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Natural $CD8^+25^+$ regulatory T cell-secreted exosomes capable of suppressing cytotoxic T lymphocyte-mediated immunity against B16 melanoma

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ABSTRACT

Natural $CD4^+25^+$ and $CD8^+25^+$ regulatory T (Tr) cells have been shown to inhibit autoimmune diseases. Immune cells secrete exosomes (EXOs), which are crucial for immune regulation. However, immunomodulatory effect of natural Tr cell-secreted EXOs is unknown. In this study, we purified natural $CD8^+25^+$ Tr cells from C57BL/6 mouse naive $CD8^+$ T cells, and *in vitro* amplified them with CD3/CD28 beads. EXOs (EXO_{Tr}) were purified from Tr cell's culture supernatants by differential ultracentrifugation and analyzed by electron microscopy, Western blot and flow cytometry. Our data showed that EXO_{Tr} had a "saucer" or round shape with 50–100 nm in diameter, contained EXO-associated markers LAMP-1 and CD9, and expressed natural Tr cell markers CD25 and GITR. To assess immunomodulatory effect, we i.v. immunized C57BL/6 mice with ovalbumin (OVA)-pulsed DCs (DC_{OVA}) plus Tr cells or EXO_{Tr}, and then assessed OVA-specific $CD8^+$ T cell responses using PE-H-$2K^b$/OVA tetramer and FITC-anti-CD8 antibody staining by flow cytometry and antitumor immunity in immunized mice with challenge of OVA-expressing $BL6\text{–}10_{OVA}$ melanoma cells. We demonstrated that DC_{OVA}-stimulated $CD8^+$ T cell responses and protective antitumor immunity significantly dropped from 2.52% to 1.08% and 1.81% ($p < 0.05$), and from 8/8 to 2/8 and 5/8 mice DC_{OVA} ($p < 0.05$) in immunized mice with co-injection of Tr cells and EXO_{Tr}, respectively. Our results indicate that natural $CD8^+25^+$ Tr cell-released EXOs, alike $CD8^+25^+$ Tr cells, can inhibit $CD8^+$ T cell responses and antitumor immunity. Therefore, EXOs derived from natural $CD4^+25^+$ and $CD8^+25^+$ Tr cells may become an alternative for immunotherapy of autoimmune diseases.

1. Introduction

Natural polyclonal $CD4^+25^+$ and $CD8^+25^+$ regulatory T (Tr) cells expressing forkhead box P3 (Foxp3) play an important role in maintenance of self-tolerance via inhibition of the antigen-dependent expansion of self-antigen-reactive T cells *in vivo* [1]. They exert non-specific immune suppression independent upon the secretion of immune suppressive cytokines but via an *in vitro* cell-to-cell contact fashion [2].

Exosomes (EXOs) are small membrane vesicles (50–100 nm in diameter) of endocytic origin, that are formed by fusion of multivesicular endosomes with the plasma membrane, followed by exocytosis [3]. EXOs can be secreted by various immune cells such as dendritic cells (DCs), T and B cells [3]. EXOs represent organelles important in intercellular communication [4]. The modulatory effect of immune cell-released EXOs in immune responses has attracted more and more attention [4]. We previously demonstrated that mature DC-released EXOs stimulate more efficient CTL responses and antitumor immunity than tumor-secreted EXOs [5]. Recently, it has been shown that transgene TGF-β-modified tolerogenic DC-secreted EXOs suppress Th17-mediated autoimmune inflammatory bowel disease via inducing $CD4^+$ Tr cells [6]. Active $CD8^+$ T cells secreting bioactive EXOs expressing T cell receptor (TCR) and Fas ligand (FasL) [7] induced apoptosis to the bystander T cells [8]. Recently, we have demonstrated that active T cell-secreted FasL-expressing EXOs are capable of inhibiting DC-stimulated CTL responses and antitumor immunity via down-regulated DC's peptide/MHC complexes and FasL-mediated DC cytolysis [9,10] However, the potential inhibitory effect of natural Tr cell-secreted EXOs in immune modulation is still elusive.

In this study, we purified EXOs from *in vitro* amplified $CD8^+25^+$ Tr cell culture supernatants, and then investigated their suppressive effect on CTL responses and antitumor immunity.

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2. Materials and methods

2.1. Reagents, cell lines and animals

The antibodies specific for CD8, CD25, GITR, Foxp3, LAMP-1 and CD9, and the ELISA kits specific for IL-10, TGF-β, IFN-γ, TNF-α and IL-4 were obtained from BD Biosciences (Mississauga, Ontario, Canada). The PE-labeled H-2K^b/OVA$_{257-264}$ tetramer was obtained from Beckman Coulter (San Diego, CA, USA). The highly lung metastatic OVA-expressing B16 melanoma cell line BL6–10$_{OVA}$ was generated in our laboratory [9]. The female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

2.2. Preparation of immune cells and exosomes

Naive CD8$^+$ T cells and ovalbumin (OVA)-pulsed splenic dendritic cells (DC$_{OVA}$) were prepared as previously described [11]. CD8$^+$CD25$^+$ Tr cells were purified from C57BL/6 mouse naive CD8$^+$ T cells by using biotin-anti-CD25 antibody (7D4) and anti-biotin MACS beads (Miltenyi Biotech, Auburn, CA, USA), followed by passing T cells through LS column (Miltenyi Biotech). Purified CD8$^+$CD25$^+$ Tr cells were cultured in RPMI 1640 medium containing IL-2 (20 U/mL) and T cell expander (CD3/CD28 beads, Invitrogen, Burlington, Ontario, Canada) at a ratio of 2:1 (cell:beads) for 5–7 days. Exosomes (EXOs) were purified from Tr cell culture supernatants by differential ultracentrifugation and termed EXO$_{Tr}$.

2.3. Tetramer staining assay

To assess the immunoregulatory effect, C57BL/6 mice ($n = 8$) were intravenously (i.v.) immunized with DC$_{OVA}$ (2×10^6 cells/mouse) plus CD8$^+$CD25$^+$ Tr cells (3×10^6 cells/mouse) or EXO$_{Tr}$ (10 μg/mouse). Six days after immunization, blood samples were stained with PE-H-2K^b/OVA$_{257-264}$ tetramer and FITC-anti-CD8 antibody and analyzed by flow cytometry.

2.4. In vivo cytotoxicity assay

Six days after immunization, an *in vivo* cytotoxicity assay was performed to assess OVA-specific CD8$^+$ CTL effector activity by co-injection of OVA-specific and control target cells at 1:1 ratio into the immunized mice as previously described [9]. Sixteen hours after target cell delivery, the residual OVA-specific target cells remaining in recipients' spleens were analyzed by flow cytometry.

2.5. Animal studies

To assess the suppression in antitumor immunity, the immunized mice were subcutaneously (s.c.) challenged with OVA-expressing 0.1×10^6 BL6–10$_{OVA}$ tumor cells at day 8 after immunization. For ethic reason, all mice with tumors that reach to a size of 1.5 cm in diameter were sacrificed.

2.6. Statistical analyses

Statistical analyses were performed with the Student's *t*-test and log-rank test using Prism software (GraphPad Software, San Diego, CA, USA) to compare variables from different groups. A value of $p < 0.05$ was considered significant.

3. Results and discussion

We first purified natural CD8$^+$25$^+$ Tr cells from C57BL/6 mouse naive CD8$^+$ T cells using biotin-anti-CD25 antibody, and then expanded CD8$^+$25$^+$ Tr cells *in vitro* with CD3/CD28 beads as previously described [12,13]. We found that Tr cells expressed bona fide Tr cell markers such as cell-surface CD25 and GITR, and intracellular Foxp3 (Fig. 1A), and secreted immunosuppressive cytokines such as IL-10 and TGF-β (Fig. 1B), which is consistent with a recent report [14] We further purified exosomes (EXOs) from Tr cell culture supernatants by differential ultracentrifugation, and then analyzed EXOs by electron microscopy, Western blot and flow cytometry. We demonstrated that Tr cell-secreted EXOs (EXO$_{Tr}$) had a typical exosomal "saucer" or round shape with a diameter of 50–100 nm (Fig. 1C), contained exosomal proteins such as LAMP-1 and CD9 (Fig. 1D) [15], and expressed Tr cell markers, but in much less content than Tr cells (Fig. 1A).

To assess immunoinhibitory effect of CD8$^+$25$^+$ Tr cells andEXO$_{Tr}$, we i.v. immunized C57BL/6 mice with co-injection of DC$_{OVA}$ plus Tr cells or EXO$_{Tr}$, and then assessed OVA-specific CTL responses by flow cytometry. We demonstrated that DC$_{OVA}$ stimulated OVA-positive CD8$^+$ T cell responses, accounting for 2.52% in the total CD8$^+$ T cell population in immunized mice 6 days after immunization (Fig. 2A). However, OVA-specific CD8$^+$ T cell responses in immunized mice with co-injection of Tr cells or EXO$_{Tr}$ significantly

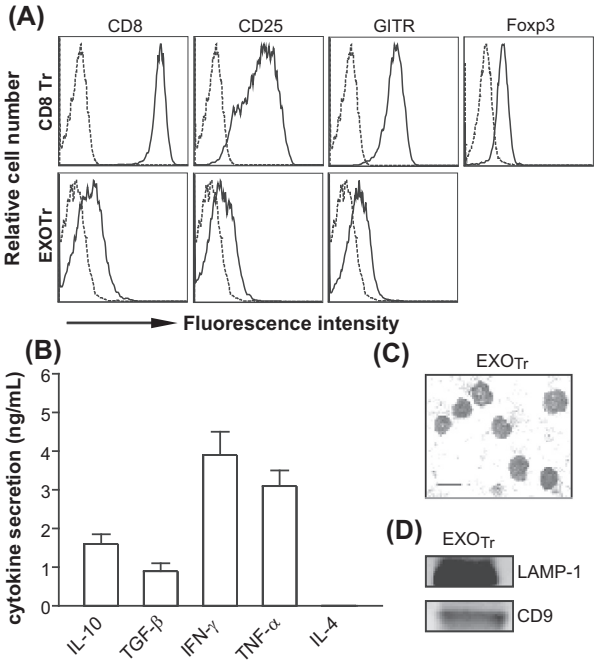

Fig. 1. Phenotypic analysis of CD8$^+$CD25$^+$ Tr cells and CD8$^+$CD25$^+$ Tr cell-secreted EXOs. (A) *In vitro* expanded CD8$^+$25$^+$ Tr cells and EXO$_{Tr}$ were stained with a panel of antibodies specific for CD8, CD25, GITR and Foxp3 (solid lines) or isotype-matched irrelevant antibodies (dotted lines) and then analyzed by flow cytometry. (B) The culture supernatant from CD8$^+$25$^+$ Tr cells was subjected to ELISA analysis for assessment of cytokine secretion. (C) Electron micrograph of EXO$_{Tr}$. EXO$_{Tr}$ were fixed in 4% paraformaldehyde, loaded onto carbon-coated formvar grids, and then fixed in 1% glutaraldehyde. After washes for three times, the EXO sample-loaded grids were stained with saturated aqueous uranyl and then examined with JEOL 1200EX electron microscope at 60 kV. Bar, 100 nm. (D) Western blot analysis of EXO$_{Tr}$. EXO$_{Tr}$ (30 μg) were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene difluoride (PVDF) membrane and stained with LAMP-1 and CD9 antibodies, respectively. Followed by incubation with IRDyeR680CW-labeled second antibody, the blots were scanned using ODYSSEY densitometer (Li-COR Bioscience). One representative experiment of two is shown.

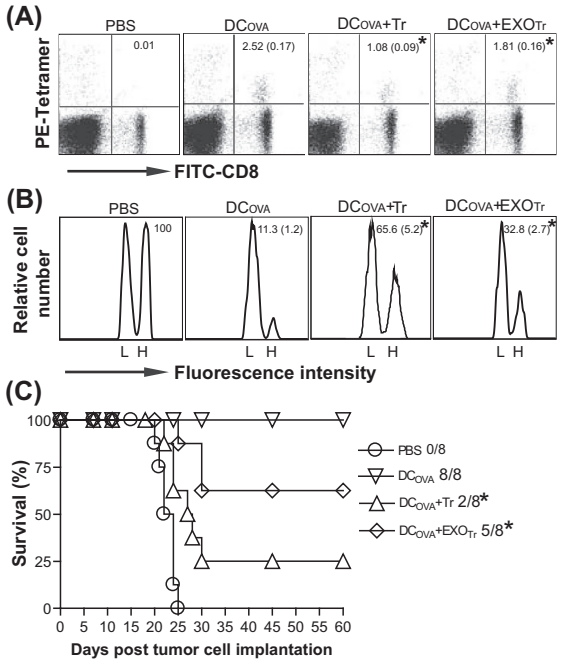

Fig. 2. $CD8^+CD25^+$ Tr cells and EXO_{Tr} suppress $CD8^+$ CTL responses and antitumor immunity. (A) *In vivo* $CD8^+$ T-cell proliferation assay. Blood samples derived from C57BL/6 mice i.v. immunized with DC_{OVA} alone or DC_{OVA} plus $CD8^+CD25^+$ Tr cells or EXO_{Tr} were assessed for OVA-specific $CD8^+$ CTL responses at day 6 post immunization using PE-tetramer and FITC-CD8 staining by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell pool. The value in parentheses represents the SD. $^*p < 0.05$ vs cohorts of DC_{OVA} group (Student's *t*-test). (B) *In vivo* cytotoxicity assay. Sixteen hours after target cell delivery, the residual OVAI peptide-pulsed $CFSE^{high}$- and the control Mut1 peptide-pulsed $CFSE^{low}$-target cells remaining in the spleens of the above immunized mice were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of OVA-specific $CFSE^{high}$ and control $CFSE^{low}$ target cells remaining in the spleens. The value in parentheses represents the SD. $^*p < 0.05$ vs cohorts of DC_{OVA} group (Student's *t*-test). (C) Animal studies. Eight days after above immunization, mice were s.c. challenged with $BL6\text{–}10_{OVA}$ tumor cells. Tumor growth and animal mortality were monitored daily for 60 days. $^*p < 0.05$ vs cohorts of DC_{OVA} group (log-rank test). One representative experiment of two is shown.

dropped to 1.08% and 1.81%, respectively ($p < 0.05$) (Fig. 2A), indicating that Tr cells and EXO_{Tr} are capable of suppressing *in vivo* DC_{OVA}-stimulated $CD8^+$ T cell responses. To assess whether these $CD8^+$ T cells are effector cytotoxic T lymphocytes (CTLs), we performed *in vivo* cytotoxicity assay. As shown in Fig. 2B, 88.7% of the $OVA_{257–264}$ peptide-pulsed $CFSE^{high}$ target cells, but none of the irrelevant control Mut1 peptide-pulsed (i.e., $CFSE^{low}$) target cells were killed over 16 h after transfer of target cells into immunized mice with or without co-injection. As expected, the injection of Tr cells or EXO_{Tr} significantly reduced the loss of $CFSE^{high}$ target cells from 88.7% in DC_{OVA}-immunized mice to 34.4% and 67.2% in DC_{OVA}-immunized mice with co-injection of Tr cells and EXO_{Tr} ($p < 0.05$), respectively (Fig. 2B), indicating that $CD8^+25^+$ Tr cells and EXO_{Tr} efficiently inhibit DC-stimulated effector CTL responses. To further assess the suppression in antitumor immunity by Tr cells and EXO_{Tr}, we challenged PBS control mice and immunized mice with OVA-expressing $BL6\text{–}10_{OVA}$ tumor 6 days after immunization. As shown in Fig. 2C, all control mice (8/8) died of tumor within 4 weeks after tumor inoculation, whereas all of the DC_{OVA}-immunized mice without co-injection (8/8) were protected from tumor cell challenge. We demonstrated that 6/8 and 3/8 of immunized mice with co-treatment of Tr cells and EXO_{Tr} died of tumor, respectively ($p < 0.05$) (Fig. 2C), indicating that $CD8^+25^+$ Tr cells and EXO_{Tr} also suppress *in vivo* $CD8^+$ CTL-mediated antitumor immunity.

Taken together, for the first time, we show that the natural $CD8^+25^+$ Tr cell-secreted EXOs are capable of inhibiting *in vivo* DC-induced CTL responses and antitumor immunity, suggesting that $CD8^+25^+$ Tr-released exosomal molecules may play a critical role in Tr cell-mediated immune suppression. $CD4^+25^+$ Tr cell suppression has been found to be associated with cell-surface inhibitory LAG-3, Gal-1, Nrp-1 and TIGI molecules [16]. However, no inhibitory molecule has been reported to be associated with $CD8^+25^+$ Tr cell immune suppression. Proteomic study has been applied to analyze EXOs derived from patient's fluid samples for biomarker discovery [15]. Proteomic analysis of $CD8^+25^+$ Tr-released EXOs are underway in our laboratory for identification of the key molecules involved in $CD8^+25^+$ Tr cell-mediated immune suppression. Since natural $CD4^+25^+$ and $CD8^+25^+$ Tr cells play an important role in maintenance of self-tolerance [1], they have been applied to inhibit autoimmune diseases [17]. Therefore, EXOs derived from natural $CD4^+25^+$ and $CD8^+25^+$ Tr cells may become an alternative for immunotherapy of autoimmune diseases.

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