CD31 promotes diffuse large B cell lymphoma metastasis by upregulating OPN and mTOR

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Research Article
Abstract

Background

Diffuse large B cell lymphoma (DLBCL) will progress to recurrence/refractory because of ongoing metastasis in various organizations (including the brain and testicles) and it is difficult for first-line chemotherapy agents to cross the physical barriers (such as the blood-brain barrier and blood-testosterone barrier). At present, the mechanism of invasion is unknown and there is no effective drug to effectively inhibit the metastasis of DLBCL.

Methods

In this study, 40 newly diagnosed DLBCL patients samples were analyzed by real-time PCR. Western-blot, immunofluorescence staining, immunohistochemical staining and RNA sequence were used to identify differentially expressed genes of different invasive DLBCL cells. Xenograft models were constructed to validate the different invasive DLBCL cells in vivo. Scanning electron microscope were used to detect the effect of overexpression CD31 DLBCL cells on integrity of blood brain barrier.

Results

Osteopontin (OPN) was upregulated in multiple metastases patients compared with single or less metastases patients. Overexpression OPN DLBCL cells didn’t respond to R-CHOP and more easily progress to recurrence/refractory. In vivo experiments, the DLBCL cells aggressiveness positively correlated with OPN. Overexpression OPN DLBCL cells formed more tumor metastases over time in mice and it also shortened mice survival time. Using RNA sequencing and in vitro studies, we showed that OPN was obviously upregulated by Platelet endothelial cell adhesion molecule-1 (CD31) through AKT pathway. CD31 also helped DLBCL foci hide from immune cells by recruiting function suppressed CD31+ memory T cells in xenografts. CD31 inhibited CD31+ memory T cells to synthetic INF-γ, TNF-α and perforin effectively by abnormally activated mTOR pathway. Finally, CD31 disrupted the tight junctions (TJ) between endothelial cells of blood-brain barrier by activating OPN-EGFR-ZO-1/ZO-2 axis, allowing DLBCL cells to metastasize to brain.

Conclusions

Overall, CD31 is a marker for DLBCL metastasis and targeting CD31 may be a valuable strategy to reduced DLBCL cells aggressiveness and prevent patients progress to recurrence/refractory because of ongoing metastasis.

Introduction
Diffuse large B-cell lymphoma (DLBCL) is a malignant cancer originating in the lymphatic system. Malignant cancers are characterized by the formation of metastases. For example, the initial tumour lesion in newly diagnosed DLBCL can either occur in only one individual lymph node or tissue, or in multiple lymph nodes or tissues throughout the body simultaneously. Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) are used as first-line treatment options for DLBCL, resulting in 1-year disease-free survival of patients with DLBCL of over 85%\cite{1}. However, aggressive DLBCL that did not benefit from the R-CHOP regimen had poor results in late stages\cite{2–3}. To help such patients, clinical researchers have continued attempting to improve the therapeutic efficacy by trying novel drugs; however, the toxicity resulting from different drug combinations have rendered their results insignificant\cite{4}. Studying and elucidating its tumour growth characteristics is a major method for accurately evaluating the aggressiveness of DLBCL\cite{5}.

OPN is a key factor influencing tumour invasion and metastasis\cite{6}. A number of studies have shown that OPN is significantly associated with the development and metastasis of leukaemia\cite{7–8}. It maybe associated with regulating the expression of matrix metalloproteinases (MMPs) such as MMP-3 and MMP-7\cite{9}.

Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) not only confers cells with the ability to migrate in the endothelial and perivascular basement membranes, but also serves as a marker for drug delivery\cite{10–11} to systems connected by tight junctions, such as the blood-brain barrier (BBB). As a transmembrane glycoprotein involved in leukocyte migration, CD31 might be a good target for endothelial-delivery drug penetration. In addition, aberrant expression of CD31 has been shown to be closely associated with the activity of B-cells\cite{12}.

Studies on the aggressiveness of DLBCL are currently very limited. CD31 was extracted on the basis that OPN could not fully explain the aggressiveness of DLBCL. Starting with OPN, we here also demonstrate that DLBCL tumour foci can only be shielded from T-cell immune activity and systemically disseminated after the aberrant activation of OPN by CD31. These results complement the role of CD31 and OPN in DLBCL. In addition, we further attempt to elucidate the mechanism of action by which CD31 mediates the interaction of DLBCL cells with immune cells and intercellular junctions.

**Methods**

**Primary Cell Culture and Reagents**

In order to get a more accurate study of diffuse large B-cell lymphoma (DLBCL), we directly detected newly diagnosed and relapsed/refractory DLBCL patient’s cells. The patient's DLBCL cells helped us to further accurately elucidate the mechanism of the DLBCL cells. We used positive magnetic bead sorting to obtain higher purity B cells from lymph nodes and tumor tissues. Tables 1 and 2 provide patients information. All operations will be carried out carefully in accordance with the instructions.
DETAChaBEAD CD19 (Dynal, Norway) was designed to release CD19 + B cells from DLBCL patients and normal donors. CD19 + B cells released according to the instructions were pure, viable, and not activated. And there were no residual magnetic beads or primary antibodies bound to the surface. The obtained DLBCL primary cell suspension was cultured in RPMI 1640 medium enriched with 100 U/ml penicillin, 100 mg/ml streptomycin and 15% fetal bovine serum (FBS). To further ensure the purity of patient DLBCL cells, we subcultured the primary cells for 4 weeks and used flow cytometry (BD Biosciences, San Jose, CA, USA) and Immunohistochemistry (CD20, CD3, CD3e, CD38, CD138, PAX-5, CD79a, Bcl-6, Bcl-2, Mum-1, CD10, C-myc, P53, Ki-67) to detect whether they were DLBCL cells. The mouse CD19 + B cells were released using the ImunoSep Mouse CD19 + cell positive selection kit (Precision BioMedicals Co., Ltd., Tianjin, CHN). We carefully followed the instructions to release CD19 + B cells from DLBCL mice model and healthy mice. The CD19 + B cells released from the DLBCL model mice also subcultured for 4 weeks and the non-proliferative cells would die. This helped us ensure the tumor-derived and monoclonal of the cells. Mouse CD4 + T cells were released using Dynabeads FlowComp Mouse CD4 (Invitrogen, Carlsbad, CA). Mouse CD8 + T cells were released using Dynabeads FlowComp Mouse CD8 (Invitrogen, Carlsbad, CA). All operations will be carried out carefully in accordance with the instructions. All cell culture plates were maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere. Cell culture media and supplements were purchased from Hyclone (Termo Fisher Scientific, Waltham, MA, USA).

Western blot analysis

Western blot was employed to detect the protein expressions of related genes in newly diagnosed, relapsed and refractory DLBCL patients, as well as in the cells transfected by siRNA or lentivirus. Briefly, cells were washed by phosphate buffer solution (PBS), collected, and then lysed in radioimmunoprecipitation (RIPA) assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP40) containing proteinase inhibitor cocktail and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The lysate was centrifuged at 12000 ×g at 4°C for 20 min. The protein concentration was determined using Pierce BCA Protein Assay Kit (Termo scientific, Waltham, MA, USA) at 562 nm. The supernatant with equal amounts of protein (50 µg protein) was fractioned using 10% SDS-PAGE and electrophoretically transferred to Hybond-enhanced chemiluminescence membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membrane was blocked with 5% non-fat skim milk (Blocking-Grade Blocker; Bio-Rad) in 1× TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) at room temperature for 1 h and then incubated with primary antibody (Abcam, Cambridge, MA, USA) at 4°C overnight. After being washed with PBS containing 0.1% Tween 20 (PBST), the membranes were incubated with horseradish peroxidase (HRP) conjugated with anti-IgG secondary antibodies (Abcam, Cambridge, MA, USA) for 45 min and visualized by an ECL Detection Kit (Bio-Rad). All experiments were conducted at least three times.

Quantitative real-time PCR and PCR arrays
Cells were washed with PBS twice and lysed with buffer RL containing a 50×Dithiothreitol (DTT) solution. Total RNA was isolated and purified from cells by RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and reverse-transcribed using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNAs were analyzed by quantitative real-time PCR using primers provided by Airui Technology Corporation (Chengdu, China) and iQ SYBR Green supermix (Bio-Rad, Singapore). The specificity, efficiency, and fidelity of PCR primers for real-time quantitative PCR were validated by checking PCR products and analyzing the melting curves. The thermal cycling conditions used in the protocol were 1 min at 94°C, followed by 40 cycles at 94°C for 10s and at 60°C for 15s. All experiments were conducted at least three times.

**Patient samples**

Forty patients who were diagnosed as DLBCL and received treatment at the Affiliated Hospital of Guiyang Medical College, Affiliated Hospital of North Sichuan Medical College and West China Hospital of Sichuan University were included in this study. All patients were classified according to the Ann Arbor Staging. Relapsed and refractory patients were identified by PET-CT and pathological biopsy after standard therapy. The clinical information of all patients is shown in Table 1. We also collected samples from 40 normal donors. We obtained all samples by ultrasound-guided puncture and resection procedures. The study was approved by the institutional review board (Affiliated Hospital of the Guiyang Medical College, Affiliated Hospital of North Sichuan Medical College and West China Hospital of Sichuan University), and written informed consent was obtained in accordance with the Declaration of Helsinki before blood donation in each case. Research protocols of this study were reviewed and approved by the Ethics Committee of West china hospital of Sichuan university and North Sichuan medical college.

**mRNA sequencing and gene expression analysis**

Nonnecrotic tissues were carefully removed from the tumors and immediately snap-frozen at −80°C until later use for gene and protein expression analysis; the remaining tissues were fixed in formalin for histologic evaluation. Changes in the target gene expression profiles and key pathway components in the tumors were examined through RNA sequencing analysis. For gene expression analysis, RNA was isolated from the snap frozen xenograft tumor tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germantown, MA, USA) with DNAse I treatment and TRIzol Reagent (Invitrogen, Carlsbad, CA), as described in the manufacturer's protocol. The quality of RNA was assessed with Qubit2.0 Fluorometer and Agilent 2100 bioanalyzer. The sequencing libraries were prepared following the supplier's protocols for sequencing mRNA samples (Illumina, Foster City, CA, USA). The FASTQ sequence reads were aligned using the human genome hg19 TopHat (v2.0.9) application with default parameters (TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions.) and Bowtie (v1.0.0) (Ultrafast and memoryefficient alignment of short DNA sequences to the human genome.). The raw counts per gene in each xenograft tumor were then normalized as fragments per kilobase per million mapped reads to represent the expression level of the gene in the tumor.
Differentially expressed genes were identified using the Bioconductor package DESeq2. A heatmap was generated through the color-coding of standardized log gene expression levels (mean, zero; SD, one). The RNA sequencing data were subjected to gene set enrichment analysis (GSEA; http://www.broadinstitute.org/gsea) to identify the enrichment or depletion of defined gene expression signatures in reference to the database (NR, SwissProt, PFAM, GO, KEGG and STRING). The genes that were significantly regulated between the experiment group and vehicle control were selected based on a false discovery rate (FDR) of < 0.05 and absolute fold change of ≥ 2 on pretransformed expression on a log2 scale.

**Hematoxylin - eosin (HE) staining**

Fresh tissues were put into the stationary solution (10% formalin). The cell protein was denatured and solidified. Fixing the tissue for 24 hours. After pruning the tissue, we put it into the embedding box and rinse it with water for 30 minutes. Using different concentrations of alcohol to dehydrate tissue blocks. Finally, the tissue blocks were placed in xylene. The transparent tissue blocks were placed in the dissolved paraffin wax and stored in the wax box. After the paraffin wax was completely immersed in the tissue, the paraffin was embedded. After the block to was cooled and solidified, we used a slice machine to slice the block. The slices were stained with hematoxylin solution for several minutes. The slices were placed in the acid and ammonia water for a few seconds respectively. After 1 hour of washing, we put it into the distilled water for a moment, dehydrating in alcohol for 10 minutes, staining for 2–3 minutes. The stained sections were dehydrated by pure alcohol and then translucented by xylene and then sealed with cover glass. All experiments were conducted at least three times.

**Scanning electron microscope**

Mice were sacrificed by cervical dislocation and we carefully isolated the fontanelles. The intact brain tissue of the mice were isolated. We bluntly isolated the vascular choroids attached to the fontanelle and brain tissue. The above parts were fixed with glutaraldehyde for 24 hours. Using 40%, 60%, 80% and 100% alcohol to dehydrate and put them in the oven for 5 minutes. Being dried, the samples were adhered to the sample holder. Place the sample holder in the steaming chamber. Opened the ventilation valve and the surface of the samples were plated with a metal film so that the electron beam could penetrate the sample to form an image. The samples were observed by scanning electron microscope. All experiments were conducted at least three times.

**Diffuse large B lymphoma cells aggressiveness experiment**

We used 3D Bar Charts to count the tissues and organs invaded by DLBCL cells in BALB/c-Nude or C57BL/6Ly5.2 mice. X axis represents group number and Y axis represents time. Z axis represents the number of tissues and organs infiltrated by DLBCL cells. The higher the 3D Bar Chart, the more tissue infiltrated by diffuse large B lymphoma cells. Aggressiveness experiments were carried out for 9 weeks at all. 1–8 groups were experiment group and 10–17 groups were control groups. We regularly dissect mice to explore tumor tissue. To count the number of tumor foci in mice every week, each group of mice were
dissected weekly to examine infiltrating organs and tissues. But there were other group of mice survived into the next week. Aggressiveness experiments were carried out for 9 weeks at all. So there were 9 batches of mice in total.

**Reagents**

Antibodies for Western blot analysis were obtained from Abcam (Cambridge, MA, USA) and secondary antibodies were purchased from Li-Cor Corp. (Lincoln, NE, USA). TRIzol reagent was bought from Life Technologies (USA). AVSTIN (99.29% purity) was obtained from Shanghai Selleck Chemicals Co., Ltd. (China). Antibodies for Immunohistochemistry analysis were obtained from Invitrogen (Carlsbad, CA) and secondary antibodies were purchased from Li-Cor Corp. (Lincoln, NE, USA). Antibodies for Immunofluorescence analysis were obtained from Abcam (Cambridge, MA, USA) and secondary antibodies were purchased from Li-Cor Corp. (Lincoln, NE, USA). The inhibitors (Odanacatib, NIK SMI1, Oroxin B, Ridaforolimus and Bevacizumab) were purchased from MedChem Express (Monmouth Junction, NJ, USA) and dissolved in DMSO. The patients DLBCL cells were treated at 5–25 µM for either inhibitor or at matched concentration of DMSO (0.1%) for 24 h.

**Virus and siRNA transfection**

Lentivirus and small interfering RNA (siRNA) targeting human were selected with Invitrogen designer software. For example, retroviruses were generated by transfecting empty plasmid vectors containing the enhanced green fluorescence protein (EGFP) or vectors containing human CD31-EGFP/siRNA-CD31-EGFP into 293FT packaging cells, using the FuGENE HD6. Lentiviral stocks were concentrated using Lenti-X concentrator, and titers were determined with Lenti-X qRT-PCR titration kit (Shanghai Innovation Biotechnology Co., Ltd., China). Finally, lentivirus-V5-D-TOPO-CD31-EGFP(L-CD31),lentivirus-V5-D-TOPO-EGFP(TOPO-EGFP), lentivirus-pRNAi-u6.2-EGFP-siCD31(siCD31), and lentivirus-pRNAi-u6.2-egfP(RNAi-EGFP) 4 recombinant lentiviral vectors were constructed:. For transduction, cells were plated onto 12-well plates at the density of $2.5 \times 10^5$ /well, infected with the lentiviral stocks at a multiplicity of infection of 10 in the presence of polybrene (10 µg/ml), and then analyzed by fluorescence microscopy (Olympus, Tokyo, Japan) and Western blotting 48 h after transduction.

**Immunohistochemistry (IHC)**

The slides were placed in a mixture of potassium dichromate and H₂SO₄ and then washed to remove residual potassium dichromate and H₂SO₄ (about an hour or so). Then soaked the slides in alcohol and placed on a rack and placed in a 37°C incubator. Added some liquid paraffin to the iron mold. The tissue was placed in paraffin After paraffin cooling, used paraffin machine section the embedded tissue. Sections were deparaffinized using xylene and various concentrations of alcohol. Cooking Sections exposed the site of the antigen and repairs the antigen. Used serum to block non-specific sites. Added primary and secondary antibodies (Abcam, Cambridge, MA, USA). Then added strept avidin-biotin (SABC). Dehydrating after added chromogen and counterstaining.

**Animals and treatments**
In the experiment, we selected two kinds of male mice (BALB/c-Nude and C57BL/6Ly5.2) for different experiments. Male C57BL/6Ly5.2 and BALB/c-Nude mice weighing 18 to 20 g were purchased from the Institute of Laboratory Animal Sciences (PUMC, Beijing, China). The mice were cultured in specific pathogen free (SPF) class animal laboratory. After adapting to the environment, the mice were divided into 16 groups randomly. Eight groups of 72 mice served as the experiment group and injected EXP-1 DLBCL cells. The other groups of mice were EXP-2 DLBCL cells group. Each mice was injected $3 \times 10^7$ DLBCL cells. All mice were injected via tail vein every days for one week. The survival time of mice were recorded and analyzed. Hematoxylin and eosin (HE)

staining was used to detect DLBCL cells infiltration in thyroid, lymph nodes, spleen, ovary and mesentery. The experiment was strictly followed the Helsinki declaration and passed the ethical examination of animal experiments in Sichuan University and North Sichuan medical college. (Ethical approval number: 2020ERO36-1 and 2021025A)

**Immunofluorescence (IF)**

The cells or tissue were washed with PBS twice. Fixed cells using a cross-linking agent such as paraformaldehyde. Permeabilize cells or tissues before adding the antibody for incubation to ensure that the antibody can reach the antigenic site. The selection of permeabilizers should fully consider the properties of antigenic proteins. The permeabilization time is generally 15min. After permeabilization, washed with PBS. Cells were blocked with blocking solution for 30 minutes. Incubated primary antibodies for 1 h at room temperature or overnight at 4°C. Then washed with PBST. Incubated with secondary antibody for 1 h at room temperature in the dark. Washed with PBST for 3 times and rinsed with distilled water. Fluorescence microscopy was performed after adding the mounting medium.

**Flow cytometry**

According to the instructions of the Lymphocyte subsets Kit (Invitrogen, Carlsbad, CA), the treated cells were stained. After being stained at room temperature for 15 minutes in the dark, cells were detected using the FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), and the data were analyzed using CellFIT software. The experiments were conducted according to the protocol provided by the manufacturer. All experiments were conducted at least three times.

**Statistical analysis**

All operations will be carried out carefully in accordance with the instructions. Statistical analyses were performed using Prism 9 (GraphPad Software). The experiments were performed in biological triplicates each time and independently repeated at least 3 times. Data are presented as the mean ± S.E.M. Student’s t-test (two-tailed) was used to compare differences between the control and experimental groups. For all statistical analyses, differences were labeled as *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

**Results**
Diffuse large B cell lymphoma patients with multiple lymph node or extra-nodal tissue metastasis have significantly overexpression of OPN

To study the genes related to tumour metastasis, we compared the genes expression in 40 normal donors and 40 newly diagnosed DLBCL patients. We found that the mRNA of Osteopontin (OPN) was significantly overexpression (Fig. 1A and Table 2). Notably, patients with multiple lymph node or extra-nodal tissue invasion (e.g., thyroid, mesentery, testis, and bone marrow) had significantly higher levels of expression of OPN mRNA (Fig. 1B). Immunohistochemical (IHC) analysis showed that these patients’ lymph nodes and extra-nodal tumour tissues were infiltrated by overexpression OPN DLBCL cells (Fig. 1C). The Western-blot showed that OPN, CD44 and ICAM-1 were also overexpression in these patients (Fig. 1D). We made only a single lymph node invasion newly diagnosed DLBCL patients as group 1 (1-DLBCL) and multiple lymph node or extra-nodal tissue invasion newly diagnosed DLBCL patients as group 2 (2-DLBCL). We detected that the OPN mRNA were significantly higher in 2-DLBCL than in 1-DLBCL (Fig. 1E). Western-blot showed that OPN was also higher in 2-DLBCL (Fig. 1F). Although only a single lymph node was involved at initial diagnosis (3-DLBCL), the OPN mRNA was significantly higher when DLBCL cells invaded multiple lymph nodes and extra-nodal tissues during treatment (4-DLBCL). In addition, these patients developed into recurrent/refractory disease at later stages (Fig. 1G). IHC also showed OPN expressing DLBCL cells in newly metastasis (Fig. 1H). We specifically identified that patients with recurrence expressed higher levels of proteins such as OPN, CD44, and ICAM-1 than normal donors (Fig. 1I). We also noticed that conventional R-CHOP treatment regimen had no significant effect on OPN in newly invaded tissues and lymph nodes (Fig. 1J).

OPN promoted the invasion of diffuse large B cell lymphoma in mice

To further determine whether OPN is a key gene for the invasion of tissues by DLBCL cells, we examined the ability of DLBCL cells with different levels of expression of OPN to infiltrate BALB/c-nude mice tissue in vivo. We observed the proliferation of DLBCL cells over time using light microscopy (Fig. 2A). We noticed that these DLBCL cells were able to invade the intestinal mesentery, testis, thyroid, lymph nodes, and spleens (Fig. 2B). We used the number of metastasis in the same time to judge the aggressiveness. In 9 weeks, we found that EXP-1 cells infiltrated more tissues than EXP-2 cells. By week 9, some mice in groups 1–8 had 9 tissues infiltrated by DLBCL cells (Fig. 2C). Western-blot indicated that EXP-1 cells expressed higher levels of OPN, CD44 and ICAM-1 (Fig. 2D). Immunofluorescence (IF) showed the metastasis expressing OPN and ICAM-1 (Fig. 2E). We observed that odanacatib (MK-0822) inhibited the OPN, AKT, ICAM-1 and PLCγ in EXP-1 cells (Fig. 2F). When mice injected with EXP-1 cells treated with odanacatib, we found that the number of infiltrated tissues was reduced over the same time. However, we noticed that the invasiveness of EXP-1 cells was still stronger in the experimental group than that of EXP-2 cells in the control group (Fig. 2G). We found odanacatib did not inhibit the MMP-3, MMP-9, CCL3, AP-1...
and EGFR in EXP-1 cells (Fig. 2H). NIK SMI1 inhibited NIK, MEK4, p-JNK1 and AP-1 in EXP-1 cells. Oroxin B inhibited p-PI3K and AP-1 and slightly elevated p-PTEN in EXP-1 cells (Fig. 2I). However, neither NIK SMI1 nor oroxin B effectively inhibited the expression of MMP-3, MMP-9, EGFR in EXP-1 cells (Fig. 2J).

We injected mice in groups 1–8 with EXP-1 cells treated with NIK SMI1 or oroxin B. Although the number of tissues infiltrated by EXP-1 cells was decreased over time, it was still higher than that in the control group (Fig. 2K). We noticed that the survival periods in the odanacatib + EXP-1, NIK SMI1 + EXP-1, and oroxin B + EXP-1 groups were less than that of the EXP-2 group (Fig. 2L).

**Multiple genes and pathways regulate OPN**

To determine the upstream genes and pathways that regulate OPN, we studied the difference gene expression. We used RNA sequencing to compare multiple tissue invasions samples with normal donors samples and performed heatmap analysis (Fig. 3A). We further performed cluster analysis to identify differential expression genes in patient samples (Fig. 3B). Next, we sequenced the 3 DLBCL patient samples with multiple tissue invasions and the 3 DLBCL samples with only 1 tissue invasion (Fig. 3C). We performed cluster analysis to identify genes with >2-fold differences in expression and found that the number of highly expressed genes was reduced compared with that in Fig. 3B (Fig. 3D). We also performed sequencing analysis of the differential genes in the DLBCL invasion mice lymph nodes (Fig. 3E). We performed cluster analysis to identify genes with >2-fold differences in expression in the disease groups (Fig. 3F). The intersection of the 3 previous clustering analyses resulted in the 92 common differential genes (Fig. 3G). Our enrichment analysis showed that these genes were associated with multiple tumour pathways (Fig. 3H). Using GSEA, we found that Akt may associated with (Fig. 3I).

The genes regulating OPN included NFATc4, CD31 and Ets-1 (Fig. 3J). We found that these genes expressed in tumour tissues invaded by DLBCL cells. But only CD31 expressed in ultrasound-guided puncture samples from refractory patients (Fig. 3K).

**CD31 promote diffuse large B cell lymphoma metastasis**

We selected non-OPN-expressing DLBCL cells and divided them into 3 groups. We upregulated their expression of CD31, NFATc4 and ETS-1 by using lentiviral vectors. We detected the 3 groups of cells by using immunofluorescence (Fig. 4A). We observed that the expression of MMP-3, MMP-9, CCL3, PPARα, ICAM-1 and EGFR was increased in all 3 groups, whereas that of E-cadherin and β-catenin was decreased in all 3 groups (Fig. 4B). Next, we determined the ability of these 3 groups of cells to invade murine tissues. We found that all 3 groups of cells invaded more than 1 tissue in the same time, compared with invading only 1 lymph node previously. The CD31 group was the most aggressive (Fig. 4C). Wright’s staining showed that only CD31 group infiltration tissues were destroyed by DLBCL cells (Fig. 4D). In the CD31 group, IHC revealed a significant infiltration of CD31 expression DLBCL cells in multiple organs (Fig. 4E). We noticed that the CD31 group had the shortest survival period (Fig. 4F). We then used siRNA to inhibit the expression of CD31 in DLBCL cells with high expression of CD31. We found the OPN, MMP-3, MMP-9, CCL3, ICAM-1 and EGFR was reduced (Fig. 4G). We also treated high CD31 expression DLBCL cells with the mTOR inhibitor ridaforolimus (MK-8669). The expression of p-mTOR was significantly inhibited along with that of OPN and ICAM, whereas that of E-cadherin and β-catenin was clearly inhibited along with that of OPN and ICAM, whereas that of E-cadherin and β-catenin was clearly inhibited.
elevated. We also treated highly CD31 expression DLBCL cells with bevacizumab (avastin) to determine whether DLBCL metastasis is associated with angiogenesis. We accordingly found that only the expression of ICAM was significantly inhibited, whereas E-cadherin and β-catenin was slightly elevated. However, when we treated highly CD31 expression DLBCL cells with the OPN inhibitor odanacatib (MK-0822), only the expression of OPN was significantly inhibited, whereas that of E-cadherin and β-catenin was slightly increased (Fig. 4H). In addition, when we treated highly CD31 expression DLBCL cells with ridaforolimus or bevacizumab combined with CD31 siRNA, the OPN, EGFR and ICAM-1 was significantly decreased. E-cadherin and β-catenin was significantly increased as the expression of CD31 decreased (Fig. 4I). Both ridaforolimus combined with CD31-siRNA and bevacizumab combined with CD31-siRNA significantly increased the survival period of mice with highly CD31-expressing DLBCL (Fig. 4J).

**CD31 blocks the normal function of T cells**

It is already known that only CD31 significantly enhanced the invasiveness of DLBCL. We hypothesized that immune function affected the invasion. We subcutaneously implanted CD31, NFATc4 and ETS-1 overexpression tumour lymph nodes into the neck of immunocompetent mice (Fig. 5A). After 3 weeks, we removed the tumour lymph nodes and observed their internal structures by HE staining. It formed a layer of cells outside the tumour lymph nodes in both the NFATc4 and ETS-1 groups. These aggregated cells encapsulated the tumour tissue. However, we did not observe the same phenomenon in the CD31 group (Fig. 5B). We also found a difference number of T cells among the 3 groups. High expression CD31 tumour lymph nodes had fewer T cells. But the number of T cells in the low expression CD31 group did not differ from NFATc4 and ETS-1 (Fig. 4C). We noticed that the CD31 group T cells carried more CD31 markers on their surface and this CD31 + T cell were memory CD8 + T cells (Fig. 5D). At 3 weeks, only the high CD31 expression tumour lymph nodes group consistently expressed OPN. The expression of OPN was significantly reduced in the NFATc4 group and ETS-1 group (Fig. 5E). We used differential gene expression analysis on memory CD8 + T cells from the high CD31 expression tumour lymph nodes. We found mTOR was activated (Fig. 5F). The mTOR pathway mainly influenced the T cell activation and differentiation (Fig. 5G). The reduced expression of IFN-γ, perforin and TNF-α in this mTOR activated CD8 + memory T cell (Fig. 5H).

**DLBCL cell invasion of mice brain is associated with CD31-OPN-EGFR pathway**

We injected high expression CD31 DLBCL cells in mice through the tail vein. After 3 weeks, we observed that the right temporal lobe of the brain of mice exhibited damage and formation of neoplastic tumour tissue. IF showed that this tumour tissue had high expression of CD31 and OPN (Fig. 6A). We hypothesized that these DLBCL cells might enter the brain by crossing the blood-brain barrier. We used HE staining to observe the mouse brain. The vascular endothelial cells of brain in the control group were structurally intact with no obvious fissures between endothelial cells and the vessel walls were relatively thick. In contrast, the vascular endothelial cells in the tumour group were not structurally intact. The vessel walls were thin and had a loose structure. We also observed that the number and nuclei of these endothelial cells constituting the blood vessels were reduced. Moreover, we found some DLBCL cells had
broken the vascular barrier and penetrated the intracranial area (red circle, Fig. 6B). IHC staining of the tumour tissue confirmed our RNA sequencing results, this DLBCL cell also expressed EGFR (Fig. 6C). The gaps between endothelial cells are closed with tight junctions, which limit the movement of material from one side to the opposite side (Fig. 6D). We noticed that the tight junctions (TJ) of the blood vessels around the tumour tissue appeared structurally abnormal. The connections between cells had loosened from closed to completely split (indicated by arrows; Fig. 6E). Endothelial cells with completely split TJ had slowly detached from the vascular membrane and their mitochondria had also become swollen with sparse internal cristae (Fig. 6F). Eventually, the 2 sides of this part of the endothelium became completely permeable to DLBCL cells. Despite that, we also observed residual intact TJ (Fig. 6G). In addition, we also found that EGFR upregulate by CD31-OPN pathway and DLBCL destroy TJ by EGFR inhibiting ZO-1 and ZO-2 expression (Fig. 6H).

Discussion

Even when the primary lesion is clinically controlled with first-line agents, unpredictable and untraceable metastases provide new niches for tumour cells. Searching for ideal markers of tumour aggressiveness plays a central role in disease progression and screening out patients with poor prognosis. The metastasis process of tumour cell involves the activation of many genes. OPN has been associated with initial diagnosis and lymph node metastasis\[^{13}\]. OPN stimulates the aberrant expression of IL-6 and CCL3. In addition, we found that highly aggressive DLBCL cells also significantly overexpressed ICAM-1, MMP-9, and EGFR. These results might explain the failure of the R-CHOP regimen to significantly prevent the formation of metastatic lesions in DLBCL cells with high expression of OPN. DLBCL cells with high expression of OPN were able to invade more tissues in less time, significantly shortening the survival period in mice. The OPN inhibitor odanacatib inhibited AKT and MEKK1\[^{14}\]. AKT is involved in the PI3K signalling pathway, which regulates the function of many downstream proteins involved in tumour cell migration\[^{15}\]. MEKK1 is a key gene that regulates cell adhesion and motility\[^{16}\]. Although OPN affect the function of MMP-3 and MMP-9\[^{17–19}\], we found that the inhibitory effect of odanacatib on MMP-3 and MMP-9 is not permanent\[^{20}\]. We used oroxin B (hypocretin-2) to upregulate the expression of PTEN and inhibit PI3K signalling\[^{21}\], but found that MMP-3, MMP-9 and EGFR were not effectively inhibited. This might be related to NFATc4, ETS-1 and CD31 are closely related to the function of OPN. Their sustained activation of OPN is accompanied by the sustained activation of the expression of MMP-3, MMP-9, and EGFR. NFATc4 is a key player in the transcription of cytokine genes and other genes essential for the immune response\[^{22–23}\]. ETS-1 contributes to invasiveness, epithelial-mesenchymal transition (EMT) and drug resistance\[^{24}\]. They induce the MMP-3 and MMP-9 via AP-1, ERK and JNK signalling\[^{25–26}\]. So NIK SMI1 and oroxin B can not inhibit the expression of MMP-3 and MMP-9 activited by ERK signalling. CD31 is a member of the immunoglobulin-immunoreceptor tyrosine-based inhibitory motif (Ig-ITIM) superfamily\[^{27}\]. Cell extravasation has been shown to require CD31-mediated ICAM-1-dependent tight adhesion and transendothelial migration\[^{28}\]. CD31 can also counteract the inhibition of ICAM-1 by the AP-1 inhibitor nobiletin\[^{29}\] and promote tumour metastasis by FAK and AKT signalling pathways\[^{30}\].
Interestingly, resistance to EGFR-mediated tumour metastasis are directly associated with AKT signalling\[^{31}\]. Sustained activation of AKT signalling by CD31 blocks NIK SMI1 and oroxin B to inhibit EGFR. E-cadherin and β-catenin are both associated with progression of cancer\[^{32}-^{33}\]. We found CD31 inhibited E-cadherin and β-catenin at the same time. In addition, only CD31 was able to induce DLBCL cells damage tissues. After using siRNA to inhibit CD31, we detected changes in mTOR, ERK, AKT, PI3K, and STAT3 pathways. Activated STAT3 is known to be associated with more advanced clinical stages of DLBCL and poorer overall survival\[^{34}\]. mTOR is a target of rapamycin involved in many B-cell functions and directly associated with STAT3\[^{35}-^{36}\]. The mTOR inhibitor ridaforolimus (MK-8669) inhibited ICAM-1 and OPN, whereas promoted E-cadherin and β-catenin. Some studies used CD31 as a marker of blood vessels. Given that patients with high tumour microvessel density have a significantly worse prognosis, we used bevacizumab (avastin) to determine whether the aggressiveness of DLBCL is associated with CD31 induced tumour vascular growth. However, the upregulation of E-cadherin and β-catenin by bevacizumab was not as pronounced as that by ridaforolimus. In recent clinical trials, bevacizumab did not show encouraging results in the treatment of aggressive DLBCL\[^{37}-^{38}\]. CD31 not only promotes the VEGF associated with the tumour-supplying microvessels, but also activates OPN, which are not fully inhibited by bevacizumab. Therefore, relying solely on CD31 as a predictor of tumour microvessel density as a therapeutic guideline for the use of bevacizumab might delay the optimal timing of treatment for patients with DLBCL. When CD31-siRNA was combined with bevacizumab or ridaforolimus, ICAM-1 and EGFR was significantly inhibited. E-cadherin and β-catenin were both significantly elevated. They also significantly prolonged the survival of mice injected with highly CD31 expressing DLBCL cells.

CD31 also has an effect on T cells. As CD31 is a molecule that can bind to itself, high expression CD31 DLBCL cells could recruit CD31 + T cells. Although we found more CD31 + T cells were clustered around the high expression CD31 tumour tissue, the total number of T cells was reduced. In contrast, more T cells were clustered around tumour tissues with low expression of CD31. We found that CD31 + T cells are primarily composed of memory CD8 + T cells. Memory CD8 + T cells are an important component of protective immunity\[^{39}\]. It is interesting to note that CD31 also could influence memory CD8 + T cells differentiation and activation through mTOR pathway. We found a significantly reduced expression of IFN-γ, TNF-α and perforin\[^{40}\] in this CD31 + CD8 + T-cells with abnormally activated mTOR. CD31 + CD8 + memory T-cells clustered around the DLBCL metastases, but they did not have any anti-tumour effects. It's worth noting that CD31 siRNA could reverse the mTOR inhibitory effect on expression of IFN-γ, TNF-α and perforin in CD31 + CD8 + T cells.

CNSL occurs in approximately 5% of patients with DLBCL and is a devastating complication that often results in death within a few months of diagnosis\[^{41}\]. Conventional regimens combining methotrexate or cytarabine are used as a prophylactic treatment\[^{42}-^{44}\]. We found that high expression CD31 DLBCL cells could enter the brain and form new metastases at the damaged brain parenchyma in mice. The neoplastic intracranial tissues had high levels of expression of CD31 and OPN. We used transmission electron microscopy to observe the disappearance of tight junctions (TJ) between endothelial cells in these abnormal blood vessels. There were fewer cristae in the endothelial cells mitochondria, indicating
the inability of these endothelial cells to maintain normal function. Several different protein complexes are assembled to form adhesive structures that form barriers between endothelial cells, such as adherens junctions (AJ), gap junctions (GJ) and TJ. CD31 induced the high expression of EGFR. EGFR caused TJ instability\[^{45}\]. Only after the downregulation of the expression of EGFR following the inhibition of the JAK/STAT3 pathway can intact tight junctions be formed, for example through the recruitment of claudins by ZO-1 and ZO-2\[^{46–47}\]. This was consistent with our previous studies showing that CD31 affects the STAT3 pathway. We therefore believe that the ability of DLBCL cells to penetrate intracranial vascular endothelial cells is also a result of the aberrant activation of CD31.

Current clinical agents cannot fully target these highly CD31-expressing DLBCL cells. This not only makes the selection of treatment options more difficult, but also worsens the prognosis of patients. Our study provides a new mechanism to explain the aggressiveness in DLBCL and offers a new perspective for its treatment.

**Conclusion**

In summary, we provide a new insight into the aggressiveness of DLBCL cells is closely associated with high expression of CD31. CD31 activates OPN through AKT pathways. CD31 also helped DLBCL foci hide from immune cells by recruiting function suppressed CD31 + memory T cells in xenografts. CD31 inhibited CD31 + memory T cells to synthetic INF-\(\gamma\), TNF-\(\alpha\) and perforin effectively by abnormally activated mTOR pathway and promoted DLBCL cells sustain expression of OPN. In addtion, CD31 activated VEGF by OPN and inhibited ZO-1/ZO-2 allowing DLBCL cells to penetrate intracranial vascular endothelial cells.

**Abbreviations**

DLBCL: Diffuse large B cell lymphoma; BBB: blood-brain barrier; OPN: Osteopontin; CD31: Platelet endothelial cell adhesion molecule-1; TJ: tight junctions; R-CHOP: Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone; MMPs: matrix metalloproteinases; EMT: epithelial-mesenchymal transition; WB: western-blot; IF: immunofluorescence; IHC: immunohistochemistry; GSEA: gene sequence enrichment analysis.

**Declarations**

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**Authors contribution**
Z. C.H designed and performed all the experiments and wrote the manuscript. S.X.S contributed to figure and text formatting. Y.Y.Y, L.L.R, D.L, Y.Y.C contributed to the sample collection. H.T, F.J.W, Y.Q contributed to the critical reading of the manuscript. X.Y.Z, X.L, J.Y.H contributed to bioinformatics analyses. Y.Q.J acquired funding, planned experiments.

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**Availability of data and materials**

All data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

The experiment was strictly followed the Helsinki declaration and passed the ethical examination of animal experiments in Sichuan University and North Sichuan Medical College. (Ethical approval number: 2020ERO36-1 and 2021025A)

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


Tables

Table 1
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<th>Total (N,%)</th>
<th>40</th>
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<tr>
<td><strong>Gender</strong></td>
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| Male       | 24 (60%)
| Female     | 16 (40%)
| **Age**    |    |
| ≤60        | 20 (50%)
| ≥60        | 20 (50%)
| **GCB/ABC**|    |
| GCB        | 20 (50%)
| ABC        | 20 (50%)
| **Ann Arbor Staging** |    |
| I-II       | 7 (17.5%)
| III-IV     | 33 (82.5%)
| **IPI Score** | Point |    |
| Low risk   | 0   | 1 (2.5%)
| 1          | 2 (5%)
| Low to medium risk | 2 | 8 (20%)
| High to medium risk  | 3 | 10 (25%)
| High risk   | 4   | 11 (27.5%)
| 5          | 8 (20%)
| **NCCN-IPI Score** |    |
| Low risk   | 0   | 0 (0%)
| 1          | 3 (7.5%)
| Low to medium risk | 2 | 0 (0%)
| 3          | 9 (22.5%)
| High to medium risk  | 4 | 8 (20%)
| 5          | 11 (27.5%)
| High risk   | 6   | 7 (17.5%)
| 7          | 2 (5%)
Table 2

<table>
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<th>Total patients, n=40</th>
<th>OPN high, n=20</th>
<th>OPN low, n=20</th>
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<tr>
<td>Median OPN level(range)</td>
<td>0.577(0.075-1.755)</td>
<td>0.8175(0.82-1.755)</td>
<td>0.3175(0.075-0.545)</td>
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<tr>
<td>Mean age, mean±SD</td>
<td>59±11</td>
<td>58±10</td>
<td>60±13</td>
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<tr>
<td>Male, n(%)</td>
<td>20(50%)</td>
<td>11(55%)</td>
<td>9(45%)</td>
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Figures
Figure 1

OPN expression is abnormal in DLBCL patients with multiple lymph node or extranodal tissue invasion. A, Real-time PCR was used to detect OPN, HNRNPU, OSM, and CXCL12 in lymph nodes of 40 normal donors and 40 newly diagnosed Diffuse Large B lymphoma (DLBCL) patients. B, Real-time PCR was used to detect the difference in OPN expression between DLBCL patients and normal donors. These 10 patients had multiple lymph nodes and tissues infiltrated by DLBCL cells. C, Immunohistochemistry (IHC) was
used to detect the expression of OPN in lymph nodes and tissues infiltrated by DLBCL. D, Western-blot was used to detect gene expression differences between DLBCL patients and normal donors. These patients had multiple lymph nodes and tissues infiltrated by DLBCL cells. E, Real-time PCR detected the difference in OPN between 1-DLBCL patients and 2-DLBCL patients. 1-DLBCL patients were DLBCL patients without multiple lymph nodes and distant tissues infiltrated by DLBCL. 2-DLBCL was DLBCL patients with multiple lymph nodes and distant tissues infiltrated by DLBCL. F, Western blot was used to detect the gene expression differences between 1-DLBCL and 2-DLBCL. G, The OPN mRNA in relapsed/refractory patients with multiple lymph nodes and distant tissues infiltrated by DLBCL. 3-DLBCL were DLBCL patients with only a single lymph node tumor lesion at newly diagnosis. 4-DLBCL were DLBCL patients with primary lymph node enlargement and multiple lymph nodes and distant tissues infiltrated by DLBCL during the treatment of these patients. H, IHC was used to detect new lymph nodes and distant tissue infiltrated by DLBCL in relapsed/refractory patients. I, Western-blot was used to detect gene expression differences during disease progression in DLBCL patients with multiple lymph nodes and distant tissue infiltration. J, In order to know whether CHOP had an effect on continuously infiltrating tissues DLBCL cells and high expression of OPN. Western-blot was used to detect the effect of R-CHOP on OPN in lymph nodes and tissues infiltrated by DLBCL cells. *p < 0.05, **p < 0.01, ***p < 0.001
OPN promoted the invasion of diffuse large B cell lymphoma in mice. A. The DLBCL models were established using BALB/C-nude mice to detect the aggressiveness of DLBCL cells from different DLBCL patients. DLBCL cells were released from DLBCL samples and primary cell culture was performed. We used DETACHaBEAD CD19 to release DLBCL cells from patient tumour tissues. To further ensure the purity of patient DLBCL cells, we subcultured the primary cells for 4 weeks and verified them using flow cytometry and immunohistochemistry (CD20, CD30, CD3, CK, CD38, CD138, PAX-5, CD79a, BCL-6, BCL-2, MUM-1, CD10, C-MYC, TP53, and MKI67). The cells were observed at different times using light microscopy. We used arrows to indicate the chronological order of our observations. B, 3 × 10^7 DLBCL cells were injected into mice through the tail vein. HE staining was used to detect tissues infiltrated by DLBCL cells. C, The number of infiltrated tissues in the EXP-1 and EXP-2 were calculated during 9 weeks. Exp-1 DLBCL cells were used in groups 1-8 and EXP-2 DLBCL cells were used in groups 10-17. D, The gene expression of EXP-1 and EXP-2 were detected by Western-blot. E, Immunofluorescence was used to detect the gene expression of DLBCL cells infiltrating different tissues. F, Western blot was used to detect the effect of OPN inhibitor Odanacatib (MK-0822) on OPN and other genes in DLBCL cells. The inhibition of OPN and other genes become more obvious with the Odanacatib treatment time increasing. The concentration of Odanacatib was 10 μmol/L. G, The effect of Odanacatib on the aggressiveness of high OPN expression DLBCL cells was detected. High OPN expression EXP-1 cells after Odanacatib treatment were used in groups 1-8. Low OPN expression EXP-2 cells were used in groups 10-17. H, Western-blot was used to detect the effect of Odanacatib on the genes of EXP-1 cells. The inhibition of MMP-3 and other genes didn't become obvious with the Odanacatib treatment time increasing. I, Western blot was used to detect the effects of NIK SMI1 and Oroxin B (Hypocretin-2) on the genes of EXP-1 cells with the inhibitors treatment time increasing. J, Western blot was used to detect the effects of NIK SMI1 (NF-κB induced kinase (NIK) inhibitors) and Oroxin B (Hypocretin-2, one of flavonoids isolated from traditional Chinese herbal medicine Oroxylum indicum (L.) Vent, selectively induces tumor-suppressive ER stress in malignant lymphoma cells and has antioxidant activity.) on the genes of EXP-1 cells with the inhibitors treatment time increasing. K, The number of infiltrated tissues of DLBCL cells with different OPN expression levels were calculated. EXP-1 had high expression of OPN and EXP-2 had low expression of OPN. In the left figure, EXP-1 cells treated by NIK SMI1 and were injected into groups 1-8. EXP-2 cells with low OPN expression were injected into groups 10-17. In the right figure, EXP-1 cells with high expression of OPN treated by Oroxin B and were injected into groups 1-8. EXP-2 cells with low OPN expression were injected into Groups 10-17. L, Effects of different treatments on the survival time of mice. *p < 0.05, **p < 0.01, ***p < 0.001
Multiple genes and pathways regulate OPN. **A**, RNA sequence was used to compare lymph node samples from 3 aggressiveness DLBCL patients and 3 normal donors. S1, S2 and S3 represent 3 DLBCL samples that invaded 8 tissues or more. N1, 2, and N3 represent 3 normal donor lymph node samples. FDR<0.05 and absolute fold change $\geq 2P0.001$. **B**, Cluster analysis was used to reveal the differentially genes in Fig.A. P$\leq 0.001$. **C**, RNA sequencing was used to compare lymph node samples from 3 DLBCL patients with extranodal tissue invasion and 3 DLBCL patients with single lymph node disease. S1, S2 and S3 are DLBCL samples of Fig.A. S4, S5, and S6 represent DLBCL patient samples without extranodal invasiveness. FDR<0.05 and absolute fold change $\geq 2P0.001$. **D**, Cluster analysis was used to reveal differentially genes in Fig.C. P$\leq 0.001$. **E**, RNA sequencing was used to compare 3 mice lymph nodes completely infiltrated with invasive DLBCL and 3 normal mice lymph nodes. M1, M2 and M3 represent 3 mice lymph nodes completely infiltrated by invasive DLBCL. C1, C2 and C3 represent 3 normal mice lymph nodes. FDR<0.05 and absolute fold change $\geq 2P0.001$. **F**, Cluster analysis was used to reveal differentially genes in Fig.E. P$\leq 0.001$. **G**, The differential genes in Fig.B, D and F were analyzed. P$\leq 0.001$. **H**, Fig.G differential genes were analyzed.
related pathways that regulate OPN. J, Upstream genes regulating OPN in Fig.1 were analyzed. K, IHC was used to detect the NFATc4, CD31 and ETS-1 in tumor tissues of mice. (*p < 0.05, **p < 0.01, ***p < 0.001)

Figure 4

CD31 promote diffuse large B cell lymphoma metastasis. A, DLBCL cells (Fig 2D) without OPN expression and invading only one lymph node in the whole process were divided into 3 groups. These 3 groups DLBCL cells were infected with Lentiviruses of CD31, NFATc4 and ETS-1 for 48 h. The immunofluorescence (IF) was used to detect the expression of CD31, NFATc4, ETS-1 and OPN. B, Western blot was used to detect the genes of three groups of DLBCL cells. C, The number of the mice tissues infiltrated by DLBCL cells in 3 groups were calculated. D, The Rayner staining was used to detected tissues infiltrated by CD31 DLBCL cells. E, Immunohistochemistry was used to detect DLBCL cells with high CD31 expression in new tumor tissues. F, The survival curves of the three groups were analyzed. G, Small interfering RNA was used to inhibit high CD31 expression DLBCL cells that invaded multiple lymphoid tissues in mice (Fig 2D). Western blot was used to detect gene expression. H, Western blot was used to detect the effects of three inhibitors (Ridaforolimus (MK-8669), Bevacizumab (Avastin) and Odanacatib (MK-0822) on genes. I, Two inhibitors (Ridaforolimus and Bevacizumab) were respectively used in combination with CD31 small interfering RNA. Western blot was used to detect gene expression. J, Two inhibitors (Ridaforolimus and Bevacizumab) were respectively used in combination with CD31 small interfering RNA. The survival curves of every groups were analyzed. (*p < 0.05, **p < 0.01, ***p < 0.001)
Figure 5

CD31 blocks the normal function of T cells. A, In order to simulate the recognition and response of immune cells to metastasis, tumor lymph nodes of patients with high expression of CD31, NFATc4 and ETS-1 were directly implanted subcutaneously into the neck of mice with normal immune function. B, Tumor lymph nodes were removed 3 weeks later. Hematoxylin-eosin staining was used to detect lymph nodes. The arrow points to a layer of cells outside the tumour lymph nodes in both the NFATc4 and ETS-1
Flow cytometry was used to analyze the percentage of T cells per 100,000 cells in 3 groups of tumor tissue of the same weight (0.5g). Low CD31 represents tumor lymph nodes with Low CD31 expression. Flow cytometry was used to analyze the surface molecular markers of T cells in tumor lymph nodes with high expression of CD31. Immunohistochemistry was used to detect the changes of OPN expression in tumor lymph nodes of the three groups before and after transplantation. The RNA sequencing was used to detecte the differentially expressed genes of T cells in CD31 tumor lymph nodes. Functional enrichment analysis of T cells differentially expressed genes. Immunofluorescence was used to detect TNF-α, Perforin and IFN-γ levels of T cells aggregated in tumor lymph nodes in the CD31 group. Low CD31 T cells represent T cells with Low expression of CD31. CD31 T cells represent T cells that express high levels of CD31. (*p < 0.05, **p < 0.01, ***p < 0.001)
DLBCL cell invasion of mice brain is associated with CD31-OPN-EGFR pathway. A, High expression CD31 DLBCL cells were used to establish central nervous system lymphoma (CNSL) models (EXP-1). Immunofluorescence (IF) was used to detect EXP-1 tumour tissue after 3 weeks. B, HE staining was used to detect the structure of skull vessels, cranial vessels and arachnoid vessels in mice. The red circle shows DLBCL cells. C, mRNA sequencing was used to detect EXP-1 differential gene.
Immunohistochemistry was used to verify EGFR expression in EXP-1. D, The transmission electron microscope (TEM) was used to observe a cross section of endothelial cells. The red circles indicates the tight junctions (TJ) that closed the spaces between endothelial cells. E, The abnormally TJ of blood vessels around the tumor tissue. As indicated by the arrows, the TJ went from being completely closed to being completely structurally broken. F, The red circle above indicates a completely split TJ. The red circles below indicates abnormal mitochondria. G, DLBCL completely destroyed the structure of endothelial cells. The two red dotted lines marks the sides of the endothelial cells. The arrow represents material moving to the opposite side. The red circles indicate complete TJ. H, A-443654 is an Akt inhibitor. EGFR-IN-42 is an EGFR inhibitor. GSEA was used to detect EGFR, ZO-1 and ZO-1. real-time PCR and Western-blot were used to detect the pathway of EGFR, ZO-1 and ZO-1. (*p < 0.05, **p < 0.01, ***p < 0.001)