Circulating exosomes from esophageal squamous cell carcinoma mediate the generation of B10 and PD-1\textsuperscript{high} Breg cells

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Abbreviations: B10, IL-10\textsuperscript{+} Breg; Breg, regulatory B cell; ESCC, esophageal squamous cell carcinoma; GO, gene ontology; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNA; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; TLR, Toll-like receptor; Treg, regulatory T cell.

Abstract
As one of the most frequently diagnosed cancers, esophageal squamous cell carcinoma (ESCC) remains the leading cause of malignancy-related death worldwide. Many studies have focused on the potential role of cancer cells in educating B cells during cancer progression. Here, we aim to explore the role of circulating exosomes from ESCC in the generation of two main regulatory B (Breg) subsets, including interleukin-10\textsuperscript{+} Bregs (B10) and programmed cell death (PD)-1\textsuperscript{high} Bregs. Firstly, we observed an elevated percentage of B10 cells in peripheral blood of ESCC patients compared with healthy controls. Then we isolated and characterized exosomes from the peripheral blood of ESCC patients and an ESCC cell line. Exosomes from ESCC patients and the ESCC cell line suppressed the proliferation of B cells and induced the augmentation of B10 and PD-1\textsuperscript{high} Breg cells. By comparing the long non-coding RNA and mRNA expression profiles in exosomes from ESCC patients or healthy controls, we identified a series of differentially expressed genes. Finally, we undertook gene annotation and pathway enrichment analyses on differentially expressed genes to explore the potential mechanism underlying the modulatory role of cancer exosomes in B cells. Our findings contribute to the study on B cell-mediated ESCC immunosuppression and shed light on the possible application of exosomes in anticancer therapies.

KEYWORDS
Breg, esophageal squamous cell carcinoma, exosome, IL-10, PD-1
1 | INTRODUCTION

As the sixth leading cause of cancer-associated death, esophageal cancer results in approximately 400,000 mortalities globally every year.\(^1\)\(^2\) In Asian countries, ESCC remains the predominant histologic type of esophageal cancer.\(^3\)\(^4\) Despite incremental advancements in diagnostics and therapeutics, its prognosis remains poor with a 5-year survival rate of 15%-25%.\(^5\) As a result, there remains an urgent need to elucidate the molecular mechanisms underlying ESCC progression. Recent studies focused on the dynamic changes of the immune system in cancer progression.\(^5\) Actually, growing evidence has raised a bidirectional regulatory role of the immune system in facilitating tumor growth, with several studies reporting that the immune system can recognize tumor cells and restrain cancer progression.\(^6\)\(^7\)

In oncoimmunology, the balance between immune-effective and immunosuppressive cells has aroused great attention. Moreover, a growing number of studies have begun to focus on how these cells regulate the tumor microenvironment. Among immune regulatory cells, robust predictive value has been endowed to the regulatory T cells that participate in tumor progression with immunosuppressive functions.\(^8\) Although current immunotherapeutic strategies focus on T cell-mediated immunity, in recent years, growing research has assigned a critical role to B cells, which could act as immunoregulatory factors in modulating carcinogenesis and cancer progression.\(^9\)

Regulatory B cells are a subset of B lymphocytes that suppress inflammatory responses and maintain immune tolerance. Regulatory B cells are consistently abundant cellular components in the tumor microenvironment, whereas its subpopulation composition and the potential regulatory effects on cancers, especially ESCC, remain poorly understood.\(^10\) Interleukin-10\(^+\) Breg cells, referred as B10, exert their function by IL-10 secretion and have unique CD19\(^+\)CD24\(^+\)CD27\(^+\) phenotypes in human.\(^11\) Great attention has been paid on the relationships between various types of cancers and B10, including gastric cancer and breast cancer.\(^12\) Recently, a novel PD-1\(^+\) regulatory B-cell subset has been identified that is associated with tumor stage and early recurrence in human hepatocellular carcinoma.\(^13\) However, the association between ESCC and B10 cells and PD-1\(^+\) Bregs remains unclear.

In the tumor microenvironment, the proliferation and differentiation of B cells can be regulated by cancer cells through exosomes in glioma and Burkitt’s lymphoma.\(^14\)\(^16\) Moreover, studies showed that proteins and RNAs that are packaged into the exosomes are highly stable.\(^17\)\(^18\) Exosomes are small membrane vesicles of endocytic origin and act as cargos to deliver specific genetic messages to the target cells and further modulate their phenotype and biological processes.\(^19\) Hence, we mainly observed the functions of exosomes from ESCC in B cell modulation.

Here, we assessed the ratio of B10 cells in the peripheral blood from ESCC patients. Moreover, we explored the modulatory effects of exosomes in the differentiation of B cells into B10 and PD-1\(^+\) B cells.

2 | MATERIALS AND METHODS

2.1 | Patients and serum samples

Thirty normal healthy volunteers and 30 ESCC patients were recruited at the Department of Oncology, First Hospital of Qinhuangdao (Qinhuangdao, China) from August 2018 to January 2019. Patients were histopathologically diagnosed by at least 2 independent senior pathologists. None of the patients had prior chemotherapy or radiotherapy, or any other serious diseases. This study was carried out according to the recommendations of the Human Investigation Committee of The First Hospital of Qinhuangdao. Written informed consent was obtained from all subjects. The clinical and pathological characteristics of the 30 patients and healthy donors are presented in Tables 1 and S1.

2.2 | Cell culture

As we previously reported, ESCC cell line (ECA109) was cultured in DMEM with 10% FBS (Gibco) in a humid environment at 37°C containing 5% CO\(_2\).\(^3\)

2.3 | Isolation and identification of exosomes

The exosomes in peripheral blood samples from patients and normal volunteers were purified using ExoQuick exosome precipitation solution (System Biosciences).

| TABLE 1 | Clinical and pathological characteristics of 30 patients with esophageal squamous cell carcinoma |
|----------|---------------------------------------------|
| Clinical parameter | N |
| Gender | |
| Male | 18 |
| Female | 12 |
| Age, years | |
| <60 | 14 |
| ≥60 | 16 |
| Family history | |
| Positive | 6 |
| Negative | 24 |
| Tobacco use | |
| Yes | 10 |
| No | 20 |
| Alcohol use | |
| Yes | 19 |
| No | 11 |
| TNM stage | |
| I | 3 |
| II | 8 |
| III | 10 |
| IV | 9 |
2.4 | Transmission electron microscopy

After fixed by glutaraldehyde and paraformaldehyde, exosomes were loaded on carbon-coated electron microscopy grids and then negatively labeled with methylamine tungstate. A transmission electron microscope was used to capture the microscopy images (Zeiss).

2.5 | Nanoparticle tracking analysis

The size distribution of isolated extracellular vesicles was measured using Nanosight (Malvern) and NTA analytical software (version 2.3; Nanosight).

2.6 | Western blot analysis

Exosomes were lysed by RIPA buffer (Roche). After the protein concentrations were quantified with a BCA Protein Concentration Kit (Beyotime), proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. Then the PVDF membrane with proteins was blocked with 5% nonfat milk and incubated with primary Abs (CD9,CD81) (Cell Signaling Technology) overnight at 4°C. After incubation with secondary Ab (Icllab), the bands were visualized by chemiluminescence (Millipore). ImageJ (https://imagej.nih.gov/ij/) was applied to quantify the intensity of the protein bands.

2.7 | Isolation and culture of B cells from PBMCs

Human PBMCs were isolated using density gradient centrifugation with Ficoll-Paque Plus (Sigma-Aldrich). Then CD19⁺ B cells were magnetically purified from the PBMCs using magnetic microbeads (Miltenyi Biotec). Isolated CD19⁺ B cells were cultured in RPMI-1640 medium supplemented with 1 μg/mL CD40L plus 5 μg/mL anti-human IgM (R&D Systems).

2.8 | Interactions between B cells and exosomes

Exosomes were labeled with a PKH26 red fluorescent marker according to the manufacturer's instructions (PKH26GL; Sigma-Aldrich). The labeled exosomes were diluted in exosome-free medium and incubated with isolated CD19⁺ B cells labeled with DAPI. The images were obtained with a Zeiss Laser Scanning Confocal Microscope (Zeiss).

2.9 | Cell proliferation assay

Isolated CD19⁺ B cells were cultured in 96-well plates at 5 × 10⁵ cells/well with exosomes for 24 hours. CCK-8 solution (20 μL) (Dojindo) was added to each well for the last 2 hours of culture. A microplate reader (Bio-Rad) was used to measure the absorbance value at 450 nm wavelength. Results were representative of 3 individual experiments.

2.10 | Flow cytometry

Single cell suspension was harvested and stained with a combination of Abs for the cell-surface markers including FITC-labeled anti-human CD19 mAb, phycoerythrin-labeled anti-human CD24 mAb, allophycocyanin-labeled anti-human CD27 mAb, allophycocyanin-labeled anti-human PD-1 mAb or related isotype Abs according to the manufacturer's instructions (BioLegend). For analysis of intracellular IL-10 expression, Cell Stimulation Cocktail (plus protein transport inhibitors) was added during the last 5 hours of culture according to the manufacturer's instructions (eBioscience). After fixed and permeabilized with an intracellular fixation and permeabilization buffer set (eBioscience), cells were incubated with corresponding human Abs. Cells were then analyzed using a BD Biosciences FACSCalibur flow cytometer. Data were processed and analyzed using FlowJo (https://www.flowjo.com/).

2.11 | Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 software. All data are presented as mean ± SD. The B10 ratio in PBMCs and IL-10 expression level in CD19⁺ B cells from ESCC patients or healthy controls were compared using the Mann-Whitney U test. The differences between 2 groups were assessed using Student’s t test. P value < .05 was considered as statistically significant.

3 | RESULTS

3.1 | Expansion of B10 cells in PBMCs of ESCC patients

We first measured the percentage of B10 cells (CD19⁺CD24hiCD27⁻) in PBMCs from ESCC patients (n = 30) and healthy donors (n = 30). Clinical data of the 30 ESCC patients are listed in Table 1. A significant augmentation of B10 cells was observed in the peripheral blood of ESCC patients compared with healthy controls (Figure 1A-C).

Moreover, we assessed the IL-10 expression on CD19⁺ B cells. Results of flow cytometry showed an elevated IL-10 production in peripheral B cells of ESCC patients than healthy donors (Figure 1D-F). These results suggested the potential modulatory role of B10 cells in ESCC progression.

3.2 | Exosome characterization

Peripheral blood-derived exosomes were visualized by electron microscopy imaging (Figure 2A-C). NTA showed that the particle size distribution of purified exosomes from the peripheral blood and ECA109 cell line, ESCC patients, and healthy controls were between 30 and 120 nm (Figure 2D-F). The expression of specific exosome markers (CD9 and CD81) on exosomes from the peripheral blood and ECA109 cell line, ESCC patients and healthy controls was validated by western blot analysis (Figure 2G). These data confirmed that exosomes were purified from the peripheral blood of ECA109 cell line, ESCC patients and healthy controls.
3.3 | Internalization of circulating exosomes by B cells

The CD19⁺ B cells were purified from the peripheral blood of healthy donors. To verify if the circulating exosomes derived from the peripheral blood or ESCC cell lines could interact with purified CD19⁺ B cells, exosomes, labeled with PKH26, were added into the medium and co-incubated with CD19⁺ B cells for 6 hours. After incubation, CD19⁺ B cells were labeled with DAPI (for nuclei) and then captured by confocal microscope. Images showed that the exosomes (red) could be internalized by CD19⁺ B cells. Moreover, the internalized exosomes mainly located in the cytoplasm of CD19⁺ B cells. These data suggested that circulating exosomes could be taken up by CD19⁺ B cells. (Figure 3)

3.4 | Circulating exosomes from ESCC inhibit proliferation of B cells

Exosomes (10 or 20 μg) were added into the medium and cocultured with B cells for 24 hours. The CCK-8 results showed that high-dose exosomes played a better role in suppressing the proliferation...
of CD19\textsuperscript{+} B cells than low-dose exosomes and the control group (Figure 4A).

### 3.5 Circulating exosomes from ESCC mediated differentiation of B cells

We next assessed the modulatory ability of circulating exosomes from ESCC patients (ESCC-Exo), healthy donors (norm-Exo), and ECA109 cells (ECA109-Exo) in the conversion of CD19\textsuperscript{+} B cells from healthy donors. After incubation for 48 hours, cells were harvested and stained with fluorescence-conjugated mAbs for flow cytometry analysis. Results showed that treatment with both circulating exosomes in ESCC patients (ESCC-Exo) and exosomes from ECA109 (ECA109-Exo) significantly increased the ratio of the B10 population (Figure 4B-E).

Similar results were also observed on PD-1 expression and IL-10 production. Figure 5 shows a significant increased expression of both IL-10 (Figure 5A-E) and PD-1 expression (Figure 5F-I) in B cells stimulated by ESCC-Exo and ECA109-Exo. These data indicate the regulatory role of circulating exosomes in the peripheral blood of ESCC patients on B10 cells and PD-1\textsuperscript{hi} Breg cell differentiation.

### 3.6 Comparison of RNA expression profiles between ESCC-Exo and norm-Exo and function annotation

The mRNAs and IncRNAs delivered by exosomes were shown to be functional, which could be translated into protein or act as competing endogenous RNAs in the recipient cells.\textsuperscript{19} To understand the biological mechanism underlying the modulatory effects of exosomes, we downloaded the high throughput sequencing data (GSE104926), including IncRNA and mRNA expression profiles, from Gene Expression Omnibus.

The mRNA and IncRNA profiling was undertaken using the Illumina sequencing platform. By inspecting the expression patterns of global IncRNA and mRNA between serum exosomes from early stage ESCC and healthy controls, we identified significantly differentially expressed IncRNAs and mRNAs. Results showed that 1331 mRNAs and 407 IncRNAs were upregulated and 175 IncRNAs and
947 mRNAs were downregulated in ESCC-Exo compared with norm-Exo, respectively.

Subsequently, we applied GO biological process enrichment and KEGG signaling pathways analysis to explore the regulatory mechanism underlying the ESCC exosomes. As shown in Figure 6A, differentially expressed mRNAs were enriched in GO terms such as the TLR4 signaling pathway (GO: 0034145) and MAPK cascade (GO: 0000165), which induced the conversion of PD-1<sup>hi</sup> Bregs. In order to explore the protein interactions and determine the hub genes, we plotted the protein-protein interaction network of differentially expressed genes enriched in the TLR4 signaling pathway and MAPK. NRAS and MAPK12 were identified as hub genes, which were considered highly correlated with other genes in the network and played critical roles in modulating conversion of PD-1<sup>hi</sup> Breg cells (Figure 6B).

Similarly, in the KEGG signaling pathway analysis, differentially expressed mRNAs were enriched in KEGG terms including the B-cell receptor signaling pathway (has 04662) and MAPK signaling pathway (Figure 6C). The protein-protein interaction network showed that MAP3K2 and RAP1B were hub genes in the MAPK signaling pathway and might participate in the differentiation of PD-1<sup>hi</sup> Breg cells (Figure 6D).

4 | DISCUSSION

In cancer progression, cancer cells generally induce an immunosuppressive microenvironment to fight against anticancer immunology. Recent studies have identified a series of immune cells, such as Tregs, myeloid-derived suppressor cells, tumor-associated macrophages, and Bregs, as key immune regulators that restrain antitumor responses and facilitate cancer progression.

Among Bregs, B10 cells have recently been reported to play key immune regulatory roles in inflammation and autoimmune disease. Here, we showed that patients with ESCC experienced an expansion of B10 cells in the peripheral blood.

It has been widely accepted that tumor cells can educate immune cells to facilitate immunomodulation and establish a stable immunosuppressive microenvironment that helps tumor cells escape from immune surveillance. Recent studies have focused on the
immunological activities of exosomes, which are secreted by tumor cells, in the tumor microenvironment. Exosomes from tumor cells could suppress T cell and natural killer cell activity, and stimulate myeloid-derived suppressor cells in a series of cancers.\textsuperscript{23-26} Here, we evaluated the immune-modulatory effects of circulating exosomes from ESCC in B cells. We found that CD19\textsuperscript{+} B cells could endocytose exosomes from the peripheral blood of ESCC. Subsequent analysis showed that ESCC-Exo suppressed the proliferation of CD19\textsuperscript{+} B cells.
and mediated their differentiation into B10 cells and the production of IL-10.

Previous studies reported that B10 cells could inhibit the production of γ-interferon and tumor necrosis factor-α in CD4⁺ T cells and monocytes in inflammation and autoimmune disease. In chronic graft-versus-host disease, B10 could provide protection by inhibiting the proliferation and suppressive effects of T cells through IL-10 secretion and cell-to-cell contact involving cytotoxic T-lymphocyte-associated protein-4. In gastric cancer, low B10 percentage along with reduced IL-10 production restrained gastric cancer progression in XELOX (capecitabine plus oxaliplatin) regimen-treated patients.

In the ESCC context, we confirmed that ESCC-Exo mediated the differentiation of B10 and evoked the production of IL-10. Based on the evidence above, we considered that these induced B10 cells might further confer the suppressive phenotype on other immune cells and increase the proportion of Bregs and Tregs by producing IL-10, hence orchestrating a positive feedback loop triggered by exosomes.

The modulatory role of PD-1 on T cells in the tumor microenvironment has been well studied. The PD-1 immune checkpoint plays a crucial role in triggering T cell exhaustion during tumor progression. Moreover, PD-1 regulates the secretion of γ-interferon, tumor necrosis factor-α, and IL-2. Existing cancer immunotherapy is focused on the blockage of the PD-L1/PD-1 immune checkpoint.
Therapeutic Abs targeting PD-1 on T cells have made great breakthroughs in clinical trials. In addition to T cells, PD-1 was found on the surface of activated monocytes, natural killer cells, dendritic cells, and B cells. In hepatocellular carcinoma, PD-1+Bregs have been identified as a new B-cell subset. Moreover, we also assessed PD-1 expression on B cells cocultured with ESCC-Exo. Our data suggested that ESCC-Exo increased the PD-1 expression in B cells and mediated the induction of PD-1+Bregs. Furthermore, combination of PD-1 on T cells and PD-L1 on antigen-presenting or cancer cells is a critical mechanism of T cell exhaustion during cancer progression. Hence, whether this mechanism has effects on PD-1+Bregs deserves further exploration. In thyroid tumors, PD-1+Bregs depressed the proliferation and regulatory effects of CD4+ and CD8+ T cells through the PD-1/PD-L1-dependent pathway. Based on these studies and our results, we speculated that a fine-tuned collaborative action among diverse populations of immune cells in the tumor microenvironment might limit the host response to cancer cells.

It has been reported that activation of the TLR4-BCL6 axis contributes to PD-1+Bregs. In addition, mRNAs in the exosomes have been reported to be functional, which could be translated into protein and exert biological effects in the recipient cells. Hence, we compared the mRNAs in exosomes from ESCC and healthy donors. By applying gene annotation and enrichment analyses, we found a series of differentially expressed mRNAs that play a role in the TLR4 and MAPK signaling pathways. A previous report showed that the activation of MAPKs in B cells exposed to multifarious stimuli coincided with the ability of the cells to express BCL6, which is activated by TLR4. Furthermore, activation of the MAPK and TLR4 pathways also enhanced the IL-10 production of B cells and mediated the conversion of B10 cells. Hence, ESCC-derived exosomes could lead to the activation of TLR4 and MAPK signaling pathways and lead to PD-1 expression and IL-10 secretion in recipient B cells.
In conclusion, our results shed light on the possible manipulation by exosomes from ESCC on the differentiation of B10 and PD-1+ Breg cells, which provides new clues to better understand the immune balance in tumor progression.

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DISCLOSURE
The authors have no conflict of interest.

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