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ORIGINAL ARTICLE

High frequency of CD74 expression in lymphomas: implications for targeted therapy using a novel anti-CD74-drug conjugate

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Abstract

CD74 is a type II transmembrane glycoprotein that functions as an MHC class II chaperone and displays diverse roles in immune responses. Recently, anti-CD74 immunotherapy has shown promise as an effective treatment strategy for lymphoid neoplasms in preclinical models. Using a human anti-CD74 antibody (SP7219), we defined the expression of CD74 protein in both normal and over 790 neoplastic hematolymphoid tissue samples. We found that CD74 is expressed broadly in normal B-cell compartments including primary and secondary lymphoid follicles and in the thymic medulla. The vast majority of lymphomas expressed CD74, including Hodgkin lymphomas (98%), B-cell lymphomas (96%), extranodal NK/T-cell lymphomas (88%), mature T-cell lymphomas (80%), and plasma cell myeloma (75%). Our findings confirm and expand previous observations regarding the expression of CD74 and suggest that CD74 expression on tumor cells may be directly targeted for immunomodulatory therapy for lymphoid and plasma cell malignancies.

Keywords: CD74; lymphoma; immunohistochemistry; immunomodulation

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Introduction

CD74, a nonpolymorphic type II integral membrane glycoprotein, is widely expressed in human immune cells, including B-cells, activated T-cell subsets, monocytes, macrophages as well as dendritic, Langerhans, stromal, epithelial, and endothelial cells [1,2]. Recent studies have also shown expression of CD74 in hematologic malignancies such as B-cell lymphomas and nonhematologic tumors such as carcinomas of gastric, renal, pulmonary, and colonic origin, thymic epithelial neoplasms, and subtypes of sarcomas [2–12]. Although CD74 was originally identified as the HLA-DR (MHC class II) invariant chain that functions as an MHC class II chaperone, extensive studies show that CD74 has diverse roles in immune responses. CD74 participates in non-MHC II protein trafficking, regulates B-cell differentiation, proliferation, and survival, and plays critical roles in T-cell development, dendritic cell motility, inflammation, and thymic selection [13–17]. CD74 also plays a role in inflammatory and immune-related disorders leading to tissue injury, such as ulcerative colitis, liver fibrosis, systemic lupus erythematosus, and Alzheimer disease [17–19]. In addition, as a high-affinity receptor for macrophage migration inhibitory factor (MIF), CD74 can function as a signaling molecule [15]. In B-cell neoplasms as well as macrophages, engagement of receptor complex CD74/CD44 by MIF leads to activation of multiple intracellular signal pathways [16].

Given the important roles of CD74 in immune responses and its broad expression in B-cell neoplasms, the use of anti-CD74 antibody as a therapeutic strategy has been intensely pursued. Preclinical data using humanized anti-CD74 monoclonal antibody, hLL1 milatuzumab, showed a direct antiproliferative effect in non-Hodgkin lymphoma (NHL) cell lines and xenografts [2]. Phase I studies in eight previously treated B-cell lymphomas, however, showed stable disease without complete or partial response [20]. To

overcome these challenges and to unleash the full potential of the target antigen, a novel antihuman CD74 antibody-drug conjugate (ADC), STRO-001, was developed. STRO-001 has shown potent *in vitro* cytotoxicity in several NHL cell lines and antitumor activity in xenograft models of diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL) [21,22].

The cell- and tissue-specific expression patterns of CD74 are likely to influence the choice and usage of this human CD74 ADC in targeted therapies. Therefore, in the current study, we characterize the expression of CD74 protein in a large cohort of well-annotated normal and neoplastic human hematolymphoid specimens using immunohistochemistry and immunofluorescence on tissue sections and cell suspensions.

Materials and methods

Generation of human anti-CD74 antibody

The human anti-CD74 antibody SP7219 was discovered by Sutro Biopharma (Sutro Biopharma, San Francisco, CA, USA) using ribosome display technology and expressed in Sutro's proprietary XpressCF+ protein production system as previously reported and detailed in supplementary materials and methods, Appendix S1 [23–25]. The biotinylated SP7219 (SP9417) was generated by conjugation of SP7219 with NHS-PEG4-Biotin (Thermo Fisher Scientific, Grand Island, NY, USA) through primary amine-based reaction. The Fluorescein-labeled SP7219 (SP9240) was generated by the conjugation of a NHS-Fluorescein (5/6-carboxyfluorescein succinimidyl ester) (Thermo Fisher Scientific) through primary amine-based reaction.

Western blotting

Adherent cells were harvested with Accutase (Innovate Cell Technologies, San Diego, CA, USA) and collected by centrifugation. The cell pellets were washed with Dulbecco's phosphate-buffered saline (PBS) and lysed using RIPA lysis buffer (Millipore, Hayward, CA, USA) on ice for 30 min. ×4 NuPAGE LDS loading dye (Thermo Fisher Scientific) was added to undiluted protein samples and about 100 ug of total protein per lane was loaded onto a 4–12% bis–tris protein gel (Thermo Fisher Scientific). Other controls loaded on the same gel included 1 and 0.1 µg of recombinant CD74 extracellular domain (R&D Systems, Minneapolis, MN, USA) and molecular weight marker from Bio-rad (Bio-rad, Hercules, CA, USA). After the proteins were transferred to PVDF membrane, the membrane was blocked with PBS + 3% nonfat dry milk for 1 h at room temperature, washed with a buffer consisting of PBS + 0.1% Tween20 + 0.2% BSA, and incubated with 5 µg/ml SP7219 or ab185065 (Abcam, Cambridge, MA, USA) at 4 °C overnight; ab185065 is an anti-sodium potassium ATPase antibody used as a plasma membrane loading control. The membranes were washed again and incubated with 1:10 000 goat antihuman Fab-HRP secondary antibody (Pierce, Thermo Fisher Scientific) for 20 min at room temperature. The membrane was washed twice, and the signal was detected with Super-Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific) per manufacturer's instructions. The membrane was developed on the Azure Biosystems (Dublin, CA, USA) c300 digital imager.

Cell lines, human bone marrow cells and FACS-based cell binding

OPM2 cells were purchased from The Leibniz Institute DSMZ (Braunschweig, Germany). Raji, RPMI-6666, SU-DHL-6 and CHO-k cells were purchased from ATCC (Manassas, VA, USA). CHO-human-CD74 cell lines were generated by stable transfection of CHO-k cells with a mammalian expression vector containing the full human CD74 sequence. All cell lines were maintained in RPMI, high glucose medium (Corning, Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 2 mM GlutaMAX (Thermo Fisher Scientific), and 1x penicillin/streptomycin (Corning).

For FACS binding assays, a total of 200 000 cells per well was incubated on ice with serial dilutions of unconjugated SP7219 for 60 min. Cells were washed twice with ice-cold FACS buffer and then incubated with 5 mg/ml Alexa 647-labeled donkey antihuman Fc antibody (Jackson ImmunoResearch, West Grove, PA, USA) on ice for another 60 min. Unstained cells and cells stained with secondary antibody alone were used as controls. Samples were then washed twice using FACS buffer and analyzed using a BD FACS Canto system (BD, Franklin Lakes, NJ, USA). FACS data were analyzed by Flowjo software (Ashland, OR, USA) and geometric mean fluorescence intensity (MFI) was fitted using nonlinear regression analysis with one site-specific binding equation on GraphPad Prism (La Jolla, CA, USA).

Whole bone marrow (BM) aspirates (3 ml each) from five healthy human donors was obtained through

AllCells, Inc. (Alameda, CA, USA). FACS binding on BM cells is detailed in supplementary materials and methods.

Tissue samples and tissue microarrays construction

Formalin fixed, paraffin-embedded tissue samples were obtained from the Department of Pathology, Stanford University Medical Center, Stanford, CA, USA. All tissues were obtained prior to treatment, and Institutional Review Board approval was obtained. For expression in normal hematopoietic tissue, whole tissue sections of normal human tonsil, lymph node, BM, thymus and spleen were used. Hematolymphoid neoplasia was classified according to the 2016 World Health Organization classification [26]. Tissue microarrays (TMAs) were constructed using a tissue arrayer (Beecher Instruments, Silver Spring, MD, USA), as previously described [27].

Immunohistochemistry

TMA, whole sections of human lymphoma and leukemia samples, and normal human hematopoietic tissue samples were sectioned at 4-μm thickness, deparaffinized in xylene, and hydrated in graduated alcohols. Antigen retrieval was done by pressure cooker in EDTA (1 mM)/Tris (5 mM) at pH 9 for 10 min. Slides were then stained with biotin conjugated anti-CD74 antibody (SP7219, Sutro Biopharma, San Francisco, CA, USA) at 1:800 dilution. Slides were developed using the advanced avidin/biotin technology method (VECTASTAIN® Elite® ABC-HRP Kit, Vector Laboratories, Burlingame, CA, USA) and coverslipped with aqueous-based mounting medium. TMAs were scored as 0, no staining; 1, uninterpretable (loss of sample tissue or high background); 2, weak staining (0–20% of cells positive and low intensity); 3, strong staining (>20% of cells positive with moderate to high intensity staining). For Table 1, only cases with score 3 were included. Two authors independently scored all samples (SZ and YN); discrepant cases were jointly reviewed and a final score assigned.

Double immunofluorescence labeling

Paraffin-embedded whole tissue sections of normal human tonsil and thymus, plasma cell myeloma (PCM), and classical Hodgkin lymphoma (CHL) were sectioned at 0.4-μM thickness, deparaffinized, and antigen retrieval was done by pressure cooker in EDTA (1 mM)/Tris (5 mM) at pH 9 for 10 min. After washing in PBS (pH 7.4), slides were stained with CD20 (antibodies used for double immunofluorescence labeling are summarized in Table 2) for 30 min. Slides were then washed in PBS (pH 7.4) and incubated in the dark for 30 min with a mixture of AlexaFluor 488 conjugated anti-CD74 (SP9240-01, 1:600 dilution) and AlexaFluor 568 labeled goat anti-mouse IgG (1:150 dilution, Invitrogen, Carlsbad, CA, USA). Slides were then washed in PBS and counterstained by incubation with Vectashield DAPI (Vector Laboratories, Burlingame, CA, USA). Finally, the slides were coverslipped with an aqueous-based mounting medium.

Table 1. Immunohistologic staining of CD74 in hematopoietic neoplasms

Tumor subtype	Positive	% Positive
Hodgkin lymphoma (n = 59)	58/59	98
Classical Hodgkin lymphoma	49/49	100
Lymphocyte predominant Hodgkin lymphoma	9/10	90
B-cell lymphoma (n = 423)	404/423	96
Follicular lymphoma	148/151	98
Grades 1 and 2	90/91	99
Grades 3 A and B	58/60	97
Diffuse large B-cell lymphoma	135/140	96
Primary mediastinal large B-cell lymphoma	20/20	100
Mantle cell lymphoma	19/21	90
Nodal marginal zone lymphoma	6/6	100
Extranodal marginal zone lymphoma	22/24	92
Splenic marginal zone lymphoma	4/5	80
Chronic lymphocytic leukemia/small lymphocytic lymphoma	36/36	100
Lymphoplasmacytic lymphoma	5/5	100
B-lymphoblastic lymphoma/leukemia	3/9	33
Posttransplant lymphoproliferative disorder	3/3	100
Burkitt lymphoma	3/3	100
Plasma cell myeloma	101/134	75
Extranodal NK/T-cell lymphoma, nasal type	84/96	88
T-cell lymphoma (n = 61)	49/61	80
T-lymphoblastic lymphoma/leukemia	5/14	36
Peripheral T-cell lymphoma, NOS	10/13	77
Angioimmunoblastic T-cell lymphoma	3/3	100
Subcutaneous panniculitis-like T-cell lymphoma	1/1	100
Anaplastic large cell lymphoma/ALK$^+$	18/18	100
Anaplastic large cell lymphoma/ALK–	12/12	100
Other hematopoietic neoplasms (n = 20)	18/20	90
Acute myeloid leukemia	12/12	100
Histiocytic neoplasms	6/8	75

Data analysis and visualization

Images of normal human hematolymphoid tissue and TMA immunohistochemical staining results were acquired using a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with ×4, ×10, ×20, and ×40, and ×60 objective lenses with numerical apertures ranging from 0.05 to 0.90. Images were

Table 2. Summary of antibodies used for double immunofluorescence labeling

Antibody	Vendor	Specie	Clone	Dilution
CD20	DAKO	Mouse	L26	1:500
CD3	Cell Marque	Rabbit	Polyclonal	1:100
CD4	Novocastra	Mouse	1F6	1:40
CD8	DAKO	Mouse	C8/144B	1:400
PD1	Cell Marque	Mouse	NAT105	1:200
HGAL	Cell Marque	Mouse	MRQ-49	1:100
CD138	Cell Marque	Mouse	B-A38	1:30
MUM1	Abcam	Mouse	MUM1p	1:80

captured with a SPOT flex mosaic 15.2 digital camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). Immunofluorescence images were acquired using a Nikon Eclipse E800 microscope and a DXM1200C Nikon digital camera (Nikon, Tokyo, Japan). Digitized images were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

Results

Specificity of the anti-CD74 antibody

The CD74 antibody SP7219 was used to evaluate CD74 expression by Western blot (Figure 1A). In this assay, SP7219 was able to detect membrane bound full length CD74 in CD74-positive cells (CHO-human-CD74, lane 2) as well as recombinant human CD74 ECD (extra-cellular domain) (lanes 4 and 5). No CD74 band was detected in CD74-negative CHO-k cells (lane 3). Anti-sodium potassium ATPase antibody was used as plasma membrane loading control (lanes 6 and 7).

In a FACS-based cell-binding assay, CD74 expression on the cell surface can be detected by 100 nM of SP7219 on CD74-positive SU-DHL-6 (DLBCL cell line) cells, but not on CD74 negative OPM-2 cells (PCM cell line) (Figure 1B). SP7219 binds to CD74 on RPMI-6666 (Hodgkin lymphoma [HL] cell line) and SU-DHL-6 cells with very high affinity (Kd around 2 nM) (Figure 1C). Direct labeling of human BM cells was performed to determine the surface expression of CD74 on B-cells, T-cells, NK-cells, and monocytes (Figure 1D,E). With an Alexa647 labeled isotype used as a background control, T-cells and NK-cells did not express CD74, whereas monocytes and B-cells expressed this molecule at significantly higher levels. The expression of CD74 was also evaluated in the Raji cell line derived from Burkitt lymphoma and OPM-2, a negative control cell line, by immunohistochemistry. Raji cells showed significant staining for CD74 localized to the cell membrane (Figure 1F) in contrast to OPM-2 cells (Figure 1G).

Expression of CD74 protein in normal hematolymphoid tissue

Immunohistologic staining of normal tonsils and lymph nodes showed strong labeling of CD74 protein in primary lymphoid follicles and in mantle zones as well as germinal centers (GCs) of secondary lymphoid follicles (Figure 2A–C). Both immunohistochemistry and flow cytometry confirmed that CD74 staining was mainly localized to cell surface membranes. Paracortical and interfollicular T-cell zones lacked CD74 staining except for a few scattered cells morphologically resembling endothelial cells and lymphoid cells. A subset of intraepithelial lymphocytes within tonsillar epithelium showed staining for CD74. In normal human spleen, strong CD74 labeling was found in the lymphocytes of primary and secondary follicles in the white pulp, while staining was absent in T-cell zones and within the red pulp (Figure 2D).

Next, CD74 staining was investigated in normal human thymus and BM. In normal thymus, prominent CD74 staining was found in the medulla, but the vast majority of cortical thymocytes lacked staining (Figure 2E). Sections of normal human BM showed CD74 staining in scattered cells that morphologically corresponded to lymphoid cells and a subset of maturing myeloid lineage cells, while erythroid precursors and megakaryocytes, as well as BM stromal elements, lacked CD74 staining (Figure 2F).

To further characterize the cell types that express CD74, double immunofluorescence labeling was carried out on tonsil and thymus sections. Double labeling for CD74 and CD20 confirmed the expression of CD74 in B-cells that comprise the primary follicles, mantle zones, and GCs of secondary follicles of tonsil (Figure 3A) and thymic medulla (Figure 3B); prominent CD74+ CD20+ cells were present in those areas with a few scattered CD74+ CD20+ cells present in the thymic cortex. In contrast, CD74+ cells lacked colocalization with CD3 in both tonsil and thymus sections (Figure 3C,D). Similarly, double labeling for CD74 and CD8 or CD4 showed that these T-cell subsets lack expression of CD74 (Figure 3E,F). In addition, double labeling for CD74 and PD1 showed no CD74+ PD1+ dual-positive cells in primary or secondary lymphoid follicles indicating that T-follicular helper (TFH) cells lack CD74 (Figure 3G). Double labeling for CD74 and HGAL showed that tonsillar GC B-cells are CD74+ HGAL+ dual-positive

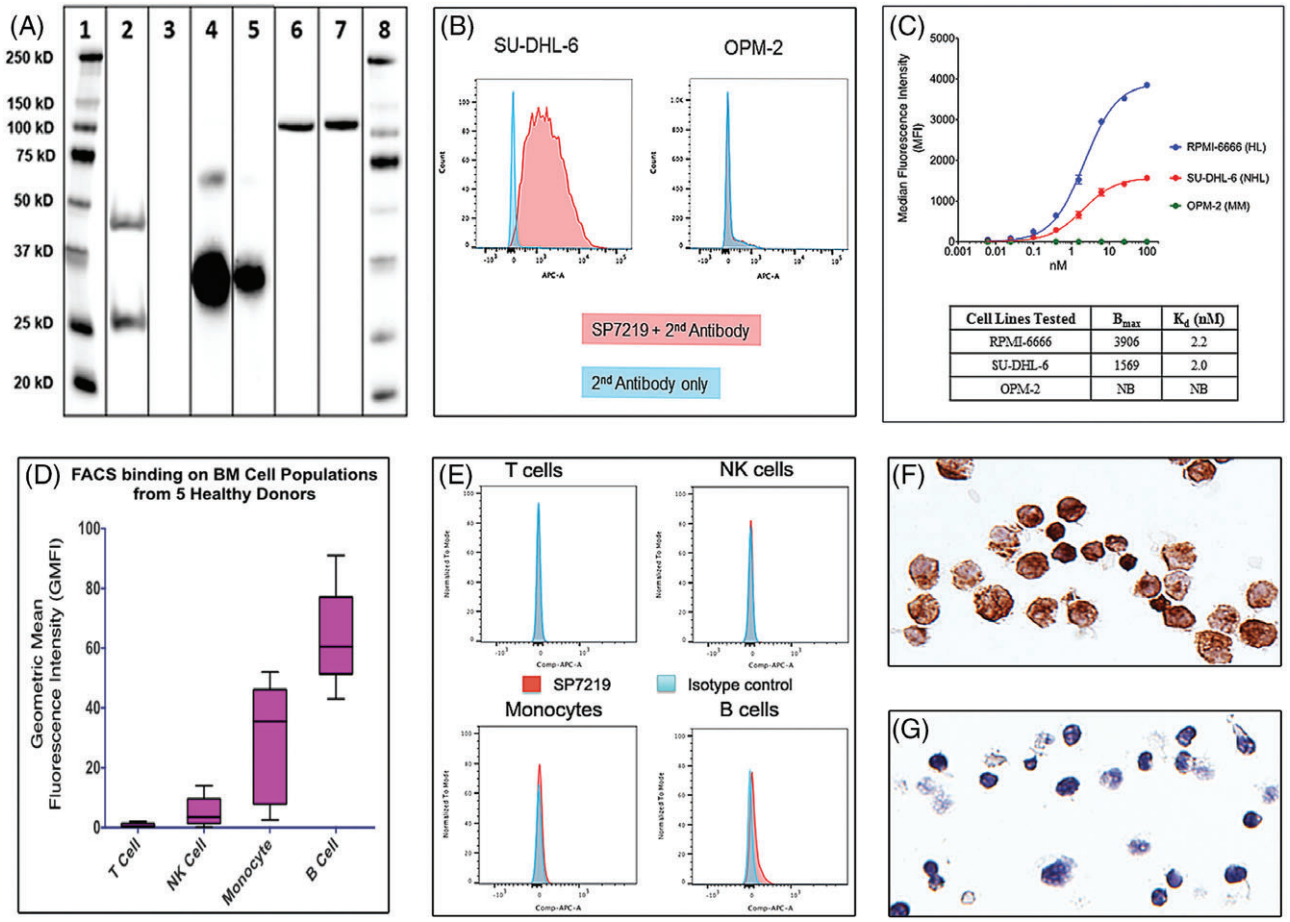

Figure 1. Specificity of anti-CD74 monoclonal antibody. CD74 protein expression was detected using the anti-CD74 monoclonal antibody SP7219. (A) Western blot analysis shows specific binding to full length human CD74 expressed in CHO-human-CD74 cells (lane 2) and recombinant CD74 extracellular domain (ECD) (lane 4, 1 μg ECD; lane 5, 0.1 μg ECD). No CD74 band was detected in CD74-negative CHO-k cells (lane 3). Anti-sodium potassium ATPase antibody was used as plasma membrane loading control (lanes 6 and 7). Lane 1 and lane 8 were loaded with molecular weight markers. (B) CD74 expression on cell surface membranes can be detected by SP7219 in a FACS-based cell-binding assay on CD74-positive SU-DHL-6 cells and not on CD74-negative OPM-2 cells. (C) SP7219 binds to CD74 on positive cell lines with very high affinity (Kd around 2 nM). (D) Detection of CD74 with an Alexa647-labeled anti-CD74 antibody, SP-7219, (n = 5) on BM T-cells, NK-cells, monocytes, and B-cells. (E) Representative histograms, including an isotype control or blocking experiment, are shown for each staining. (F) Immunostaining shows CD74-specific staining in Raji cells (positive control). (G) Immunostaining shows no CD74 staining in OPM-2 cells (negative control).

(Figure 3H), while double labeling for CD74 and CD138 showed that plasma cells lack CD74 (Figure 3I). Finally, double labeling for CD74 and MUM1/IRF4 showed that post GC, B-cells lack CD74 (Figure 3J).

CD74 is expressed in the majority of B-cell NHL and PCM

A total of 793 lymphomas of different subtypes were evaluated for expression of CD74 and the results are summarized in Table 1 and illustrated in Figures 4 and 5. In a broad survey of 423 B-cell lymphomas, the majority expressed CD74 (404/423, 96%; Figure 4A–F). Staining was present on cell surface membranes although weak cytoplasmic staining for CD74 was evident in a subset of cases. Like normal thymic medullary B-cells, all primary mediastinal large B-cell lymphomas (PMBCL, 20/20; 100%) showed strong labeling for CD74 with intense membrane staining highlighting large atypical cells (Figure 4A). The vast majority of DLBCL (135/140; 96%) was positive for CD74 and showed a range of moderate to strong staining (Figure 4B). Staining distribution was similar among GC (62), non-GC (66), and unclassifiable (12) subtypes of DLBCL. Similarly, follicular

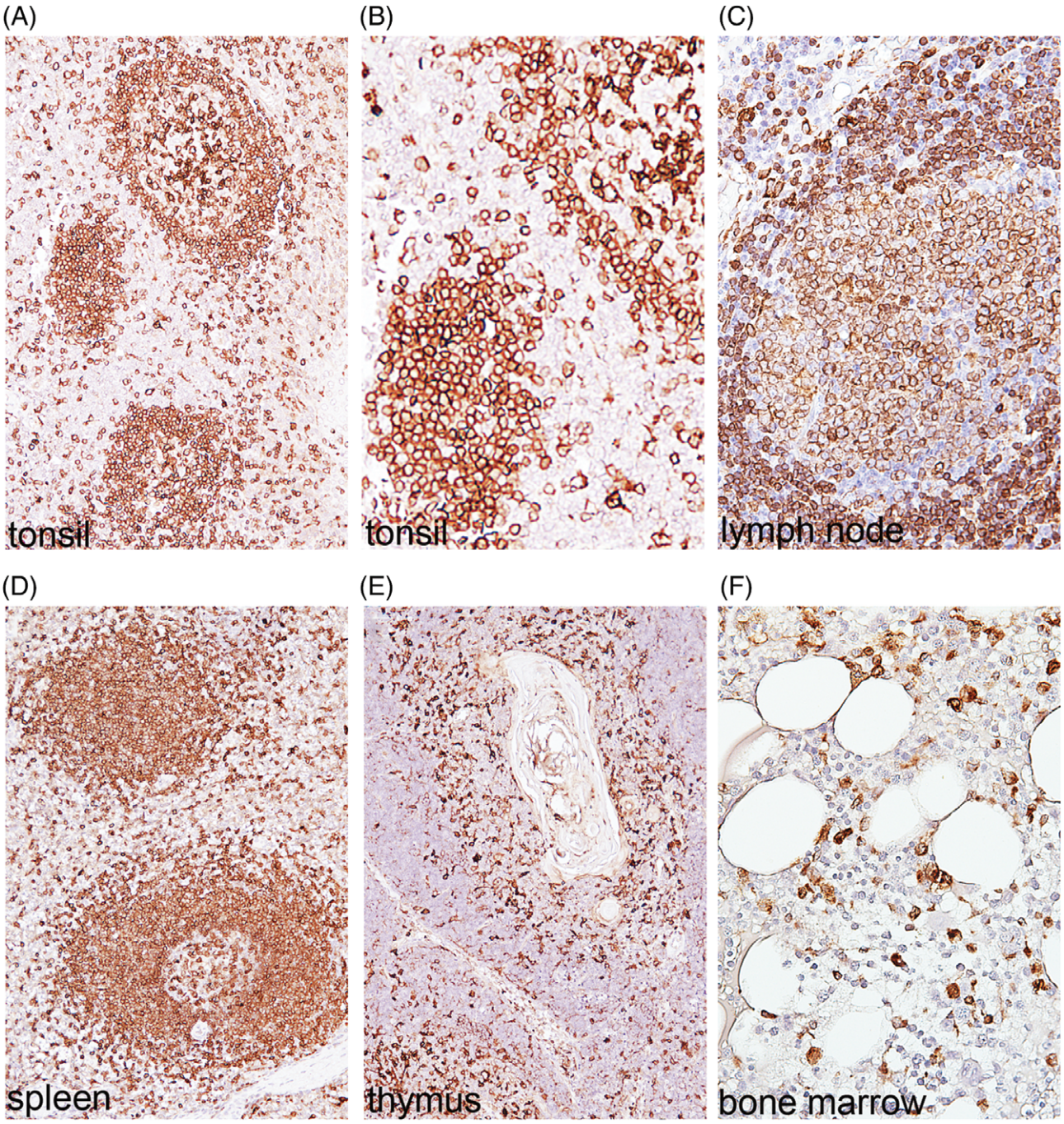

Figure 2. CD74 immunostaining in normal lymphoid tissues. Low- and high-magnification images of normal human tonsil, lymph node, and splenic tissues show CD74-specific staining in B-cells of primary follicles, marginal and mantle zones of secondary follicles, and GCs (A–D). Normal thymus shows prominent CD74-positive cells in the medulla and scattered CD74-positive cells in the cortex (E). Bone marrow shows lack of CD74 staining in trilineage hematopoietic precursors although scattered lymphoid cells show staining (F).

lymphoma (FL) also showed a range of moderate to strong staining (148/151, 98%; Figure 4C). The intensity as well as numbers of positive cells did not correlate with the grade of FL. Most MCLs showed robust expression of CD74 (19/21, 90%; Figure 4D). This MCL cohort included cyclin D1-positive and negative cases, and all three cyclin D1-negative cases expressed CD74. All cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL, 36/36, 100%; Figure 4E), Burkitt lymphomas (3/3, 100%)

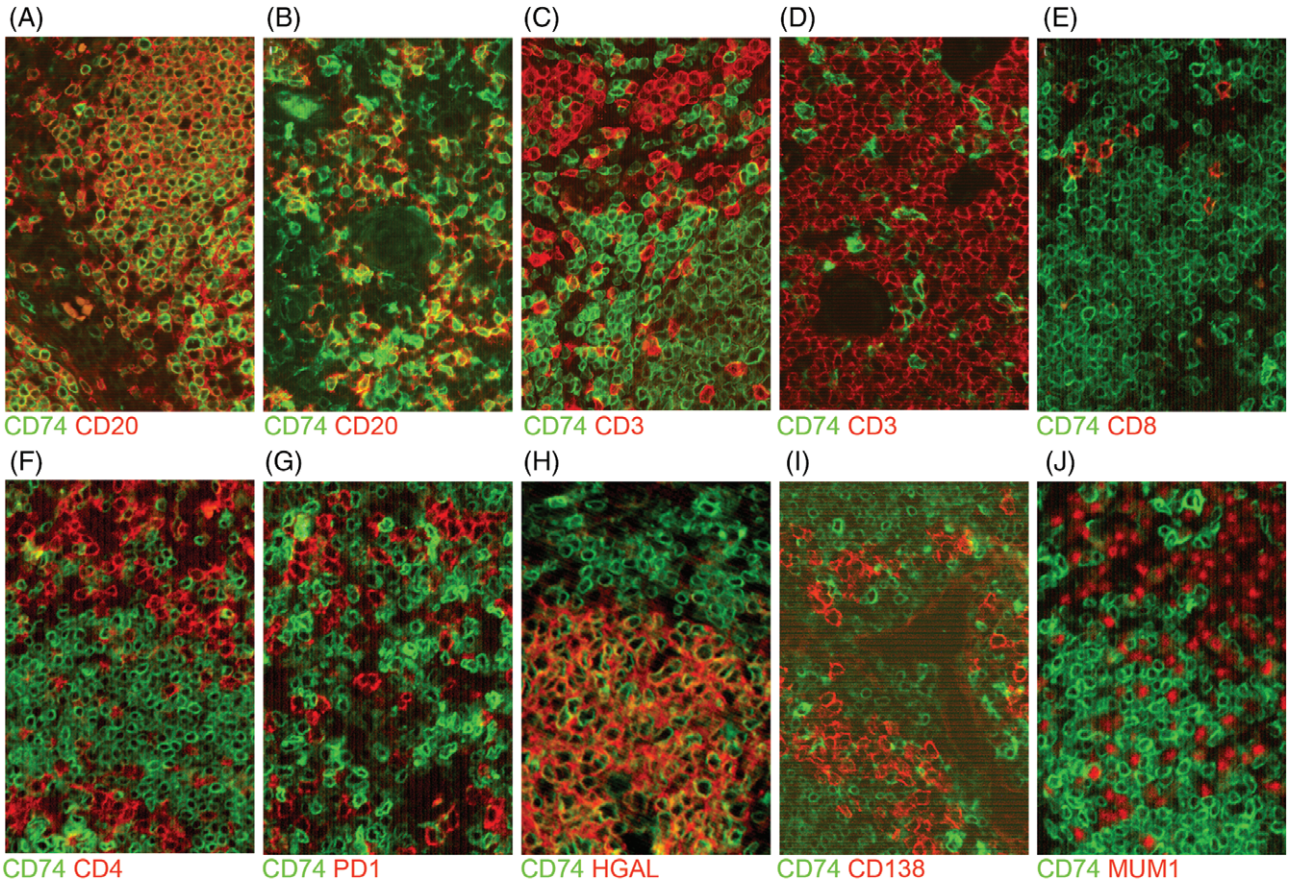

Figure 3. Double immunofluorescence labeling of CD74 in normal lymphoid subsets. Double-immunofluorescence labeling of tonsil and thymus shows colocalization of CD74 (green, membrane/cytoplasmic staining in panels) with $CD20^{+}$ B-cells (red, membrane staining) in primary follicle, mantle zone, and GC of secondary follicle (A) and in the medulla of thymus (B); in contrast, CD3 (red, membrane staining) shows no colocalization with CD74 in intrafollicular T-cell areas or within follicles (C); similarly, no colocalization of CD74 and CD3 is seen in cells of the medulla (D). In addition, no colocalization of CD74 and CD8 or CD4 or PD1 is seen in tonsillar cells (E–G). Colocalization of CD74 and HGAL is seen in tonsillar GC cells (H); whereas no colocalization of CD74 and CD138 or MUM1 is seen in tonsillar cells (I–J).

and lymphoplasmacytic lymphoma (5/5, 100%) showed moderate to strong staining for CD74. In addition, all nodal marginal zone lymphomas (MZLs) showed strong staining for CD74. Similarly, extranodal MZL (Figure 4F) and splenic MZL showed 92 and 80% positivity for CD74, respectively. In contrast, B-lymphoblastic leukemia/lymphoma (B-ALL/LBL) showed CD74 staining in a smaller subset of cases (3/9, 33%).

Among PCM, a total of 101 of 134 cases (75%) showed staining for CD74 (Figure 4G). Double immunofluorescence labeling for CD74 and MUM1 confirmed colocalization in neoplastic cells of PCM (Figure 4H).

Hodgkin lymphoma subtypes express CD74

All 49 cases of CHL showed CD74 staining in Hodgkin/Reed–Sternberg (HRS) cells in a membrane and Golgi pattern (Figure 5A). Although by immunohistochemistry CD74 staining was localized to the membrane and cytoplasm, immunofluorescence staining of CD74 showed crisp membranous localization with faint cytoplasmic staining. CD74 staining was not seen in the background inflammatory infiltrate, including infiltrating T-lymphocytes. Double-immunofluorescence labeling for CD74 and MUM1 confirmed colocalization in typical HRS cells (Figure 5A, inset). Moreover, this cohort included a significant proportion of cases that lacked CD15 staining. In most cases of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (9/10, 90%), CD74 highlighted the lymphocyte predominant (LP) cells (Figure 5B).

CD74 is expressed in the majority of NK-cell and T-cell lymphomas

The majority of lymphomas derived from NK-cells and T-cells showed staining for CD74, albeit with a

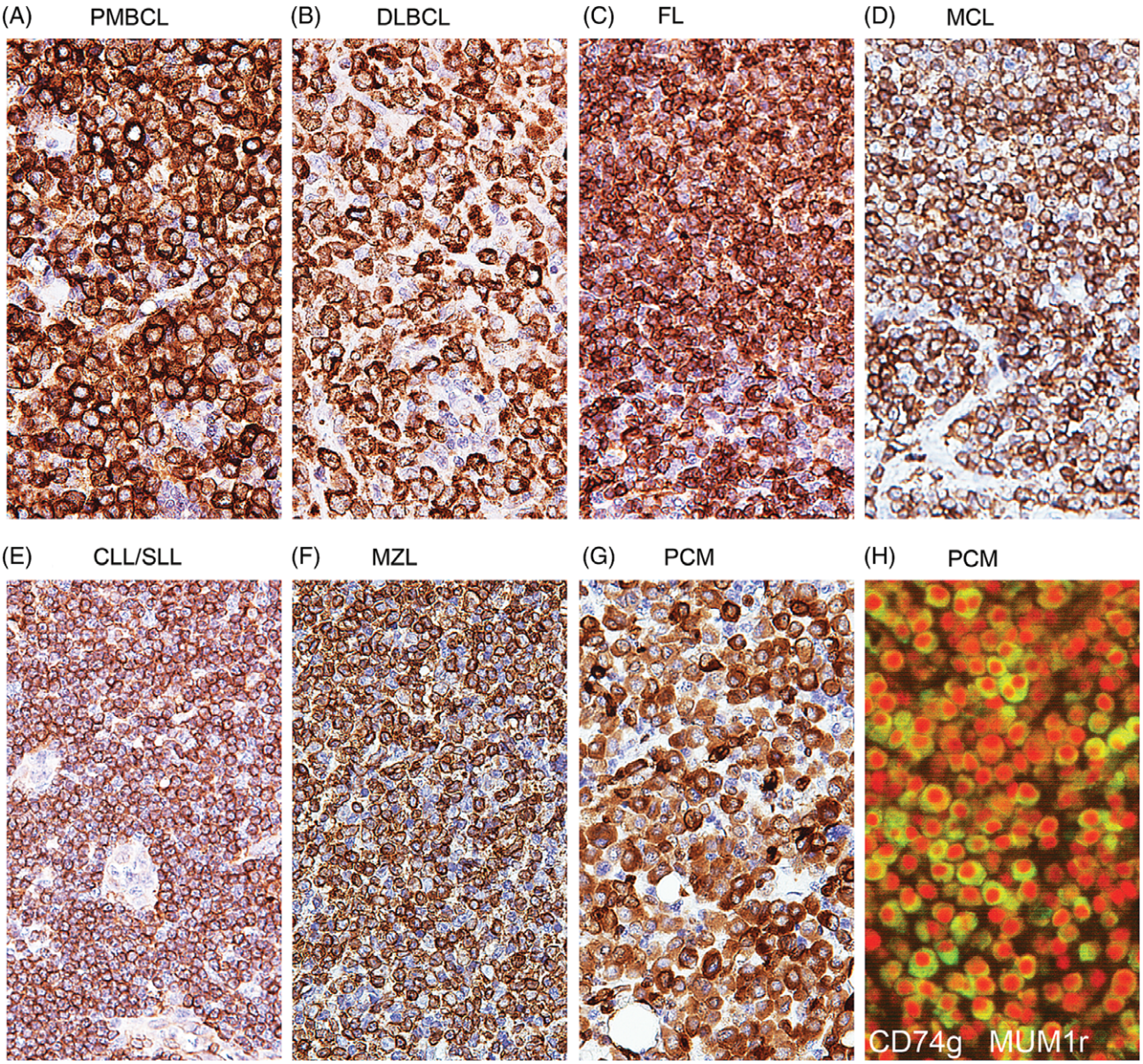

Figure 4. CD74 immunostaining in hematopoietic neoplasms. Immunohistologic staining for CD74 in PMBCL (A), DLBCL (B), FL (C), MCL (D), CLL/SLL (E), and extranodal MZL (F) shows intense membrane and faint cytoplasmic staining in neoplastic cells. Like most B-cell lymphomas, PCM (G) also shows intense membrane and cytoplasmic staining in neoplastic cells; double-immunofluorescence labeling of PCM shows colocalization of CD74 (green, membrane staining) and MUM1 (red, nuclear staining) in neoplastic cells (H). Original magnification ×400 for all panels.

wide range of staining intensities. Most extranodal NK/T-cell lymphoma, nasal type, showed intense staining for CD74 (84/96, 88%; Figure 5C). CD74 was expressed across multiple histologic subtypes of mature T-cell lymphomas. Among anaplastic large cell lymphomas (ALCL), both ALK+ ALCL (18 cases) and ALK− ALCL (12 cases) showed intense CD74 expression in the large pleomorphic cells (Figure 5D). Three cases of angioimmunoblastic T-cell lymphoma (AITL), and a case of subcutaneous panniculitis-like T-cell lymphoma also showed staining for CD74. Among peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS), 77% of cases showed staining for CD74 (10/13; Figure 5E). Of the 10 CD74-positive PTCL cases, subtyping showed 6 cases were CD4+, 2 cases were CD8+, 1 case was CD4-CD8-, and 1 case was not further subtyped; in addition, 2 cases had a cytotoxic immunophenotype,

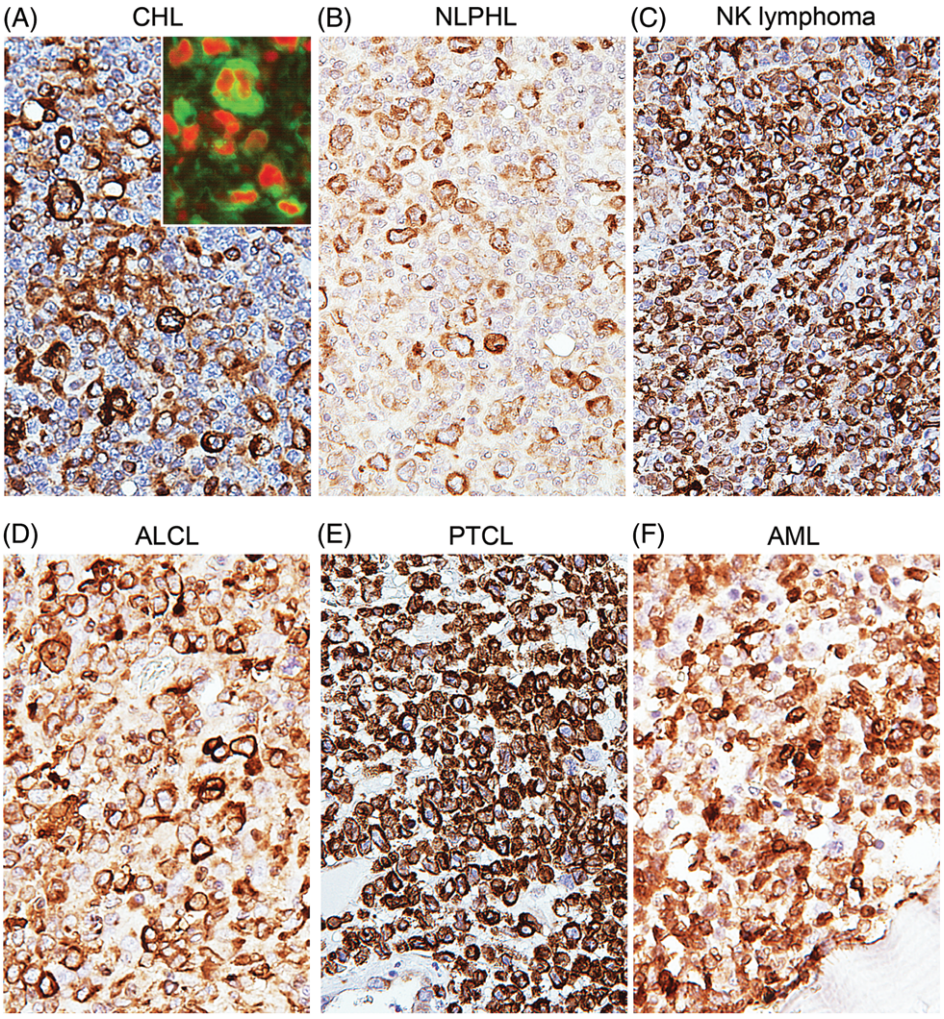

Figure 5. CD74 immunostaining in Hodgkin, T-cell, and NK-cell lymphomas and in AML. Immunohistochemical stain for CD74 on paraffin-embedded tissue sections of CHL (inset: sections of CHL stained with antibodies to CD74 (green, cell membrane) and MUM1 (red, nuclear) shows colocalization in R–S cells; original magnification ×600) (A), and NLPHL (B), extranodal NK/T-cell lymphoma, nasal type (C), ALK+ ALCL (D), PTCL, NOS (E), and AML (F). Original magnification ×400 for all panels.

2 cases expressed CD30 in 30–50% of neoplastic cells and another case showed a TFH phenotype. In contrast to CD74 expression in high numbers of mature T-cell lymphoma categories, only a minority of T-lymphoblastic lymphoma/leukemia (T-ALL/LBL, 5/14, 36%) showed staining for CD74, indicating less frequent CD74 expression in malignancies derived from immature T-cells.

CD74 is expressed in other hematopoietic and nonhematopoietic neoplasms

Although not the main focus of our investigation, a limited number of acute myeloid leukemias (AMLs) and histiocytic neoplasms were included in our analysis. All 12 AML cases tested showed intense expression of CD74 in immature blasts (Figure 5F). These AML cases included three with myelodysplasia-related changes, two with 11q23 abnormalities, one with t(9;22) translocation arising from chronic myeloid leukemia and the remainder were AML without specific genetic abnormalities (AML, not otherwise specified). CD74 was also expressed in examples of histiocytic neoplasms including Langerhans cell histiocytosis (3/3, 100%), histiocytic sarcoma (2/2, 100%) and Rosai-Dorfman disease (1/3, 33%) (Table 1).

To assess CD74 expression in nonhematopoietic tissue, a total of 121 samples from various organs and tissue types and corresponding tumors were stained for CD74. Staining for CD74 was not detected in the

majority of normal nonhematopoietic tissue or tumors tested; however, CD74 was expressed in various types of tissue resident macrophages, including alveolar macrophages and Kuppfer cells, which are strongly positive (see supplementary material, Figure S1). In brain tissue, normal glial and neuronal cells lacked staining for CD74 whereas the tumor cells of two cases of glioblastoma multiforme were CD74 positive. Similarly, one hepatocellular carcinoma and three ovarian carcinomas showed staining for CD74 in tumor cells (see supplementary material, Table S1).

Discussion

Recently, CD74 has emerged as a potential therapeutic target and data from preclinical models indicate that anti-CD74 immunotherapy holds promise as an efficacious choice for patients with lymphoma. Using a novel anti-CD74 antibody SP7219, we evaluated the distribution of CD74 in a large cohort of diagnostic tissue samples obtained from patients with hematopoietic neoplasms. Our findings confirm and extend previous observations and bring to light new subtypes of hematopoietic tumors that are likely to benefit from anti-CD74 therapy.

In normal B-cells, CD74 is expressed in primary follicles, GC of secondary follicles, mantle and marginal zones, and in the thymic medulla. In accordance with this broad range of expression across normal B-cell compartments, CD74 was expressed in the vast majority of B-cell lymphomas. Several studies show that the CD74/MIF complex can induce multiple signaling pathways and potentiate CD74/MIF biological functions, including leukocytic integrin activation, cell proliferation, anti-apoptosis, and induction of pro-inflammatory gene expression [16,28]. Much of this deregulation of signaling pathways results in the genesis of B-cell lymphomas [29]. The robust expression of CD74 in most B-cell lymphomas in our study underscores the potential of harnessing this molecule for targeted therapy.

Our results show that CD74 is highly expressed in PMBCL as previously reported in one study [4]. PMBCL has specific clinical and pathological features related to overexpression of an NF-κB activation signature and nuclear localization of NF-κB transcription factor complexes [30]. In normal B-cells, CD74 signaling has been shown to promote cell survival through CD44/PI3K/AKT-mediated NF-κB activation. In addition, CD74/MIF initiated Src/PI3K/AKT pathway activates the phosphorylation of the pro-apoptotic proteins, BAD and FOXO3a [16]. Given the roles played by CD74/MIF signaling in anti-apoptosis pathways, it is likely that CD74/MIF signaling potentiates pro-survival signals to malignant B-cells in PMBCL.

CD74 is a marker of HRS cells of CHL. In 1992, Sarker reported that most HRS cells showed diffuse cytoplasmic staining for LN2 (CD74) with a few HRS cells demonstrating paranuclear deposits [8]. Recently, proteomic analyses of CHL cell line supernatant found high MIF expression and significantly elevated levels of MIF protein in the plasma of CHL patients [31]. Our results support those observations and show that SP7219 exhibits strong membrane staining of HRS cells. Like CHL, we also found CD74 expression in LP cells of NLPHL.

PMBCL and CHL share many clinical and molecular characteristics including deregulation of the NF-κB signaling pathway, which is constitutively active in PMBCL and CHL. In two independent gene expression profiling (GEP) studies, over one-third of genes, including genes related to NF-κB signaling, were shared between PMBCL and CHL [32,33]. Taken together, the CD74/MIF ligand–receptor complex likely activates NF-κB signaling leading to cell proliferation, immune escape, and tumor cell survival in PMBCL and CHL.

Preclinical studies of milatuzumab have shown potent efficacy in hematopoietic neoplasms primarily occupying the BM niche, such as CLL/SLL, PCM, and AML [2,34–36]. All CLL/SLL in the current study expressed CD74. Retention of B-cells within the marrow niche is dependent on CD74/MIF/CXCR4 complex, which mediates B-cell migration toward the BM stroma, whereas attachment is largely attributed to very late antigen (VLA)-4, an integrin that binds to both alternatively spliced fibronectin and vascular cell adhesion molecule-1 (VCAM-1) on marrow stromal cells [16,37]. CD74/MIF is also known to play a key role in the regulation of VLA-4 expression [34]. Advanced stage CLL cells express higher levels of CD74 and VLA-4 compared to early stage CLL cells [38]. These data together suggest that CD74/MIF may regulate CLL homing and survival. Indeed, CD74-blockade using milatuzumab successfully inhibits *in vivo* homing of CLL cells to the BM niche and thereby reduces Bcl-2 overexpression and survival of CLL cells [38].

We found that CD74 was expressed in 75% of PCM, an incurable disease characterized by accumulation of malignant plasma cells within the BM. PCM harbors a spectrum of structural chromosomal changes, including multiple recurrent IGH reciprocal translocations such as t(11;14)(q13;q32), t(4;14)(p16;q32), and

t(14;16)(q32;q23), which result in increased expression of cyclin-D1, the membrane receptor FGFR3, and the oncogene C-MAF [26,39,40]. Genome sequencing studies of PCM found frequent mutations in genes involved in RNA processing, protein translation, and the unfolded protein response [41]. In addition, sequencing analysis also found multiple mutations in NF-κB-associated genes that may affect up to one-third of PCM cases [41–44]. Given that the CD74/MIF complex plays a key role in the regulation of B-cell survival [13], it is not surprising that the CD74/MIF complex is involved in the pathogenesis and progression of PCM. Furthermore, the lack of expression of CD20 in PCM limits the use of rituximab [35], which is a frontline therapy for diffuse large B-cell and other B-cell lymphomas [29]. Therefore, developing novel therapeutic approaches to eradicate malignant plasma cells is necessary. Our data showing that CD74 is expressed in 75% of PCM suggest that patients with myeloma would benefit from therapeutic antibodies that can engage the MIF-CD74 signaling pathway.

Perhaps the most novel finding of our study was the expression of CD74 in a subset of T- and NK-derived lymphomas. CD74 was expressed across multiple histologic subtypes of mature T-cell lymphoma, including PTCL, NOS, AITL, ALK+ALCL, and ALK−ALCL and subcutaneous panniculitis-like T-cell lymphoma. Similar to B-ALL/LBL, only a minority of T-ALL/LBL showed staining for CD74. There was a significant proportion of extranodal NK/T-cell lymphoma, nasal type that showed moderate to strong CD74 staining. While the effect of CD74 signaling to promote B-cell proliferation and survival is well studied, much less is known about the biologic role of CD74 expression by normal human T- and NK-cells.

CD74 was also highly expressed in a small cohort of AML cases we tested. Additional studies are warranted to further validate these findings and correlate with clinical and molecular subtypes of AML. Furthermore, in 121 nonhematopoietic samples, several carcinomas from hepatocellular and ovarian derivation were positive, despite the absence of CD74 staining in normal tissue counterparts. These findings raise the possibility that anti-CD74 may be a potential targeted therapy in nonhematopoietic tumors and warrants further investigation in these malignancies. The lack of CD74 staining in normal epithelium, liver, brain, and gastrointestinal tract among other normal human tissue types suggests that anti-CD74 is likely to have minimal off-target effects.

In conclusion, we have shown in a large cohort of well-annotated human samples that CD74 is expressed in a wide variety of lymphoid neoplasms and PCM. Our data provide sufficient evidence to support the use of anti-CD74 antibodies for targeted therapy in these neoplasms.

Author contributions statement

SZ and YN designed and performed experiments, analyzed data, and wrote the paper. AM, AY, JH, HC, and XL developed the antihuman CD74 antibody-drug conjugate, performed Western blotting and flow cytometry and analyzed data. AM reviewed data and the paper.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. CD74 immunostaining in nonlymphoid tissues

Table S1. CD74 staining in nonhematopoietic tissues and neoplasms