategy for cytometric analysis of effector T cell apoptosis and activation.



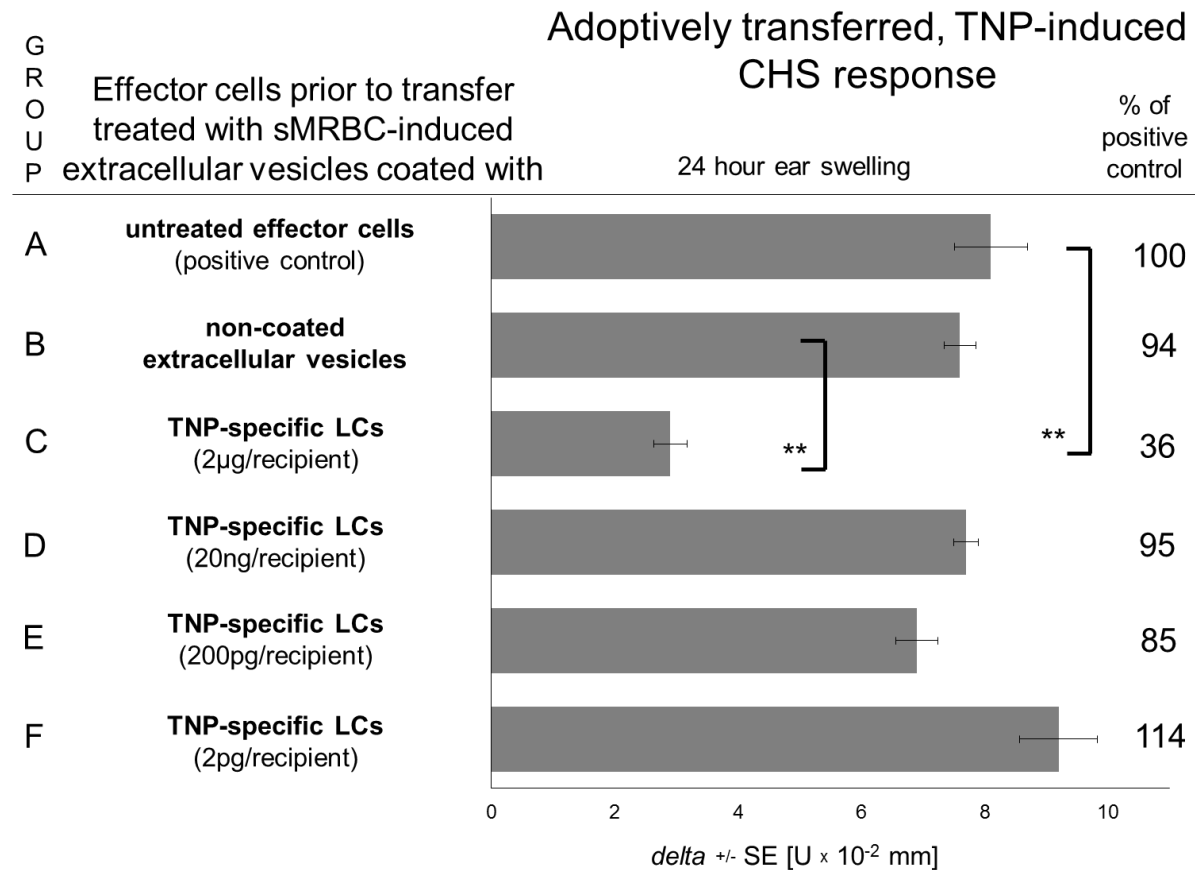
Suppl. Fig. 2. Mice tolerized with syngeneic mouse red blood cells (sMRBC) prior to elicitation of contact hypersensitivity (CHS) reaction express similar activation of lymph node and spleen effector T cells as sensitized mice. (A) Ear swelling response of mice that were only challenged with picryl chloride (PCL) in group A or of mice that were either PCL-sensitized in group B or administered with sMRBC and PCL-sensitized in group C prior to elicitation of contact hypersensitivity (CHS) reaction by ear challenge with PCL. (B) The percentage (left panel) of either CD69+ (upper graph) or CD25+ (lower graph) cells among CD3+CD4+ population of T cells from lymph nodes and spleens of mice from the above groups A, B and C, and the geometric mean of fluorescence (right panel) emitted by fluorochrome conjugated to antibodies against either CD69 (upper graph) or CD25 (lower graph) and analyzed among population of CD3+CD4+ T cells from lymph nodes and spleens of mice from the above groups A, B and C. One-way ANOVA with RIR Tukey test (n=5). *p < 0.05.



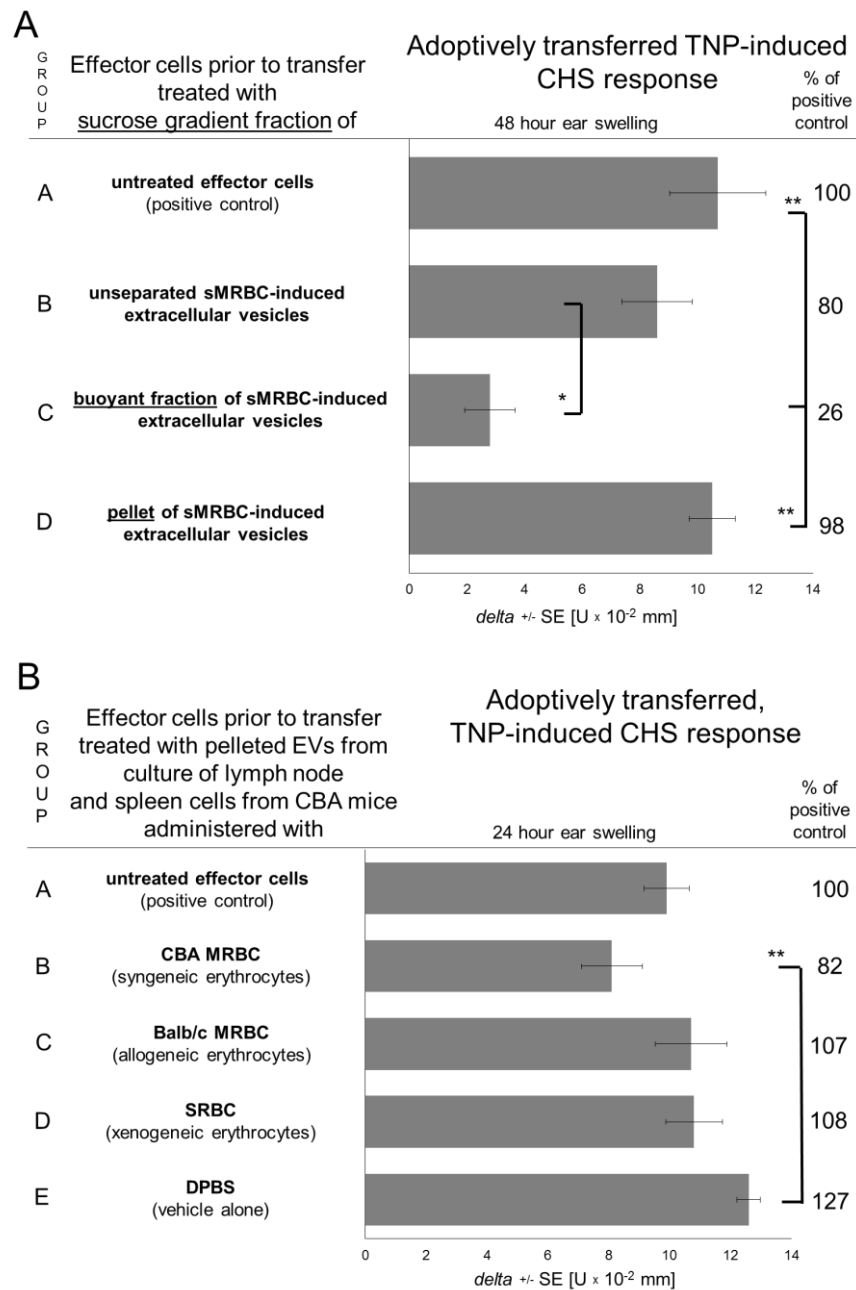
Suppl. Fig. 3. Syngeneic red blood cell (sMRBC)-induced extracellular vesicles (EVs) influence the activation of human T cells by antigen-presenting cells. Mean fluorescence intensity (MFI) normalized to the value obtained while analyzing control group of EV-untreated cells, reflecting the expression of CD69 by Jurkat T cells cultured with either sMRBC-induced EVs and anti-CD3 and anti-CD28 monoclonal antibodies (upper panel) or superantigen E (SEE)-stimulated Raji B cells and sMRBC-induced EVs or EVs from lymph nodes and spleen cells of naive mice (lower panel). Cytometric plot legend: grey – unstained cells; red line – unstimulated T cells; blue line – stimulated T cells; orange line – T cells stimulated in the presence of sMRBC-induced EVs (n=4).



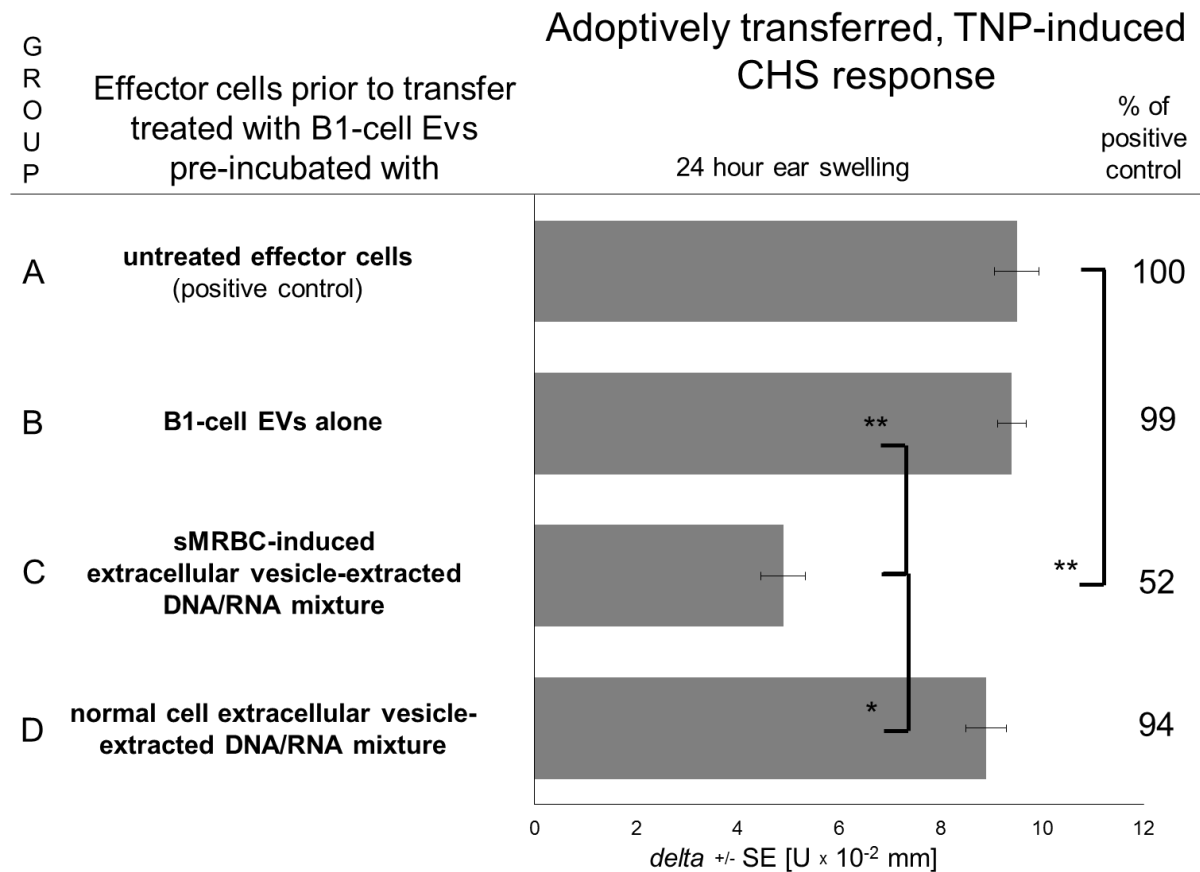
Suppl. Fig. 4. Syngeneic red blood cell (sMRBC)-induced extracellular vesicles (EVs) influence IL-2-dependent viability of mouse T cells. The percent of viable HT-2 cells after treatment with increasing doses either EVs from lymph nodes and spleen cells of naive mice in group A or sMRBC-induced EVs in group B, cultured in the presence of IL-2. Two-tailed Student's T test (n=3). **p < 0.01, ***p < 0.005.



Suppl. Fig. 5. Suppression of contact hypersensitivity (CHS) by syngeneic red blood cell (sMRBC)-induced extracellular vesicles (EVs) coated with trinitrophenol (TNP)-specific antibody light chains (LCs) is dose-dependent. Ear swelling response of mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with either sMRBC-induced EVs alone in group B, or after supplementation with decreasing doses of LCs in groups C-F. One-way ANOVA with RIR Tukey test (n=5). **p < 0.01.



Suppl. Fig. 6. Lymph node and spleen cells of mice intravenously administered with syngeneic red blood cells (sMRBC) release extracellular vesicles (EVs) suppressing contact hypersensitivity (CHS) to trinitrophenyl (TNP) hapten. (A) Ear swelling response of mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with either unseparated sMRBC-induced EVs in group B or EV fractions resulting from sucrose gradient separation in groups C and D. (B) Ear swelling response of CBA mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with either sMRBC-induced EVs in group B, or EVs derived by lymph node and spleen cells of mice injected with non-syngeneic erythrocytes or DPBS alone in groups C-E. One-way ANOVA with RIR Tukey test (n=5). *p < 0.05, **p < 0.01.



Suppl. Fig. 7. Contact hypersensitivity (CHS) to trinitrophenyl (TNP) hapten is suppressed by B1 lymphocyte-derived extracellular vesicles (EVs) supplemented with syngeneic red blood cell (sMRBC)-induced EV-extracted DNA/RNA. Ear swelling response of mouse recipients of unaffected CHS effector cells in group A or of CHS effector cells treated with B1 cell-derived EVs alone in group B or B1 cell EVs supplemented with DNA/RNA extracted from either sMRBC-induced EVs in group C or EVs derived from naive mice in group D. One-way ANOVA with RIR Tukey test (n=5-6). * $p < 0.05$, ** $p < 0.01$.