Laboratory Investigation (2017) 97, 1095–1102

EMMPRIN (CD147) is induced by C/EBPβ and is differentially expressed in ALK+ and ALK− anaplastic large-cell lymphoma

Janine Schmidt[1], Irina Bonzheim[1], Julia Steinhilber[1], Ivonne A Montes-Mojarro[1], Carlos Ortiz-Hidalgo[2], Wolfram Klapper[3], Falko Fend[1] and Leticia Quintanilla-Martínez[1]

Anaplastic lymphoma kinase-positive (ALK+) anaplastic large-cell lymphoma (ALCL) is characterized by expression of oncogenic ALK fusion proteins due to the translocation t(2;5)(p23;q35) or variants. Although genotypically a T-cell lymphoma, ALK+ ALCL cells frequently show loss of T-cell-specific surface antigens and expression of monocytic markers. C/EBPβ, a transcription factor constitutively overexpressed in ALK+ ALCL cells, has been shown to play an important role in the activation and differentiation of macrophages and is furthermore capable of transdifferentiating B-cell and T-cell progenitors to macrophages *in vitro*. To analyze the role of C/EBPβ for the unusual phenotype of ALK+ ALCL cells, C/EBPβ was knocked down by RNA interference in two ALK+ ALCL cell lines, and surface antigen expression profiles of these cell lines were generated using a Human Cell Surface Marker Screening Panel (BD Biosciences). Interesting candidate antigens were further analyzed by immunohistochemistry in primary ALCL ALK+ and ALK− cases. Antigen expression profiling revealed marked changes in the expression of the activation markers CD25, CD30, CD98, CD147, and CD227 after C/EBPβ knockdown. Immunohistochemical analysis confirmed a strong, membranous CD147 (EMMPRIN) expression in ALK+ ALCL cases. In contrast, ALK− ALCL cases showed a weaker CD147 expression. CD274 or PD-L1, an immune inhibitory receptor ligand, was downregulated after C/EBPβ knockdown. PD-L1 also showed stronger expression in ALK+ ALCL compared with ALK− ALCL, suggesting an additional role of C/EBPβ in ALK+ ALCL in generating an immunosuppressive environment. Finally, no expression changes of T-cell or monocytic markers were detected. In conclusion, surface antigen expression profiling demonstrates that C/EBPβ plays a critical role in the activation state of ALK+ ALCL cells and reveals CD147 and PD-L1 as important downstream targets. The multiple roles of CD147 in migration, adhesion, and invasion, as well as T-cell activation and proliferation suggest its involvement in the pathogenesis of ALCL.
Laboratory Investigation (2017) **97,** 1095–1102; doi:10.1038/labinvest.2017.54; published online 5 June 2017

Anaplastic lymphoma kinase-positive (ALK+) anaplastic large-cell lymphoma (ALCL) is characterized by the translocation t(2;5)(p23;q35) that juxtaposes the nucleophosmin (*NPM*) gene to the *ALK* gene leading to the generation of the chimeric protein NPM-ALK.[1] Several signaling pathways, including the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, are activated by the constitutive expression of the NPM-ALK fusion protein, leading to proliferation, prolonged tumor cell survival, cytoskeletal rearrangement, and cell migration.[2] A central target gene of the JAK/STAT signaling pathway is the transcription factor C/EBPβ, which is overexpressed in ALK+ ALCL.[3–6] C/EBPβ belongs to the CCAAT/enhancer binding protein family of transcription factors and members of this family have been related to cellular processes such as differentiation of adipocytes and cells of the myelomonocytic lineage,[7,8] control of metabolism,[9] inflammation,[10,11] and cellular proliferation.[12] In particular, C/EBPβ has been shown to play a role in the activation and differentiation of macrophages.[13,14] In this context, it is important to note that C/EBPβ is capable of transdifferentiating B-cell and T-cell progenitors *in vitro*, leading to loss of B- or T-cell-specific markers and to a rapid and efficient reprogramming of these cells to macrophages.[15,16]

[1]Institute of Pathology and Neuropathology and Comprehensive Cancer Center Tübingen, University Hospital Tübingen, Eberhard-Karls-University, Tübingen, Germany; [2]Departamento de Patología, Centro Médico ABC (The American British Cowdray Medical Center), Mexico City, Mexico and [3]Hematopathology Section, University Hospital Schleswig-Holstein Campus Kiel/Christian-Albrechts University Kiel, Kiel, Germany
Correspondence: Professor L Quintanilla-Martinez, MD, Institute of Pathology and Neuropathology and Comprehensive Cancer Center Tübingen, University Hospital Tübingen, Eberhard-Karls-University, Liebermeisterstr. 8, 72076 Tübingen, Germany.
E-mail: Leticia.Quintanilla-Fend@med.uni-tuebingen.de

Received 9 December 2016; revised 12 April 2017; accepted 14 April 2017

ALCL cells are supposed to derive from activated T cells, as indicated by the presence of clonal TCR-gene rearrangements and the expression of interleukin-2 receptor alpha (CD25) and CD30, a member of the tumor necrosis factor receptor superfamily.[17,18] Despite the T-cell genotype, ALK+ ALCL frequently demonstrates an unusual phenotype with loss of most T-cell markers, of the T-cell receptor (TCR) and associated molecules,[19] as well as the unusual expression of mono- and histiocytic surface antigens. The monocytic markers CD13 and CD33 as well as CD68, and rarely the myeloid antigen CD15 have been detected in ALCL cells[20,21] and their expression is more prevalent in ALK+ ALCL.[22]

On the basis of the central role of C/EBPβ in ALK+ ALCL pathogenesis and on the fact that C/EBPβ has the potential to transdifferentiate thymocytes to macrophages, we wanted to investigate whether the overexpression of C/EBPβ is responsible for the unique phenotype of ALCL. Therefore, extensive surface antigen expression profiles of two ALK+ ALCL cell lines with and without specific knockdown of C/EBPβ were generated and selected antigens further examined in primary ALCL cases.

MATERIALS AND METHODS

Cell Culture and Patient Samples

The ALK+ ALCL (t(2;5)(p23;q35)-positive) cell lines SUDHL-1, SUP-M2, and SR786; the ALK− ALCL cell line Mac-1; the mantle cell lymphoma cell lines Rec-1, Jeko-1, and Granta; the promyelocytic leukemia cell line HL-60; the diffuse large B-cell lymphoma cell line SUDHL-4; the multiple myeloma cell line KMS-12; the T-cell acute lymphoblastic lymphoma cell line Jurkat; and the Burkitt lymphoma cell line Raji were cultured as previously described.[3] HeLa and HEK293T cells were cultured in DMEM (Invitrogen GmbH, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FCS; PAA Laboratories GmbH, Pasching, Austria) and 100 μg/ml penicillin/streptomycin (Invitrogen). One hundred fifteen formalin-fixed, paraffin-embedded ALCL primary samples (97 ALK+ and 18 ALK−) were collected from the files of the Institute of Pathology and Neuropathology, University Hospital Tübingen, Germany, Institute of Pathology, University of Kiel, Germany, and Department of Pathology, the American British Cowdray Medical Center, Mexico City, Mexico. All cases were comprehensively immunophenotyped, as part of the diagnostic work-up, and were classified following the recommendations of the World Health Organization classification for tumors of haematopoietic and lymphoid tissues in ALK+ and ALK− ALCL.[23]

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissue sections on the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using Ventana reagents. Primary antibody detection was carried out with the iView diaminobenzidine detection kit (Ventana). ALCL primary cases had been previously studied in paraffin sections with a panel of antibodies to assess tumor cell phenotype. All cases were stained for CD30 (Dako, Glostrup, Denmark, dilution 1:50), ALK (Dako, dilution 1:400), C/EBPβ (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:200), and CD147 (MEM-M6/1; Abcam, Cambridge, UK, dilution 1:1500). Eighty-one ALK+ ALCL cases were stained from tissue microarrays, whereas 34 cases were stained from whole sections. In addition, as control for the normal stain of CD147, three tonsils and two lymph nodes were used. In addition, 22 cases were stained for PD-L1 (28-8, Abcam, dilution 1:50). The use of human tissue samples was approved by the local ethics committee of the University of Tübingen (approval 620/2011BO2).

Virus Production and Lentiviral Infection

For production of the replication-defective lentiviral virions, the packaging cell line HEK293T was co-transfected with the packaging plasmid pCMVΔR8.9, the envelope plasmid containing the G-Protein of vesicular stomatitis virus and the transfer vector (pFUGW).[24] The transfer vector includes the reporter gene EGFP driven by an internal ubiquitine-C promoter and the specific shRNA under the control of the human H1 promoter. The pFUGW derivate vectors with the specific C/EBPβ-shRNA inserted were named 'pF-C/EBPβ,' vectors without the shRNA insert were called 'pF-S.' The lentiviral infection of KiJK and Karpas 299 cells (1×10^6 cells per ml) was performed in 12-well plates in the presence of Polybren (8 μg/ml; Sigma, St Luis, MO, USA). Cells were centrifuged at 800 *g* and 32 °C for 90 min. Subsequently the cells were incubated for 24 h at 37 °C in a CO_2 incubator.

Cytofluorimetric Analysis of Infected Cells

Three days after infection, transduction efficiency and cell viability of infected cells and control cells were analyzed by flow cytometry. Cells were washed in phosphate-buffered saline (PBS; Applied Biosystems Foster City, CA, USA) and resuspended in PBS with 5% FCS and 1 μg/ml Propidium iodide (PI; Sigma, ST. Luis, MO, USA). Infected cells were detected by means of GFP positivity and the percentage of dead cells was determined using PI. Data were evaluated with WinMDI 2.9 software.

RNA Isolation and Real-Time Quantitative PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five hundred nanograms of total RNA extracted from the cell lines KiJK and Karpas 299 were transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and a mix of Oligo(dT) primers (Promega, Madison, WI, USA) and random hexamers (Roche Applied Science, Penzberg, Germany) in a final volume of 20 μl. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using a LightCycler 480 System for detection

(Roche). For the quantification of C/EBPβ, we used the C/EBPβ gene expression assay from Applied Biosystems (C/EBPβ: Hs00270923_s1; Foster City, CA, USA). TATA box-binding protein was used as housekeeping gene as previously described[25]. PCR was carried out using 4 μl cDNA with the LightCycler 480 Probes Master (Roche) and uracil-DNA glycosylase (Roche) in a 20 μl final reaction mixture. After initial incubation at 40 °C for 10 min and 95 °C for 10 min, samples were amplified for 55 cycles at 95 °C for 15 s, followed by 1 min at 60 °C. Data were analyzed using the ΔC_t method as described elsewhere[4]. The average threshold cycle (C_t) was determined from duplicates.

Isolation of CD3$^+$ T Cells from Human Blood

Peripheral blood mononuclear cells were obtained from human whole-blood samples using density gradient centrifugation with FicoLite-H separation medium (Linaris, Wertheim-Bettingen, Germany) according to the Miltenyi Biotec protocol. The isolation of CD3$^+$ cells was carried out by positive selection with CD3 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) followed by magnetic separation with a VarioMACS separator according to the manufacturer's instructions.

Human Cell Surface Marker Screening Panel

For generation of surface marker expression profiles of the cell lines KiJK and Karpas 299, cells were analyzed for the expression of 242 surface antigens using the BD Lyoplate Human Cell Surface Marker Sceening Panel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

Cells were cultivated for 2 weeks to allow full development of phenotypical changes induced from C/EBPβ knockdown. To investigate the surface marker expression, transduced cells were incubated with the reconstituted primary antibodies and the Alexa Fluor 647-conjugated goat anti-mouse IgG and goat anti-rat IgG secondary antibodies (Invitrogen) according to the BD Lyoplate protocol. An amount of 1.55×10^5 (Karpas 299) and 3×10^5 (KiJK) cells were used per sample. All samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences). Interpretation of the data was carried out with WinMDI 2.9 to obtain the geometric mean value (gmv) of each of the tested 242 surface antigens. According to the gmv, surface marker expression was defined as follows: No expression gmv: 1–5, weak expression gmv: 5–29, moderate expression gmv: 30–49, and strong expression gmv: ≥ 50.

Western Blot Analysis

Cell lysis and immunodetection were performed as described[4] and separation was done using a 4–12% SDS-acrylamide gradient gel (Criterion XT 4–12% bis-tris gel; Bio-Rad, Hercules, CA, USA). Protein extracts of CD3$^+$ cells were further concentrated using Amicon Ultra-2 10 K filter membranes (Millipore, Schwalbach/Ts., Germany) according to the manufacturer's instructions. Immunoreagents used for western blot were the mouse monoclonal CD147 antibody HIM6 (1:100; BD Bioscience) and the mouse monoclonal anti-α-Tubulin (1:5000; Sigma) as loading control.

RESULTS

Successful Knockdown of C/EBPβ

Two ALK+ ALCL cell lines, KiJK and Karpas 299, were transduced with C/EBPβ shRNA or the empty vector. Flow cytometric analysis for GFP showed that C/EBPβ shRNA was effectively transduced into the cell lines with a high infection rate of almost 100% (Supplementary Figure 1a). After 3 days, C/EBPβ was successfully downregulated as demonstrated by qPCR (Supplementary Figure 1b). Downregulation was maintained over a period of 2 weeks as tested in preliminary experiments (data not shown).

Expression Profiles of KiJK and Karpas 299

To analyze the influence of C/EBPβ on the phenotype of the KiJK and Karpas 299 cells, surface antigen expression was investigated using the Human Cell Surface Marker Screening Panel. Empty virus (pF-S) infected KiJK and Karpas 299 cells showed strong-to-moderate expression of CD25, CD30, CD43, CD45, CD46, CD47, CD98, CD146, CD147, and CD162 (see Supplementary Tables S1A and S2).

For several antigens, differential expression in the two cell lines was observed (see Supplementary Table S1B). CD227 and CD274 were detected at higher levels in KiJK cells. Several antigens could only be detected in Karpas 299 (CD4, CD18, CD41b, and CMRF-56) or in KiJK cells (CDw93 and CD166), respectively. Both Karpas 299 and KiJK showed a very strong expression of human leukocyte antigens (HLA) of class I and II but with a markedly stronger expression of both classes in Karpas 299 cells.

Neither cell line showed expression of the T-cell-specific $\alpha\beta$- and $\gamma\delta$-TCRs, nor of the TCR-associated CD3 antigen. Expression of other T-cell-specific antigens, including CD2, CD4, CD5, CD7, and CD8, was absent from KiJK cells, whereas Karpas 299 cells only showed a weak expression of CD4, CD4v4, and CD5 (see Supplementary Table S1C). Of note, the monocytic surface antigens CD13, CD15, and CD33 were not detected in any of the cell lines (see Supplementary Table S1D). A weak expression of CD63 (LAMP-3/MLA1) was demonstrated in both cell lines and of CD123 (IL3R) and CD15s (sialyl Lewis X) in KiJK cells.

The Activation Markers CD25, CD30, CD98, CD147, CD227, and HLA Class I are Regulated by C/EBPβ

To identify surface antigens, which are potentially regulated by C/EBPβ, we compared the geometric mean values of the expressed antigens with the corresponding values in the C/EBPβ shRNA-infected cells (pF-C/EBPβ). CD25 (IL-2-RA), CD30 (Ki-1), CD98 (CD98HC and LAT), CD147 (EMMPRIN), CD227 (MUC1/EMA), CD274 (programmed death ligand 1), CD305 (inhibitory leukocyte-associated Ig-like receptor, LAIR-1), and HLA class I antigens were downregulated after C/EBPβ knockdown indicating regulation

Table 1 Surface antigens expressed in empty virus (pF-S) and C/EBPβ shRNA (pF-C/EBPβ) infected KiJK and Karpas 299 cells

	Geometric mean values (ratio)					
Antigen(s)	KiJK pF-S	KiJK pF-C/EBPβ		Karpas 299 pF-S	Karpas 299 pF-C/EBPβ	
CD25 (IL2RA)	51	46	(0.9)	126	71	(0.56)
CD30 (TNFRSF8)	235	188	(0.8)	227	113	(0.5)
CD98 (4F2HC, 4F2LC)	216	166	(0.77)	209	149	(0.71)
CD147 (EMMPRIN)	220	169	(0.77)	239	178	(0.74)
CD227 (MUC1/EMA)	227	122	(0.54)	34	25	(0.74)
CD274 (PDCD1LG1)	50.9	44	(0.86)	25.39	14.48	(0.57)
HLA-A, -B, -C	223	196	(0.88)	402	306	(0.76)

Geometric mean values detected by flow cytometry represent the expression of the respective antigen. The corresponding ratio is given in brackets.

through C/EBPβ (Table 1). The strongest effect was seen for CD25 (ratio pF-C/EBPβ/pF-S: 0.56) and CD30 (0.5) in Karpas 299, and CD227 (0.54) in KiJK cells. Downregulation of CD25 after C/EBPβ knockdown was also shown on mRNA level in both cell lines (data not shown). Moderate downregulation was detected for CD98, CD147, CD227, and HLA-A, -B, -C in Karpas 299 (CD98: 0.71; CD147: 0.74; CD227: 0.74; HLA-A, -B, -C: 0.76) and for CD98, CD147, CD30, CD25, and HLA-A, -B, -C in KiJK cells (CD98: 0.77; CD147: 0.77; CD30: 0.8; CD25: 0.9; HLA-A, -B, -C: 0.86), respectively.

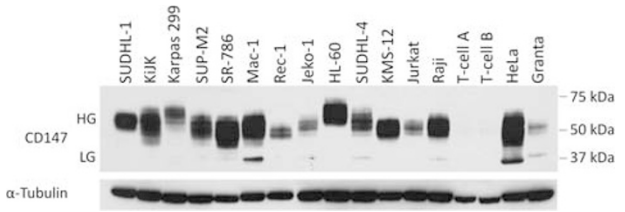

Figure 1 CD147 expression in different lymphoma and leukemia cell lines and in CD3$^+$ T cells. Western blot analysis of CD147 expression in SUDHL-1, KiJK, Karpas 299, SUP-M2, SR786, Mac-1, Rec-1, Jeko-1, HL-60, SUDHL-4, KMS-12, Jurkat, Raji, and Granta cells as well as in two different CD3$^+$ T-cell samples (A and B). The epithelial cell line HeLa was selected as positive control for CD147 expression. Each lane contains 30 μg of protein extract. Alpha-tubulin was used as a loading control. HG, high glycosylated CD147 form (~40–70 kDa); LG, low glycosylated CD147 form (~35 kDa).

CD147/Extracellular Matrix Metalloproteinase Inducer is Strongly Expressed in Lymphoma/Leukemia Cell Lines but Not in Normal CD3+ T Cells

CD147 (EMMPRIN) expression was studied further because of its strong expression and regulation by C/EBPβ in ALK+ ALCL cell lines and its possible tumor biological significance. Therefore, CD147 expression was analyzed by western blot in several lymphoma and leukemia cell lines, as well as in normal CD3$^+$ T cells (Figure 1). Strong expression of the high glycosylated (HG) active form was shown in ALK+ ALCL cell lines (SUDHL-1, KiJK, Karpas 299, SUP-M2, and SR786), in the ALK− ALCL cell line Mac-1 and in the B-cell lymphoma cell lines (HL-60, SUDHL-4, KMS-12, and Raji). Weaker expression of the HG-form was detected in the mantle cell lymphoma (Rec-1, Jeko-1, and Granta), and the T-cell acute lymphoblastic lymphoma cell lines (Jurkat). The low glycosylated (LG) inactive form was strongly expressed in Mac-1 and HeLa, and weakly expressed in SUDHL-4, Raji, and Granta. In normal CD3$^+$ T-cell samples, no expression of CD147 was detected. The expression of CD147 was investigated in normal tonsils and lymph nodes. In both tissues, CD147 was weakly to moderately positive in the germinal center B cells but negative in the B cells of the mantle zone. Normal T cells were CD147 negative, corroborating the western blot findings (Figure 2a and b).

CD147 and PD-L1 Expression in ALK+ and ALK− ALCL Primary Cases

All 115 ALCL cases were analyzed for CD147. ALK+ ALCL cases were homogeneously CD147 positive with rather strong (Figure 2c) or moderate (Figure 2d) membranous staining in the tumor cells (86 cases). Only 11 cases revealed a weak expression (Figure 2e). In contrast, ALK− ALCL showed predominantly a weak expression (13 cases) with five cases being strongly positive comparable to the ALK+ ALCL cases (89 *vs* 28%, $P<0.0001$) (Figure 3). The ALK+ ALCL cases were also CD30 and C/EBPβ positive (Figure 4, upper panel). In contrast, ALK− cases were CD30 positive but C/EBPβ negative (Figure 4, lower panel). Twenty-two ALCL cases (10 ALK+ and 12 ALK−) were also stained for PD-L1. The 10 ALK+ cases showed a strong homogeneous membranous PD-L1 expression, whereas 10 of 12 ALK− ALCL showed only a weak, heterogeneous expression (Figure 4). Two cases showed strong expression comparable to the ALK+ cases.

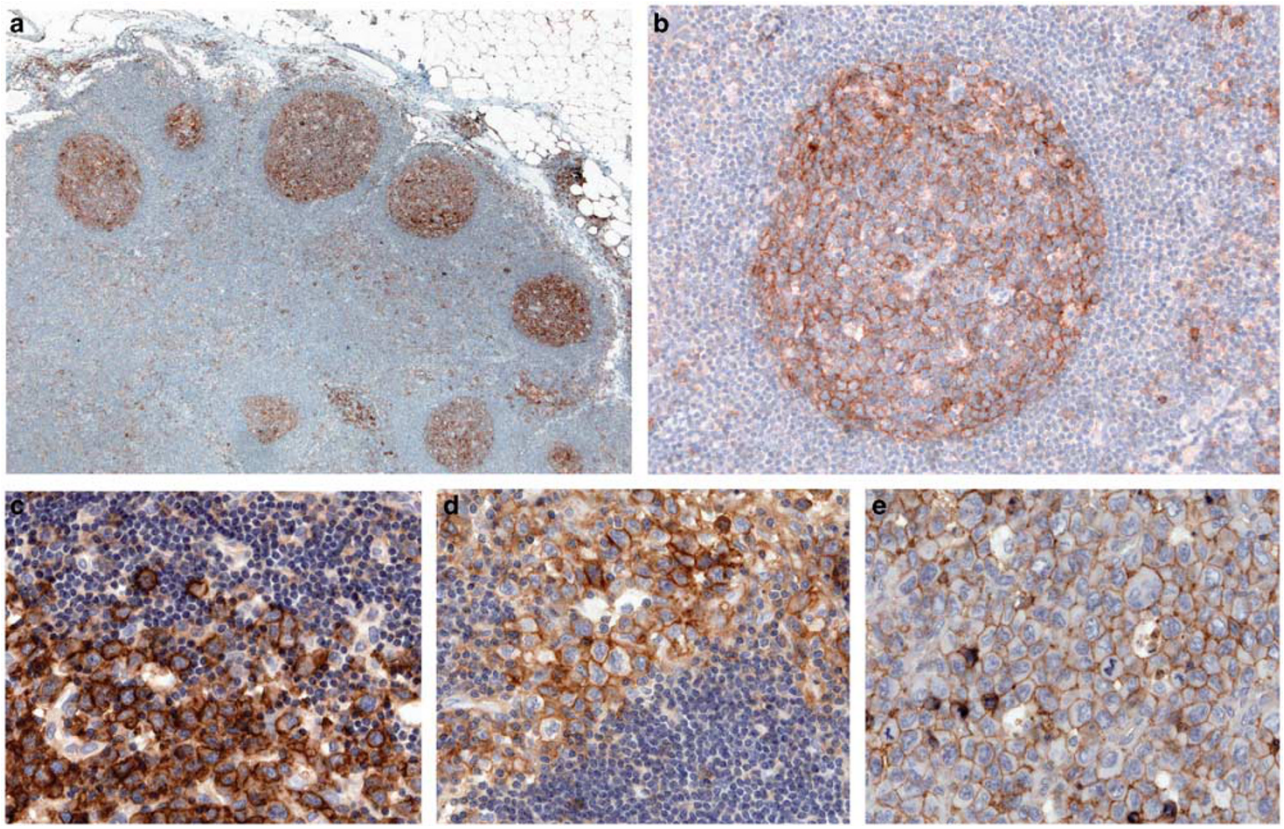

Figure 2 CD147 expression in normal lymph node and in primary ALK+ ALCL cases. (**a**) CD147 expression in normal lymph node is positive in germinal center B cells. The B cells in the mantle zone and the normal T cells in the interfollicular area are negative (×50). (**b**) Higher magnification to show the membraneous staining in the B cells (×200). (**c**) ALK+ ALCL case with strong (+++) expression of CD147 (×400). (**d**) ALK+ ALCL case with moderate (++) expression of CD147 (×400). **(e)** ALK+ ALCL case with weak (+) expression of CD147 comparable to the expression of the B cells in the germinal centers.

DISCUSSION

In this study, we analyzed the surface marker expression profiles of two ALK+ ALCL cell lines with and without C/EBPβ knockdown to investigate the role of this transcription factor in the expression of lineage-specific and activation markers in ALK+ ALCL. Knockdown of C/EBPβ revealed regulation of activation markers but did not influence the expression of lineage-specific surface markers, in the sense of re-expression of T-cell markers or downregulation of monocytic markers. Of the C/EBPβ-regulated antigens, CD147 (EMMPRIN) was further investigated due to its novelty and key role in T-cell activation, proliferation, migration, invasion, and adhesion, and was found to be strongly expressed in ALK+ ALCL primary tumors.

Analysis of the expression of 242 surface antigens with the Human Cell Surface Marker Screening Panel revealed the lack of most T-cell lineage markers in the two ALK+ ALCL cell lines KiJK and Karpas 299, but also showed generally weak or absent expression of monocytic markers, including those which previously had been shown in primary ALCL cases.[20,21] After knockdown of C/EBPβ, neither re-expression of T-cell-specific antigens nor downregulation of monocytic markers could be observed. Altogether, this indicates that C/EBPβ expression alone is not responsible for the unusual phenotype of ALK+ ALCL and that other factors are responsible for the loss of T-cell surface antigens.

However, C/EBPβ knockdown did have an effect on the surface antigen profile, resulting in downregulation of CD25, CD30, CD98, CD147, CD227, CD274, CD305, and MHC class I in both ALCL cell lines. Interestingly, all of these antigens, except for MHC class I, are considered activation markers, and some could play a role in suppression of the anti-tumor T-cell response (CD274 and CD305).[26–31] CD25, CD30, CD98, and CD147 are upregulated in T cells as well as in macrophages upon activation.[26,27,29,32,33] CD227 (MUC1/EMA), characteristically strongly expressed in ALCL, is also upregulated upon T-cell stimulation and affects TCR-mediated downstream signaling events.[28,34] The mechanisms for the observed downregulation of activation markers following C/EBPβ-knockdown in ALCL remain to be identified. Nevertheless, C/EBPβ is an important regulator of cytokine production in macrophages, and ALCL has been shown to produce a variety of pro-inflammatory cytokines.[35,36] This could indicate that knockdown of C/EBPβ results in reduced secretion of pro-inflammatory

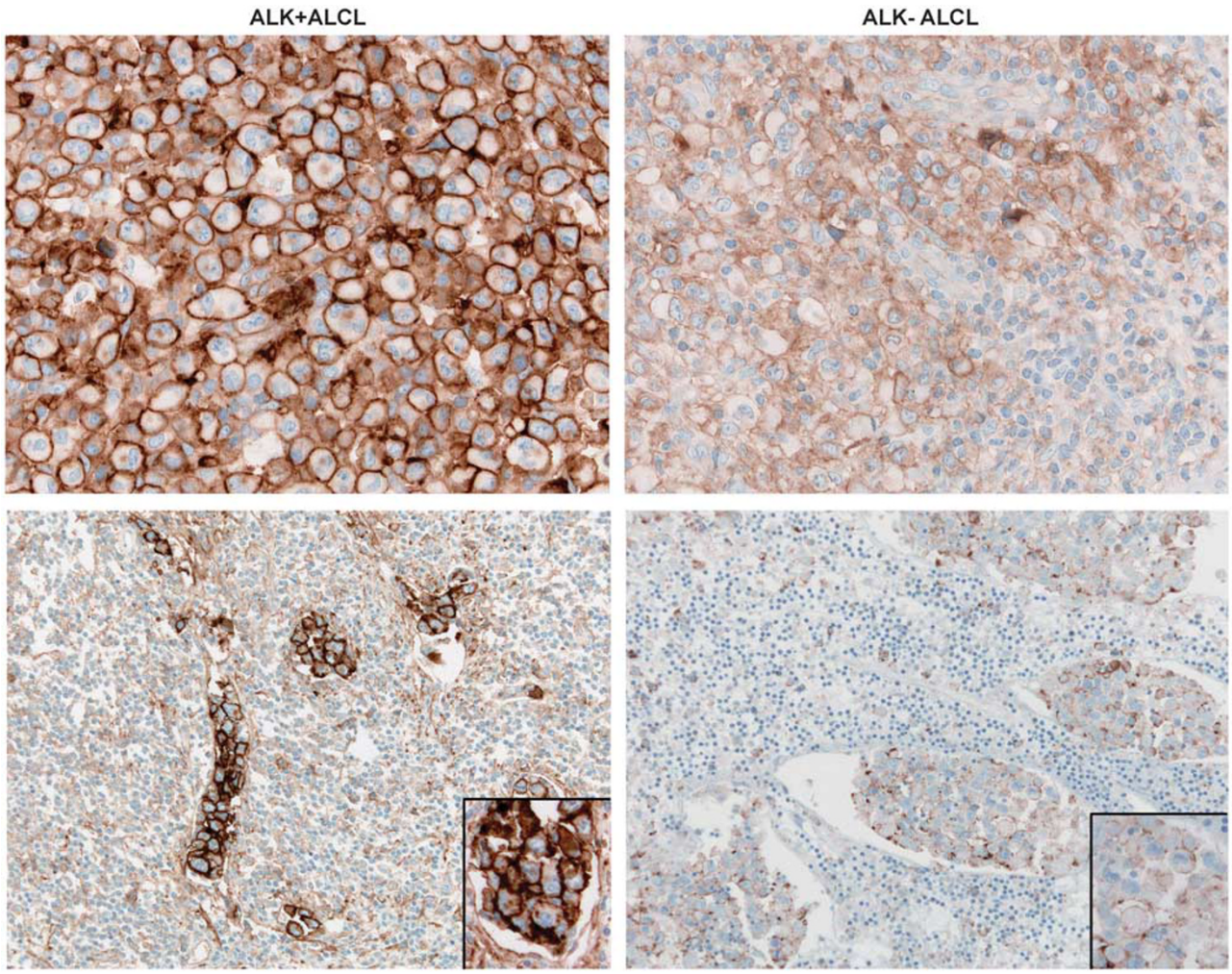

Figure 3 CD147 expression in primary ALK+ and ALK− ALCL cases. ALK+ ALCL cases (left) show a strong CD147 expression both in cases with diffuse infiltration as in cases with sinusoidal infiltration. In contrast, ALK− ALCL cases (right) show a weaker expression of CD147 (upper panel ×400, lower panel ×200, inset ×400).

cytokines, which in turn leads to reduced autocrine stimulation and downregulation of activation markers.

An interesting candidate antigen expressed by ALK+ ALCL and regulated by C/EBPβ is CD147 or extracellular matrix metalloproteinase inducer (EMMPRIN). Although CD147 is upregulated in T cells upon activation, its expression was rather strong in ALK+ ALCL cases when compared with ALK− ALCL cases. CD147 is a transmembrane glycoprotein of the immunoglobulin superfamily that can be heavily glycosylated. Whereas unglycosylated CD147 has a molecular weight of 27 kDa, there is a mature highly glycosylated form (43–66 kDa), which is considered the biologically functional form (HG), and an immature low glycosylated (32 kDa) form (LG).[37] CD147 is expressed in a wide variety of cell types including hematopoietic (leukocytes, erythrocytes, and platelets), epithelial, endothelial, and tumor cells. Recent studies have shown that CD147 has multiple roles in T cells, including development, activation, proliferation, migration, adhesion, and invasion, which are also important steps for the pathogenesis of various diseases.[38] Interestingly, CD147 has been proposed to be a marker of activated T-regulatory cells (aT_{regs}), which in addition express FoxP3 and high levels of CD25.[39] In fact, CD147 or EMMPRIN is expressed in a variety of solid tumors,[40] and has been previously detected in ALCL by IHC.[41] In this study, we now show that CD147 is strongly expressed in ALK+ ALCL primary cases and that there is a difference in the expression of CD147 between ALK+ *vs* ALK− ALCL cases, further supporting the role of C/EBPβ in the induction of CD147 expression.

One of the main functions of CD147 is the stimulation of matrix metalloproteinase (MMP) production in stromal cells.[42–44] MMPs catalyze the degradation of extracellular matrix proteins, which promotes cancer cell invasion and metastasis by destroying basement membranes that serve as natural barriers for tumor spread.[45] Induction of MMP can be considered an important oncogenic aspect of EMMPRIN

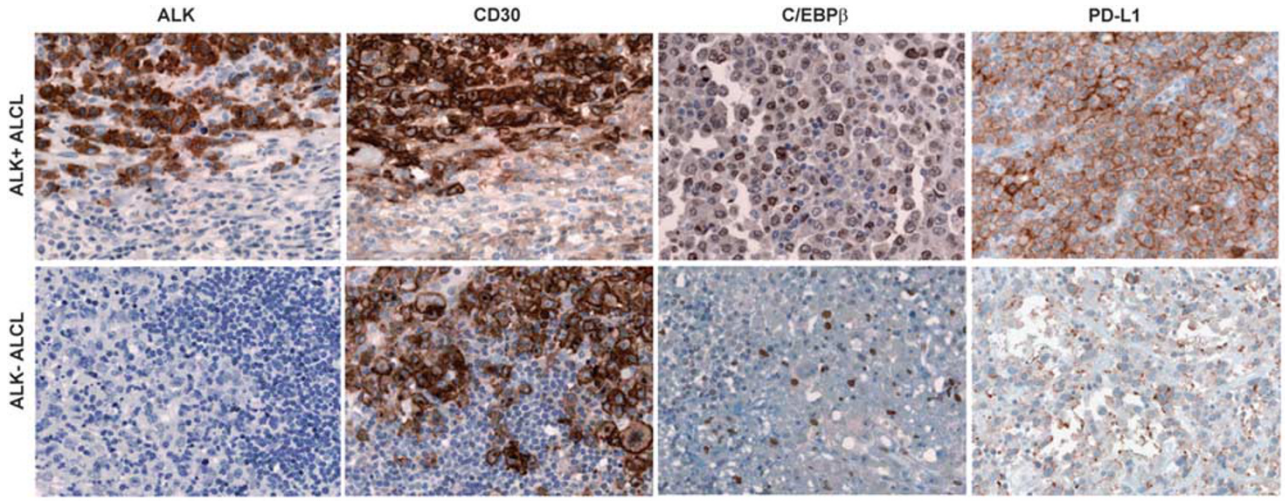

Figure 4 Immunophenotype of primary ALK+ and ALK− ALCL cases. ALK, CD30, C/EBPβ, and PD-L1 immunostaining in ALCL. The ALK+ ALCL primary case (upper panel) shows nuclear and cytoplasmic ALK expression, strong nuclear positivity for C/EBPβ and membranous expression for PD-L1. The ALK− case (lower panel) is negative for ALK and for C/EBPβ, with reactive histiocytes serving as internal positive controls for C/EBPβ staining. Only heterogeneous and weak surface expression of PD-L1 is detected (original magnification ×400).

function. Another interesting function of CD147 is favoring T-cell adhesion that might explain in part the characteristic cohesive morphology of ALCL.

A further aspect of EMMPRIN function of special relevance for ALK+ ALCL is the recent observation that the calcium-binding protein S100A9 is a specific ligand of EMMPRIN and enhances MMP production, migration, and invasiveness of melanoma cells dependent on EMMPRIN binding.[46] S100A9 is overexpressed in a variety of human neoplasms and has been associated with promoting tumor cell invasion and migration.[47] In ALCL, we recently observed that S100A9 is overexpressed both on RNA and protein level, and its expression is directly regulated by C/EBPβ.[5] This could indicate that C/EBPβ plays a central role for ALK+ ALCL progression by promoting both S100A9 expression and signaling through CD147/EMMPRIN.

Another surface molecule with potential relevance for ALCL pathogenesis modulated in its expression by C/EBPβ knockdown, and with a differential expression in ALK+ and ALK− ALCL is programmed death ligand 1 (PD-L1; CD274), which has recently gained prominence as therapeutic target in a variety of human neoplasms. Of interest, expression of CD274 has previously been reported in ALCL cell lines, in contrast to the majority of B-NHL and B-cell lymphoma cell lines.[30,48] In addition, it was shown in ALK+ ALCL that expression of PD-L1 depends on NPM-ALK expression and enzymatic activity.[49] In accordance with these findings, we could demonstrate that primary ALK+ ALCL cases strongly express PD-L1, whereas ALK− ALCL cases show a generally weak expression by IHC. This finding indicates that PD-L1 expression in ALCL cells can mediate suppression of anti-tumor response. Accordingly, in co-cultures of irradiated ALCL cells and allogeneic T cells, T-cell proliferation, and IFN-γ secretion were markedly increased after addition of blocking antibody against CD274.[30]

In conclusion, our study demonstrates that C/EBPβ contributes to the activated phenotype of ALK+ ALCL cells and may be involved in suppressing anti-tumor response through induction of PD-L1 (CD274). The differential expression of EMMPRIN (CD147) in ALK+ ALCL could contribute to the oncogenic role of C/EBPβ by promoting invasiveness through induction of MMP production, possibly in conjunction with the C/EBPβ target gene S100A9. Further functional studies are required to further elucidate the precise role of EMMPRIN (CD147) in ALK+ ALCL.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGMENTS

This study was supported in part by the Deutsche Forschungsgemeinschaft (SFB 685, TP8) to LQM and FF. WK was supported by the Kinderkrebsinitiative Buchholz, Holm-Seppensen (KKI). IAMM was supported by the MSCA ITN ALKATRAS in the EU Horizon 2020 framework program.

AUTHOR CONTRIBUTIONS

LQ-M and FF designed the study, supervised the experimental work, analyzed data, and wrote the manuscript. JaS performed the experimental work and helped writing the manuscript. JuS and IAM-M helped performing the experimental work. IB analyzed data and supervised the experimental work. WK and CO-H provided primary ALCL cases for analysis.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Morris SW, Kirstein MN, Valentine MB, *et al.* Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994;263:1281–1284.
2. Kinney MC, Higgins RA, Medina EA. Anaplastic large cell lymphoma: twenty-five years of discovery. Arch Pathol Lab Med 2011;135:19–43.

3. Anastasov N, Bonzheim I, Rudelius M, *et al.* C/EBPbeta expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway. Haematologica 2010;95:760–767.
4. Quintanilla-Martinez L, Pittaluga S, Miething C, *et al.* NPM-ALK-dependent expression of the transcription factor CCAAT/enhancer binding protein beta in ALK-positive anaplastic large cell lymphoma. Blood 2006;108:2029–2036.
5. Bonzheim I, Irmler M, Klier-Richter M, *et al.* Identification of C/EBPbeta target genes in ALK+ anaplastic large cell lymphoma (ALCL) by gene expression profiling and chromatin immunoprecipitation. PLoS ONE 2013;8:e64544.
6. Steinhilber J, Bonin M, Walter M, *et al.* Next-generation sequencing identifies deregulation of microRNAs involved in both innate and adaptive immune response in ALK+ ALCL. PLoS ONE 2015; 10:e0117780.
7. Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. J Biol Chem 1998;273:30057–30060.
8. Scott LM, Civin CI, Rorth P, *et al.* A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood 1992;80:1725–1735.
9. Wang ND, Finegold MJ, Bradley A, *et al.* Impaired energy homeostasis in C/EBP alpha knockout mice. Science 1995;269:1108–1112.
10. Cardinaux JR, Allaman I, Magistretti PJ. Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. Glia 2000;29:91–97.
11. Poli V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem 1998;273:29279–29282.
12. Greenbaum LE, Li W, Cressman DE, *et al.* CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 1998;102:996–1007.
13. Natsuka S, Akira S, Nishio Y, *et al.* Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood 1992;79:460–466.
14. Tanaka T, Akira S, Yoshida K, *et al.* Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell 1995;80:353–361.
15. Laiosa CV, Stadtfeld M, Xie H, *et al.* Reprogramming of committed T cell progenitors to macrophages and dendritic cells by C/EBP alpha and PU.1 transcription factors. Immunity 2006;25:731–744.
16. Xie H, Ye M, Feng R, *et al.* Stepwise reprogramming of B cells into macrophages. Cell 2004;117:663–676.
17. Foss HD, Anagnostopoulos I, Araujo I, *et al.* Anaplastic large-cell lymphomas of T-cell and null-cell phenotype express cytotoxic molecules. Blood 1996;88:4005–4011.
18. Stein H, Mason DY, Gerdes J, *et al.* The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 1985;66:848–858.
19. Bonzheim I, Geissinger E, Roth S, *et al.* Anaplastic large cell lymphomas lack the expression of T-cell receptor molecules or molecules of proximal T-cell receptor signaling. Blood 2004;104:3358–3360.
20. Carbone A, Gloghini A, Volpe R, *et al.* KP1 (CD68)-positive large cell lymphomas: a histopathologic and immunophenotypic characterization of 12 cases. Hum Pathol 1993;24:886–896.
21. Juco J, Holden JT, Mann KP, *et al.* Immunophenotypic analysis of anaplastic large cell lymphoma by flow cytometry. Am J Clin Pathol 2003;119:205–212.
22. Bovio IM, Allan RW. The expression of myeloid antigens CD13 and/or CD33 is a marker of ALK+ anaplastic large cell lymphomas. Am J Clin Pathol 2008;130:628–634.
23. Swerdlow S, Campo E, Harris N. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press: Lyon, 2008.
24. Anastasov N, Klier M, Koch I, *et al.* Efficient shRNA delivery into B and T lymphoma cells using lentiviral vector-mediated transfer. J Hematop 2009;2:9–19.
25. Specht K, Kremer M, Muller U, *et al.* Identification of cyclin D1 mRNA overexpression in B-cell neoplasias by real-time reverse transcription-PCR of microdissected paraffin sections. Clin Cancer Res 2002;8: 2902–2911.
26. Chadburn A, Inghirami G, Knowles DM. The kinetics and temporal expression of T-cell activation-associated antigens CD15 (LeuM1), CD30 (Ki-1), EMA, and CD11c (LeuM5) by benign activated T cells. Hematol Pathol 1992;6:193–202.
27. Komada H, Imai A, Hattori E, *et al.* Possible activation of murine T lymphocyte through CD98 is independent of interleukin 2/interleukin 2 receptor system. Biomed Res 2006;27:61–67.
28. Mukherjee P, Tinder TL, Basu GD, *et al.* MUC1 (CD227) interacts with lck tyrosine kinase in Jurkat lymphoma cells and normal T cells. J Leukoc Biol 2005;77:90–99.
29. Uchiyama T, Broder S, Waldmann TA. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. J Immunol 1981;126:1393–1397.
30. Andorsky DJ, Yamada RE, Said J, *et al.* Programmed death ligand 1 is expressed by non-hodgkin lymphomas and inhibits the activity of tumor-associated T cells. Clin Cancer Res 2011;17:4232–4244.
31. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2008;83:799–803.
32. Andreesen R, Brugger W, Lohr GW, *et al.* Human macrophages can express the Hodgkin's cell-associated antigen Ki-1 (CD30). Am J Pathol 1989;134:187–192.
33. Scheibenbogen C, Keilholz U, Richter M, *et al.* The interleukin-2 receptor in human monocytes and macrophages: regulation of expression and release of the alpha and beta chains (p55 and p75). Res Immunol 1992;143:33–37.
34. Agrawal B, Krantz MJ, Parker J, *et al.* Expression of MUC1 mucin on activated human T cells: implications for a role of MUC1 in normal immune regulation. Cancer Res 1998;58:4079–4081.
35. Hsu SM, Waldron J, Xie SS, *et al.* Hodgkin's disease and anaplastic large cell lymphoma revisited. 1. Unique cytokine and cytokine receptor profile distinguished from that of non-hodgkin's lymphomas. J Biomed Sci 1995;2:302–313.
36. Siebert S, Amos N, Williams BD, *et al.* Cytokine production by hepatic anaplastic large-cell lymphoma presenting as a rheumatic syndrome. Semin Arthritis Rheum 2007;37:63–67.
37. Bai Y, Huang W, Ma LT, *et al.* Importance of N-glycosylation on CD147 for its biological functions. Int J Mol Sci 2014;15:6356–6377.
38. Hahn JN, Kaushik DK, Yong VW. The role of EMMPRIN in T cell biology and immunological diseases. J Leukoc Biol 2015;98:33–48.
39. Landskron J, Tasken K. CD147 in regulatory T cells. Cell Immunol 2013;282:17–20.
40. Nabeshima K, Iwasaki H, Koga K, *et al.* Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Int 2006;56:359–367.
41. Thorns C, Feller AC, Merz H. EMMPRIN (CD 147) is expressed in Hodgkin's lymphoma and anaplastic large cell lymphoma. An immunohistochemical study of 60 cases. Anticancer Res 2002;22:1983–1986.
42. Guo H, Zucker S, Gordon MK, *et al.* Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem 1997;272:24–27.
43. Kataoka H, DeCastro R, Zucker S, *et al.* Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase. Cancer Res 1993;53:3154–3158.
44. Sameshima T, Nabeshima K, Toole BP, *et al.* Glioma cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts. Cancer Lett 2000;157:177–184.
45. Liotta LA, Tryggvason K, Garbisa S, *et al.* Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980;284:67–68.
46. Hibino T, Sakaguchi M, Miyamoto S, *et al.* S100A9 is a novel ligand of EMMPRIN that promotes melanoma metastasis. Cancer Res 2013;73: 172–183.
47. Srikrishna G. S100A8 and S100A9: new insights into their roles in malignancy. J Innate Immun 2012;4:31–40.
48. Yamamoto R, Nishikori M, Tashima M, *et al.* B7-H1 expression is regulated by MEK/ERK signaling pathway in anaplastic large cell lymphoma and Hodgkin lymphoma. Cancer Sci 2009;100:2093–2100.
49. Marzec M, Zhang Q, Goradia A, *et al.* Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). Proc Natl Acad Sci USA 2008;105:20852–20857.