雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ota Y, Hishima T, Mochizuki M, Kodama Y, Moritani S, Oyaizu N, Mine S, Ajisawa A, Tanuma J, Uehira T, Hagiwara S, Yajima K, Koizumi Y, Shirasaka T, Kojima Y, Nagai H, Yokomaku Y, Shiozawa Y, Koibuchi T, Iwamoto A, Oka S, Hasegawa H, Okada S, Katano H.	Classification of AIDS-related lymphoma cases between 1987 and 2012 in Japan based on the WHO Classification of Lymphomas, fourth edition.	***Cancer Med***	3(3)	143-153	2014
Katano H, Hishima T, Mochizuki M, Kodama Y, Oyaizu N, Ota Y, Mine S, Igari T, Ajisawa A, Teruya K, Tanuma J, Kikuchi Y, Uehira T, Shirasaka T, Koibuchi T, Iwamoto A, Oka S, Hasegawa H, Okada S, Yasuoka A	The prevalence of opportunistic infections and malignancies in autopsied patients with human immunodeficiency virus infection in Japan.	***BMC Infect Dis***	14	229	2014
Kojima Y, Hagiwara S, Uehira T, Ajisawa A, Kitanaka A, Tanuma J, Okada S, Nagai H.	Clinical outcomes of AIDS-related Burkitt lymphoma: a multi-institution retrospective survey in Japan.	***Jpn J Clin Oncol.***	44(4)	318-23,	2014
Goto H, Kojima Y, Matsuda K, Kariya R, Taura M, Kuwahara K, Nagai H, Katano H, Okada S.	Efficacy of anti-CD47 antibody-mediated phagocytosis with macrophages against primary effusion lymphoma.	***Eur J Cancer.***	50(10)	:1836-46	2014
Goto H, Kudo E, Kariya R, Taura M, Katano H, Okada S	Targeting VEGF and interleukin-6 for controlling malignant effusion of primary effusion lymphoma.	***J Cancer Res Clin Oncol.***	141	465-474	2015
Okada S, Goto H, and Yotsumoto M.	Current status of treatment for primary effusion lymphoma.	***Intractable Rare Dis Res***	3(3)	65-74	2014
Nagai H	Recent advances in Hodgkin lymphoma: interim PET and molecular targeted therapy	***Jpn J Clin Oncol.***		Epub ahead of print]	2014

Morishima S, Nakamura S, Yamamoto K, Miyauchi H, Kagami Y, Kinoshita T, Onoda H, Yatabe Y, Ito M, Miyamura K, Nagai H, Moritani S, Sugiura I, Tsushita K, Mihara H, Ohbayashi K, Iba S, Emi N, Okamoto M, Iwata S, Kimura H, Kuzushima K, Morishima Y.	Increased T-cell responses to Epstein-Barr virus with high viral load in patients with Epstein-Barr virus-positive diffuse large B-cell lymphoma.	***Leuk Lymphoma.***		[Epub ahead of print]	2014
Kojima Y, Ohashi H, Nakamura T, Nakamura H, Yamamoto H, Miyata Y, Iida H, Nagai H.	Acute thrombotic thrombocytopenic purpura after pneumococcal vaccination.	***Blood Coagul Fibrinolysis***	25(5)	512-4,	2014
Yamada M, Katano H, Yotsumoto M, Hashimoto H, Muramatsu T, Shiotsuka M, Fukutake K, Kuroda M	Unique expression pattern of viral proteins in human herpesvirus 8-positive plasmablastic lymphoma: a case report	***Int J Clin Exp Pathol***	7	6415-6418	2014
Ogawa Y, Watanabe D, Hirota K, Ikuma M, Yajima K, Kasai D, Mori K, Ota Y, Nishida Y, Uehira T, Mano M, Yamane T, Shirasaka T.	Rapid multiorgan failure due to large B-cell lymphoma arising in human Herpesvirus-8-associated Multicentric Castleman's Disease in a patient with human immunodeficiency virus infection	***Intern Med.***	53(24)	2805-9	2014
Yajima K, Uehira T, Otera H, Koizumi Y, Watanabe D, Kodama Y, Kuzushita N, Nishida Y, Mita E, Mano M, Shirasaka T.	A case of non-cirrhotic portal hypertension associated with anti-retroviral therapy in a Japanese patient with human immunodeficiency virus infection	***J Infect Chemother***	20(9)	582-5	2014
Ohnishi K, Sakamoto N, Kobayashi K, Iwabuchi S, Nakamura-Uchiyama F, Ajisawa A, Yamauchi Y, Takeshita N, Yamamoto Y, Tsunoda T, Yoshimura Y, Tachikawa N, Uehira T.	Subjective adverse reactions to metronidazole in patients with amebiasis	***Parasitol Int.***	63(5)	698-700	2014
Okasaki M1, Kubota K, Minamimoto R, Miyata Y, Morooka M, Ito K, Ishiwata K, Toyohara J, Inoue T, Hirai R, Hagiwara S, Miwa A.	Comparison of 11C-4'-thiothymidine, 11C-methionine, and 18F-FDG PET/CT for the detection of active lesions of multiple myeloma	***Ann Nucl Med. 2014 Nov 25***		In press	2015

Yotsu RR1, Hagiwara S, Okochi H, Tamaki T.	Case series of patients with chronic foot ulcers treated with autologous platelet-rich plasma	***J Dermatol***		In press	2015
Fujiwara S, Imadome K, and Takei M.	Modeling EBV infection and pathogenesis in new-generation humanized mice.	***Exp Mol Med***	47	e136	2015
Matsuda G, Imadome K-I, Kawano F, Mochizuki M, Ochiai N, Morio T, Shimizu N, and Fujiwara S.	Cellular immunotherapy with ex vivo expanded cord blood T cells in a humanized mouse model of EBV-associated lymphoproliferative disease.	***Immuno-therapy***	in press		2015
Yoshimori M, Imadome KI, Komatsu H, Wang L, Saitoh Y, Yamaoka S, Fukuda T, Kurata M, Koyama T, Shimizu N, Fujiwara S, Miura O, Arai A.	CD137 expression is induced by Epstein-Barr virus infection through LMP1 in T or NK cells and mediates survival promoting signals.	***PLoS ONE***	9 (11)	e112564	2014
Siddiquey MN, Nakagawa H, Iwata S, Kanazawa T, Suzuki M, Imadome KI, Fujiwara S, Goshima F, Murata T, Kimura H.	Anti-tumor effects of suberoylanilide hydroxamic acid on Epstein-Barr virus-associated T- and natural killer-cell lymphoma.	***Cancer Sci***	105 (6)	713-72	2014
Taura M, Kudo E, Kariya R, Goto H, Matsuda K, Hattori S, Vaeteewoottacharn K, McDonald F, Suico MA, Shuto T, Kai H, and Okada S.	COMMD1/Murr1 reinforces HIV-1 latent infection through IκB-α stabilization.	***J Virol***	89(5)	2643-2658,	2015
Gotoh K, Kariya R, Alam MM, Matsuda K, Hattori S, Maeda Y, Motoyama K, Kojima A, Arima H and Okada S.	The antitumor effects of methy-β-cyclodextrin against primary effusion lymphoma via the depletion of cholesterol from lipid rafts.	***Biochem Biophys Res Comm***	455(3-4)	285-289	2014
Gotoh K, Kariya R, Matsuda K, Hattori S, Vaeteewoottacharn K, and Okada S.	A novel EGFP-expressing Nude mice with complete loss of lymphocytes and NK cells to study tumor-host interactions.	***BioSci Trends***	8(4)	202-205	2014
Kariya R, Matsuda K, Gotoh K, Vaeteewoottacharn K, Hattori S, and Okada S.	Establishment of Nude mice with complete loss of lymphocytes and NK cells and Application for in vivo bio-imaging.	***In vivo***	28(5)	779-784	2014
Onodera R, Motoyama K, Tanaka N, Ohyama A, Okamatsu A, Higashi T, Kariya R, Okada S, and Arima H.	Involvement of Autophagy in Antitumor Activity of Folate-appended Methyl-β-cyclodextrin.	***Sci Rep***	4	4417	2014

Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, Mizukami T, Takizawa K, Araki K, Sugamura K, Yamaguchi K, Watanabe T, Hamaguchi I.	Identification of TL-Om1, an ATL Cell Line, as a Reference Material for Human T-Lymphotropic Virus 1 Quantitative PCR.	***J Clin Microbiol***	53(2)	587-596	2015
Matsuda Y, Kobayashi-Ishihara M, Fujikawa D, Ishida T, Watanabe T, Yamagishi M.	Epigenetic Heterogeneity in HIV-1 Latency Establishment.	***Sci Rep***	5	7701	2015
Takahashi R; Yamagishi M, Nakano K, Yamochi T, Yamochi T, Fujikawa D, Nakashima M, Tanaka Y, Uchimaru K, Utsunomiya A, Watanabe T.	Epigenetic deregulation of *Ellis Van Creveld* confers robust Hedgehog signaling in adult T-cell leukemia.	***Cancer Sci***	105(9)	1160-1169	2014
今村顕史、加藤博史、照屋勝治、上平朝子、矢嶋敬史郎、四本美保子、岡田誠治、片野晴隆.	エイズに合併するカポジ肉腫などのHHV-8関連疾患に対する治療の手引き.	日本エイズ学会誌	16(1)	42-51	2014
永井宏和	ホジキンリンパ腫の治療	臨床血液	55(10)	1941-51	2014
永井宏和	ホジキンリンパ腫（血液疾患の分子標的療法）	日本臨床	72(6)	1099-1103	2014
上平朝子、西田恭治.	連載 エイズに見られる感染症と悪性腫瘍(14)進行性多巣性白質脳症	化学療法の領域	30(12)	2152-9	2014
吉岡巖、金宮健翁、木下竜弥、鄭則秀、原田泰規、上平朝子、白阪琢磨、岡聖次.	抗HIV薬Atazanavir内服患者に発生した尿路結石症の検討	泌尿器外科	27(11)	1823-7	2014
笠井大介、廣田和之、伊熊素子、小川吉彦、矢嶋敬史郎、渡邊大、西田恭治、上平朝子、白阪琢磨.	HIV感染症患者に合併した結核に関する検討	日本呼吸器学会誌	4(1)	66-71	2015

European Journal of Cancer (2014) 50, 1836–1846

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Efficacy of anti-CD47 antibody-mediated phagocytosis with macrophages against primary effusion lymphoma

Hiroki Goto [a], Yuki Kojima [b], Kouki Matsuda [a], Ryusho Kariya [a], Manabu Taura [a], Kazuhiko Kuwahara [c], Hirokazu Nagai [b], Harutaka Katano [d], Seiji Okada [a,*]

[a] *Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto, Japan*
[b] *Department of Hematology, National Hospital Organization Nagoya Medical Center, Nagaoya, Japan*
[c] *Department of Immunology, Graduate School of Life Sciences, Kumamoto University, Kumamoto, Japan*
[d] *Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan*

Received 6 November 2013; received in revised form 20 December 2013; accepted 4 March 2014
Available online 9 April 2014

KEYWORDS
AIDS
CD47
Macrophage
Mouse model
Primary effusion lymphoma

Abstract ***Background:*** Recently, the critical role of CD47 on the surface of resistant cancer cells has been proposed in their evasion of immunosurveillance. Primary effusion lymphoma (PEL) is a subtype of aggressive non-Hodgkin lymphoma that shows serous lymphomatous effusion in body cavities, especially in advanced acquired immunodeficiency syndrome (AIDS). PEL is resistant to conventional chemotherapy and has a poor prognosis. In this study, we evaluated the effect of anti-CD47 antibody (Ab) on PEL *in vitro* and *in vivo*.
Methods: Surface CD47 of PEL cell lines was examined by flow cytometry. Efficacy of knocking down CD47 or anti-CD47 Ab-mediated phagocytosis against PEL was evaluated using mouse peritoneal macrophages and human macrophages *in vitro*. Primary PEL cells were injected intraperitoneally into NOD/Rag-2/Jak3 double-deficient (NRJ) mice to establish a direct xenograft mouse model.
Results: Surface CD47 of PEL cell lines was highly expressed. Knocking down CD47 and anti-CD47 Ab promoted phagocytic activities of macrophages in a CD47 expression-dependent manner *in vitro*. Treatment with anti-CD47 Ab inhibited ascite formation and organ invasion completely *in vivo* compared with control IgG-treated mice.
Conclusion: CD47 plays the pivotal role in the immune evasion of PEL cells in body cavities. Therapeutic antibody targeting of CD47 could be an effective therapy for PEL.

1. Introduction

Primary effusion lymphoma (PEL) is a rare subtype of non-Hodgkin lymphoma (NHL) that occurs most com-

* *Corresponding author:* Address: Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan. Tel.: +81 96 373 6522; fax: +81 96 373 6523.
E-mail address: okadas@kumamoto-u.ac.jp (S. Okada).

http://dx.doi.org/10.1016/j.ejca.2014.03.004
0959-8049/

monly in human immunodeficiency virus-1 (HIV-1)-infected patients, and shows malignant effusions in body cavities [1,2]. Kaposi sarcoma-associated herpesvirus (KSHV)/human herpesvirus-8 (HHV-8) contributes to the oncogenesis of PEL [3]. PEL displays a number of constitutively activated signaling pathways in survival and growth [4–6]. PEL develops rapid resistance to conventional chemotherapy and has a poor prognosis [7]. Although rituximab, a chimeric anti-CD20 antibody (Ab), has demonstrated a significant survival advantage for B-cell NHL in combination with standard chemotherapy [8–10], rituximab does not play a significant therapeutic role in PEL because PEL generally lacks CD20 expression [1]. The lack of optimal treatment for PEL demands innovative therapeutic approaches that target its specific molecules.

CD47 is a ubiquitously expressed transmembrane protein with numerous functions [11,12]. As one of several functions, CD47 serves as the ligand for signal regulatory protein alpha (SIRPα), which is expressed on the surface of phagocytic cells, including macrophages and dendritic cells [13,14]. The interaction of CD47 and SIRPα initiates a signal transduction cascade, resulting in inhibition of phagocytosis [15,16]. Various types of resistant tumours appear to upregulate the expression of CD47 as a mechanism of immune evasion [17–22]. Therapeutic antibody targeting of CD47 enabled efficient phagocytosis of leukemic stem cells and various resistant tumours by macrophages; however, the possibility of utilising peritoneal macrophages for anti-CD47 Ab therapy has not been elucidated. Moreover, the efficacy of anti-CD47 Ab against PEL was not investigated.

In the current study, we identified CD47 as a therapeutic target in PEL and evaluated the effect of anti-CD47 Ab-mediated phagocytosis with macrophages on PEL using a direct xenograft mouse model.

2. Materials and methods

2.1. Cell lines

BCBL-1, BC-1, TY-1 and Raji cells were maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5% CO_2. BC-2, BCP-1 and RM-P1 cells were maintained in RPMI 1640 supplemented with 20% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified incubator at 37 °C and 5% CO_2.

2.2. Short interfering RNA (siRNA) and transfection

CD47 siRNA and MISSION siRNA Universal Negative Control SIC-001 were purchased from Sigma-Aldrich (St. Louis, MO, USA). For siRNA transfection, Neon transfection system (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocol. The transfected cells were incubated in a humidified incubator at 37 °C and 5% CO_2 for 48 h, and then CD47 protein expression was determined by flow cytometry. The sequences for the CD47 siRNA were 5′-ACAAG UCCACUGUCCCCACdTdT-3′ (sense) and 5′-GUGG GGACAGUGGACUUGUdTdT-3′ (antisense).

2.3. Antibody preparation

The anti-human CD47 (B6H12.2) hybridoma was obtained from the ATCC (Rockville, MD, USA). Hybridoma cells were cultured under standard conditions and antibodies were purified by Protein G.

2.4. Flow cytometry

Cells were stained with the following Abs: human CD3-fluorescein isothiocyanate (FITC) from Beckman Coulter (Fullerton, CA, USA); human CD14-Alexa Fluor (AF) 647, human CD19-FITC, human CD20-phycoerythrin (PE), human CD45-Pacific blue (PB), mouse CD49b/DX5-allophycocyanine (APC), human CD56-FITC, mouse CD45-AF 700 from BioLegend (San Diego, CA, USA); mouse CD11b/Mac-1-PE from BD Biosciences (San Jose, CA, USA). Anti-CD47 Ab was conjugated with HiLyte Fluor (HF) 647 labelling kit (Dojindo Laboratories, Kumamoto, Japan). After staining, cells were washed twice, resuspended in staining medium (PBS with 3% FBS and 0.05% sodium azide) and immediately analysed on an LSR II flow cytometer (BD Biosciences). Data were analysed with FlowJo software (Tree Star, San Carlos, CA, USA).

2.5. Preparation of mouse and human macrophages, and isolation of human normal B-cells

Mouse peritoneal macrophages were isolated from 10-week-old Balb/c mice that had been injected intraperitoneally with 2 ml of 4.05% (w/v) thioglycollate medium (BD Diagnostic Systems, Sparks, MD, USA) 3 d prior to peritoneal lavage with 10 ml PBS. The collected cells were washed with RPMI 1640 medium. Macrophages were enriched by adherence to plates for 1 h at 37 °C in RPMI 1640 medium with 1% FBS. Then, non-adherent cells were removed by extensive washing with PBS. The percentage of CD11b + CD49b– mouse peritoneal macrophages prepared by this method was more than 93.0%.

Human M-CSF-induced macrophages were prepared, as described previously [23]. The percentage of CD14 + CD56– human macrophages prepared by this method was more than 95.0%.

Human normal B-cells were isolated from peripheral blood mononuclear cells (PBMC) by negative selection using B-cell isolation kit II (Miltenyi Biotec, Bergisch

Gladbach, Germany), as per the manufacturer's instructions. After isolation, the percentage of CD19 + B-cells was more than 95.0%.

Informed consent was obtained for the collection of human peripheral blood from healthy volunteers. Approval for this study was obtained from the Kumamoto University Medical Ethics Committee.

2.6. In vitro phagocytosis assay

Macrophages, 1×10^5, were plated in each well of a 12-well tissue culture plate in RPMI1640 containing 10% FBS. After 24 h, media were replaced with RPMI1640 containing 10% FBS. PEL cells were fluorescently labelled with a carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) according to the manufacturer's protocol. Then, 5×10^5 CFSE-labelled cells were added to the macrophage-containing wells along with siRNA, 10 μg/ml IgG1 isotype (Biolegend) or anti-CD47 Ab, and incubated for 2 h. The cells were washed and harvested from each well using trypsin/EDTA. Cell suspensions were stained with a mouse macrophage Ab anti-mouse CD11b/Mac-1 or anti-human CD14 and analysed on an LSR II flow cytometer. Phagocytosed PEL cells were defined as either CFSE + mouse CD11b + or CFSE + human CD14 + cells when incubated with murine or human macrophages, respectively.

2.7. Tetrazolium dye methylthiotetrazole (MTT) assay

The antiproliferative activities of anti-CD47 Ab against PEL cell lines were measured by the MTT method (Sigma–Aldrich), as published [24].

2.8. Direct xenograft mouse model

NOD Rag-2/Jak3 double-deficient mice (NRJ mice) [25] were housed and monitored in our animal research facility according to institutional guidelines.

A 39-year-old HIV-1-infected Japanese man who developed acute pericardial tamponade was admitted to the hospital. Cytological analysis of pericardial effusion showed PEL. Pericardial effusion collected by pericardiocentesis was separated using Pancoll (PAN Biotech, Aidenbach, Germany). Freshly isolated PEL cells (1×10^7) were injected intraperitoneally into 8- to 12-week-old NRJ mice. Mice developed ascites promptly within 14 d. A portion of the ascites was collected immediately postmortem via paracentesis; these cells are referred to as 'PEL-NRJ' cells. For the xenograft mouse model, 1×10^7 PEL-NRJ cells suspended in 200 μl PBS were injected into the peritoneal cavities of additional NRJ mice without *ex vivo* culture. The mice were then treated with intraperitoneal injections of mouse control IgG (Sigma-Aldrich) or anti-CD47 Ab on day 3 after cell inoculation (100 μg/mouse, every other day). Tumour burden was evaluated by measuring the volume of ascites on day 14. Organ invasions were evaluated by immunohistochemistry and flow cytometry. Investigation was carried out according to the institutional guidelines approved by the ethics committee of the Graduate School of Life Sciences, Kumamoto University and the National Hospital Organization Nagoya Medical Center.

2.9. Polymerase chain reaction (PCR) analysis

Total RNA was isolated from cells using RNAiso plus (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. Semiquantitative PCR was carried out with an RT-PCR kit (Takara Bio) using the recommended protocol. The oligonucleotide primers used in semiquantitative PCR are described in Supplementary Table S1.

2.10. Immunohistochemistry

To investigate the expression of HHV-8 ORF73 (LANA) protein, tissue samples were stained, as published [24]. The number of LANA-positive cells was counted in ten microscopic fields at ×200 and the results averaged.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The statistical significance of the differences observed between experimental groups was determined using Student's *t*-test, and $p < 0.05$ was considered significant.

3. Results

3.1. Expression of CD47 on the surface of PEL cell lines

We first examined PEL cell surface expression of CD47 and compared the expression level between 6 PEL cell lines and PBMC from 6 healthy donors by flow cytometry. As shown in Fig. 1A and B, the expression levels of CD47 on the surface of PEL cell lines were higher or compatible with those of PBMC and CD19 + PBMC.

3.2. Knocking down CD47 increases phagocytosis

To investigate the role of CD47 in macrophage phagocytosis of PEL, we determined the effect of knocking down CD47 on the phagocytosis of BCBL-1 cells *in vitro*. At 48 h post-transfection of CD47 siRNA, expression of CD47 was decreased in a dose-dependent manner (Fig. 2A). We prepared mouse and human mac-

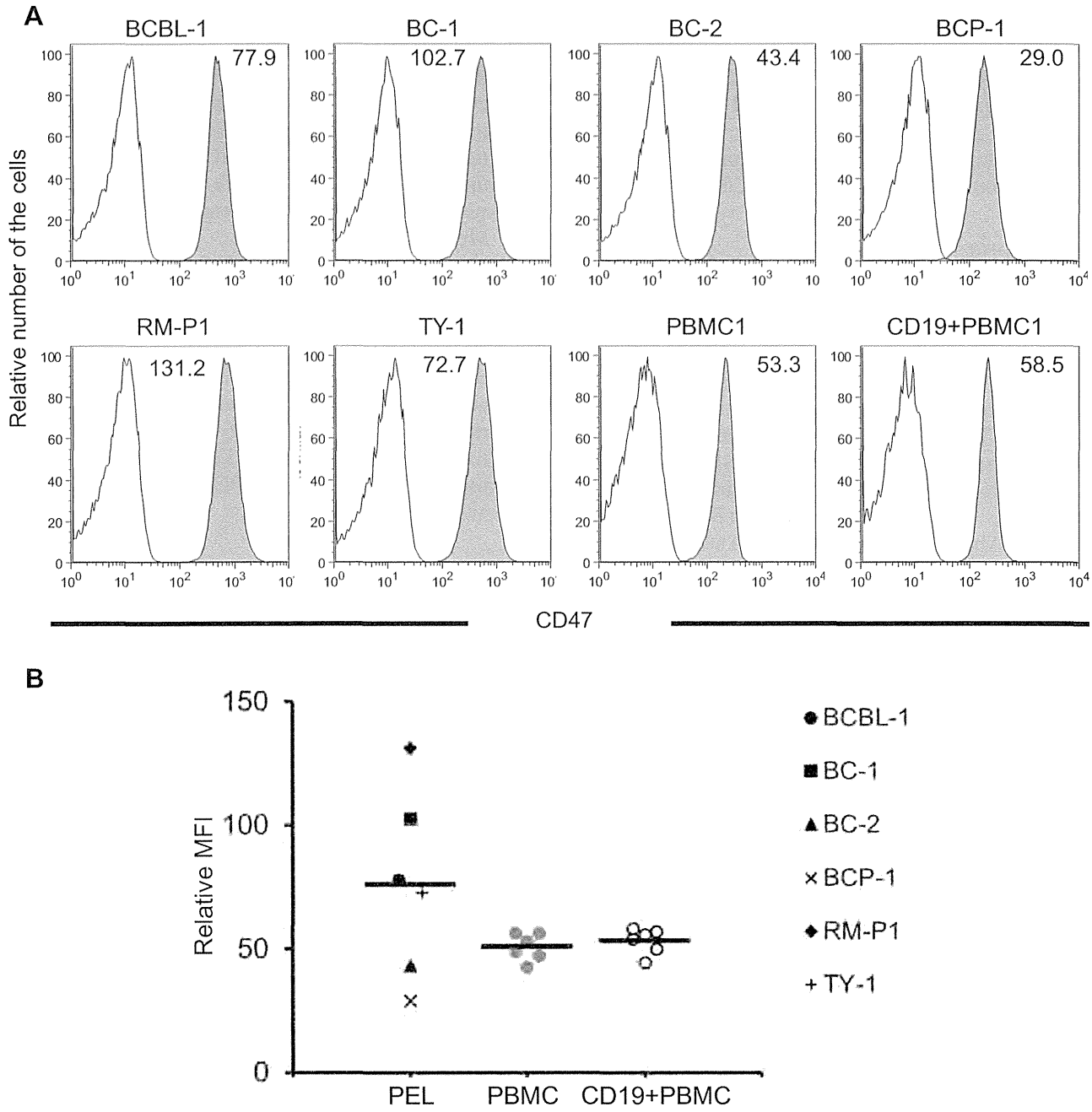

Fig. 1. Primary effusion lymphoma (PEL) cell lines express a high level of cell-surface CD47. (A) Representative plot of surface CD47 expression by flow cytometry. Grey histograms show antibody-stained cells; white histograms are isotype controls. (B) Expression levels of CD47 on the surface of PEL cell lines, peripheral blood mononuclear cells (PBMC) and CD19 gated PBMC. Numbers of CD47 expression indicate the relative mean fluorescence intensity (MFI) by flow cytometry.

rophages (Supplementary Fig. S1), and evaluated the effect of CD47 expression on the macrophage phagocytosis. As shown in Fig. 2B and C, transfection of CD47 siRNA increased the phagocytosis of BCBL-1 in comparison to control siRNA in a dose-dependent manner.

These results show that expression of CD47 in PEL is essential for evasion of macrophage phagocytosis.

3.3. Anti-CD47 Ab enables phagocytosis in vitro

We investigated whether PEL cells could be eliminated by anti-CD47 Ab mediated-macrophage phagocytosis. As shown in Supplementary Fig. S2, the MTT assay showed that anti-CD47 Ab did not have a direct antitumour effect against PEL cell lines. Murine or human macrophages were incubated with

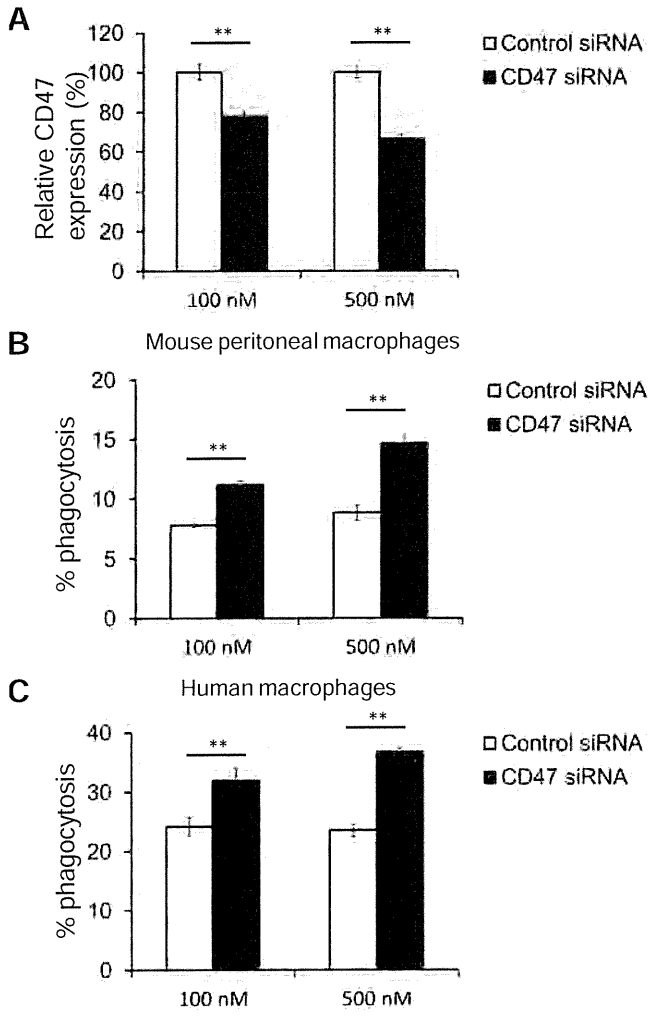

Fig. 2. Phagocytosis of primary effusion lymphoma (PEL) is dependent on CD47. (A) Relative CD47 expression levels were quantified by comparing mean fluorescence intensity (MFI) with knockdown BCBL-1 cells. The phagocytic activities of mouse peritoneal macrophages (B) and human macrophages (C) are shown after co-culture with CD47 or control siRNA-transduced BCBL-1 cells for 2 h. The numbers indicate the percentage of target cells ingested by macrophages. Values are the mean ± standard deviation (SD) ($n = 3$). $^{**}p < 0.01$ when compared with relative CD47 expression or% phagocytosis.

CFSE-labelled PEL cells in the presence of IgG1 isotype control or anti-CD47 Ab. Anti-CD47 Ab enabled increased the phagocytosis of PEL cells by mouse peritoneal macrophages (Fig. 3B) and human macrophages (Fig. 3A and C) compared with treatment with an IgG1 isotype control. In contrast, anti-CD47 Ab treatment had a slight effect on human normal B-cells (Fig. 3D). In addition, the expression of CD47 was significantly correlated with the fold increase of phagocytic activity (Fig. 3E and F). Anti-CD47 Ab enhanced the phagocytosis of PEL in a CD47 expression-dependent manner.

These results suggest that anti-CD47 Ab promotes macrophage phagocytosis of PEL cells.

3.4. Direct xenograft mouse model

To evaluate the *in vivo* efficacy of anti-CD47 Ab, freshly isolated PEL cells were transferred into NRJ mice. After primary PEL cells proliferated in NRJ mice, PEL cells were collected from ascites to obtain 'PEL-NRJ' cells (Fig. 4A).

As depicted in Fig. 4B, HHV-8 latent protein mRNAs (*LANA*, *v-FLIP*, *v-cyclin*) were positive in PEL-NRJ cells on semiquantitative PCR analysis. Fig. 4C shows that PEL-NRJ cells expressed post-germinal centre B-cell/plasma cell-associated antigen (CD38) and CD45 but not T-cell-associated marker (CD3), B-cell-associated markers (CD19, CD20). These phenotypes were consistent with those of previously described PEL [1]. In addition, PEL-NRJ cells expressed a high level of cell-surface CD47.

3.5. Anti-CD47 Ab suppresses the development of PEL in vivo

NRJ mice were inoculated intraperitoneally with 1×10^7 PEL-NRJ cells. PEL-NRJ cells produced profuse ascites within 2 weeks of inoculation (Fig. 5A).

Anti-CD47 Ab-treated mice seemed to show no apparent change. The volume of ascites was significantly lower than that of control IgG-treated mice on day 14 (0.0 ± 0.0 ml versus 6.3 ± 4.0 ml, $n = 7$; Fig. 5B). Moreover, the body weight gain of anti-CD47 Ab-treated mice significantly decreased compared with that of control IgG-treated mice on day 14 (0.4 ± 0.4 g versus 8.0 ± 4.6 g, $n = 7$; Fig. 5C).

Organ invasion by PEL-NRJ cells on day 14 was evaluated by haematoxylin–eosin staining, LANA immunostaining and flow cytometry. It was found that mice inoculated intraperitoneally with PEL-NRJ cells exhibited invasion into the liver and lungs without macroscopic lymphoma formation (Fig. 6A). As depicted in Fig. 6B, the number of LANA-positive cells in anti-CD47 Ab-treated mice was significantly reduced compared with control IgG-treated mice (0 ± 0 versus 9.8 ± 5.8 cells per field magnification in the liver, 0 ± 0 versus 8.4 ± 5.5 cells per field magnification in lungs, $\times 200$, $n = 5$ for each group). Fig. 6C and D show the presence of PEL-NRJ cells in the spleen using flow cytometry. The rate of PEL-NRJ cells in the spleen of anti-CD47 Ab-treated mice was significantly reduced compared with control IgG-treated mice ($0 \pm 0 \times 10^4$ versus $6.43 \pm 4.24 \times 10^4$, $n = 5$ for each group; Fig. 6D).

These findings demonstrated that anti-CD47 Ab significantly inhibited the growth and infiltration of PEL cells *in vivo*.

4. Discussion

PEL is an incurable and aggressive B-cell lymphoma that is characterised by malignant effusion in body cav-

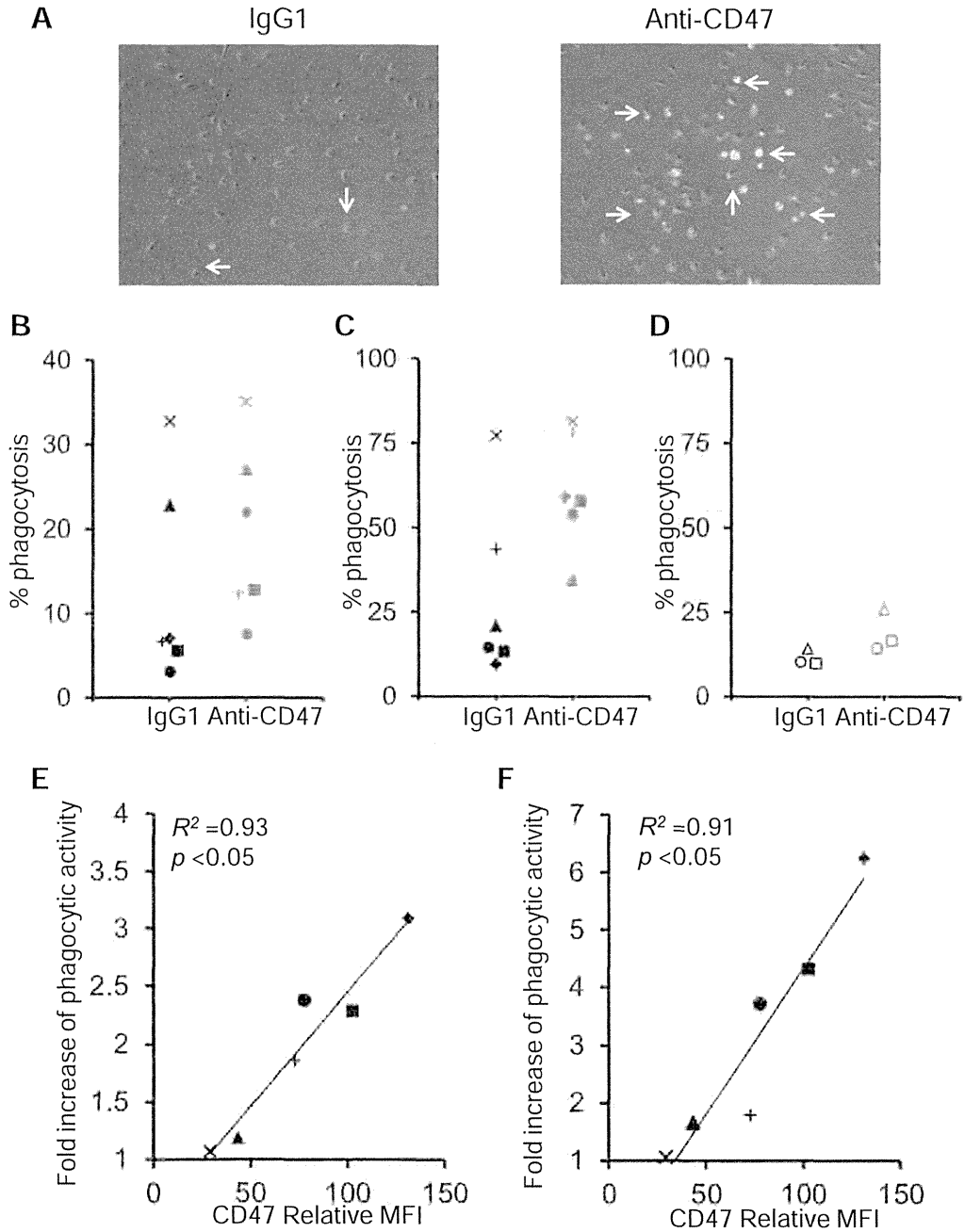

Fig. 3. Anti-CD47 Ab enables phagocytosis of primary effusion lymphoma (PEL) cells by macrophages *in vitro*. (A) BCBL-1 cells were labelled by carboxyfluorescein succinimidyl ester (CFSE) and incubated with human macrophages in the presence of IgG1 isotype control or anti-CD47 Ab for 2 h and then examined by fluorescence microscopy. Representative photomicrographs are shown with BCBL-1 cells (green) and arrows indicating macrophages containing phagocytosed BCBL-1 cells. The phagocytic activities of mouse peritoneal macrophages (B) and human macrophages (C, D) are shown after co-culture with PEL cells or human normal B-cell in the presence of IgG1 isotype control or anti-CD47 Ab for 2 h. The numbers indicate the percentage of target cells ingested by macrophages. Correlations between CD47 expression and the fold increase of phagocytic activity with mouse peritoneal macrophages (E) and human macrophages (F) are shown (●: BCBL-1; ■: BC-1; ▲: BC-2; ×: BCP-1; ♦: RM-P1; +: TY-1; ○: normal B-cell 1; □: normal B-cell 2; △: normal B-cell 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ities. PEL is spread by lymphomatous effusion and acquires resistance to treatment. There is therefore a need for a novel strategy to overcome resistance and eradicate lymphoma cells in body cavities.

In the present study, we investigated the role of CD47 in PEL and examined the preclinical feasibility of anti-CD47 Ab therapy. PEL cells expressed a high level of CD47 (Fig. 1). Phagocytosis of PEL is dependent on

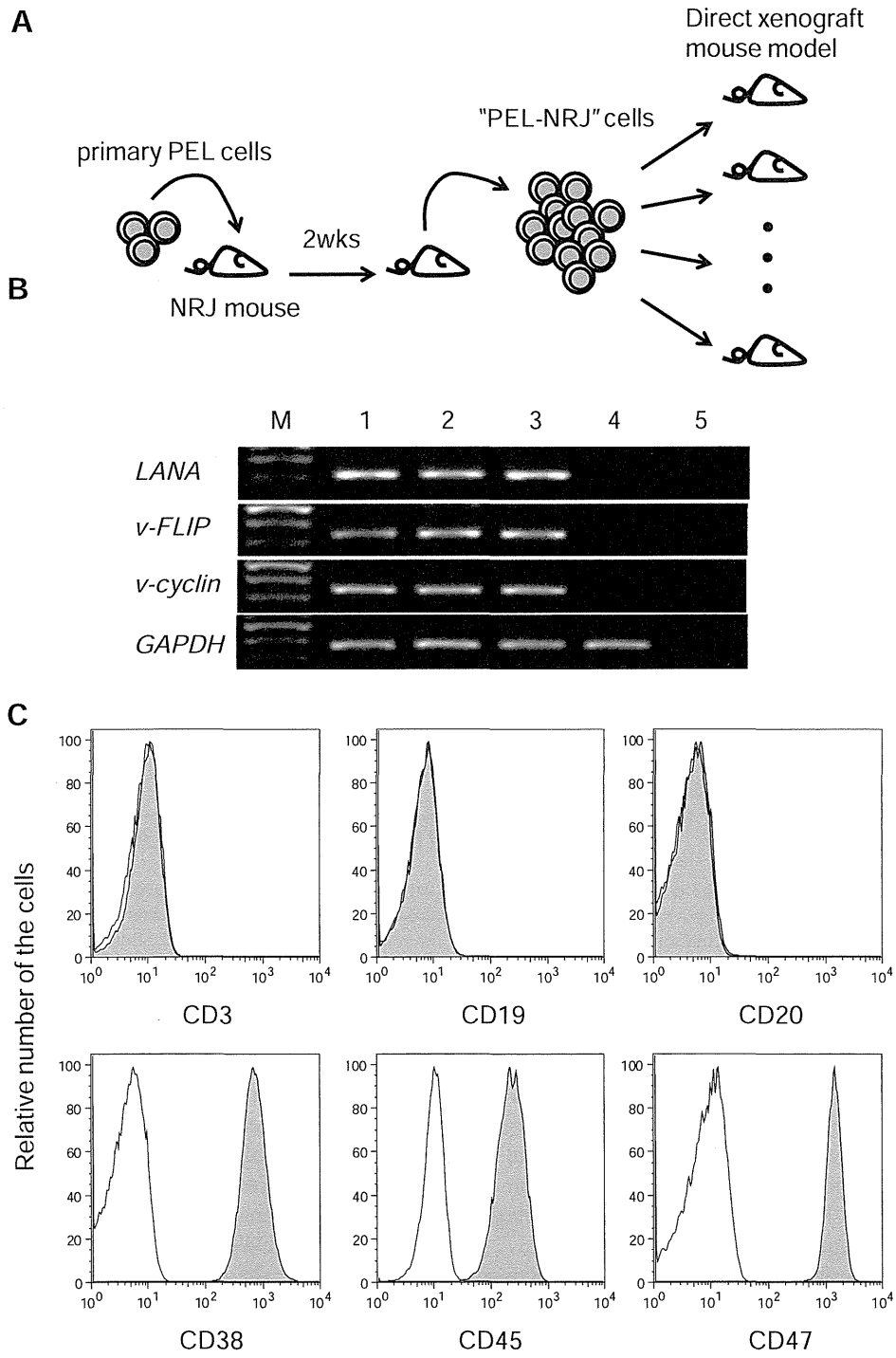

Fig. 4. Direct xenograft mouse model and phenotypes of primary effusion lymphoma-NOD Rag-2/Jak3 double-deficient (PEL-NRJ). (A) Experimental scheme of establishment of direct xenograft mouse model. After primary PEL cells were transferred and proliferated in NRJ mice, PEL cells were collected from ascites to obtain 'PEL-NRJ' cells. Subsequently, NRJ mice were inoculated intraperitoneally with PEL-NRJ cells. (B) HHV-8 mRNAs (*LANA*, *v-FLIP*, *v-cyclin*) demonstrated by semiquantitative polymerase chain reaction (PCR). GAPDH was used as an internal control. Lane 1, PEL-NRJ; lane 2, original lymphoma cells; lane 3, BCBL-1, HHV-8-positive cell line established from PEL; lane 4, Raji, HHV-8-negative cell line established from Burkitt's lymphoma; lane 5, water; M, molecular weight marker. (C) Flow cytometer profile of PEL-NRJ. Representative example of immunophenotype of PEL-NRJ. Grey histograms show antibody-stained cells; white histograms are isotype controls.

CD47 expression (Figs. 2 and 3E and F). The high level expression of CD47 on the surface of PEL is considered to contribute to immune evasion and resistance.

As shown in Fig. 3, anti-CD47 Ab efficiently promoted the phagocytosis of PEL cells. In some cell lines, such as BC-2 and BCP-1, which expressed a relatively

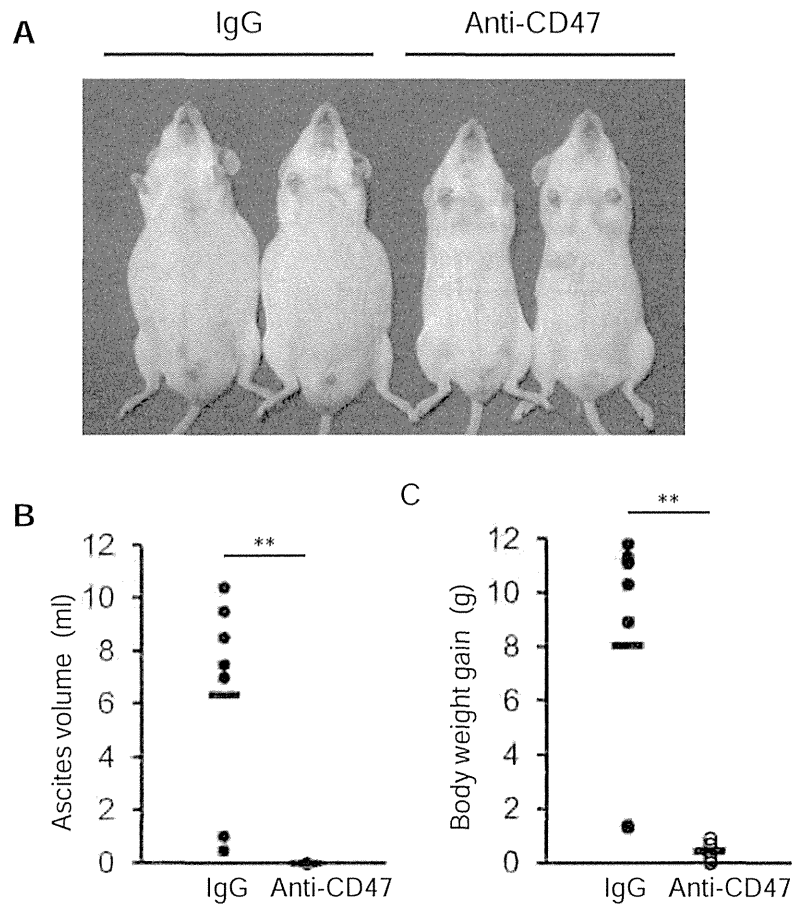

Fig. 5. Treatment of NOD Rag-2/Jak3 double-deficient (NRJ) mice with anti-CD47 Ab suppresses ascites of primary effusion lymphoma (PEL) *in vivo*. (A) Photograph of control IgG-treated and anti-CD47 Ab-treated mice 14 d after inoculation with PEL-NRJ cells intraperitoneally. (B) Volume of ascites 14 d after inoculation with PEL-NRJ cells in the mice is shown as the mean ± standard deviation (SD) of seven mice. (C) Body weight gain of mice 14 d after inoculation with PEL-NRJ cells in control IgG-treated and anti-CD47 Ab-treated mice is shown as the mean ± SD of seven mice. $^{**}p < 0.01$ when compared with ascites volume or body weight gain.

low level of CD47, the upregulation of phagocytic activity was low. However, the basal susceptibility of phagocytosis by macrophages was high and immunotherapy with macrophages was effective. The difference in the 'eat me' signal, such as with phosphatidylserine [26,27] and calreticulin [28,29] might be related to phagocytic activity without anti-CD47 Ab treatment.

NRJ mice display not only complete deficiency of mature T/B lymphocytes and complement protein but also complete deficiency of NK cells, such as NOD/Scid/common γ-deficient (NOG) mice [30,31]. Moreover, NOD-specific SIRPα polymorphism contributes to the resultant signaling, causing NOD macrophages not to engulf human grafts [32,33]. This immunodeficient nature provides the ideal microenvironment for the engraftment of PEL cells. When PEL-NRJ cells were inoculated intraperitoneally into NRJ mice, they were engrafted within 2 weeks. The engraftment and ascite formation of PEL-NRJ cells *in vivo* are earlier than those of previously reported cell lines, such as BCBL-1 (data not shown). *In vitro* cell-culture conditions can lead to HHV-8 and host gene-expression signatures that differ from patterns observed *in vivo* [34–36]. A direct xenograft mouse model is considered to reflect PEL pathogenesis. Interestingly, PEL-NRJ cells expressed a high level of CD47 expression compared with that of PEL cell lines (PEL-NRJ, relative MFI = 242.0, PEL cell lines, mean relative MFI = 76.2 ± 37.5). The high level of CD47 expression preserved in PEL-NRJ might be one of the differences between primary cells and cell lines, contributing to prompt engraftment *in vivo*. Taken together, the results of *in vivo* analysis using this mouse model provide strong validation for the effectiveness of targeting CD47.

The therapeutic efficacy of anti-CD47 Ab has been reported using human macrophages derived from peripheral blood and mouse macrophages derived from bone marrow [17–22]. Here, we focused on utilising peritoneal macrophages to eradicate PEL cells by anti-CD47 Ab for the first time. Anti-CD47 Ab enabled efficient phagocytosis of PEL cells *in vitro* and *in vivo*. This therapeutic strategy is reasonable because PEL cells proliferate specifically in body cavities, such as the peritoneal cavity, in the presence of peritoneal macro-

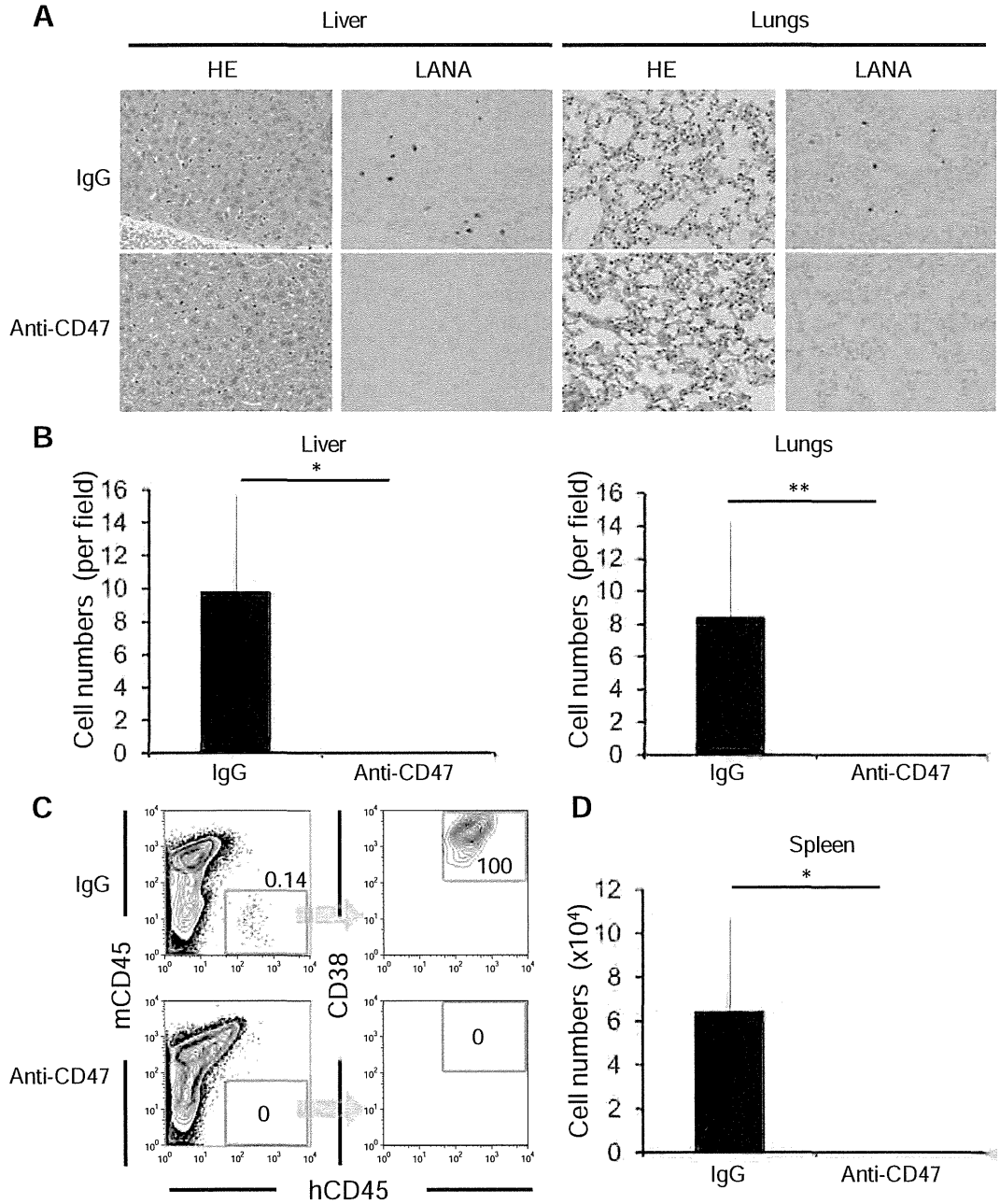

Fig. 6. Invasion of primary effusion lymphoma-NOD Rag-2/Jak3 double-deficient (PEL-NRJ) cells into the organs *in vivo*. (A) Hematoxylin–eosin staining and immunohistochemical staining using anti-LANA (PA1-73N Ab) were performed to detect PEL-NRJ cells in the liver and lungs. (B) LANA+ cell numbers per field (mean ± standard deviation (SD) of five mice) in the liver and lungs of control IgG-treated and anti-CD47 Ab-treated mice. (C) Representative flow cytometric profiles with the ratio of human CD45+ CD38+ cells in the spleen of control IgG-treated and anti-CD47 Ab-treated mice. (D) Human CD45+ CD38+ cell numbers per spleen (mean ± SD of five mice) in the spleen of control IgG-treated and anti-CD47 Ab-treated mice. $^{*}p < 0.05$; $^{**}p < 0.01$ when compared with cell numbers.

phages. In this study, we also evaluated the efficacy of human PBMC-derived macrophages other than mouse peritoneal macrophages (Figs. 2 and 3). Targeting therapy of CD47 was effective also in these macrophages, indicating the efficacy of targeting CD47 not only in peritoneal macrophages but also in other macrophages.

In most cancers, M1 macrophages are able to suppress tumour growth, whereas M2 macrophages inhibit adaptive immunity and augment tumour progression. Targeting CD47 has been reported to have the potential to change the behaviour of resident M2 macrophages to suppress tumour growth [37]. In this study, we assessed the efficacy of human M-CSF-induced macrophages, which correspond approximately to M2 macrophages [38], on PEL. Our results have also shown that anti-CD47 Ab therapy is considered to be effective for PEL even in the presence of M2 macrophages.

In the current study, we could not clarify the toxicity of human peritoneal macrophages against normal cells because we analysed the phagocytic activity of mouse peritoneal macrophage using a mouse model. Blocking anti-mouse CD47 Ab does not cause significant toxicity in mice, and blocking anti-human CD47 Ab does not affect phagocytosis of normal human peripheral blood cells and CD34+ bone marrow cells *in vitro* [17,19]. The toxicity of anti-CD47 Ab on normal B-cells was tolerable (Fig. 3D); therefore, anti-CD47 Ab-mediated phagocytosis with peritoneal macrophages might be tolerated and applicable for treatment. The safety of targeting CD47 to body cavities needs to be further validated *in vivo*.

Extranodal dissemination of NHL has been reported to require CD47 and is inhibited by anti-CD47 Ab [39]. In the PEL xenograft mouse model, anti-CD47 Ab therapy also prevented the formation of lymphoma invasion into the organs (Fig. 6).

In conclusion, CD47 is a critical regulator of the growth of PEL in the body cavity and PEL dissemination. Given that PEL is aggressive and resistant to conventional chemotherapy, our data provide preclinical evidence that antibody targeting of CD47 could be an effective therapeutic strategy for eliminating PEL cells.

Conflict of interest statement

None declared.

Acknowledgements

We thank Ms. I. Suzu and Ms. S. Fujikawa for technical assistance and Ms. Y. Endo for secretarial assistance. This work was supported in part by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour, and Welfare of Japan (H25-AIDS-I-002), the Global COE program, 'Global Education and Research Center Aiming at the Control of AIDS', Grants-in-Aid for Science Research (No. 25114711) from the Ministry of Education, Science, Sports, and Culture of Japan, and the scholarship for the Graduate School of Medical Sciences, Kumamoto University, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.03.004.

References

[1] Nador RG, Cesarman E, Chadburn A, et al. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood 1996;88:645–56.

[2] Ansari MQ, Dawson DB, Nador R, et al. Primary body cavity-based AIDS-related lymphomas. Am J Clin Pathol 1996;105:221–9.

[3] Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 1995;332:1186–91.

[4] Uddin S, Hussain AR, Al-Hussein KA, et al. Inhibition of phosphatidylinositol 3'-kinase/AKT signaling promotes apoptosis of primary effusion lymphoma cells. Clin Cancer Res 2005;11:3102–8.

[5] Uddin S, Hussain AR, Manogaran PS, et al. Curcumin suppresses growth and induces apoptosis in primary effusion lymphoma. Oncogene 2005;24:7022–30.

[6] Hussain AR, Ahmed SO, Ahmed M, et al. Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. PLoS One 2012;7:e39945.

[7] Boulanger E, Gerard L, Gabarre J, et al. Prognostic factors and outcome of human herpesvirus 8-associated primary effusion lymphoma in patients with AIDS. J Clin Oncol 2005;23:4372–80.

[8] Vose JM, Link BK, Grossbard ML, et al. Phase II study of rituximab in combination with chop chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. J Clin Oncol 2001;19:389–97.

[9] Vose JM, Link BK, Grossbard ML, Czuczman M, Grillo-Lopez A, Fisher RI. Long-term update of a phase II study of rituximab in combination with CHOP chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. Leuk Lymphoma 2005;46:1569–73.

[10] Cheson BD, Leonard JP. Monoclonal antibody therapy for B-cell non-Hodgkin's lymphoma. N Engl J Med 2008;359:613–26.

[11] Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol 2001;11:130–5.

[12] Sick E, Jeanne A, Schneider C, Dedieu S, Takeda K, Martiny L. CD47 update: a multifaceted actor in the tumour microenvironment of potential therapeutic interest. Br J Pharmacol 2012;167:1415–30.

[13] Blazar BR, Lindberg FP, Ingulli E, et al. CD47 (integrin-associated protein) engagement of dendritic cell and macrophage counterreceptors is required to prevent the clearance of donor lymphohematopoietic cells. J Exp Med 2001;194:541–9.

[14] Barclay AN, Brown MH. The SIRP family of receptors and immune regulation. Nat Rev Immunol 2006;6:457–64.

[15] Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. J Exp Med 2001;193:855–62.

[16] Okazawa H, Motegi S, Ohyama N, et al. Negative regulation of phagocytosis in macrophages by the CD47-SHPS-1 system. J Immunol 2005;174:2004–11.

[17] Majeti R, Chao MP, Alizadeh AA, et al. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 2009;138:286–99.

[18] Jaiswal S, Jamieson CH, Pang WW, et al. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009;138:271–85.

[19] Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. Cell 2010;142:699–713.

[20] Chao MP, Alizadeh AA, Tang C, et al. Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. Cancer Res 2011;71:1374–84.

[21] Willingham SB, Volkmer JP, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci U S A 2012;109:6662–7.

[22] Kim D, Wang J, Willingham SB, Martin R, Wernig G, Weissman IL. Anti-CD47 antibodies promote phagocytosis and inhibit the growth of human myeloma cells. Leukemia 2012;26:2538–45.
[23] Chihara T, Hashimoto M, Osman A, et al. HIV-1 proteins preferentially activate anti-inflammatory M2-type macrophages. J Immunol 2012;188:3620–7.
[24] Goto H, Matsuda K, Srikoon P, et al. Potent antitumor activity of zoledronic acid-induced Vgamma9Vdelta2 T cells against primary effusion lymphoma. Cancer Lett 2013;331:174–82.
[25] Goto H, Kariya R, Shimamoto M, et al. Antitumor effect of berberine against primary effusion lymphoma via inhibition of NF-kappaB pathway. Cancer Sci 2012;103:775–81.
[26] Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2207–16.
[27] Hoffmann PR, de Cathelineau AM, Ogden CA, et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J Cell Biol 2001;155:649–59.
[28] Gardai SJ, McPhillips KA, Frasch SC, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell 2005;123:321–34.
[29] Chao MP, Jaiswal S, Weissman-Tsukamoto R, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. Sci Transl Med 2010;2:63ra94.
[30] Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100:3175–82.
[31] Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 2005;174:6477–89.
[32] Takenaka K, Prasolava TK, Wang JC, et al. Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. Nat Immunol 2007;8:1313–23.
[33] Yamauchi T, Takenaka K, Urata S, et al. Polymorphic Sirpa is the genetic determinant for NOD-based mouse lines to achieve efficient human cell engraftment. Blood 2013;121:1316–25.
[34] Parravicini C, Chandran B, Corbellino M, et al. Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Am J Pathol 2000;156:743–9.
[35] Staudt MR, Kanan Y, Jeong JH, Papin JF, Hines-Boykin R, Dittmer DP. The tumor microenvironment controls primary effusion lymphoma growth in vivo. Cancer Res 2004;64:4790–9.
[36] Sarosiek KA, Cavallin LE, Bhatt S, et al. Efficacy of bortezomib in a direct xenograft model of primary effusion lymphoma. Proc Natl Acad Sci U S A 2010;107:13069–74.
[37] Edris B, Weiskopf K, Volkmer AK, et al. Antibody therapy targeting the CD47 protein is effective in a model of aggressive metastatic leiomyosarcoma. Proc Natl Acad Sci U S A 2012;109:6656–61.
[38] Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol 2006;177:7303–11.
[39] Chao MP, Tang C, Pachynski RK, Chin R, Majeti R, Weissman IL. Extranodal dissemination of non-Hodgkin lymphoma requires CD47 and is inhibited by anti-CD47 antibody therapy. Blood 2011;118:4890–901.

Katano et al. BMC Infectious Diseases 2014, 14:229
http://www.biomedcentral.com/1471-2334/14/229

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

The prevalence of opportunistic infections and malignancies in autopsied patients with human immunodeficiency virus infection in Japan

Harutaka Katano[1*], Tsunekazu Hishima[2], Makoto Mochizuki[3,4], Yoshinori Kodama[5], Naoki Oyaizu[6], Yasunori Ota[6], Sohtaro Mine[1,3], Toru Igari[3], Atsushi Ajisawa[7], Katsuji Teruya[8], Junko Tanuma[8], Yoshimi Kikuchi[8], Tomoko Uehira[9], Takuma Shirasaka[9], Tomohiko Koibuchi[10], Aikichi Iwamoto[10,11], Shinichi Oka[8], Hideki Hasegawa[1], Seiji Okada[12] and Akira Yasuoka[13]

Abstract

Background: Opportunistic infections and malignancies such as malignant lymphoma and Kaposi sarcoma are significant complications of human immunodeficiency virus (HIV) infection. However, following the introduction of antiretroviral therapy in Japan in 1997, the incidence of clinical complications has decreased. In the present study, autopsy cases of HIV infection in Japan were retrospectively investigated to reveal the prevalence of opportunistic infections and malignancies.

Methods: A total of 225 autopsy cases of HIV infection identified at 4 Japanese hospitals from 1985–2012 were retrospectively reviewed. Clinical data were collected from patient medical records.

Results: Mean CD4 counts of patients were 77.0 cells/μL in patients who received any antiretroviral therapy during their lives (ART (+) patients) and 39.6 cells/μL in naïve patients (ART (–) patients). Cytomegalovirus infection (142 cases, 63.1%) and *pneumocystis* pneumonia (66 cases, 29.3%) were the most frequent opportunistic infections, and their prevalence was significantly lower in ART (+) patients than ART (–) patients. Non-Hodgkin lymphoma and Kaposi sarcoma were observed in 30.1% and 16.2% of ART (–) patients, and 37.9% and 15.2% of ART (+) patients, respectively. Malignant lymphoma was the most frequent cause of death, followed by cytomegalovirus infection regardless of ART. Non-acquired immunodeficiency syndrome (AIDS)-defining cancers such as liver and lung cancer caused death more frequently in ART (+) patients (9.1%) than in ART (–) patients (1.5%; $P = 0.026$).

Conclusions: The prevalence of infectious diseases and malignancies were revealed in autopsy cases of HIV infection in Japan. The prevalence of cytomegalovirus infection and *pneumocystis* pneumonia at autopsy were lower in ART (+) patients than ART (–) patients. Higher prevalence of non-AIDS defining malignancies among ART (+) patients than ART (–) patients suggests that onsets of various opportunistic infections and malignancies should be carefully monitored regardless of whether the patient is receiving ART.

Keywords: AIDS, Opportunistic infections, Autopsy, Antiretroviral therapy

* Correspondence: katano@nih.go.jp
[1]Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
Full list of author information is available at the end of the article

Background

Opportunistic infections such as *Pneumocystis jirovecii* pneumonia (PCP), cytomegalovirus (CMV), non-tuber culous mycobacteria (NTM), and fungal infections are frequently found in patients with acquired immunodeficiency syndrome (AIDS) [1]. The most frequent opportunistic infection among patients with AIDS is CMV infection, which commonly causes retinitis, pneumonia, and gastrointestinal tract ulcers. PCP is also a frequent infectious disease in the lungs of patients with AIDS. Additionally, malignancies such as non-Hodgkin lymphoma (NHL) and Kaposi sarcoma (KS) are significant complications. NHL in particular is not easily controlled and is a frequent AIDS-associated cause of death. Interestingly, KS has only been reported in homosexual patients, and patients with multifocal KS lesions have a poor prognosis.

The introduction of antiretroviral therapy (ART) has drastically changed the incidence of opportunistic infections in patients infected with human immunodeficiency virus 1 (HIV-1), resulting in a decline in mortality rates [2-7]. ART has decreased the frequencies of CMV, PCP, and NTM infections in patients with AIDS [7]; however, the frequency of NHL has not changed dramatically [8]. Additionally, non-AIDS-defining malignancies such as liver, lung, and gastric cancers have been observed in patients with AIDS, regardless of ART [9]. A recent study demonstrated that low CD4 counts at ART initiation was associated with a greater risk of KS and lymphoma, whereas other cancers increased over time with ART, likely reflecting an increased risk of cancer with aging [10], low CD4 counts, and cigarette smoking [11-13].

Although mortality rates have decreased dramatically with the use of ART, its effect in many patients with AIDS is limited, and AIDS-associated complications remain a leading cause of death [14,15]. Additionally, untreated HIV-1-positive patients with severe AIDS-defining illnesses frequently visit hospitals and often rapidly succumb to suddenly aggressive progression of their illness [16,17]. Systematic pathological analysis of autopsy cases can provide useful information related to the cause of death and the distribution of pathogens in patients. However, there have been few reports describing the prevalence of infectious diseases and malignancies in autopsied patients with HIV infection [1,18]. A previous study using samples from autopsied patients with HIV infection during 1982–1998 demonstrated the prevalence of CMV, PCP, and NTM infections decreased during the study period [18]. The same study reported that, although the prevalence of KS was unchanged, the prevalence of NHL increased during the study period [18]. To the best of our knowledge, there are no reports demonstrating changes in the prevalence of opportunistic infections in autopsy cases of HIV infection following the introduction of ART after 2000.

In the present study, autopsy cases of HIV infection in Japan were retrospectively investigated to determine the prevalence of opportunistic infections and malignancies often found in patients with AIDS, including non-AIDS-defining malignancies. Additionally, the association of ART use with the prevalence of opportunistic infections and malignancies was investigated.

Patients and methods

Patients

The present study was approved by the Institutional Review Board of the National Institute of Infectious Diseases (Approval No. 356) and of four hospitals in Japan: Tokyo Metropolitan Komagome Hospital, National Center for Global Health and Medicine, Research Hospital, the Institute of Medical Science, the University of Tokyo, and Osaka National Hospital. Each hospital enrolled in the present study is a central hospital for AIDS treatment in Tokyo and Osaka, and has performed more than 15 autopsies of patients infected with HIV. According to a national autopsy survey by the Japan Pathology Society, 828 patients infected with HIV were autopsied in Japan from 1987–2009. During the period 1985–2009, 215 patients infected with HIV were autopsied at the 4 aforementioned hospitals. Thus, the number of cases in this study covered approximately 26% of all autopsied HIV cases. Ten cases autopsied in the period 2010–2012 were added to the 215 cases, making a total of 225 patients analyzed in this study (Table 1), of which 95.1% were male. The patients' ages at death ranged from 12 to 80 years, with a mean age of 44.4 years (median 44 years). Among them, 35.6% were homosexual, and 29.3% received ART (Table 1). The mean CD4 count at the last blood examination before death was 51.5 cells/μL (range: 0–560 cells/μL; median: 13.5 cells/μL). ART was introduced in Japan in 1997. In this study, ART was defined as any combination of therapy that included two nucleoside or nucleotide reverse transcriptase inhibitors plus a non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or abacavir (another nucleotide reverse transcriptase inhibitor). Additionally, ART (+) patients were defined as patients who received any ART during their lifetime, whereas ART (−) patients were as patients who did not receive ART.

Methods

Pathological findings were collected from autopsy records. CMV infection was determined by the infiltration of large cells with typical inclusion bodies. Infections by other viral agents such as hepatitis B virus, herpes simplex virus, hepatitis C virus, JC virus (causing progressive multifocal leukoencephalopathy), and varicella zoster virus were confirmed by immunohistochemistry or polymerase chain reaction. HIV encephalopathy was defined by morphological features indicating the presence of syncytial

Table 1 Characteristics of the patients infected with HIV

Factors	Groupings	Total patients		ART (–) patients		ART (+) patients		*P* value
		n	%	n	%	n	%	
Total		225*	100%	136	100%	66	100%	
Sex	Male	214	95.1%	128	94.1%	63	95.5%	0.695**
	Female	11	4.9%	8	5.9%	3	4.5%	
Age at death	<10 years	0	0.0%	0	0.0%	0	0.0%	**0.028*** **
	11–20	2	0.9%	2	1.5%	0	0.0%	
	21–30	30	13.3%	22	16.2%	6	9.1%	
	31–40	60	26.7%	34	25.0%	19	28.8%	
	41–50	69	30.7%	48	35.3%	14	21.2%	
	51–60	34	15.1%	18	13.2%	14	21.2%	
	61–70	24	10.7%	10	7.4%	10	15.2%	
	71–80	5	2.2%	1	0.7%	3	4.5%	
	>81	0	0.0%	0	0.0%	0	0.0%	
	Unknown	1	0.4%	1	0.7%	0	0.0%	
Risk factor	Homosexual	80	35.6%	52	38.2%	24	36.4%	0.800**
	Heterosexual	38	16.9%	24	17.6%	10	15.2%	
	Blood product	37	16.4%	29	21.3%	7	10.6%	
	Other	9	4.0%	5	3.7%	4	6.1%	
	Unknown	61	27.1%	26	19.1%	21	31.8%	
CD4 count before death	<50 cells/μL	122	54.2%	80	58.8%	33	50.0%	0.639***
	51–100	25	11.1%	11	8.1%	14	21.2%	
	101–200	13	5.8%	4	2.9%	9	13.6%	
	201–300	5	2.2%	5	3.7%	0	0.0%	
	301–400	3	1.3%	1	0.7%	2	3.0%	
	>401	4	1.8%	1	0.7%	3	4.5%	
	Unknown	53	23.6%	34	25.0%	5	7.6%	

ART, antiretroviral therapy. ART (+) patients were defined as patients who received any ART during their lifetime, whereas ART (–) patients were as patients who did not receive any ART in their lifetime.
*Total number of patients = 225 and included 23 patients with unknown ART status.
**P values were calculated for the rates of male or homosexuals between ART (–) and (+) patients by Chi-square test.
***P values were calculated for age or CD4 counts between all ART (–) and (+) patients by Mann–Whitney *U*-test. Bold font indicates statistical significance.

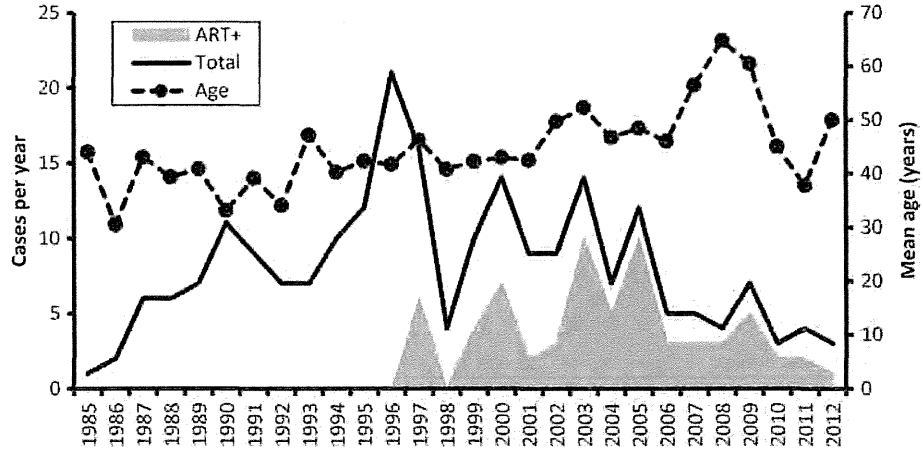

Figure 1 Annual number and mean age of AIDS-related autopsies. The solid line indicates total number of AIDS autopsies in each year. The gray area indicates the number of patients on ART in these autopsy cases. The broken bar indicates the mean age.

giant cells and detection of HIV-1 antigen by immunohistochemistry in the brain. Bacterial infection was identified by Gram stain, and in some cases, species of bacteria were identified by bacterial cultures. Tuberculosis and NTM infection were determined by acid-fast stain and/or PCR. Fungal and protozoan infections such as PCP, toxoplasma, *Candida*, *Aspergillus*, and *Cryptococcus* infection, were determined morphologically using Grocott's methenamine silver stain, periodic acid-Schiff stain, or/and immunohistochemistry. The histological sub-typing of malignant lymphoma was based on the World Health Organization classification, fourth edition. KS was confirmed by immunohistochemistry for Kaposi sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen 1. Causes of death were determined by pathologists at each hospital based on the severity, distribution, and type of illness in the pathological findings of autopsy. Clinical data, such as age at autopsy, sex, risk factors, CD4 cell counts at the last blood examination before death, and use of ART in their lifetime were collected from medical records. Analysis of statistical significance was carried out using Mann–Whitney *U*-test for non-parametric two-sample analysis and Chi-squared test for contingency table analysis.

Results

After the introduction of ART in Japan in 1997, the total number of autopsies conducted on patients with HIV infection has slowly decreased whereas the mean age at autopsy has increased slightly (Figure 1). After 1997, 66

Table 2 Infectious diseases and malignancies in AIDS-associated autopsies

	All patients		ART (−) patients		ART (+) patients			
	n	%	n	%	n	%	*P* values	
Total	225	100.0%	136	100.0%	66	100.0%		
Infectious diseases								
Cytomegalovirus	142	63.1%	97	71.3%	25	37.9%	**<0.001**	
Pneumocystis jirovecii pneumonia	66	29.3%	43	31.6%	11	16.7%	**0.024**	
Non-tuberculous mycobacterium	31	13.8%	20	14.7%	8	12.1%	0.618	
Candida	25	11.1%	17	12.5%	6	9.1%	0.474	
Aspergillus	24	10.7%	17	12.5%	4	6.1%	0.160	
Human immunodeficiency virus encephalopathy	21	9.3%	13	9.6%	6	9.1%	0.915	
Cryptococcus	16	7.1%	11	8.1%	3	4.5%	0.526	Y
Hepatitis B virus	12	5.3%	6	4.4%	5	7.6%	0.549	Y
Herpes simplex virus	12	5.3%	1	0.7%	1	1.5%	0.816	Y
Toxoplasmosis	11	4.9%	9	6.6%	3	4.5%	0.789	Y
Hepatitis C virus	9	4.0%	3	2.2%	5	7.6%	0.147	Y
Progressive multifocal leukoencephalopathy	8	3.6%	4	2.9%	2	3.0%	0.684	Y
Tuberculosis	6	2.7%	4	2.9%	0	0.0%	0.385	Y
Varicella zoster virus	4	1.8%	2	1.5%	2	3.0%	0.835	Y
Multicentric Castleman disease	2	0.9%	1	0.7%	1	1.5%	0.816	Y
Malignancies								
Non Hodgkin lymphoma	71	31.6%	41	30.1%	25	37.9%	0.272	
Kaposi sarcoma	38	16.9%	22	16.2%	10	15.2%	0.852	
Endocervical cancer	0	0.0%	0	0.0%	0	0.0%	-	
Non-AIDS defining malignancies	20	8.9%	10	7.4%	10	15.2%	0.082	
Hepatic cancer	8	3.6%	4	2.9%	4	6.1%	0.495	Y
Lung cancer	6	2.7%	2	1.5%	4	6.1%	0.174	Y
Leukemia	2	0.9%	0	0.0%	2	3.0%	0.200	Y
Hodgkin lymphoma	2	0.9%	1	0.7%	1	1.5%	0.816	Y
Gastric cancer	1	0.4%	1	0.7%	0	0.0%	0.711	Y
Other cancer	3	1.3%	3	2.2%	0	0.0%	0.551	Y

P values were calculated by Chi-square test. Y indicates the use of Chi-square test with Yates correction. Bold font indicates statistical significance. ART, antiretroviral therapy. Because more than one illness was detected in patients, the numbers of all illness are greater than the total number.

of 126 patients (52.6%) received ART during their lifetime. The mean age at death of patients on ART was 47.3 years, which was significantly higher than that of ART naïve patients (42.6 years; $P = 0.028$; Mann–Whitney *U*-test). Mean CD4 counts of ART (−) and (+) patients at the last blood examination before death were not significantly different (39.6 and 77.0 cells/μL, respectively, $P = 0.63$, Mann–Whitney *U*-test).

CMV was the most commonly identified pathogen among the autopsy cases (Table 2) and was detected in various organs, the most frequent being the adrenal gland (Figure 2A). PCP and NTM were also common pathogens found in the lungs of autopsied patients. *Candida albicans* was frequently detected in the gastrointestinal tract and oral cavity (Figure 2B). The prevalence of CMV and PCP was significantly lower in ART (+) patients than in ART (−) patients (Table 2). There was no significant difference in the prevalence of other opportunistic infections such as NTM and *Candida* or prevalence of HIV encephalopathy between ART (+) and (−) patients (Table 2).

Malignancies were identified in 50.2% (113/225) of all cases (Table 2). NHL was the most frequent malignancy with a lower prevalence in ART (−) patients (30.1%) than ART (+) patients (37.9%); however, the difference was not significant (Table 2). Diffuse large B-cell lymphoma was the most frequent histological subtype of NHL followed by Burkitt lymphoma, primary effusion lymphoma, and plasmablastic lymphoma (Table 3). Epstein–Barr virus positivity in lymphoma cases was significantly lower in ART (+) patients compared with ART (−) patients ($P = 0.001$, Chi-square test). KS was frequently found in the skin as well as other sites such as the gastrointestinal tract upon autopsy. In addition to NHL and KS, non-AIDS-defining malignancies such as Hodgkin lymphoma (HL), hepatic cancer, lung cancer, and leukemia were also observed in 20 patients. The prevalence of non-AIDS-defining malignancies was higher in ART (+) patients compared with ART (−) patients (Table 2).

The lung was the most frequent target for pathogens in patients with AIDS and 173 (76.9%) autopsy cases demonstrated the presence of lung-related illnesses (Table 4), which were significantly more frequent in ART (−) patients (112/136, 82.4%) than ART (+) patients (42/66, 63.6%) ($P = 0.003$, Chi-square test). CMV then PCP was the most frequently observed lung-related illnesses. The brain was the second most frequently affected organ in the autopsy cases. Although the brain was not investigated in 53 autopsies, 85 of the remaining 172 cases (49.4%) had brain-related illnesses, with CMV infection the most common, followed by lymphoma and HIV encephalopathy (Table 5). However, there was no significant difference in the rate of brain-related illnesses in ART (+) (37.8%, 17 of 45) or ART (−) patients (52.7%, 58/110) ($P = 0.091$, Chi-square test).

We also investigated the direct causes of death in the autopsied patients (Table 6). Lymphoma was the most frequent cause of death, followed by CMV infection. Non AIDS-defining cancers as a cause of death were significantly different between ART (−) (2, 1.5%) and ART (+) patients (6, 9.1%) ($P = 0.026$; Chi-square test with Yates correction). The prevalence of CMV, pneumonia, PCP, and NTM as a cause of death were lower in ART (+) patients compared with ART (−) patients, but no significant differences were observed between the groups.

Discussion

In the present study, we measured the prevalence of infectious disease and malignancy in autopsy cases of

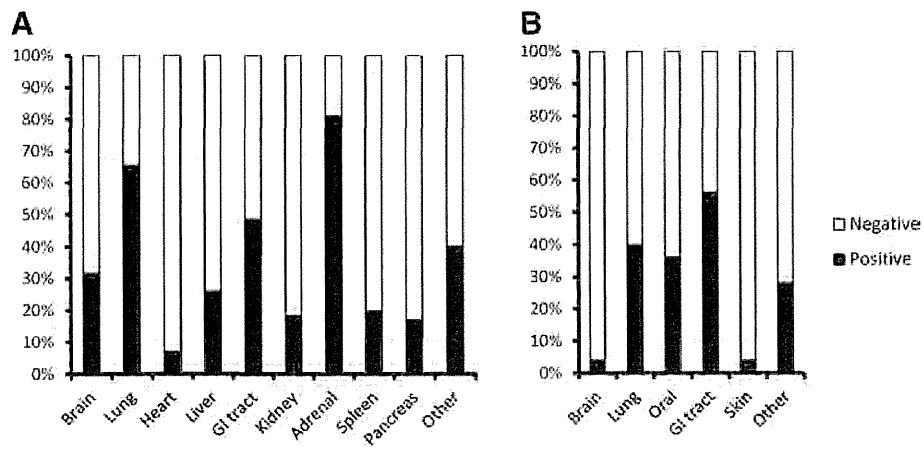

Figure 2 Distribution of cytomegalovirus and *Candida albicans*. **(A)** CMV positive rate in each organ. Black bar indicates the CMV positive rate in each organ from 142 CMV-positive patients. Because CMV was detected in more than one organ per patient, the sum of the black bars is over 100%. **(B)** The positive rate of *Candida albicans* in each organ. Black bar indicates the positive rate of *Candida albicans* per organ from 25 *Candida albicans*-positive patients.