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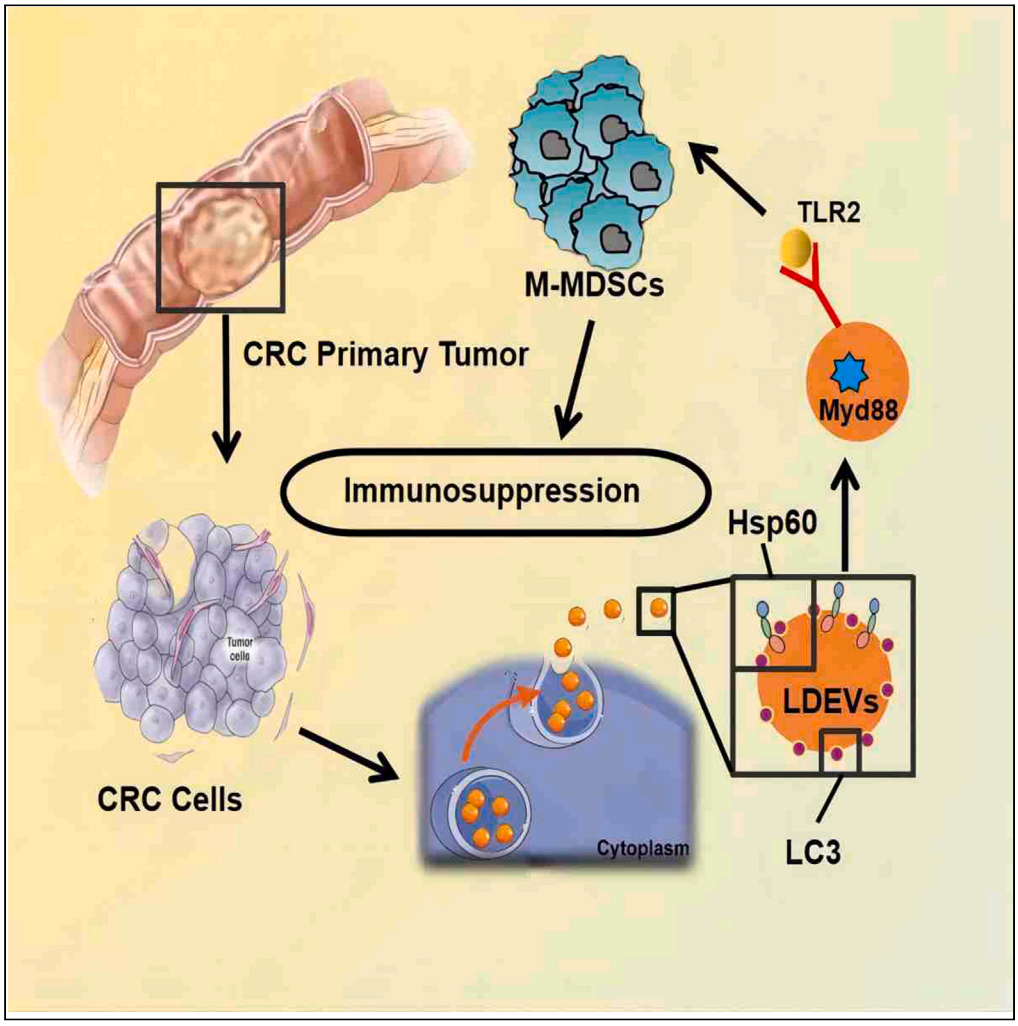

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Highlights

CRC cell-derived LDEVs mediated M-MDSCs formation both *in vitro* and *in vivo*

LDEVs triggered MDSCs induction via TLR2-MYD88 and Hsp60 was the surface ligand of LDEVs that induced M-MDSCs

LDEVs induced MDSCs inhibited T cells division via both cell contact and non-contact manners

LDEVs levels correlated with circulating M-MDSCs accumulation, IL-10 and arginase secretion in clinical CRC patients

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LC3-dependent extracellular vesicles promote M-MDSC accumulation and immunosuppression in colorectal cancer

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SUMMARY

For a long time, myeloid-derived suppressor cells (MDSCs) dilated in circulation system of colorectal cancer (CRC) patients have been puzzling clinicians. Various evidence shows that MDSCs constitute the bulk of immunosuppression in CRC, which is related to tumor growth, adhesion, invasion, metastasis, and immune escape. However, the mechanisms underlying these cells formation remain incompletely understood. In this study, we reported that CRC cell-derived LC3-dependent extracellular vesicles (LDEVs)-mediated M-MDSCs formation via TLR2-MYD88 pathway. Furthermore Hsp60 was the LDEVs surface ligand that triggered these MDSCs induction. In clinical studies, we reported that accumulation of circulating M-MDSCs as well as IL-10 and arginase1 secretion were reliant upon the levels of tumor cell-derived LDEVs in CRC patients. These findings indicated how local tumor cell-derived extracellular vesicles influence distal hematopoiesis and provided novel justification for therapeutic targeting of LDEVs in patients with CRC.

INTRODUCTION

For a long time, colorectal cancer (CRC) patients circulating massively expanded myeloid-derived suppressor cells (MDSCs) has been perplexing clinicians.[1] Accumulating evidence indicates that MDSCs play important roles in the progression of CRC and are associated with tumor cell growth, adhesion, invasion, metastasis, and immune escape.[2,3]

Although the definition of MDSCs remains debatable, most investigators subdivide these cells into two different subsets, monocytic MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs), based on the phenotype and morphology.[4,5] To understand the mechanisms of MDSCs accumulation and immunosuppression in CRC are of important clinical implications.[6,7] Numerous studies over the past decade viewed as abnormal secretion of cytokines facilitates MDSCs differentiation.[8] However, statistically significant MDSCs accumulation could be detected at an early stage of CRC, when the minimal tumor could not secrete enough cytokines to influence medullary hematopoiesis.[2,9,10] Therefore, there must be some specific mediators triggering the differentiation of MDSCs in CRC patients, which remain unidentified.

Notably, recent research has emerged extracellular vesicles (EVs) as messengers of cells communication and provided new insight into how the vesicles may influence the non-cell autonomous exchange of information between cells.[11,12] EVs can be secreted by all types of tumor cells and participate in a wide range of pathological processes in the progression of cancer.[13] There are two major categories of EVs participate in CRC progression: LC3-dependent extracellular vesicle (LDEVs) and exosomes, which could be distinguished by heterogeneous membrane profile and size.[14,15] An understanding of whether tumor cells utilize EVs as an approach to mediate intercellular transfer and immune reactions from one cell to nearby and distant cells may contribute to the use of EVs as novel biomarkers or to the manipulation of them for use in therapeutic applications.

Hence, in this study, we scrutinized how local tumor cell-derived EVs influences distal hematopoiesis and provide novel justification for therapeutic targeting of LDEVs in patients with CRC.

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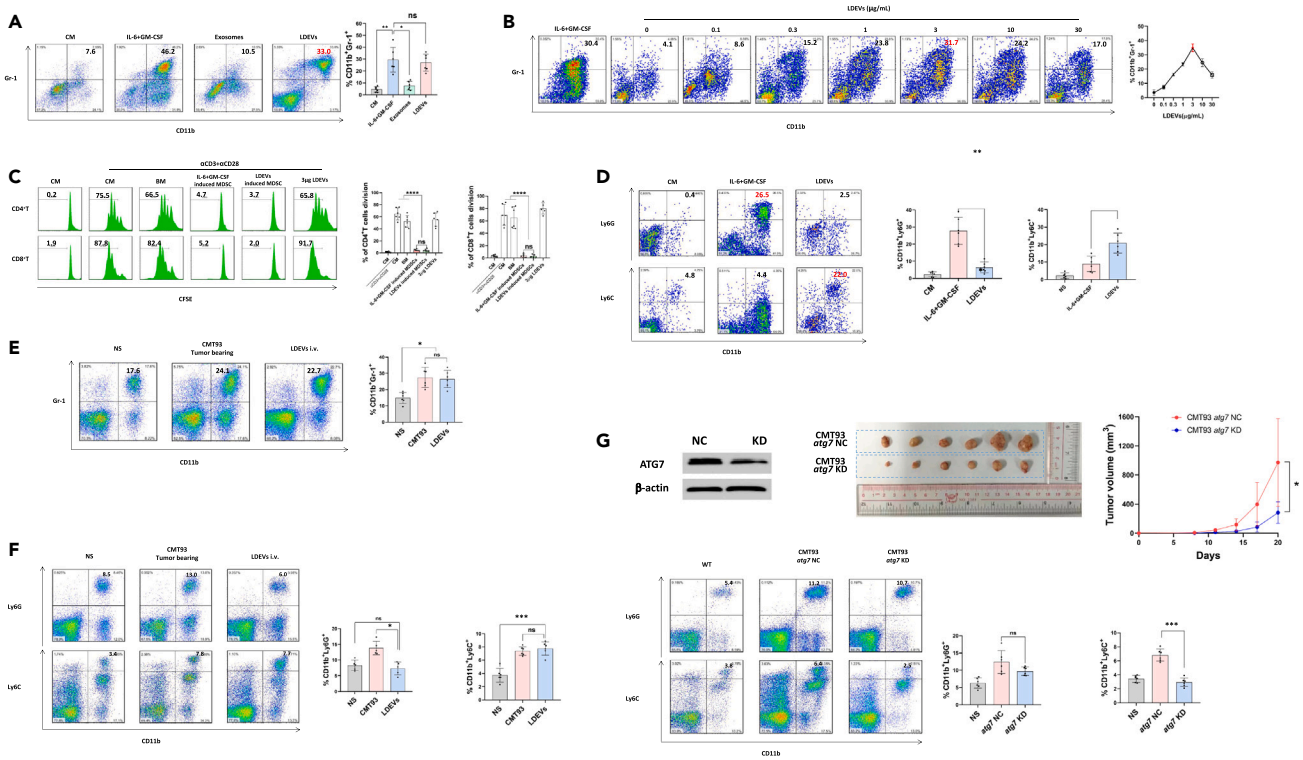

Figure 1. LDEVs induced M-MDSCs *in vitro* and *in vivo*

(A and B) BM cells treated with control media (CM), cytokines (IL-6 and GM-CSF), exosomes, LDEVs (A) or with different doses of LDEVs (B) for 4 days. The percentage of $CD11b^{+}Gr\text{-}1^{+}$ cells were assessed by flow cytometry.

(C) CFSE-labeled spleen cells were co-cultured with cytokines or LDEVs pre-treated BM cells stimulated with anti-CD3/anti-CD28 for 3 days. CFSE dilution was determined by flow cytometry.

(D) BM cells treated with control media (CM), cytokines or LDEVs for 4 days. The percentage of $CD11b^{+}Ly6G^{+}$ or $CD11b^{+}Ly6C^{+}$ cells were assessed by flow cytometry. (E and F) C57BL/6 mice were injected s.c. with CMT93 cells to establish tumor bearing models or injected i.v. with LDEVs every other day ten times (n = 6 per group). On the 20th day, the frequencies of MDSCs and the two subtypes in peripheral blood were analyzed by flow cytometry.

(G) Control (NC) or *atg7* KD CMT93 cells were inoculated s.c. into C57BL/6 mice (n = 6 per group). On the 20th day, the frequencies of two subtypes of MDSCs in peripheral blood were analyzed by flow cytometry. Data (mean ± SEM) represent 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

RESULTS

CRC cell-derived LDEVs mediated M-MDSCs formation both *in vitro* and *in vivo*

To reveal whether CRC cell-derived EVs mediated MDSCs formation, two major kinds of EVs, LDEVs,[14] and exosomes,[15] were enriched from CMT93 culture media and then cultured with mouse bone marrow (BM) cells separately. Apparently, LDEVs treated BM cells exhibited the same phenotype as cytokines (IL-6 and GM-CSF) induced MDSCs (Figures 1A, S1, and S3). Dose analysis showed that the proportion of $CD11b^{+}Gr\text{-}1^{+}$ cell peaked after incubation with 3 μg/mL LDEVs. But high concentrations of LDEVs lead to cell death, which might be caused by overstimulation (Figures 1B and S5). Subsequently, the suppressive function was defined via examining the LDEVs induced MDSCs' capacity in inhibiting T cell proliferation with anti-CD3 and anti-CD28 stimulation.[4] LDEVs endows these BM cells with a substantial suppressive activity similar as cytokines, which allow their denomination as MDSCs (Figure 1C). However, further investigation of the subfractions of these cells showed that LDEVs treated BM cells resulted in a profound expansion of $CD11b^{+}Ly6C^{+}$ M-MDSCs, which is in contrast with cytokines mainly induced $CD11b^{+}Ly6G^{+}$ PMN-MDSCs (Figures 1D and S2).

These findings were next validated in the murine models. In LDEVs treated (i.v.) mice, the frequency of MDSCs in peripheral blood were also markedly increased as CMT93 tumor bearing mice (Figure 1E). Interesting, i.v. LDEVs didn't influence PMN-MDSCs accumulation but M-MDSCs in mouse peripheral blood (Figure 1F). Furthermore, *atg7*, a core gene for LDEVs formation, was knocked down to abolish the generation of LDEVs from tumor tissue.[14] In the mice bearing *atg7* knockdown (KD) CMT93 tumors, the frequency of M-MDSCs cells in peripheral blood were significantly reduced as compared to those in the mice bearing control tumors, whereas the frequency of PMN-MDSCs were less affected (Figure 1G). Taken together, these results demonstrated that LDEVs were participating in modulating M-MDSCs formation both *in vitro* and *in vivo*.

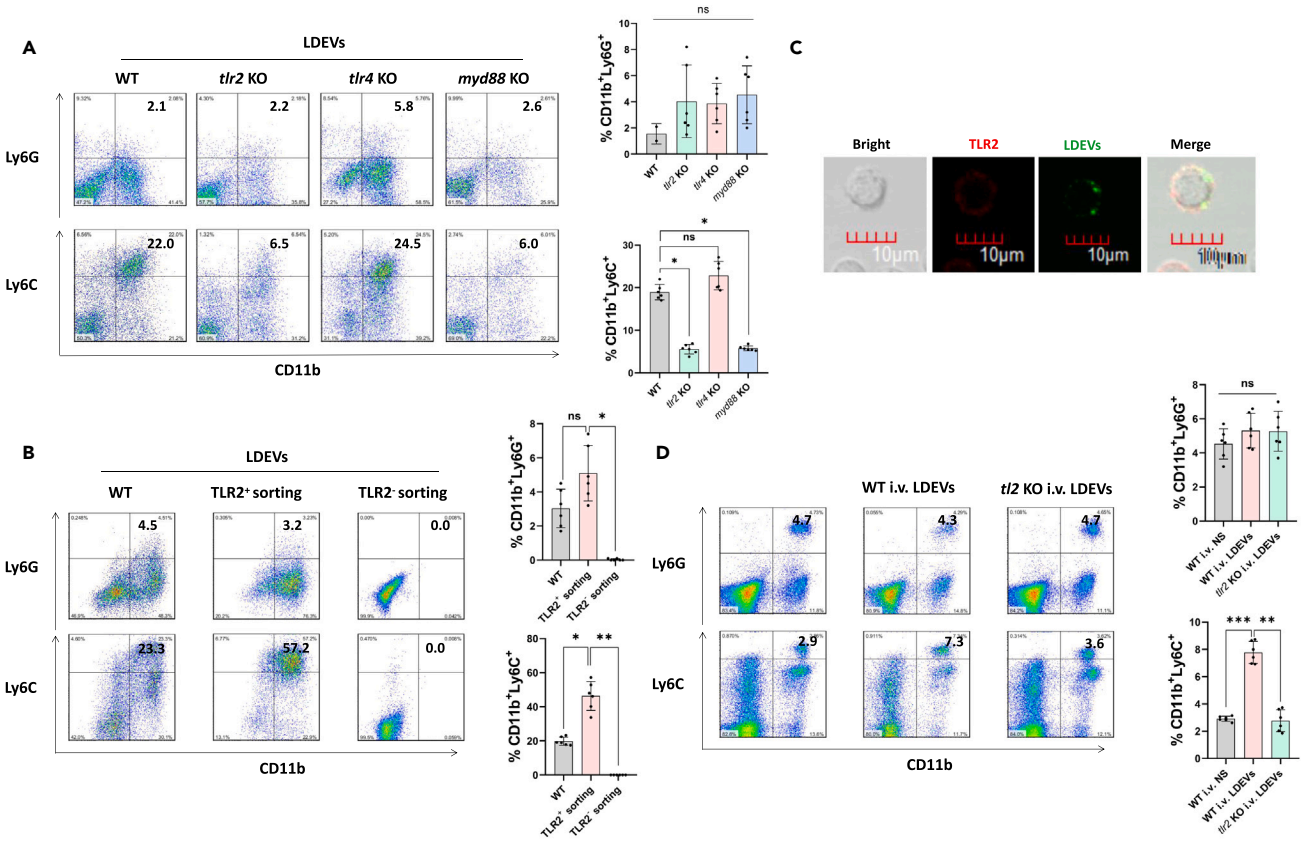

Figure 2. LDEVs induced M-MDSCs via TLR2-MyD88 pathway

(A and B) WT, *tlr2* KO, *tlr4* KO, *myd88* KO mice BM cells or purified $TLR2^+$ and $TLR2^-$ BM cells were co-cultured with LDEVs (3 μg/mL) for 4 days. Then the percentage of two subtypes of MDSCs was assessed by flow cytometry.

(C) C57BL/6 mice were injected i.v. with CFSE-labeled LDEVs for 24 h. And then BM cells were stained for TLR2 and analyzed by confocal microscopy.

(D) C57BL/6 mice were injected i.v. with NS or LDEVs, as well as *tlr2* KO mice were injected i.v. with LDEVs every other day ten times (n = 6 per group). On the 20th day, the frequencies of two subtypes of MDSCs in peripheral blood were analyzed by flow cytometry. Data (mean ± SEM) represent 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

LDEVs triggered MDSCs induction via TLR2-MYD88

LDEVs have been verified to initiate multiple pattern recognition receptors (PRRs) in tumor progression.[16,17] Among these well characterized PRRs, functional TLR2 and TLR4 were high expressed on early hematopoietic progenitor cells and can be triggered directly in an absence of exogenous growth or differentiation factors.[18] Therefore, we examined whether LDEVs induce BM cells differentiate to MDSCs via this manner. BM cells of mice genetically deficient in *tlr2* (*tlr2* knockout [KO]), tlr4 (*tlr4* KO) or *myd88* (*myd88* KO) were treated with LDEVs. *tlr2* KO and *myd88* KO BM cells were completely defective in MDSCs induction in response to LDEVs, while *tlr4* KO as well as the wild-type (WT) controls did not impact the frequency of MDSCs (Figure 2A). Subsequently, WT BM cells were sorted to TLR2 positive and TLR2 negative groups, and then treated with LDEVs, respectively. Compared with TLR2 negative BM cells, only TLR2 positive BM cells could be induced to M-MDSCs by LDEVs (Figure 2B). After mice were i.v. CFSE-labeled LDEVs for 24 h, TLR2 on the surface of BM cells was in direct contact with LDEVs obviously (Figure 2C). In agreement with the previous finding, *tlr2* KO mice i.v. LDEVs had a significant reduction of M-MDSCs compared to WT mice (Figure 2D). Collectively, these data proved that LDEVs induced M-MDSCs differentiation in a TLR2-MYD88-dependent manner.

Hsp60 was the surface ligand of LDEVs that induced M-MDSCs

To further identify the molecular components of LDEVs that were responsible for activating TLR2, we firstly seek out the levels of several TLR2 ligands (including HSP27, HSP60, HSP70, HSP90, and HMGB1) on the surface of LDEVs.[13,19] Figure 3A showed these ligands were high presented remarkably. Subsequently, LDEVs were pre-treated with the antibodies of these potential ligands and then co-cultured with BM cells. Blocking of HSP60, but not HSP27, HSP90, HSP70, or HMGB1 on the surface of LDEVs partially diminished LDEVs induced M-MDSCs (Figures 3B and S4). To verify the roles of Hsp60 in M-MDSCs induction *in vivo*, *Hsp60* knockdown CMT93 cell line (*hsp60* KD) and its negative control (*hsp60* NC) were established. The results showed LDEVs isolated from *hsp60* KD or control CMT93 cells were injected to mice

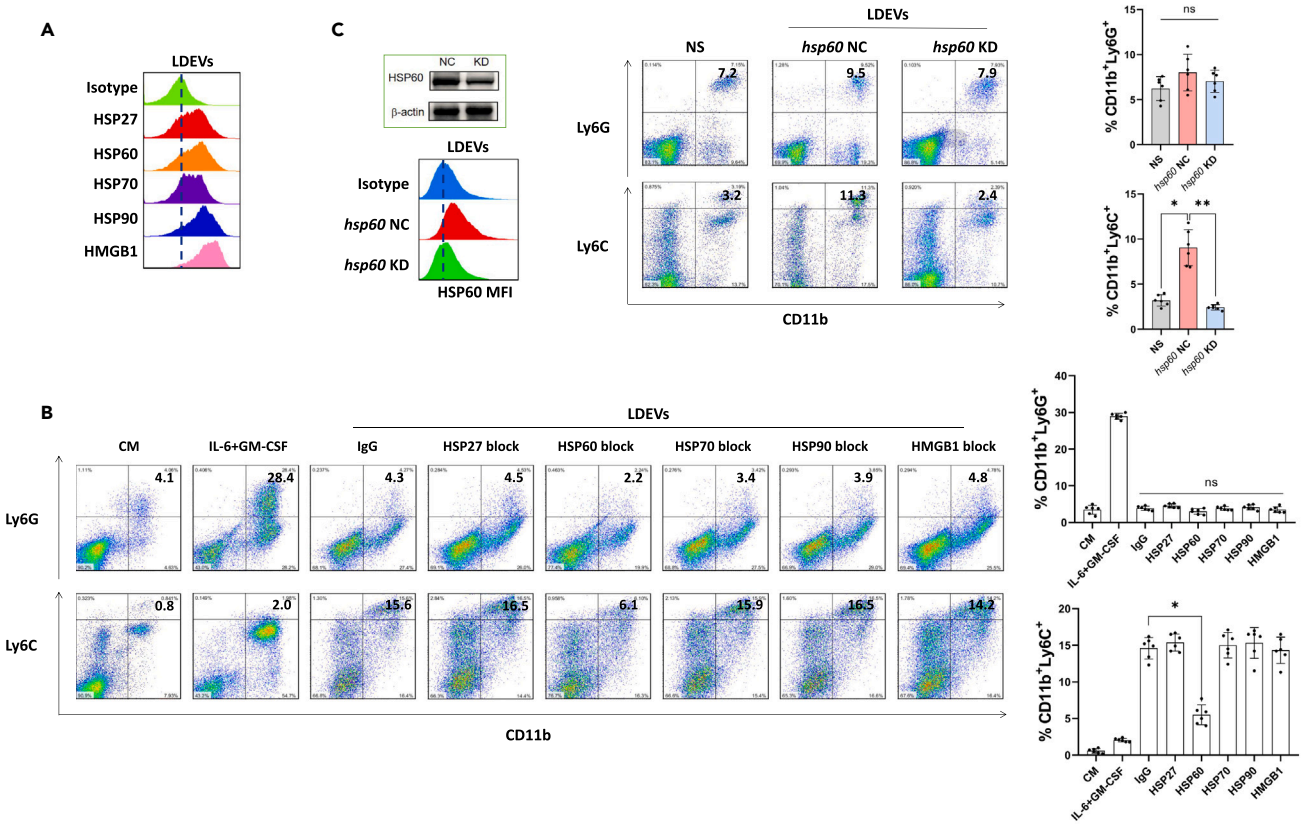

Figure 3. HSP60 on LDEVs triggered M-MDSCs induction

(A) Flow cytometric determination of TLR2 ligands (HSP27, HSP60, HSP70, HSP90, or HMGB1) expression levels on the surface of LDEVs from CMT93 cells.

(B) BM cells treated with LDEVs (3 μg/mL) or TLR2 ligands antibody pretreated LDEVs for 4 days. Then the percentage of two subtypes of MDSCs was assessed by flow cytometry.

(C) BM cells were co-cultured with NS, LDEVs from control (NC) or *hsp60* KD CMT93 cells for 4 days. Then the percentage of two subtypes of MDSCs was assessed by flow cytometry. Data (mean ± SEM) represent 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

separately. In the mice i.v. *hsp60* KD LDEVs, the frequency of circulating M-MDSCs markedly decreased compared with the mice i.v. *hsp60* NC LDEVs (Figure 3C). These results confirmed the key role of HSP60 on the surface of LDEVs in mediating M-MDSCs induction.

LDEVs induced MDSCs inhibited T cells division via both cell contact and non-contact manners

Based on the different MDSC subtypes between LDEVs and cytokines induced, it seemed possible that LDEVs induced MDSCs might perform distinct suppressive functions compared to cytokines induced. Thus, we determined the functional impact by co-culturing CFSE-labeled PBMCs with multiple proportion dilution of MDSCs induced by LDEVs or cytokines with anti-CD3 and anti-CD28 stimulation.[4,5] When the MDSCs: PBMCs was 1:4, cytokines induced MDSCs restored T cells division. Until the proportion was 1:8, a significant reduction of T cells proliferation was observed in LDEVs induced MDSCs (Figure 4A). These confirmed that LDEVs induced MDSCs have stronger suppressive capacity than cytokines induced on per cell basis. Previous researches have reported mechanisms of M-MDSCs immunosuppression commonly include producing suppressive metabolites and upregulating inhibitory ligands.[4,20] We firstly scanned the potential mechanisms affiliated with T cell inhibition that dependent cell contact. LDEVs induced MDSCs upregulated the expression of PD-L1 as well as cytokines. However, LDEVs induced MDSCs upregulated the expression of CTLA-4 and had no impact on reactive oxygen species (ROS) production, which were totally opposite to cytokines induced MDSCs (Figure 4B). And then the 3μm microporous transwells were used to disrupted cell contact in the co-cultured system. In contrast to the cytokines, suppressive activity of LDEVs induced MDSCs was undisrupted (Figure 4C). Therefore, LDEVs induced MDSCs performed immunosuppression via both cell contact and non-contact manners.

LDEVs levels correlated with circulating M-MDSCs accumulation, IL-10 and arginase secretion in clinical CRC patients

Samples of peripheral blood were collected from 28 CRC patients at initial diagnosis to verify the correlating between circulating LDEVs and M-MDSCs. Remarkably, large amount of LDEVs accumulated in patients' peripheral blood compared to healthy donors. And these LDEVs

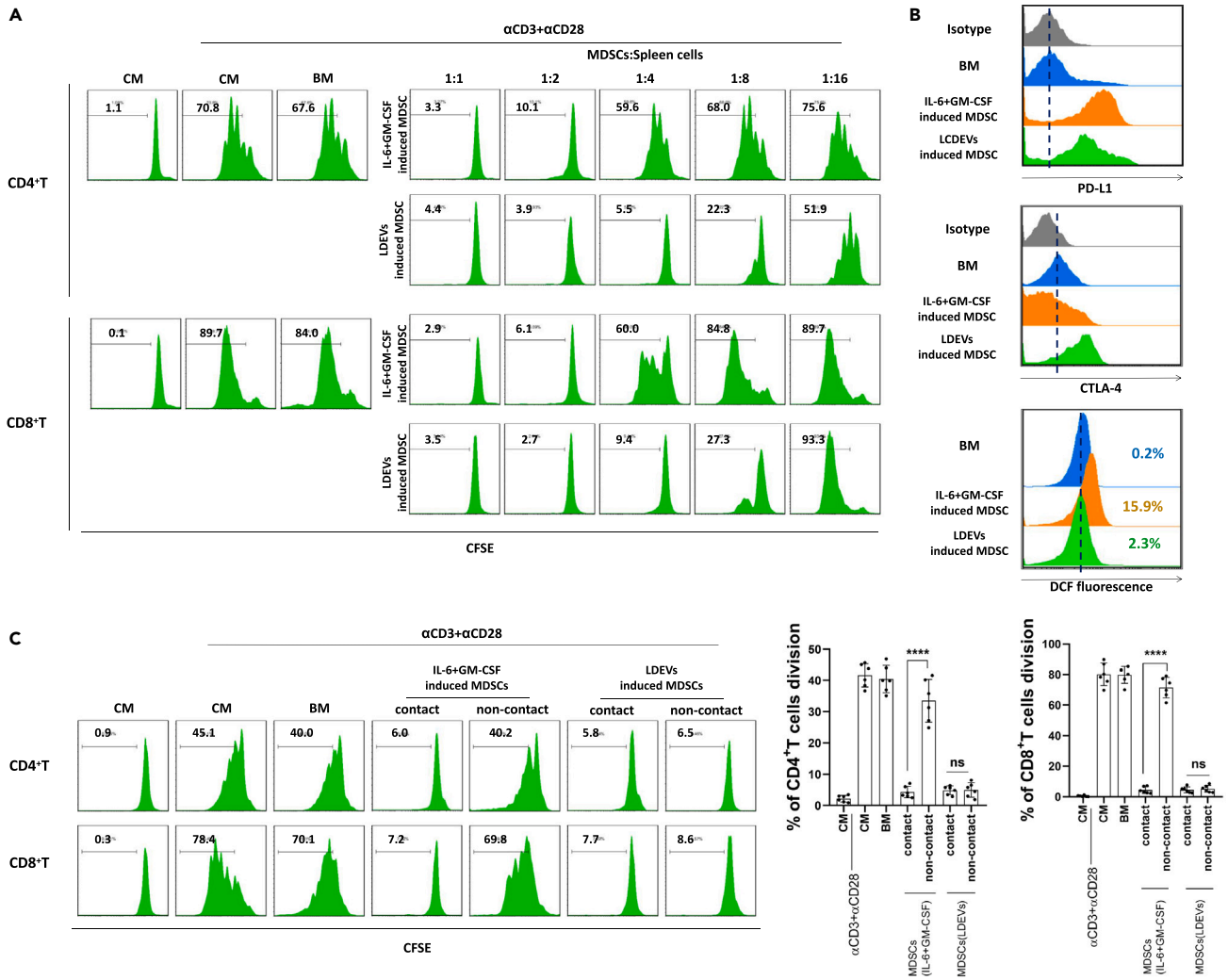

Figure 4. LDEVs induced MDSCs inhibited T cells division via both cell contact and non-contact manners

(A) CFSE-labeled spleen cells were co-cultured with multiple proportion dilution of cytokines or LDEVs pre-treated BM cells stimulated with anti-CD3/anti-CD28 for 3 days. CFSE dilution was determined by flow cytometry.

(B) The PD-1, CTLA-4 and intracellular ROS and of cytokines or LDEVs pre-treated BM cells were detected by flow cytometry.

(C) In the transwell experiment, cytokines or LDEVs induced MDSCs and CFSE-labeled PBMCs were added in the upper and lower chambers, respectively. Three days later, CFSE dilution was determined by flow cytometry. Data (mean ± SEM) represent 3 independent experiments. ****, $p < 0.0001$; ns, not significant.

high-expressed EPCAM, which indicated these vehicles were released by CRC tumor cells.[21] Furthermore, the levels of LDEVs had no connection with patients gender, age, or neoplasms staging (Figures 5A–5C). Although frequencies of MDSCs in peripheral blood were significantly elevated in CRC patients, the levels of LDEVs were only related to M-MDSCs frequencies significantly (Figure 5D). Subsequently, the levels of CRC patients' serum cytokine profiles were detected. The elevated levels of secretion of interleukin-10 (IL-10) and arginase could be obvious in CRC patients. And the baseline signature of IL-10 and arginase was associated with circulating LDEVs levels (Figure 5E). These results validated the correlations between LDEVs and M-MDSCs accumulation in clinical CRC patients, and partly explored the potential mechanisms of non-contact suppressive functions.

DISCUSSION

There was no doubt of how circulating MDSCs promoted CRC immunosuppression and tumor microenvironment (TME) development.[22,23] In contrast, there was little mechanisms explained how these cells come from. Here, we demonstrated that CRC cells-derived LDEVs loading and secretion directly induced M-MDSCs differentiation both *in vitro* and *in vivo* via TLR2-MyD88 pathway. Notable, LDEVs induced M-MDSCs have stronger suppressive capacity than cytokines induced on per cell basis. And these M-MDSCs could inhibit T cells division via both

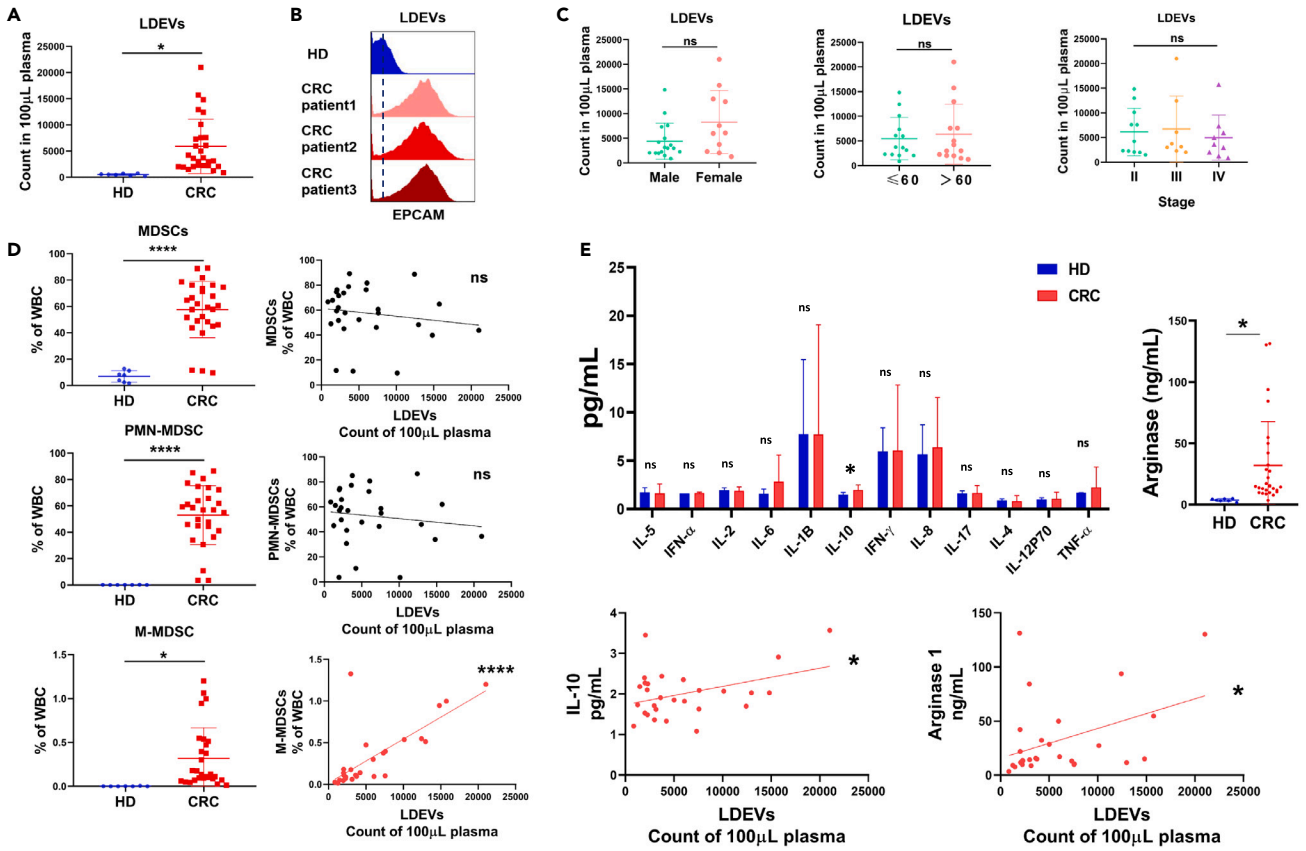

Figure 5. LDEVs levels correlated with circulating M-MDSCs accumulation, IL-10 and arginase secretion in clinical CRC patients

(A) Counters of LDEVs in 100μL plasma of healthy donners (HD) and CRC patients were analyzed by flow cytometry.

(B) And then the EPCAM levels expression on these LDEVs were assessed by flow cytometry.

(C) Statistical analysis of the correlations between the counters of LDEVs and the clinical characteristics of CRC patients.

(D) The proportion of MDSCs was significantly increased in CRC patients' peripheral blood compared with the healthy donors. Statistical analysis of the correlations between the counters of LDEVs and the proportion of circulating MDSCs of CRC patients.

(E) Levels of IL-1β, IL-2,IL-4, IL-5, IL-6, IL-8, IL-10, IL-12P70, IL-17, IFN-α, IFN-γ, TNF-α and arginase 1 in plasma of healthy donors (HD) and CRC patients were detected by ELISA. Statistical analysis of the correlations between the counters of LDEVs and the significant elevated cytokines. Associations between two groups were analyzed by student *t* tests. *, $p < 0.05$; ****, $p < 0.0001$; ns, not significant.

cell contact and non-contact manners. Remarkably, new research finds M-MDSCs correlate with immunosuppression and higher risk of tumor recurrence in CRC.[24] This might partly explain why sometimes clinical anti-PD-1 or anti-PD-L1 therapy failure while the PD-1 or PD-L1 was high expressed.[25]

Clear phenotypic characterization of MDSCs relies on multicolor flourescence activated cell sorting (FACS) analysis. However, the most pressing unmet issues of this method made it infeasible wide use in clinical: samples should be detected in time with expensive instrument. And the protocol is tedious and technology challenge to in grass-roots medical staffs.[4] Exciting, large amount of LDEVs could be easily detected and absolute counted in 100μL CRC patients' plasma. And these EVs would be potential predictive markers for *in vivo* assessment of M-MDSCs in CRC patient, which is important to immunological evaluation and disease monitor in future in prospective investigation for CRC treatment.

Limitations of the study

Limited sample size utilized in this research contributes to the lack of refined analysis of correlation between LDEVs expression and clinical characteristics. In addition, In the search for mechanisms of LDEVs induced MDSCs immunosuppression, we found that the high expression of PD-L1 and CTLA4 might mediate the cell contact-dependent immunosuppression, and the significant secretion of IL-10 and arginase 1 might mediate the non-contact immunosuppression. How these mechanisms work needs further verification. At last, as LDEVs loading and secretion is a universal biological phenomenon in tumor progression, future studies should incorporate more clinical cases to clarify whether this correlation is coincident for pan cancer.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Y.G., Q.L., Q.H., and Q.W. conducted most of the experiments and wrote the manuscript. H.S. and H.J. prepared the reagents and chemicals. L.L. and D.X. performed the cell and animal experiment analyses. L.Z., L.X., Y.Z., and S.C. collected and analyzed the clinical specimens. J.Y. and X.Z. designed the study. X.Z. also edited and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD3	BD Biosciences	Cat #340949
Anti-CD28	BD Biosciences	Cat #560684
Anti-Hsp27	Sigma Aldrich	Cat #SAB4501457
Anti-Hsp60	Sigma Aldrich	Cat #PLA0269
Anti-Hsp70	Sigma Aldrich	Cat #SAB4200714
Anti-Hsp90	Sigma Aldrich	Cat #MABS1327
Anti-HMGB1	Sigma Aldrich	Cat #SAB2108675
Anti-atg7	CST	Cat #8558
Anti-β-catin	CST	Cat #4967
CD11b-FITC	Biolegend	Cat #101206
Ly6G-PE	Biolegend	Cat #127608
Ly6C-APC	Biolegend	Cat #128012
Gr-1-APC	Biolegend	Cat #108412
CD4-APC	Biolegend	Cat #100412
CD8-APC	Biolegend	Cat #100712
Bacterial and virus strains		
sh-RNA lentiviral vectors	Sigma Aldrich	Cat #SHC004
Biological samples		
L-6	PeproTech	Cat #P08887
GM-CSF	PeproTech	Cat #P09920
DMEM	Gbico	
RMPI 1640	Gbico	
Chemicals, peptides, and recombinant proteins		
carboxy-fluoroscein-diacetate, succinimidyl ester	Invitrogen Molecular Probe	
Critical commercial assays		
Cytokine profiles ELISA kits	Biolegend	
Experimental models: Cell lines		
LDEVs treated BM cells	This paper	
Exosomes treated BM cells	This paper	
IL-6+GM-CSF treated BM cells	This paper	
TLR2 positive BM cells	This paper	
TLR2 negative BM cells	This paper	
Hsp60 KD CMT93 cell line	This paper	
Hsp60 NC CMT93 cell line	This paper	
Experimental models: Organisms/strains		
LDEVs treated mice	Laboratory Animal Center of Zhejiang University	
CMT93 tumor bearing mice	Laboratory Animal Center of Zhejiang University	
Atg7 KD CMT93 tumor bearing mice	Laboratory Animal Center of Zhejiang University	
Atg7 NC CMT93 tumor bearing mice	Laboratory Animal Center of Zhejiang University	
C57BL/6 female wild type mice	Laboratory Animal Center of Zhejiang University	

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tlr2 KO mice	Laboratory Animal Center of Zhejiang University	
Tlr4 KO mice	Laboratory Animal Center of Zhejiang University	
Myd88 KO mice	Laboratory Animal Center of Zhejiang University	
Software and algorithms		
Graphpad Prism8		
SPSS 22		
LEGENDplex 8.0.		

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaofeng Zhang (xfzhang837@163.com)

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon reasonable request.
- No original code was reported in this paper.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

C57BL/6 female wild type, *tlr2* KO, *tlr4* KO and *myd88* KO mice were purchased from the Laboratory Animal Center of Zhejiang University (Zhejiang, China). Mice were maintained in the barrier facility at Zhejiang University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Animal models

Mice were subcutaneously inoculated with CMT93, CMT93 *atg7* NC or CMT93 *atg7* KD cells (2×10^5 cells/100μL/mouse) as tumor bearing mice models. Mice were injected with LDEVs (30 μg/100 μL/mouse, Qod/10 days) as LDEVs i.v. mice models. On day 20, circulating PMN-MDSC and M-MDSC were detected by flow cytometry. And tumor growth was monitored over time. Tumor volume was estimated from two perpendicular axes using a caliper (volume=1/2(length × width^2)).

Cell culture

CMT93 cells (ATCC, CCL-223) were cultured in DMEM (Gbico) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gbico). To generate stable knockdowns, cells were transduced with lentiviral vectors (Sigma Aldrich) expressing shRNAs targeting *atg7* (5′-CCAGCTCTGAACTCAATAATA-3′), *Hsp60* (5′-CCTGCTAATGAAGACCAGAAA-3′) and non-targeting shRNA (5′-TTCTCCGAACGTGTCACGTAA-3′).

The proteins samples extracted from these cells were prepared using RIPA lysis buffer with protease inhibitor. 30μg of protein loaded per well and immune blotted overnight at 4°C with antibody against atg7 (Catalog #8558, CST), HSP60 (Catalog #PLA0269, Sigma Aldrich) and β-actin (Catalog #4967, CST). Mice bone marrow cells and spleen cells were cultured in RMPI 1640 (Gbico) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

CRC samples

Peripheral blood was collected from 7 healthy donors(n=7,3 as male and 4 as female) and 28 patients(n=28,13 as male and 15 as female)) aged 18-70 years old with CRC at the time of first diagnosis at Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, Hangzhou, China, from April 2021 to July 2021; these patients did not receive any pre-operative chemo-radiotherapy. This study was approved by the Research Ethics Committee of the Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine and followed the Declaration of Helsinki. All participants signed written informed consents before the study. Then the phenotype and

percentage of M-MDSCs ($CD11b^{+}HLA\text{-}DR^{-/LO}CD14^{+}CD15^{-}$) and PMD-MDSCs ($CD11b^{+}HLA\text{-}DR^{-/LO}CD14^{-}CD15^{+}$), and the levels of LDEVs were detected in flow cytometric applications.

METHOD DETAILS

EVs preparation and characterization

EVs were isolated from CMT93 cells culture supernatants according to standard differential centrifugation protocols.[26,27] Briefly, supernatants were centrifuged at 2000 rpm for 10 min to pellet whole cells. Then supernatants were centrifuged 4000 rpm for 20 min to pellet cellular debris and apoptotic bodies. The supernatants were further centrifuged at 10,000 g for 30 min to harvest large EVs (LDEVs-containing), and then centrifuged at 100,000 g in an ultracentrifuge for 2 h to harvest exosomes. The large EVs pellet isolated with magnetic beads combined with LC3b antibody for LDEVs. Two kinds of EVs pellet were washed three times with PBS and quantified the total protein content via BCA assay. The purity of LDEVs was analyzed by flow cytometry.

MDSCs induction and characterization *in vitro*

Mouse bone marrow cells were obtained and co-cultured with IL-6 (40 ng/mL, PeproTech) plus GM-CSF (40 ng/mL, PeproTech), LDEVs or exosomes for 4 days in a 24-well plate.[8] Then the phenotype and percentage of these cells were detected in flow cytometric applications. The following Abs were used for flow cytometry analysis of mouse cells: CD11b-FITC, Ly6G-PE, Ly6C-APC, Gr-1-APC (Biolegend). In some cases, 10μg LDEVs were pre-treated with HSP27, HSP60, HSP70, HSP90, HMGB1 antibodies (10 μg, Sigma Aldrich) separately for 24h in 4°C and then washed three times with PBS for follow-up study.

CFSE labeling and proliferation assay

PBMCs from mouse spleen cells were stained with 0.5 mM carboxy-fluoroscein-diacetate, succinimidyl ester (CFSE), according to manufacturer's instructions (Invitrogen Molecular Probe).[28] CFSE-labeled PBMCs were stimulated with coated 2 μg/mL anti-CD3 and 2 μg/mL anti-CD28 mAb (BD Biosciences) and cocultured at 1:1 ratio with BM cells, cytokines or LDEVs induced MDSCs in a 24-well plate for 3 days. Then cells were stained with CD4-APC or CD8-APC (Biolegend), and CFSE signal of gated lymphocytes was analyzed.

Co-localization assay

Mice were intravenous injection of CFSE labeled-LDEVs for 24 hours (30 μg/100 μL/mouse). Then bone marrow cells were stained with TLR2-PE and analyzed by confocal microscopy (OLYMPUS FV1000).

Elisa

Cytokine profiles in serum were quantified using ELISA kits according to the manufacturer's protocol. ELISA sets were purchased from Biolegend and analyzed by LEGENDplex 8.0.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyses were carried out using GraphPad Prism 8 and SPSS 22. Associations between two groups were analyzed by student *t* tests. All data are presented as the mean ± SEM and $p < 0.05$ was defined as statistically significant.