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NOVEL CHARACTERIZATION OF bEnd.3 CELLS THAT EXPRESS LYMPHATIC VESSEL ENDOTHELIAL HYALURONAN RECEPTOR-1

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ABSTRACT

Murine bEnd.3 endothelioma cell line has been widely used in vascular research and here we report the novel finding that bEnd.3 cells express lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and vascular endothelial growth factor receptor-3 (VEGFR-3). Moreover, these cells express progenitor cell markers of Sca-1 and CD133. Upon stimulation with tumor necrosis factor-alpha (TNF-α), the bEnd.3 cells demonstrate enhanced formation of capillary-type tubes, which express LYVE-1. As the bEnd.3 cell line is derived from murine endothelioma, we further examined human tissues of endothelioma and identified lymphatic vessels in the tumor samples which express both LYVE-1 and podoplanin. Moreover, a significantly higher number of lymphatic vessels were detected in the endothelioma samples compared with normal control. Taken together, this study not only redefines bEnd.3 cells for vascular research, but also indicates a broader category of human diseases that are associated with lymphatics, such as endothelioma.

Keywords: bEnd.3 cell, LYVE-1, lymphatics endothelioma

The bEnd.3 endothelial cell line has been widely used for blood vascular research (1-4). These cells are originally generated from mouse endothelioma, a benign neoplasm of the endothelial tissue (5,6). To date, it has not been studied whether these cells express other recently identified endothelial cell molecules, such as lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). LYVE-1 was identified in 1997 by searching EST databases for sequences homologous to the hyaluronan (HA) receptor CD44 and it is one of most employed endothelial markers for lymphatic identification and detection (7).

In parallel to the blood circulatory, the lymphatic network is found in most tissues in the body and plays vital roles in many functions, including immune surveillance, body fluid regulation, and fat absorption (8,9). The lymphatic capillaries are distinguishable from blood capillaries for their large interendothelial gaps, discontinuous basement membrane, and lack of pericytes (10,11). Scores of disorders are associated with lymphatic dysfunction, such as cancer metastasis, inflammatory and immune diseases, hypertension, obesity, AIDS and lymphedema (8,9,12-16). Unfortunately, to date, there is still little effective treatment for most lymphatic disorders. It is therefore a field with an urgent demand for new experimental tools and therapeutic protocols.

In this study, we provide the first evidence that the bEnd.3 cells express LYVE-1, a widely used endothelial marker for lymphatic research. Additionally, these cells express vascular endothelial growth factor receptor-3 (VEGFR-3) (17), another molecule largely restricted to lymphatic endothelial expression, and progenitor cell markers of CD133 and Sca-1 (8,18,19). We also show that upon the stimulation of tumor necrosis factor-alpha (TNF-α), the bEnd.3 cells increase their ability in formation of capillary-type tubes, which express LYVE-1. Furthermore, we demonstrate that lymphatic vessels are present in human tumor tissues of endothelioma, and the number of lymphatic vessels in endothelioma tumor tissues is significantly increased compared to normal tissue controls. Taken together, this study not only defines new features of bEnd.3 cells, but also identifies a lymphatic component of the human disease of endothelioma, which warrants further investigation.

MATERIALS AND METHODS

Cell Culture

The bEnd.3 cells (ATCC, VA, USA) were maintained in EGM-2-MV Medium (Lonza, Switzerland) containing 10% fetal bovine serum and supplemented with SingleQuot Kit (Lonza, Switzerland) according to the standard protocol recommended by the manufacturer.

Antibodies

The following primary and secondary antibodies and isotype controls were used: FITC-conjugated rat-anti-mouse CD31, rat IgG1, rabbit IgG, Rhodamine-conjugated donkey anti-rabbit secondary antibody, and FITC-conjugated goat-anti-rat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The purified rabbit-anti-mouse LYVE-1, rabbit-anti-human LYVE-1, rabbit-anti-mouse CD133, and FITC-conjugated rat-anti-mouse Sca-1 antibodies were purchased from Abcam Inc. (Cambridge, MD, USA). The mouse-anti-human CD31 and D2-40 antibodies were purchased from DAKO (Carpinteria, CA, USA) and the purified rat-anti-mouse VEGFR-3 antibody was a product of R&D Systems, Inc. (Minneapolis, MN, USA). FITC conjugated rat-anti-mouse isotype control and purified rat-anti-mouse CD16/CD32 Fc Block™ antibodies were purchased from BD Bioscience (San Jose, CA, USA).

Flow Cytometric Assay

The experiment was performed according to the standard protocol. Briefly, cells were cultured in until 80% confluent. Cells were then trypsinized, washed, and incubated with the Fc Block™ to block non-specific binding. After incubations with the primary and secondary antibodies, they were re-suspended in basal medium and filtered by 40 μm mesh (BD Bioscience, San Jose, CA, USA) before the analysis with the EPICS XL Flow Cytometer (Beckman-Coulter, Miami, FL, USA). The experiments were repeated at least three times.

Immunocytofluorescent Microscopic Assay

This assay was performed according to the standard protocol. Briefly, cells were seeded on slide chambers (BD Bioscience, San Jose, CA, USA) and incubated with 2% BSA with 0.5% Fc Block™ to block non-specific bindings. After incubation with the first antibody, for direct staining, cells were mounted on the slides using the DAPI mounting medium (Vector Lab, Burlingame, CA, USA). For indirect staining, cells were further incubated with the secondary antibody before mounting. The slides were examined and photographed by a Zeiss Axioplan 2e microscope (Carl Zeiss Inc., Germany). The experiments were repeated at least three times.

RNA Isolation and PCR

RNA was isolated from the cells using RNeasy Plus Mini Kit (Qiagen Inc., Valencia,

TABLE 1 Details of the Primer Sequences Used in the Study	
Primer name	**Sequence**
Mouse GAPDH forward	ACCACAGTCCATGCCATCAC
Mouse GAPDH reverse	TCCACCACCCTGTTGCTGTA
Mouse CD31 forward	CAAACCGTATCTCCAAAGCC
Mouse CD31 reverse	TCTGTGAATGTTGCTGGGTC
Mouse LYVE-1 forward	TCCTCGCCTCTATTTGGAC
Mouse LYVE-1 reverse	ACGGGGTAAAATGTGGTAAC
Mouse VEGFR-3 forward	GCTACCACTGCTACTACAAG
Mouse VEGFR-3 reverse	GATAATCCCAGTCGAAGGTG
Mouse Sca-1 forward	CTCTGAGGATGGACACTTCT
Mouse Sca-1 reverse	GGTCTGCAGGAGGACTGAGC
Mouse CD133 forward	TGCTTCTTTTGTATGTGCCG
Mouse CD133 reverse	AGCGAATTCTCGATGCTGTT

CA, USA) according to the manufacturer's protocol. Superscript VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) was used to generate the first strand cDNA. Taq DNApolymerase and deoxynucleotides were purchased from New England Biolab (Ipswich, MA, USA) and the primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA). The details of the primer sequences used in this study are summarized in *Table 1* (18,20,21). All thermal cycles were carried out in a Mastercycler ep system (Eppendorf, Germany). The experiments were repeated at least three times.

Three-Dimensional Culture and Tube Formation Assay

Matrigel (BD Bioscience, San Jose, CA, USA) was prepared according to manufacturer's protocol. The cells, in either TNF-α (100 ng/ml; R&D Systems, Minneapolis, MN, USA) or control medium treated, were gently added to the well and monitored continuously for 24 hours under an Zeiss Observer A1 (Carl Zeiss Inc., Germany) microscope. Tubes were fixed and stained as described above. Triplet experiments were repeated 6 times with similar results.

Human Tissue Immunohistochemical Assay

The experiment was performed according to the standard protocol. Briefly, 4 μm formalin-fixed and paraffin-embedded tissue sections from human tissues of pulmonary endothelioma and normal control tissues (n = 6) were stained with hematoxylin and eosin (H&E) or using the primary and secondary antibodies after antigen retrieval. Signals were detected by the EnVision Plus system (DAKO, Carpinteria, CA, USA) according to manufacturer's protocol. For quantitative analysis, the total numbers of

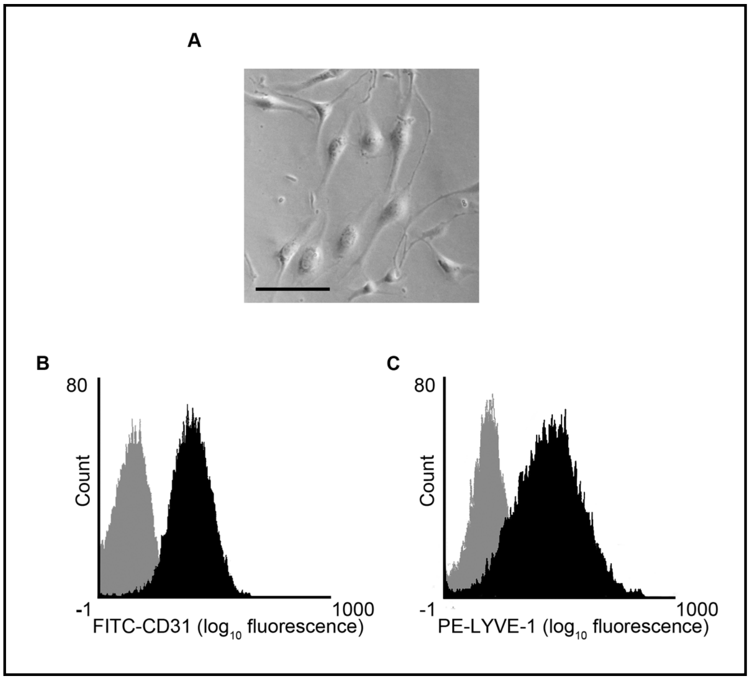

Fig. 1. bEnd.3 cells express CD31 and LYVE-1. (A) bEnd.3 cells formed an adherent monolayer in culture with elongated spindle shape. Scale bar, 100 µm. (B and C) bEnd.3 cells expressed CD31 (90%; B) and LYVE-1 (80%; C) as detected by flow cytometry. Gray, isotype control; black, CD31 (B) or LYVE-1 (C) staining.

lymphatic vessels from three different regions were counted and the average numbers were calculated and compared accordingly. Student *t* test was used for the determination of significance levels between different groups using Prism software (GraphPad, La Jolla, CA). The differences were considered statistically significant when $p < 0.05$.

RESULTS

bEnd.3 Cells Express LYVE-1, VEGFR-3, and CD31

As shown in *Fig. 1A*, bEnd.3 cells formed an adherent monolayer in culture with an elongated spindle shape. We first performed a flow cytometric assay and investigated the expression of CD31 (pan-endothelial cell marker) and LYVE-1 in these cells. Our results showed that the bEnd.3 cells expressed CD31, confirming their endothelial cell lineage (*Fig. 1B*). More interestingly, these cells also expressed LYVE-1 (*Fig. 1C*).

To further confirm CD31 and LYVE-1 expression in bEnd.3 cells and to investigate whether these cells also express VEGFR-3, we next performed a series of immunocyto-fluorescent microscopic and RT-PCR assays for a detection at both protein and mRNA levels. Our results from this set of experiments showed that in addition to CD31 and LYVE-1, the bEnd.3 cells expressed VEGFR-3, and the results were consistent between the immunocytofluorescent microscopic (*Fig. 2A-C*) and RT-PCR (*Fig. 2D*) assays.

bEnd.3 Cells Express Progenitor Cell Markers

We next assessed the expression of CD133 and Sca-1, two progenitor cell markers (8,18,19), in bEnd.3 cells. As validated by a series of immunocytofluorescent microscopic and RT-PCR assays, both proteins and

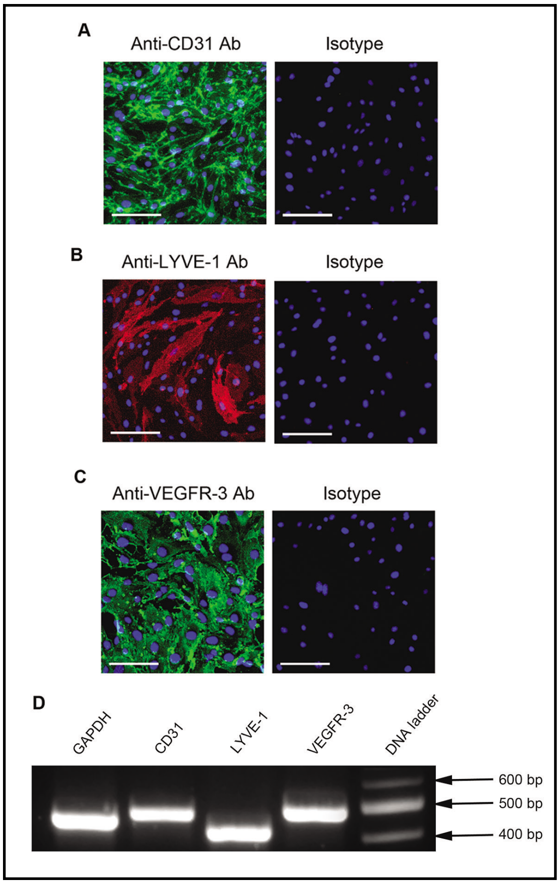

Fig. 2. bEnd.3 cells express other lymphatic endothelial cell markers. (A to C) Immunocytofluorescent micrographs demonstrating the protein expression profiles of CD31 (green; A), LYVE-1 (red; B), VEGFR-3 (green; C) but not Prox-1 (not shown) in bEnd.3 cells. No signals were detected in the isotype negative controls. Blue: DAPI nuclei staining. Scale bars, 100 μm. (D) mRNA expression profiles of the lymphatic markers from reverse transcription followed by PCR further confirmed the results.

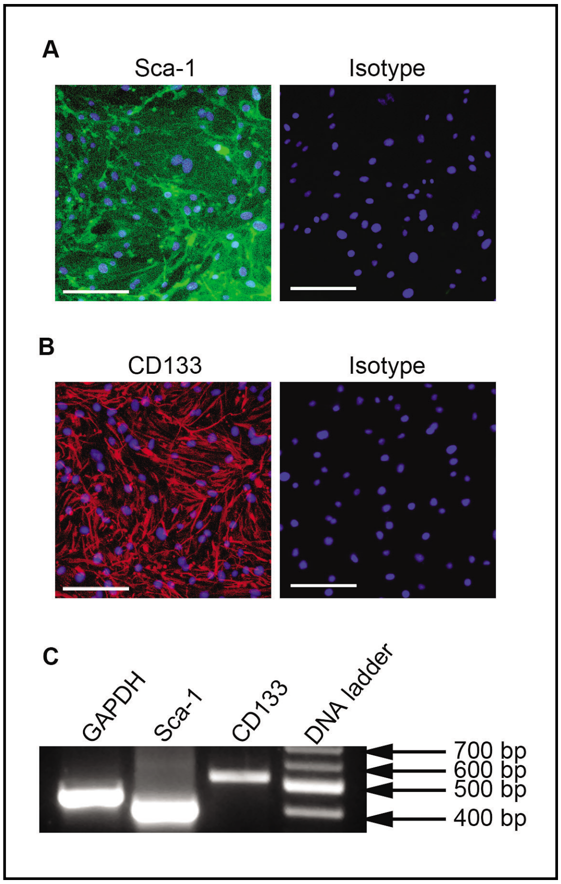

Fig. 3. bEnd.3 cells express progenitor cell markers. (A and B) Representative immunocytofluorescent micrographs demonstrating the protein expression of Sca-1 (green; A) and CD133 (red; B) in bEnd.3 cells. No signals were detected in the isotype negative controls. Blue: DAPI nuclei staining. Scale bars, 100 μm. (C) mRNA expression profiles of Sca-1 and CD133 from reverse transcription followed by PCR further confirmed the results.

mRNAs of these molecules were detected on the bEnd.3 cells (*Fig. 3*).

bEnd.3 Cells Increase Tube Formation upon TNF-α Stimulation

To further examine whether the bEnd.3 cells are able to form capillary structures and how this is modulated by an inflammatory stimulation, we next employed a three dimensional matrigel cell culture system to compare capillary tube formation of these cells between TNF-α treated and control conditions. The bEnd.3 cells demonstrated very limited ability in forming tubular structures under the control condition (*Fig. 4A*), as reported previously (22). However, after TNF-α stimulation, these cells assembled into well-organized capillary network (*Fig. 4B*), which expressed LYVE-1 (*Fig. 4C*).

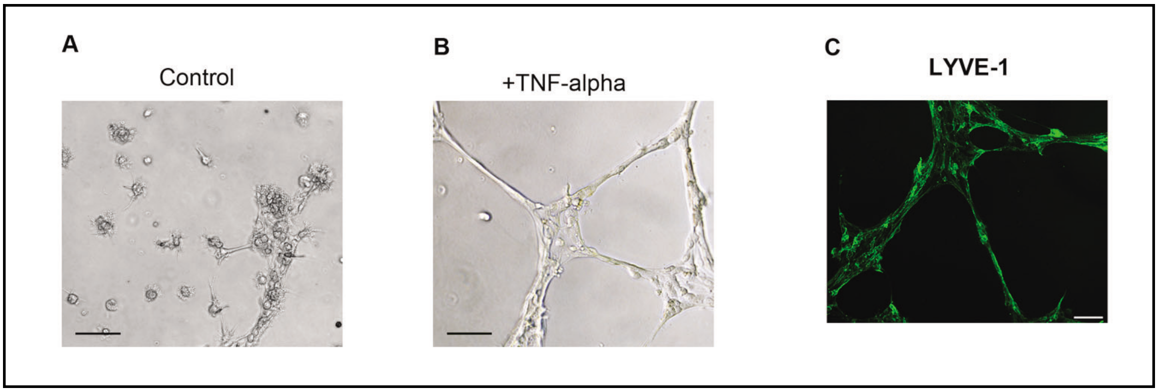

Fig. 4. bEnd.3 cells up-regulate lymphatic tube formation after TNF-α stimulation. (A and B) Representative pictures of three-dimensional Matrigel cultures demonstrating the significant difference in tube formation between the normal (A) and inflamed condition (B). While bEnd.3 cells showed limited organization and tubular network capability under normal control condition, these cells were well-assembled into a tubular network 24 hours after TNF-α treatment. (C) Representative immunocytofluorescent micrograph confirming that the tubular network formed by the bEnd.3 cells expressed LYVE-1 (green). Scale bars, 100 μm.

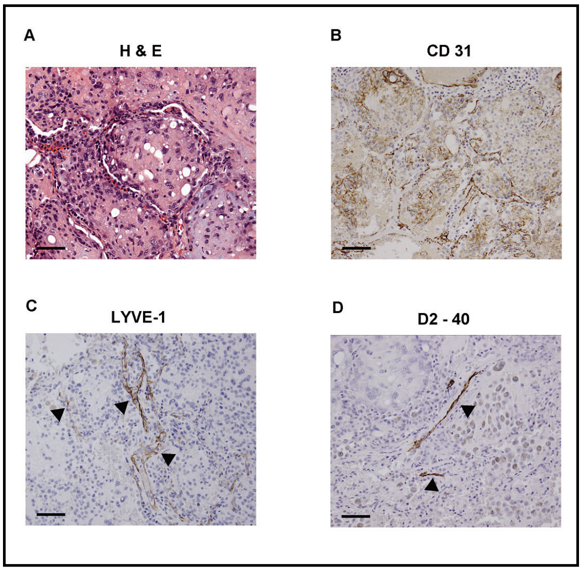

Fig. 5. Human endothelioma contains lymphatic vessels. Representative immunohistochemical micrographs showing (A) histology of endothelioma; (B) CD31 staining; (C) and (D) the presence of lymphatic tubular structures (indicated by arrowheads) that express LYVE-1 (C) and podoplanin (D). Scale bars, 100 μm.

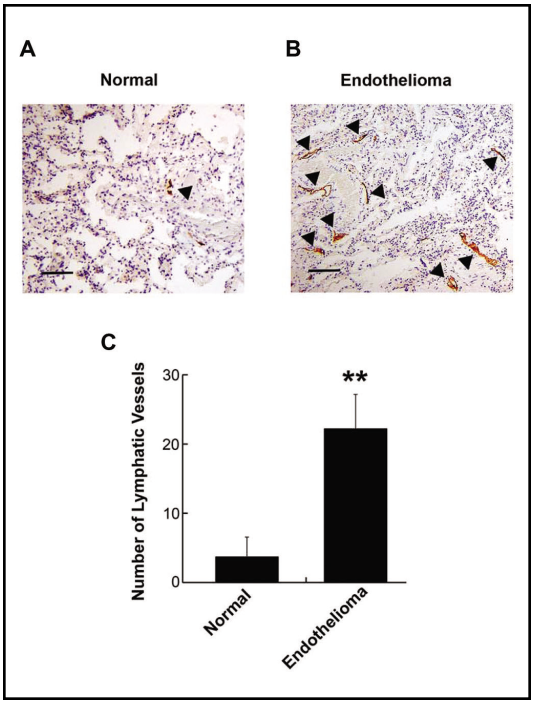

*Fig. 6. Lymphatic vessels are significantly increased in human endothelioma. Representative immunohistochemical micrographs with D2-40 staining showing increased number of lymphatic vessels (indicated by arrowheads) within human endothelioma tissue (B) compared with normal control tissue (A). Scale bars, 200 μm. Quantitative analysis is presented in (C) (**p< 0.01).*

Human Endothelioma Contains Lymphatic Vessels

Since the bEnd.3 cell line was generated from mouse endothelioma, we assessed the possibility that human vascular disorder of endothelioma may contain a lymphatic component. As shown in *Fig. 5*, our results from a series of immunohistochemical assays showed that in addition to CD31, LYVE-1 was detected in human samples of endothelioma. This presence of lymphatic vessels was also confirmed by immunostaining with D2-40, an antibody specifically recognizing human lymphatic marker of podoplanin (23,24).

Lymphatic Vessels Are Increased in Human Endothelioma

As demonstrated in *Fig. 6*, our further analysis on human tissues of endothelioma showed that compared to normal tissue controls, the number of lymphatic vessels within endothelioma tumor samples was significantly increased. This finding indicates that human disease of endothelioma is associated with pathological lymphangiogenesis, which warrants further investigation as well.

DISCUSSION

In this study, we have shown that LYVE-1 is expressed in bEnd.3 cells, and that human endothelioma contains a lymphatic component. Since LYVE-1 has been widely used as a lymphatic endothelial marker, our finding on its expression in bEnd.3 cells may indicate a lymphatic trait of these cells. Allied to this notion is these cells' expression of VEGFR-3, another widely used marker for lymphatic endothelial cells. However, since LYVE-1 and VEGFR-3 are occasionally found on non-lymphatic endothelial cells, such as macrophages and certain blood endothelial cells (25-27), it is yet to be determined whether these cells are blood, lymphatic, or mixed. Moreover, since molecular lymphatic research is still at its early stage, we currently do not have an adequate knowledge to fully categorize the endothelial cells. It is considered that blood and lymphatic endothelial cells share the same progenitor and the lymphatic endothelial cells are derived from a subset of cardinal vein cells during development (28). It is possible that there exist different populations of endothelial cells, and these populations express all or some of the endothelial markers that have been or yet to be identified. For example, we recently came across a new finding that the Schlemm's Canal of the eye expresses Prox-1 but not LYVE-1 or podoplanin (29). Moreover, though LYVE-1 has been widely used as a molecular marker for endothelial cells, its function still remains largely unknown. Since we have now identified its expression in the bEnd.3 cells, we may start to utilize these cells to investigate LYVE-1-related functions in endothelial cells. Since the bEnd.3 cell line has been extensively used in the past to study various molecular factors and pathways, a revisit of these preexisting data may provide some insightful information linking LYVE-1 to other factors or pathways as well.

Furthermore, the bEnd.3 cells are originated from mouse endothelioma of the brain, one of few tissues in the body which are devoid of lymphatic vessels. Our data showing that these cells may carry some lymphatic-like features indicate that lymphatic abnormality may occur in the brain under certain pathological conditions, such as inflammation. Allied to this notion are cumulative data from us and other researchers showing that pathologic lymphangiogenesis occurs in the cornea, another alymphatic tissue under normal condition (13). Similar pathologic process may occur in the brain, which warrants further exploration. Also, our data show that an increased number of lymphatic vessels is present in human endothelioma. Though current treatment of this type of tumor is focused on blood vessels, a future combination with antilymphatic therapy may offer more effective management of this disease, which warrants further investigation.

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