**Fig. 5.** Lysis of autologous adult T-cell leukemia/lymphoma (ATL) cells by Tax-specific CTL. Tax301–309 peptide-expanded cells from ATL patient 7 (left panel), Tax11–19 peptide-expanded cells from patient 8 (middle panel) and Tax301–309-stimulated patient 14 (right panel) were evaluated for cytotoxicity by a standard 4-h chromium[51] release assay. Lysis was restricted to human leukocyte antigen (HLA)-A*24:02 or HLA-A2 and Tax-positive target cells. Each result represents three independent experiments.

AC. However, in these reports, the nature of the antigens recognized by the CTL is not determined, or the target cells are HTLV-1-infected T-cell lines rather than primary ATL tumor cells. In contrast, the present study clearly demonstrated that Tax antigen expressed by ATL cells was a significant target for CTL from ATL patients in an autologous setting. In addition, Tax expression was observed only in actively cycling ATL cells. This could only be noticed in primary ATL cells from patients, because established ATL cell lines or HTLV-1-immortalized lines, which are commonly used for many types of experiments, are, of course, continuously dividing. These findings collectively demonstrate that the main obstacle to successful immunotherapy targeting Tax, with its very limited expression in ATL cells, could be overcome. Whether primary ATL cells express Tax has been examined in several other studies using tumor cells from patients'

blood.[11,12,33] However, the present study demonstrated that most primary ATL cells in the blood are in a quiescent state, in which they express little or no Tax. Proliferating ATL cells are probably mostly to be found in lymph nodes in humans, not in the blood,[35] and these should, therefore, express substantial levels of Tax. Thus, the present findings indicate that Tax is a promising molecular target for immunotherapy in ATL patients, such as adoptive T-cell therapy and/or active vaccination.

As mentioned above, the efficiency of in vitro Tax-CTL expansion depended on HTLV-1 disease status. There was a trend towards superior expansion of Tax-CTL in HTLV-1 AC, ATL patients with the indolent variant and ATL patients who were in treatment-induced remission compared with newly diagnosed ATL patients with the aggressive variant. These observations indicate that host immune responses against Tax
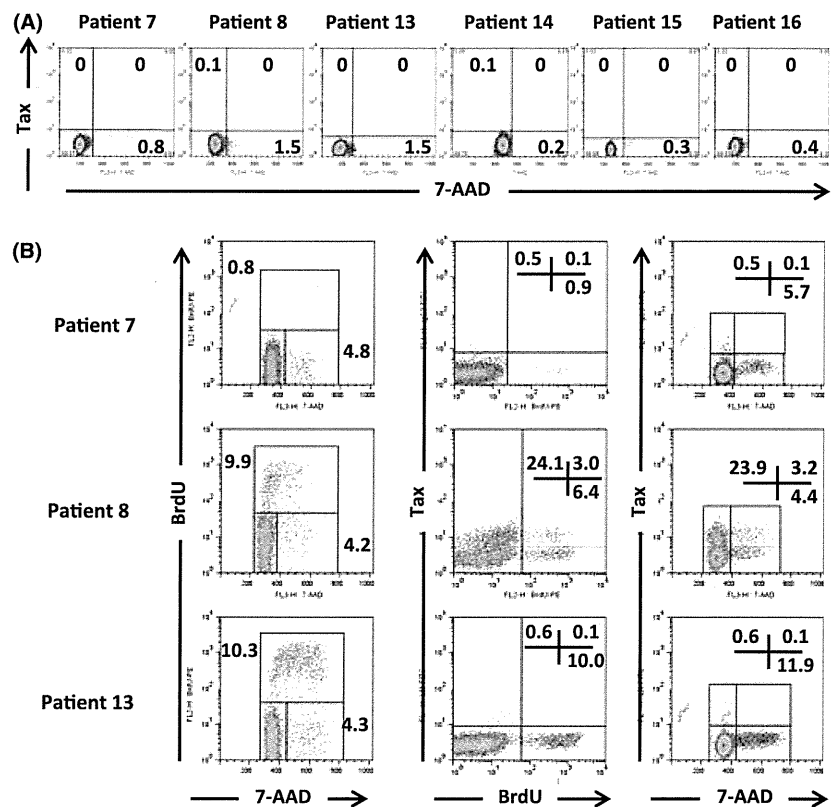


**Fig. 6.** Tax expression in adult T-cell leukemia/lymphoma (ATL) cells induced by short-term culture. (A) Lack of Tax expression in primary ATL cells from peripheral blood of patients 7, 8, 13, 14, 15 and 16. Cells were in a quiescent state as determined by 7-ADD staining. (B) Cell cycle status and Tax expression of short-term cultured primary ATL cells. Tax expression was induced when cells were actively cycling. Each result represents three independent experiments.
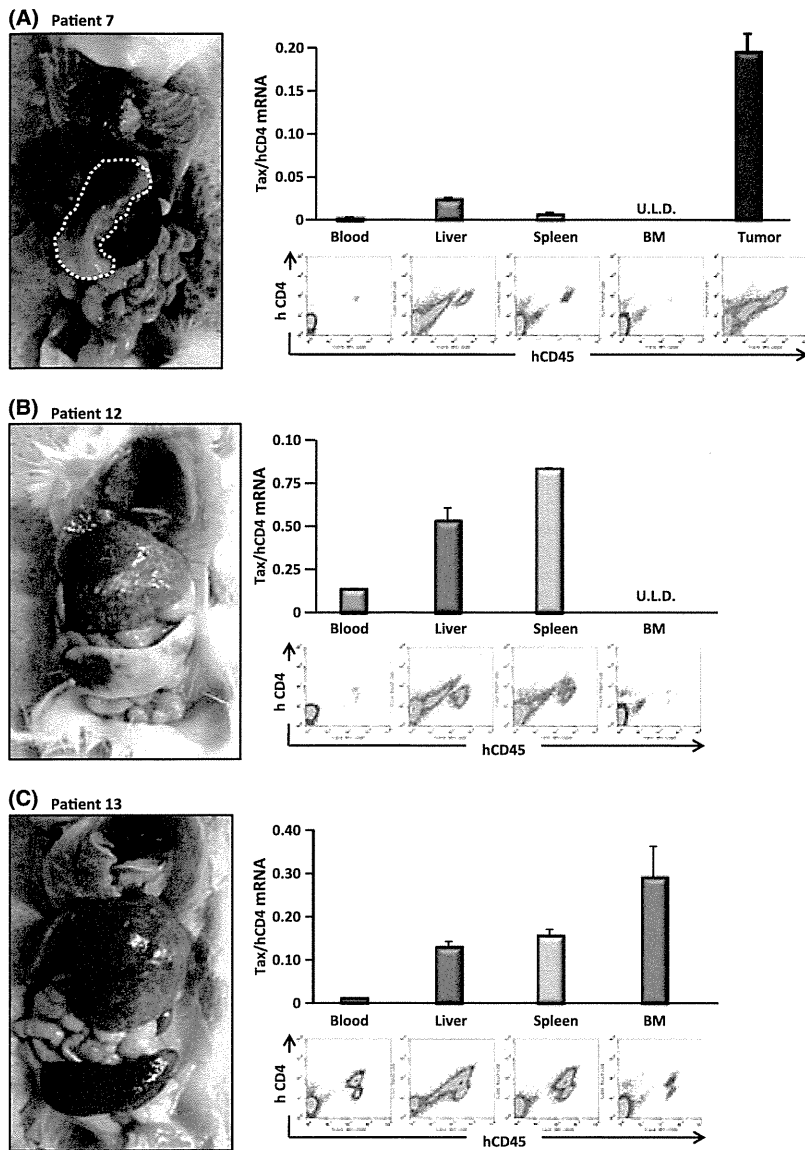
**Fig. 7.** Tax expression in primary adult T-cell leukemia/lymphoma (ATL) cell-bearing NOG mice. Tax expression of ATL cells in each affected organ of NOG mice bearing primary ATL cells from patient 7 (A), 12 (B) and 13 (C) were evaluated. NOG mice with cells from patient 7 presented with large intraperitoneal tumor masses demarcated by the white dotted lines. *Tax/human CD4* mRNA values of the cells from each organ are presented as bar graphs, where the value for TL-Su was set at unity. Flow cytometric analysis of the cells from each organ determined by human CD45 and CD4 expression is presented. Columns, mean of triplicate experiments; bars, standard deviation. BM, bone marrow; U.L.D., under limit of detection.

play an important role in maintaining the stable status of HTLV-1 AC, indolent ATL patients and ATL in remission. In addition, quantitative and/or functional reduction of Tax-CTL should lead to progression from HTLV-1 AC to ATL, or from indolent to aggressive ATL, or to relapse in ATL patients. Furthermore, the present observations suggest that restoration of substantial anti-Tax responses in some appropriate manner will lead to improvement of ATL disease status.

The efficient expansion of Tax-CTL from PBMC of ATL patients in remission suggests that reducing the number of tumor cells before Tax-targeted immunotherapy could be a crucial factor for successful induction/augmentation of antigen-specific CD8-positive CTL. We have reported that the humanized anti-CCR4 mAb KW-0761 (mogamulizumab) exerted clinically significant antitumor activity in relapsed ATL patients.[36,37] In addition, consistent with the fact that CCR4 is expressed not only on Th2 cells, but also on Treg cells,[21,38–40] KW-0761 treatment resulted in a significant and lasting decrease in CD4+CD25+FOXP3+ cells, including both the tumor cells and endogenous non-ATL Treg cells.[37] Reduction or suppression of Treg cells is expected to be a promising strategy for boosting antitumor immunity in cancer patients, as observed in studies with ipilimumab.[41,42] In fact, Tax-CTL were efficiently expanded from PBMC of patients 2 and 3 who were in CR after KW-0761 treatment. Thus, combining Tax-targeted immunotherapy following reduction of ATL cells and endogenous Treg cell depletion by KW-0761 treatment would be an ideal strategy for ATL immunotherapy.

The efficient expansion of Tax-CTL from PBMC of patients in remission after allogeneic HSCT is consistent with the report that Tax-specific CD8-positive T cells contribute to graft-versus-ATL effects.[13,43] Therefore, Tax-targeted immunotherapy after allogeneic HSCT should be therapeutically effective without increased graft-versus-host disease, which is a frequent and serious complication of this modality.

In conclusion, the present study not only provides a strong rationale for selecting Tax as a possible target for ATL immunotherapy but also contributes to our understanding of the immunopathogenesis driving progression from HTLV-1 AC to ATL, and to devising strategies for preventing this by targeting Tax.

## Disclosure Statement

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## References

1 Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood* 1977; **50**: 481–92.
2 Matsuoka M, Jeang KT. Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer* 2007; **7**: 270–80.
3 Hino S, Sugiyama H, Doi H *et al.* Breaking the cycle of HTLV-I transmission via carrier mothers' milk. *Lancet* 1987; **2**: 158–9.
4 Ishida T, Ueda R. Immunopathogenesis of lymphoma: focus on CCR4. *Cancer Sci* 2011; **102**: 44–50.
5 Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). *Br J Haematol* 1991; **79**: 428–37.
6 Akagi T, Ono H, Shimotohno K. Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. *Blood* 1995; **86**: 4243–9.
7 Kannagi M, Sugamura K, Kinoshita K, Uchino H, Hinuma Y. Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient. *J Immunol* 1984; **133**: 1037–41.
8 Arnulf B, Thorel M, Poirot Y *et al.* Loss of the ex vivo but not the reinducible CD8+ T-cell response to Tax in human T-cell leukemia virus type 1-infected patients with adult T-cell leukemia/lymphoma. *Leukemia* 2004; **18**: 126–32.
9 Kannagi M, Sugamura K, Sato H, Okochi K, Uchino H, Hinuma Y. Establishment of human cytotoxic T cell lines specific for human adult T cell leukemia virus-bearing cells. *J Immunol* 1983; **130**: 2942–6.
10 Kannagi M, Harada S, Maruyama I *et al.* Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells. *Int Immunol* 1991; **3**: 761–7.
11 Takeda S, Maeda M, Morikawa S *et al.* Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer* 2004; **109**: 559–67.
12 Kannagi M, Harashima N, Kurihara K *et al.* Tumor immunity against adult T-cell leukemia. *Cancer Sci* 2005; **96**: 249–55.
13 Harashima N, Kurihara K, Utsunomiya A *et al.* Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. *Cancer Res* 2004; **64**: 391–9.
14 Ishida T, Utsunomiya A, Iida S *et al.* Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res* 2003; **9**: 3625–34.
15 Ri M, Iida S, Ishida T *et al.* Bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on upregulation of Noxa and functional repression of Mcl-1. *Cancer Sci* 2009; **100**: 341–8.
16 Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood* 1975; **45**: 321–34.
17 Lee B, Tanaka Y, Tozawa H. Monoclonal antibody defining tax protein of human T-cell leukemia virus type-I. *Tohoku J Exp Med* 1989; **157**: 1–11.
18 Ishida T, Iida S, Akatsuka Y *et al.* The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-Cell leukemia/lymphoma. *Clin Cancer Res* 2004; **10**: 7529–39.
19 Mori F, Ishida T, Ito A *et al.* Potent antitumor effects of bevacizumab in a microenvironment-dependent human lymphoma mouse model. *Blood Cancer J* 2012; **2**: e67, doi:10.1038/bcj.2012.12 [Epub ahead of print].
20 Ishii T, Ishida T, Utsunomiya A *et al.* Defucosylated humanized anti-CCR4 monoclonal antibody KW-0761 as a novel immunotherapeutic agent for adult T-cell leukemia/lymphoma. *Clin Cancer Res* 2010; **16**: 1520–31.
21 Ishida T, Ueda R. CCR4 as a novel molecular target for immunotherapy of cancer. *Cancer Sci* 2006; **97**: 1139–46.
22 Kozako T, Arima N, Toji S *et al.* Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8+ T cell in adult T cell leukemia patients. *J Immunol* 2006; **177**: 5718–26.
23 Kannagi M, Hasegawa A, Kinpara S *et al.* Double control systems for human T-cell leukemia virus type 1 by innate and acquired immunity. *Cancer Sci* 2011; **102**: 670–6.
24 Shimizu Y, Takamori A, Utsunomiya A *et al.* Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages. *Cancer Sci* 2009; **100**: 481–9.
25 Kawano N, Shimoda K, Ishikawa F *et al.* Adult T-cell leukemia development from a human T-cell leukemia virus type I carrier after a living-donor liver transplantation. *Transplantation* 2006; **82**: 840–3.
26 Suzuki S, Uozumi K, Maeda M *et al.* Adult T-cell leukemia in a liver transplant recipient that did not progress after onset of graft rejection. *Int J Hematol* 2006; **83**: 429–32.
27 Yano H, Ishida T, Inagaki A *et al.* Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. *Int J Cancer* 2007; **120**: 2052–7.
28 Choi EM, Chen JL, Wooldridge L *et al.* High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J Immunol* 2003; **171**: 5116–23.
29 Betts MR, Price DA, Brenchley JM *et al.* The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. *J Immunol* 2004; **172**: 6407–17.
30 Itoh Y, Hemmer B, Martin R, Germain RN. Serial TCR engagement and down-modulation by peptide: MHC molecule ligands: relationship to the quality of individual TCR signaling events. *J Immunol* 1999; **162**: 2073–80.
31 Benlalam H, Linard B, Guilloux Y *et al.* Identification of five new HLA-B*3501-restricted epitopes derived from common melanoma-associated antigens, spontaneously recognized by tumor-infiltrating lymphocytes. *J Immunol* 2003; **171**: 6283–9.
32 Yoshikawa T, Nakatsugawa M, Suzuki S *et al.* HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. *Cancer Sci* 2011; **102**: 918–25.
33 Kurihara K, Harashima N, Hanabuchi S *et al.* Potential immunogenicity of adult T cell leukemia cells in vivo. *Int J Cancer* 2005; **114**: 257–67.
34 Ito A, Ishida T, Utsunomiya A *et al.* Defucosylated anti-CCR4 monoclonal antibody exerts potent ADCC against primary ATLL cells mediated by autologous human immune cells in NOD/Shi-scid, IL-2R gamma(null) mice in vivo. *J Immunol* 2009; **183**: 4782–91.
35 Umino A, Nakagawa M, Utsunomiya A *et al.* Clonal evolution of adult T-cell leukemia/lymphoma takes place in the lymph nodes. *Blood* 2011; **117**: 5473–8.
36 Yamamoto K, Utsunomiya A, Tobinai K *et al.* Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma. *J Clin Oncol* 2010; **28**: 1591–8.
37 Ishida T, Joh T, Uike N *et al.* Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase ii study. *J Clin Oncol* 2012; **30**: 837–42.
38 Imai T, Nagira M, Takagi S *et al.* Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; **11**: 81–8.
39 Iellem A, Mariani M, Lang R *et al.* Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+) CD25(+) regulatory T cells. *J Exp Med* 2001; **194**: 847–53.
40 Ishida T, Ishii T, Inagaki A *et al.* Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res* 2006; **66**: 5716–22.
41 Hodi FS, O'Day SJ, McDermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711–23.
42 Robert C, Thomas L, Bondarenko I *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011; **364**: 2517–26.
43 Ishida T, Hishizawa M, Kato K *et al.* Allogeneic hematopoietic stem cell transplantation for adult T-cell leukemia-lymphoma with special emphasis on preconditioning regimen: a nationwide retrospective study. *Blood* 2012; doi: 10.1182/blood-2012-03-414490 [Epub ahead of print].

# ORIGINAL ARTICLE

# Potent antitumor effects of bevacizumab in a microenvironment-dependent human lymphoma mouse model

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We established a mouse model of microenvironment-dependent human lymphoma, and assessed the therapeutic potential of bevacizumab, an antitumor agent acting on the microenvironment. NOD/Shi-*scid*, IL-2R$\gamma^{null}$ (NOG) mice were used as recipients of primary tumor cells from a patient with diffuse large B-cell lymphoma (DLBCL), which engraft and proliferate in a microenvironment-dependent manner. The lymphoma cells could be serially transplanted in NOG mice, but could not be maintained in *in vitro* cultures. Injection of bevacizumab together with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) significantly increased necrosis and decreased vascularization in the tumor, compared with CHOP alone. Levels of human soluble interleukin-2 receptor (sIL2R) in the serum of bevacizumab + CHOP-treated mice (reflecting the DLBCL tumor burden) were significantly lower than in CHOP recipients. Mice receiving bevacizumab monotherapy also showed significant benefit in terms of tumor necrosis and vascularization, as well as decreased serum sIL2R concentrations. The present DLBCL model reflects the human DLBCL *in vivo* environment more appropriately than current mouse models using established tumor cell lines. This is the first report to evaluate the efficacy of bevacizumab in such a tumor microenvironment-dependent model. Bevacizumab may be a potential treatment strategy for DLBCL patients.

## INTRODUCTION

Tumors develop in a complex and dynamic microenvironment. Surrounding or within the tumor nests, stromal cells, endothelial cells, innate immune cells and other lymphocytes are present that interact with each other and with the tumor cells. A large body of evidence has accumulated in the past decade demonstrating that this complex tumor microenvironment regulates tumor growth, invasion, and metastasis.[1] Angiogenesis is one of the most important phenomena within the tumor microenvironment; cancer cells have the ability to recruit and generate new blood vessels through the secretion of angiogenic factors. Tumor angiogenesis ensures that cells in the interior of the tumor receive sufficient nutrients and oxygen to survive. Blocking tumor angiogenesis would therefore severely restrict tumor growth.[2] Early experiments using mouse xenografts indicated that antibody-mediated inhibition of vascular endothelial growth factor (VEGF), which promotes the proliferation and migration of vascular endothelial cells and vessel sprouting, could severely inhibit angiogenesis and tumor growth.[3] These and other studies led to the development of the anti-VEGF neutralizing antibody bevacizumab for therapeutic use.[4] However, a current crucial problem in the research field of anti-tumor microenvironment agents such as bevacizumab is the lack of suitable small animal models. To the best of our knowledge, all preclinical testing of the antitumor activity of bevacizumab in mice *in vivo* has been performed using established tumor cell lines, which by definition can be maintained *in vitro* in culture.

Such tumor cells have thus been selected for survival in the absence of any microenvironment, including the vascular system. Using these established lines in mouse xenograft models therefore seems less relevant for the evaluation of the antitumor activities of anti-angiogenesis agents. Hence, the first objective of the present study was to overcome this problem. We aimed to establish a mouse model in which primary tumor cells from a patient engraft and proliferate in a microenvironment-dependent manner, using NOD/Shi-*scid*, IL-2R$\gamma^{null}$ (NOG) mice as recipients.[5,6]

Bevacizumab is currently approved world-wide for the treatment of several types of solid tumors such as colorectal cancer, breast cancer, non-small cell lung cancer, renal cell cancer and glioblastoma.[7–15] Many aspects of pathological angiogenesis have been extensively studied in many types of solid tumors. However, the precise role of these processes in pathogenesis of hematological malignancies is still under active investigation, and in this context, bevacizumab is not currently approved for the treatment of hematological malignancies in the United States, Europe, or Japan. Thus, the second aim of the present study was to evaluate the therapeutic potential of bevacizumab with or without systemic chemotherapy for hematological neoplasia, using newly established primary tumor cell-bearing NOG mice. We selected diffuse large B-cell lymphoma (DLBCL) as the target disease because this represents the most common type of malignant lymphoma and accounts for ~30–40% of all cases in adults.[16,17]

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## MATERIALS AND METHODS

### Animals

NOG mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan) and used at 6–8 weeks of age. All of the *in vivo* experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia, Second Edition, and were approved by the Ethics Committee of the Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences.

### Immunopathological analysis

We assessed the affected lymph nodes of 50 patients with DLBCL by immunopathology. The patients provided written informed consent in accordance with the Declaration of Helsinki, and the present study was approved by the institutional Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Hematoxylin and eosin staining and immunostaining using anti-human CD20 (L26; DAKO, Glostrup, Denmark), CD25, (4C9; Novocastra, Wetzlar, Germany), CD3 (SP7; SPRING BIOSCIENCE, Pleasanton, CA, USA), VEGF-A (sc-152, rabbit polyclonal, Santa Cruz, Heidelberg, Germany), Alpha-Smooth Muscle Actin (α-SMA; 1A4; DAKO), von Willebrand Factor (Rabbit polyclonal, DAKO), CD31 (JC70A, DAKO), CD10 (56C6; Novocastra), BCL-6 (LN22; Novocastra) and MUM1/IRF4 (M-17, Santa Cruz) were performed on formalin-fixed, paraffin-embedded sections. The presence of Epstein-Barr virus encoded RNA (EBER) was examined by *in situ* hybridization using EBER Probe (Leica microsystems, Newcastle, UK) on formalin-fixed, paraffin-embedded sections. DLBCL cases were categorized into germinal center B-cell (GCB) or non-GCB phenotypes using formalin-fixed, paraffin-embedded sections according to Hans' Algorithm.[18] VEGF-A expression levels were categorized according to the following formula: 3+ positive if $\geqslant$ 50%, 2+ positive if $< 50 \geqslant 30\%$, 1+ positive if $< 30 \geqslant 10\%$ and negative if $< 10\%$ of the DLBCL tumor cells were stained with the corresponding antibody. Nine $\times 100$ high-power fields (HPF) of hematoxylin and eosin tumor specimens were randomly selected and the area of tumor necrosis (%) was calculated by Image J software[19] and then averaged. Nine $\times 100$ HPF of von Willebrand Factor-stained tumor specimens were randomly selected and the numbers of vessels (per $mm^2$) were calculated by Image J software[19] and then averaged.

### Primary DLBCL cell-bearing mouse model

The affected lymph node cells from a patient with DLBCL were suspended in RPMI-1640. The tumor cell donor provided written informed consent before sampling in accordance with the Declaration of Helsinki, and the present study was approved by the institutional Ethics Committee of Nagoya City University Graduate School of Medical Sciences. CD3-negative subsets were isolated using anti-human CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and the autoMACS Pro Separator (Miltenyi Biotec) according to the manufacturer's instructions. Immunopathological analysis of the patient's affected lymph node revealed that the DLBCL type was non-GCB (CD10 −, BCL-6 − and MUM1/IRF4 +), and VEGF expression was 1+ positive. Six to 8 weeks after intraperitoneal (i.p.) injection, NOG mice presented with i.p. masses and splenomegaly. Cells from these i.p. masses were suspended in RPMI-1640 and i.p. inoculated into other NOG mice, which then presented with features identical to those of the first mice.

### DLBCL cell lines

DB and HT were purchased from DSMZ (Braunschweig, Germany). KARPAS422, OCI-LY19, Farage, Toledo, Pfeiffer and RL were purchased from ATCC (Manassas, VA, USA).

### Quantitative reverse transcription PCR

Total RNA was isolated with RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Reverse transcription from the RNA to first strand cDNA was carried out using High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. *Human VEGF-A* (Hs00900055_m1), *VEGF-R1* (Hs00176573_m1), *VEGF-R2* (Hs00911700_m1) and β-actin (Hs99999903_m1) mRNA were amplified using TaqMan Gene Expression Assays with the aid of an Applied Biosystems StepOnePlus according to the manufacturer's instructions. The quantitative assessment of the mRNA of interest was done by dividing its level by that of β-actin and expressing the result relative to Human Testis Total RNA (Clontech,

Mountain View, CA, USA) as 1.0. All expressed values were averages of triplicate experiments.

### Monoclonal antibodies and flow cytometry

The following monoclonal antibodies (mAbs) were used for flow cytometry: MultiTEST CD3 (clone SK7) FITC/CD16 (B73.1) + CD56 (NCAM 16.2) PE/CD45 (2D1,) PerCP/CD19 (SJ25C1) APC Reagent (BD Biosciences, San Jose, CA, USA), PerCP-conjugated anti-human CD45 mAb (2D1, BD Biosciences), APC-conjugated anti-CD19 mAb (HIB19, BD Biosciences), PE-conjugated anti-CD25 mAb (M-A251, BD Biosciences), PE-conjugated VEGF-R1 mAb (49560, BD Biosciences), PE-conjugated VEGF-R2 mAb (89106, R&D Systems Inc., Minneapolis, MN, USA) and the appropriate isotype control mAbs. Whole blood cells from mice were treated with BD FACS lysing solution (BD Biosciences) for lysing red blood cells . Cells were analyzed by a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star Inc., Ashland, OR, USA).

### Cell proliferation assay

Proliferation of the DLBCL cell lines, which express both VEGF-A and VEGF-R in the presence of different concentrations of bevacizumab for 48 h, was assessed using the CellTiter 96 Aqueous One Solution cell proliferation assay kit (Promega Corporation, Madison, WI, USA) as described previously.[20] Proliferation of the NOG DLBCL cells with or without human interleukin-2 at a final concentration of 100 IU/ml was also assessed in the same manner.

### Primary DLBCL cell-bearing mice treated with CHOP + bevacizumab

Tumor cells from the i.p. masses were suspended in RPMI-1640, and $1.0 \times 10^7$ were i.p. inoculated into each of 10 NOG mice. The mice were divided into two groups of five each for treatment with bevacizumab + CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) or CHOP alone, 2 days after tumor inoculation. Bevacizumab (10 mg/kg) or control (saline) was i.p. injected into the mice 2, 9, 16, 23, 30, 37 and 44 days after tumor cell inoculations. CHOP was given i.p. 30 days after tumor inoculations at doses as follows: cyclophosphamide, 40 mg/kg; doxorubicin, 3.3 mg/kg; vincristine, 0.5 mg/kg; prednisolone, 0.2 mg/kg.[21] Therapeutic efficacies were evaluated 49 days after tumor inoculation. Bevacizumab was purchased from Chugai Pharmaceutical Co., Ltd, Tokyo, Japan; cyclophosphamide and vincristine were purchased from Shionogi Pharmaceutical Co., Ltd, Osaka, Japan; doxorubicin was from Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan and prednisolone was from Nippon Kayaku Co., Ltd, Tokyo Japan.

### Primary DLBCL cell-bearing mice treated with bevacizumab

A total of $1.0 \times 10^7$ tumor cells were i.p. inoculated into each of 18 NOG mice, divided into two groups of nine each for bevacizumab or control. Bevacizumab (10 mg/kg) or control (saline) was i.p. injected into the mice after 3, 10, 17, 24, 31, 38 and 45 days, and therapeutic efficacies were evaluated 47 days after tumor inoculations.

### Human sIL2R measurement

The concentration of human soluble interleukin-2 receptor (sIL2R) in mouse serum was measured by enzyme-linked immunosorbent assay using the human sIL2R immunoassay kit (R&D Systems, Inc.) according to the manufacturer's instructions.

### Statistical analysis

The differences between groups regarding the tumor necrosis area, vascular number, percentage of lymphoma cells in mouse spleen cell suspensions and human sIL2R concentrations in mouse serum were examined with the Mann–Whitney $U$ test. All analyses were performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). In this study, $P < 0.05$ was considered significant.

## RESULTS

### VEGF-A expression in DLBCL

VEGF-A expression by DLBCL cells in the lymph node lesions according to GCB or non-GCB phenotypes are shown in Figure 1a.
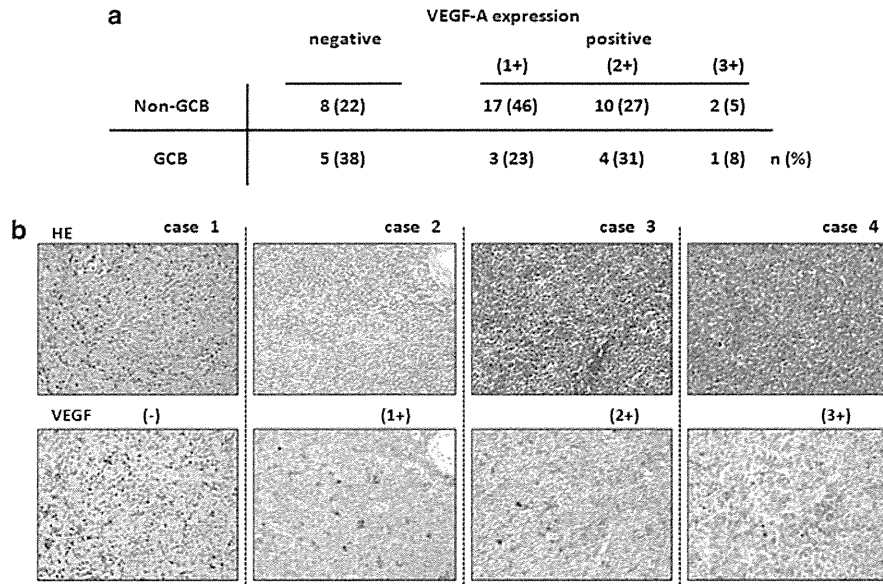
| a | | VEGF-A expression | | |
| | negative | positive | | |
| | | (1+) | (2+) | (3+) |
| Non-GCB | 8 (22) | 17 (46) | 10 (27) | 2 (5) |
| GCB | 5 (38) | 3 (23) | 4 (31) | 1 (8)   n (%) |

Figure 1. VEGF-A expression in DLBCL. (a) VEGF-A expression of DLBCL cells in the lymph node lesions according to GCB or non-GCB phenotypes. VEGF-A expression was categorized based on the percentage of DLBCL cells stained as follows: ⩾50%, 3+ positive; 30–49%, 2+ positive; 10–29%, 1+ positive; <10%, negative. (b) Cases 1, 2, 3 and 4 are representative of VEGF-A negative, 1+, 2+ and 3+ positive categories, respectively. Photomicrographs with hematoxylin and eosin (HE; upper panels) and VEGF-A staining (lower panels) are shown.

Immunopathological features of four cases from each group stratified by VEGF-A expression are shown in Figure 1b. Differences in VEGF-A expression levels between the two DLBCL groups (GCB versus non-GCB) did not achieve significance (Fisher's exact test).

Establishment of the primary DLBCL cell-bearing NOG mouse model

The macroscopic appearance of a primary DLBCL cell-bearing NOG mouse is shown in Figure 2a, demarcating the i.p. mass and splenomegaly by thin white dotted lines. Flow cytometric analysis demonstrated that the mass mainly consisted of human cells expressing CD19 and CD25 (Figure 2b). Immunopathological analysis revealed that it consisted of large atypical cells with irregular and pleomorphic nuclei, and blood vessels. The cells were CD20+, but CD3-negative (Figure 2c). These findings are consistent with DLBCL. The cells were in addition positive for CD25 and negative for EBER (data not shown). VEGF expression was 1+ positive. The DLBCL cells were also positive for MUM1/ IRF4, but negative for CD10 and BCL-6 (data not shown), and were thus classified as non-GCB phenotype. These immuno-pathological findings on the NOG DLBCL cells were identical to those of the donor DLBCL.

Blood vessels in the tumor tissue were stained by anti-α-SMA Ab (Figure 2c). Vascular endothelial cells in the tumor tissue were stained by anti-von Willebrand Factor Ab, but not by anti-CD31 mAb (data not shown). These results indicated that blood vessels in the tumor originated from the mouse, because anti-α-SMA and von Willebrand Factor Ab used in the present study recognized the corresponding protein derived from both human and mice, whereas the anti-CD31 mAb recognized the corresponding human but not murine protein (data not shown).

DLBCL cell infiltration into spleen, liver and bone marrow was seen both by flow cytometry (Figure 2d, upper panels) and pathological analyses (Figure 2d, lower panels).

The tumor cells recovered from mice receiving the primary lymphoma cells were serially i.p. transplanted into other NOG mice. This procedure of transfer from mouse to mouse was repeated successfully until at least the fifth passage. The

macroscopic features of the animals and the immunopathological findings for the tumor changed little through these serial passages. We could passage tumor cells that had been kept frozen until use, as well as those freshly isolated (data not shown). In contrast, these DLBCL cells could not be maintained in vitro in culture (data not shown).

VEGF-A, VEGF-R1 and -R2 expression in DLBCL cell lines

VEGF-A mRNA expression was detected in all eight DLBCL cell lines tested and in NOG DLBCL cells from i.p. masses (Figure 3a, left panel). VEGF-R1 mRNA expression was present only in two (OCI-Ly19 and Toledo) of the DLBCL cell lines and in the NOG DLBCL cells (Figure 3a, right panel). No VEGF-R2 mRNA expression was detected in any of the eight DLBCL cell lines tested, or in the NOG DLBCL cells (data not shown). Flow cytometry demonstrated that VEGF-R1 protein was also expressed in the two lines with mRNA (OCI-Ly19 and Toledo, Figure 3b), consistent with the results from reverse transcription PCR. VEGF-R1 expression in NOG DLBCL cells as assessed by flow cytometry was very weak (Figure 3b) and VEGF-R2 was not expressed at all in any of the DLBCL cell lines tested, or in NOG DLBCL cells (data not shown), also consistent with the reverse transcription PCR results.

Bevacizumab-mediated anti-proliferative activity against DLBCL cells in vitro

Bevacizumab did not directly block the proliferation of OCI-Ly19 and Toledo cells in vitro, despite their expression of both VEGF-A and VEGF-R1 (Figure 3c, upper panels). Neither did it inhibit NOG DLBCL cells, with or without the addition of interleukin-2 (Figure 3c, lower panels).

CHOP + bevacizumab has significantly greater therapeutic efficacy than CHOP alone in primary DLBCL cell-bearing NOG mice

Treatment with CHOP + bevacizumab resulted in an increased percentage of tumor necrosis in the primary DLBCL cell-bearing NOG mice (mean 12.7%, median 11.1%, range 5.2–18.7%), compared with CHOP alone (mean 1.8%, median 1.5%, range 1.0–2.7%, P = 0.0090; Figure 4a, left panel). An example of
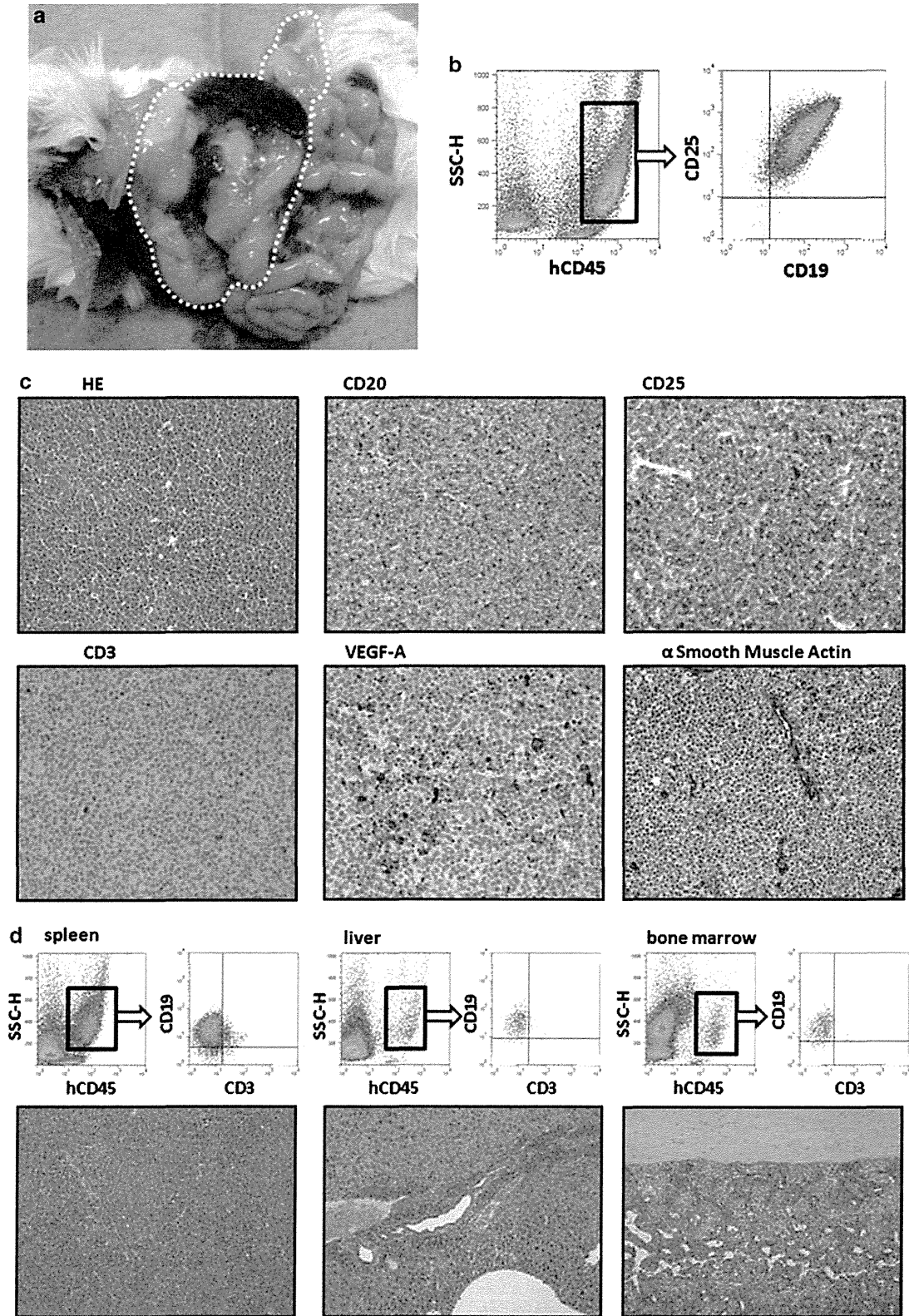
**Figure 2.** Primary DLBCL cell-bearing NOG mouse model. (a) Macroscopic appearance of a primary DLBCL cell-bearing NOG mouse. The intraperitoneal mass is demarcated by a thin white dotted line. (b) Human CD45 + cells in the mass determined by human CD19 and CD25 expression. (c) Immunohistochemical images of the intraperitoneal mass. (d) Human CD45 + cells of each organ determined by human CD3 and CD19 expression (upper panels). Photomicrographs with hematoxylin and eosin (HE) staining of each organ (lower panels).

calculating the percentage necrotic area is presented in Figure 4a, right panels. CHOP + bevacizumab treatment resulted in decreased vasculature in the tumor tissues (41.9, 40.9, 32.5–51.3/mm$^2$; (mean, median, range)), compared with CHOP alone (66.3, 71.8, 40.7–79.5/mm$^2$, $P = 0.0472$; Figure 4b, left panel). An example of this calculation is presented in Figure 4b, right-hand panels.
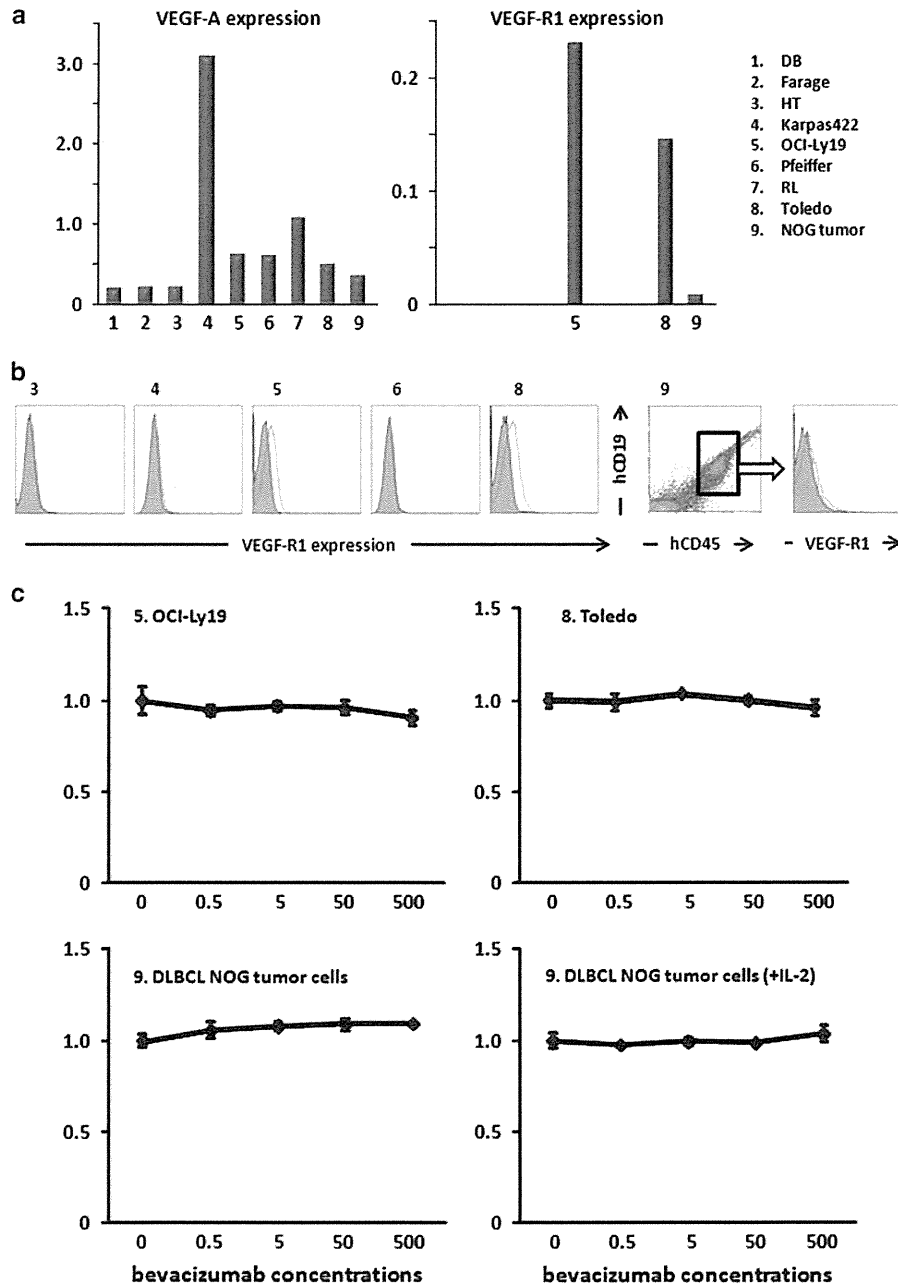
**Figure 3.** VEGF-A, VEGF-R1 and VEGF-R2 expression in DLBCL cell lines. (a) Quantitative reverse transcription (RT)-PCR analysis for VEGF-A and VEGF-R1 in eight DLBCL cell lines, and NOG DLBCL cells from the intraperitoneal mass. (b) Flow cytometry for VEGF-R1 in DLBCL cell lines, and NOG DLBCL cells from the intraperitoneal mass. (c) Bevacizumab has no direct anti-proliferative activity against DLBCL cell lines (OCI-Ly19 and Toledo) expressing both VEGF-A and VEGF-R1 (upper panels), and NOG DLBCL cells (lower panels), *in vitro*. Each result represents three independent experiments.

As sIL2R appears in the serum, concomitant with its increased expression on cells,[22] we measured human sIL2R concentrations as a surrogate marker reflecting the tumor burden of the human CD25-expressing DLBCL. Treatment with CHOP + bevacizumab showed significantly greater therapeutic efficacy as demonstrated by sIL2R concentrations in the primary DLBCL cell-bearing NOG mice (44.6, 46.1, 28.5–59.2 × $10^3$ pg/ml), compared with CHOP alone (83.5, 78.1, 49.5–119.3 × $10^3$ pg/ml, $P = 0.0283$; Figure 4c).

The percentages of DLBCL cells in spleen cell suspensions of CHOP and CHOP + bevacizumab-treated mice were 14.1%, 11.4%, 10.2–20.7%, and 26.1%, 24.6% and 19.0–34.6%, respectively. This difference was statistically significant ($P = 0.0163$; Figure 3d, left panel). An example of the calculation is shown in Figure 4d, right panels.

**Macroscopic and microscopic findings in mice with or without bevacizumab therapy**

The appearance of primary DLBCL cell-bearing control mice (treated with saline) or those treated with bevacizumab alone is shown in Figure 5a, upper and lower panels, respectively. Tumor masses are demarcated by thin white dotted lines. Photomicrographs of tumor tissue from each mouse are also shown (Figure 5b).
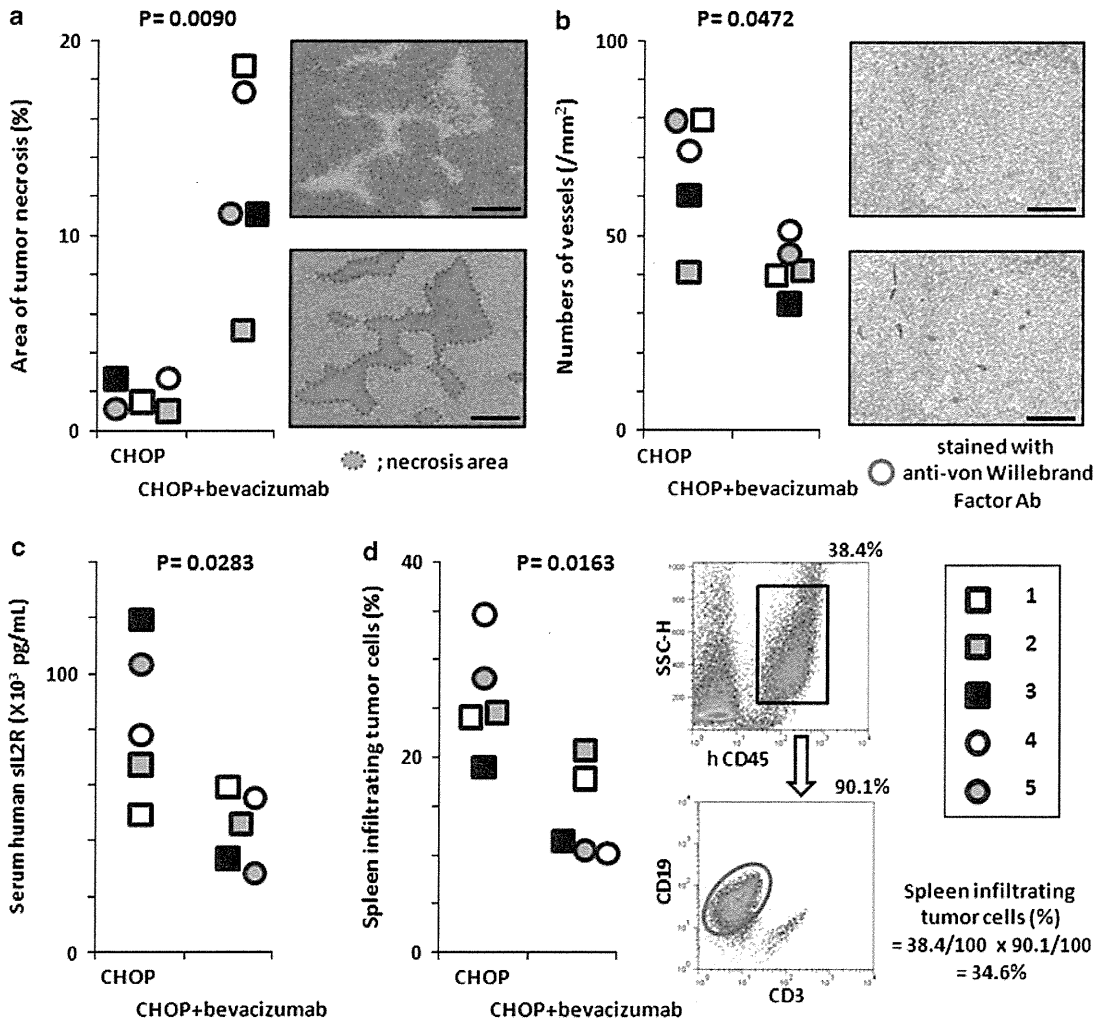
**Figure 4.** CHOP + bevacizumab has greater therapeutic efficacy than CHOP alone. (a) Area of tumor necrosis (%) of each primary DLBCL-bearing NOG mouse. The CHOP + bevacizumab-treated mice had significantly greater tumor necrosis than CHOP-treated mice (left panel). An example of a calculation for tumor necrosis area (%) by means of Image J software is shown (scale bar, 200 μm; right panels). (b) Numbers of vessels (per mm$^2$) of each primary DLBCL-bearing NOG mouse. The CHOP + bevacizumab recipients had significantly fewer than CHOP recipients (left panel). An example of such a calculation by means of Image J software is shown (scale bar, 200 μm; right panels). (c) Serum sIL2R concentrations of each primary DLBCL-bearing NOG mouse. The CHOP + bevacizumab recipients had significantly lower levels of sIL2R than CHOP recipients. (d) Spleen-infiltrating tumor cells (%) of each primary DLBCL-bearing NOG mouse. The CHOP + bevacizumab recipients had significantly lower levels of spleen-infiltrating tumor cells than CHOP recipients (left panel). An example of a calculation of spleen-infiltrating tumor cells (%) is shown (right panels).

## Bevacizumab therapy alone has significant therapeutic efficacy in primary DLBCL cell-bearing NOG mice.

Treatment with bevacizumab alone significantly increased the percentage tumor necrotic area (7.5, 4.8, 2.1–18.7%) compared with control mice (2.0, 1.7, 0.1–5.6%, $P = 0.0070$; Figure 6a). This was also the case when considering vascularization of the tumor tissues (46.2, 43.1, 33.6–60.0/mm$^2$, compared with 66.7, 64.8, 50.1–99.9/mm$^2$ in controls, $P = 0.0070$; Figure 6b). Treatment with bevacizumab showed significantly greater therapeutic efficacy as demonstrated by sIL2R concentrations in the primary DLBCL cell-bearing NOG mice (187.6, 185.2, 5.0–350.8 × 10$^3$ pg/ml), compared with controls (459.6, 482.8, 201.5–689.5 × 10$^3$ pg/ml, $P = 0.0041$; Figure 6c).

The percentages of DLBCL cells in spleen cell suspensions of bevacizumab- and saline-treated mice were 13.1, 14.6, 0.1–27.5% and 18.7%, 18.8%, 4.0–31.7%, respectively, but this difference was not statistically significant (data not shown).

## DISCUSSION

In the present study, we have achieved two goals: first, to establish a novel mouse model using NOG recipients engrafted with primary DLBCL cells from a patient, in which the tumor cells survive and proliferate in a murine microenvironment-dependent manner; second, to document that bevacizumab possesses significant therapeutic efficacy in these primary DLBCL cell-bearing mice.

NOG mice have severe, multiple immune dysfunctions, such that human immune cells engrafted into them retain essentially the same functions as in humans.[23,24] In the present system, primary DLBCL cells expressing CD19, CD20 and CD25 formed large i.p. masses, and markedly infiltrated into different organs such as spleen, liver and bone marrow. The presented features were very similar to the donor DLBCL patient. The lymphoma cells were positive for VEGF-A and therefore it would be expected that the interaction of VEGF-A produced by tumor cells with VEGF-R2
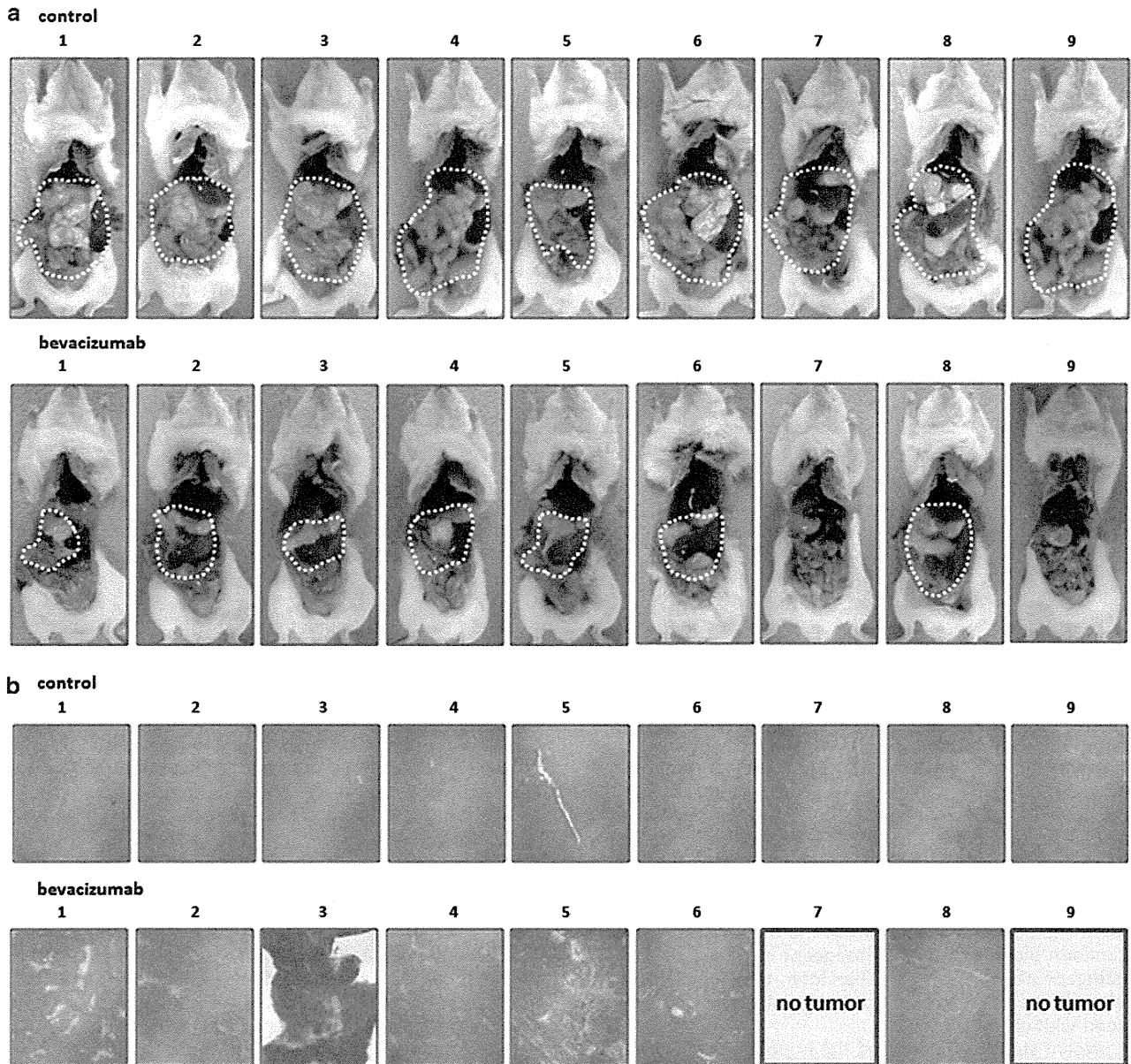
**Figure 5.** Macroscopic and microscopic findings of mice with or without bevacizumab therapy. (a) Macroscopic appearance of mice treated with saline (control; upper panels) or bevacizumab (lower panels). Tumor masses are demarcated by thin white dotted lines. (b) Photomicrographs with hematoxylin and eosin (HE) staining of saline (control; upper panels) or bevacizumab-treated tumor (lower panels).

on host (mice) endothelial cells should have an important role in tumor angiogenesis, leading to tumor cell survival and proliferation supported by receiving sufficient nutrients and oxygen, as reported by other investigators.[25–27] To the best of our knowledge, this is the first report of primary DLBCL cell-bearing mice, in which the DLBCL cells can be maintained by serial transplantations, but cannot be maintained in vitro in culture. This indicates that the microenvironment is indispensable for tumor survival; thus the present DLBCL model should better reflect the human DLBCL in vivo environment, compared with other mouse models using established tumor cell lines. Therefore, this model should provide a powerful tool for understanding the pathogenesis of DLBCL and, furthermore, for the one which can be used not only to evaluate novel cytotoxic anti-DLBCL cell agents, but also antitumor agents targeting the microenvironment, including bevacizumab, more appropriately,

in vivo. The observed significant antitumor activities of bevacizumab combined with CHOP therapy were expected, because bevacizumab is known only to be of benefit to patients with metastatic colorectal, non-small cell lung and metastatic breast cancer, when combined with chemotherapy.[8–10] The effect observed in mice receiving bevacizumab + CHOP, as demonstrated by the increased tumor necrosis area and reduced vasculature in the tumor tissue, was consistent with the conventional antitumor mechanism of bevacizumab, which neutralizes the human VEGF-A produced by the tumor cells, but not murine VEGF-A.[28] It then inhibits the growth of new blood vessels and thus starves tumor cells of necessary nutrients and oxygen.[29] This should lead to a reduced tumor burden, as indicated by the sIL2R concentrations measured. It was also reported that lymphoma cell growth was promoted in an autocrine manner via VEGF-A/VEGF-R1 or VEGF-A/VEGF-R2
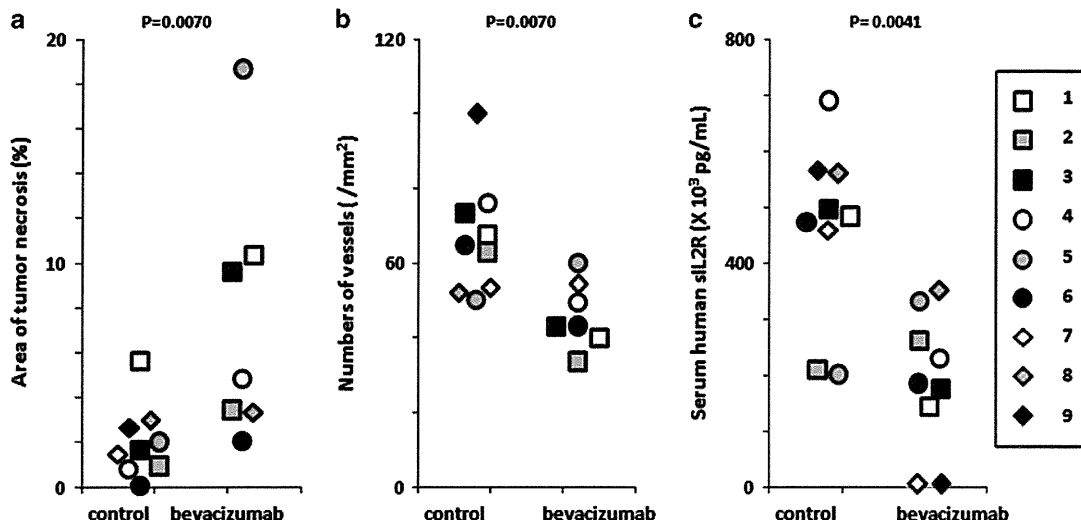
**Figure 6.** Bevacizumab therapy has significant therapeutic efficacy in the DLBCL mice. (a) Area of tumor necrosis (%) of each primary DLBCL-bearing NOG mouse. The bevacizumab-treated mice had significantly more tumor necrosis than controls. (b) Vessel numbers (per $mm^2$) of each primary DLBCL-bearing NOG mouse. The bevacizumab recipients had significantly fewer vessel numbers than controls. (c) Serum sIL2R concentrations of each primary DLBCL-bearing NOG mouse. The bevacizumab recipients had significantly lower levels of sIL2R than controls.

interactions,[25] but the present in vitro data did not support that observation. In the present study, significant effects of bevacizumab alone were also observed, as demonstrated by the increased area of tumor necrosis and reduced vasculature in the tumor tissue, leading to a degree of antitumor therapeutic efficacy as demonstrated by reduced tumor burden indicated by serum sIL2R concentrations. In contrast to combination therapy, bevacizumab alone was not active when assessed by the percentages of DLBCL cells in the spleen. One possible explanation for this is that the spleen is likely to be more richly vascularized compared with the tumor mass, and thus bevacizumab alone has little starvation effect on the tumor cells therein. It has been reported that VEGF-targeted therapy can 'normalize' the tumor vascular network and that this can lead to a more uniform blood-flow, with subsequent increased delivery of chemotherapeutic agents.[30–32] Therefore, the tumor cells in spleen might be efficiently reduced only when bevacizumab is combined with chemotherapy.

The present study demonstrated the importance of angiogenesis for the pathogenesis of VEGF-expressing DLBCL. Ganjoo et al.[33] reported that VEGF expression was detected in 42–60% of tumor cells in DLBCL and Gratzinger et al.[34] found that 60% of cases showed strong VEGF immunoreactivity, defined as VEGF expression in >30% of the tumor cells. These reports together with our present study indicate that targeting angiogenesis would be a promising strategy for at least a subgroup of DLBCL patients whose tumors depend to a large extent on angiogenesis via VEGF for survival and proliferation. In fact, bevacizumab as a single agent has been reported to have modest clinical activity in patients in the setting of relapsed aggressive non-Hodgkin lymphoma[35] and in combination with rituximab-CHOP in first-line treatment.[33] However, a phase III clinical study evaluating the efficacy and safety of bevacizumab together with rituximab plus CHOP in patients with DLBCL (MAIN trial) could not be completed after a safety and efficacy analysis of the first 720 patients. We believe that this result of the MAIN trial does not necessarily have to lead to the conclusion that bevacizumab is ineffective in DLBCL. Analogously, the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib, failed to yield a significantly improved overall survival in patients with refractory non-small cell lung cancer,[36] but did show therapeutic benefit in a subgroup of patients with mutated epidermal growth factor receptor.[37–39] In the case of

mAb targeting the epidermal growth factor receptor, both panitumumab and cetuximab also provide clinical benefits only to a subgroup of colorectal cancer patients with wild-type KRAS and BRAF.[40] These findings indicate that we should develop novel treatment strategies based on tumor biology and not on tumor category. DLBCL is a highly heterogeneous category with respect to biology, morphology and clinical presentation,[16] as are non-small cell lung cancer or colorectal cancer. Therefore, further investigations are warranted to determine which subgroups of DLBCL patients will benefit from bevacizumab therapy.

In conclusion, using NOG mice as recipients, we have established a novel model in which primary DLBCL cells from a patient engraft and proliferate in a murine microenvironment-dependent manner. The present DLBCL model should more truly reproduce the human DLBCL in vivo environment, compared with any other current models, which use established tumor cell lines. This is the first report to evaluate the efficacy of bevacizumab in such a tumor microenvironment-dependent model. Bevacizumab therapy could be a potential treatment strategy for that subgroup of DLBCL depending to a large extent on angiogenesis via VEGF for tumor survival and proliferation.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

## REFERENCES

1 Sautès-Fridman C, Cherfils-Vicini J, Damotte D, Fisson S, Fridman WH, Cremer I et al. Tumor microenvironment is multifaceted. Cancer Metastasis Rev 2011; 30: 13–25.

2 Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007; 6: 273–286.

3 Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993; 362: 841–844.

4 Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 2004; 3: 391–400.

5 Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K et al. NOD/SCID/ γcnull mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002; 100: 3175–3182.

6 Ito M, Kobayashi K, Nakahata T. NOD/Shi-scid IL2rγnull (NOG) mice more appropriate for humanized mouse models. Curr Top Microbiol Immunol 2008; 324: 53–76.

7 Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med 2003; 349: 427–434.

8 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350: 2335–2342.

9 Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 2006; 355: 2542–2550.

10 Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med 2007; 357: 2666–2676.

11 Vredenburgh JJ, Desjardins A, Herndon 2nd JE, Marcello J, Reardon DA, Quinn JA et al. Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. J Clin Oncol 2007; 25: 4722–4729.

12 Saltz LB, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. J Clin Oncol 2008; 26: 2013–2019.

13 Hochster HS, Hart LL, Ramanathan RK, Childs BH, Hainsworth JD, Cohn AL et al. Safety and efficacy of oxaliplatin and fluoropyrimidine regimens with or without bevacizumab as first-line treatment of metastatic colorectal cancer: results of the TREE Study. J Clin Oncol 2008; 26: 3523–3529.

14 Reck M, von Pawel J, Zatloukal P, Ramlau R, Gorbounova V, Hirsh V et al. Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAiL. J Clin Oncol 2009; 27: 1227–1234.

15 Miles DW, Chan A, Dirix LY, Cortés J, Pivot X, Tomczak P et al. Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol 2010; 28: 3239–3247.

16 Nogai H, Dörken B, Lenz G. Pathogenesis of non-Hodgkin's lymphoma. J Clin Oncol 2011; 29: 1803–1811.

17 A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. Blood 1997; 89: 3909–3918.

18 Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004; 103: 275–282.

19 Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics Int 2004; 11: 36–42.

20 Ishida T, Iida S, Akatsuka Y, Ishii T, Miyazaki M, Komatsu H et al. The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-Cell leukemia/lymphoma. Clin Cancer Res 2004; 10: 7529–7539.

21 Mohammad RM, Wall NR, Dutcher JA, Al-Katib AM. The addition of bryostatin 1 to cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy improves response in a CHOP-resistant human diffuse large cell lymphoma xenograft model. Clin Cancer Res 2000; 6: 4950–4956.

22 Waldmann TA. The IL-2/IL-2 receptor system: a target for rational immune intervention. Immunol Today 1993; 14: 264–270.

23 Ito A, Ishida T, Yano H, Inagaki A, Suzuki S, Sato F et al. Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2Rgamma(null) mouse model. Cancer Immunol Immunother 2009; 58: 1195–1206.

24 Ito A, Ishida T, Utsunomiya A, Sato F, Mori F, Yano H et al. Defucosylated anti-CCR4 monoclonal antibody exerts potent ADCC against primary ATLL cells mediated by autologous human immune cells in NOD/Shi-scid, IL-2R gamma(null) mice in vivo. J Immunol 2009; 183: 4782–4791.

25 Wang ES, Teruya-Feldstein J, Wu Y, Zhu Z, Hicklin DJ, Moore MA. Targeting autocrine and paracrine VEGF receptor pathways inhibits human lymphoma xenografts in vivo. Blood 2004; 104: 2893–2902.

26 Borgström P, Hillan KJ, Sriramarao P, Ferrara N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res 1996; 56: 4032–4039.

27 Ruan J, Hajjar K, Rafii S, Leonard JP. Angiogenesis and antiangiogenic therapy in non-Hodgkin's lymphoma. Ann Oncol 2009; 20: 413–424.

28 Yu L, Wu X, Cheng Z, Lee CV, LeCouter J, Campa C et al. Interaction between bevacizumab and murine VEGF-A: a reassessment. Invest Ophthalmol Vis Sci 2008; 49: 522–527.

29 Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer 2008; 8: 579–591.

30 Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005; 307: 58–62.

31 Gerber HP, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 2005; 65: 671–680.

32 Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov 2011; 10: 417–427.

33 Ganjoo KN, An CS, Robertson MJ, Gordon LI, Sen JA, Weisenbach J et al. Rituximab, Bevacizumab and CHOP (RA-CHOP) in untreated diffuse large B-cell lymphoma: safety, biomarker and pharmacokinetic analysis. Leuk Lymphoma 2006; 47: 998–1005.

34 Gratzinger D, Zhao S, Marinelli RJ, Kapp AV, Tibshirani RJ, Hammer AS et al. Microvessel density and expression of vascular endothelial growth factor and its receptors in diffuse large B-cell lymphoma subtypes. Am J Pathol 2007; 170: 1362–1369.

35 Stopeck AT, Unger JM, Rimsza LM, Bellamy WT, Iannone M, Persky DO et al. A phase II trial of single agent bevacizumab in patients with relapsed, aggressive non-Hodgkin lymphoma: Southwest oncology group study S0108. Leuk Lymphoma 2009; 50: 728–735.

36 Thatcher N, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, von Pawel J et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced nonsmall-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). Lancet 2005; 366: 1527–1537.

37 Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010; 362: 2380–2388.

38 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 2004; 350: 2129–2139.

39 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304: 1497–1500.

40 Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol 2010; 28: 1254–1261.

IMMUNOBIOLOGY

# Cancer/testis antigens are novel targets of immunotherapy for adult T-cell leukemia/lymphoma

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Adult T-cell leukemia/lymphoma (ATLL) is an intractable hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1), which infects approximately 20 million people worldwide. Here, we have explored the possible expression of cancer/testis (CT) antigens by ATLL cells, as CT antigens are widely recognized as ideal targets of cancer immunotherapy against solid tumors. A high percentage (87.7%) of ATLL cases (n = 57) expressed CT antigens at the mRNA level: NY-ESO-1 (61.4%), MAGE-A3 (31.6%), and MAGE-A4 (61.4%). CT antigen expression was confirmed by immunohistochemistry. This contrasts with other types of lymphoma or leukemia, which scarcely express these CT antigens. Humoral immune responses, particularly against NY-ESO-1, were detected in 11.6% (5 of 43) and NY-ESO-1–specific CD8^{+} T-cell responses were observed in 55.6% (5 of 9) of ATLL patients. NY-ESO-1–specific CD8^{+} T cells recognized autologous ATLL cells and produced effector cytokines. Thus, ATLL cells characteristically express CT antigens and therefore vaccination with CT antigens can be an effective immunotherapy of ATLL. (*Blood*. 2012;119(13): 3097-3104)

## Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1).[1,2] HTLV-1 is endemic in southwestern Japan, Africa, South America, and the Caribbean Islands, and approximately 20 million people worldwide are infected.[3] A total of 5% of the infected persons develop ATLL after a long latency period.[2] ATLL cells are CD4-positive; and the majority, if not all, of them express the transcription factor FoxP3 (Forkhead Box P3), CD25, CTLA-4, and CCR4 (CC chemokine receptor 4), and are functionally immunosuppressive, thus phenotypically and functionally resembling naturally occurring regulatory T cells (Tregs).[3-9] Because of its immunosuppressive property and resistance to conventional chemotherapy, aggressive ATLL has a poor prognosis with a mean survival time of less than 1 year.[2,8] A recent phase 3 trial of a dose-intensified multidrug chemotherapy for untreated ATLL patients (acute, lymphoma, and unfavorable chronic types) showed a median progression-free and overall survival of only 7.0 and 12.7 months, respectively.[10] This and other reports indicate that chemotherapy alone is of limited success for ATLL and mostly fails to cure the disease.[10,11] Allogeneic hematopoietic stem cell transplantation has been introduced over the past decade as a potential therapy for ATLL with a long-term remission in only a small fraction of patients who are young, well controlled in disease progression, and have an appropriate stem cell source.[12] More effective strategies to treat ATLL are therefore required.

Several HTLV-1 components have been explored as targets for immunotherapy of ATLL. HTLV-1 Tax, which is crucial for ATLL oncogenesis, has generally been considered to be a main target of the host's cellular immune responses. Yet, the frequency of Tax expression in HTLV-1 infected cells reduces in the course of disease progression, and *Tax* transcripts are detected only in approximately 40% of the established ATLL cases,[13] thus limiting Tax-targeted immunotherapy to a subset of patients. HBZ (HTLV-1 bZIP factor), another HTLV-1 component, which contains an N-terminal transcriptional activation domain and a leucine zipper motif at its C-terminal, also plays an important role in the proliferation of ATLL cells and is detectable in almost all ATLL cases.[2] However, CD8^{+} T cells specific for HBZ could only recognize peptide-pulsed target cells but not ATLL cells themselves.[14] Furthermore, HTLV-1 is transmitted mainly from mothers to infants through breast milk, and such vertical infection in early life may induce tolerance to the virus and result in insufficient HTLV-1–specific T-cell responses.[15,16] For these reasons, targeting the HTLV-1 components alone may be insufficient for successful immunotherapy of ATLL, necessitating identification of novel tumor-associated target antigens for the immunotherapy.

The expression of cancer/testis (CT) antigens, of which more than 100 have been identified so far, is normally limited to human germ line cells in the testis and in various types of human cancers.[17,18] This restricted expression pattern in normal tissues makes them ideal cancer antigens for tumor immunotherapy.
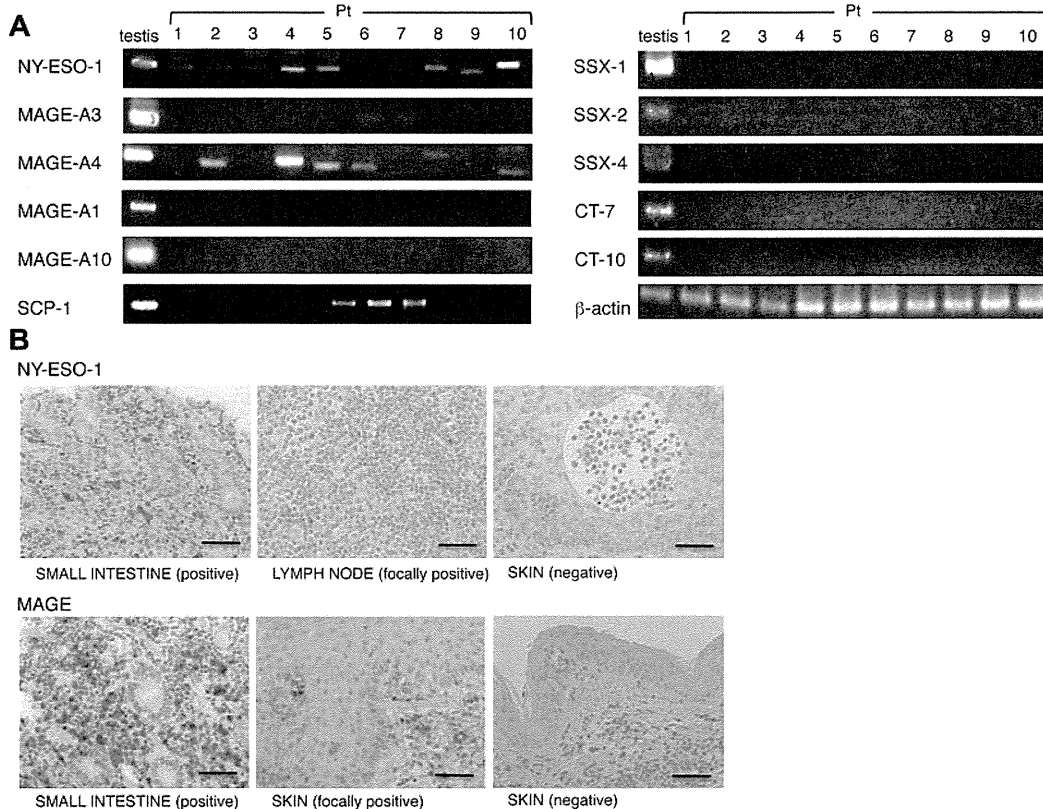
— 48 —

**Figure 1. NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed by primary ATLL cells.** (A) Representative results of RT-PCR analysis for mRNA expression of. NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4, and SCP-1. (B) ATLL samples were subjected to immunohistochemical staining for NY-ESO-1 (E978) mAb and pan-MAGE (57B) mAb. Bar represents 50 μm. These experiments were performed independently at least twice with similar results.

NY-ESO-1 and MAGE family antigens, a subset of CT antigens, are indeed able to elicit spontaneous humoral and cellular immune responses in cancer patients.[17-19] Clinical trials of CT antigen vaccination are currently under way with several types of vaccine formulation, including peptide, protein, and DNA, and some of the treated patients have experienced clinical benefits from vaccination.[19,20] This promising result of CT antigens as a target of tumor immunotherapy has prompted intensive studies of their expression in a wide range of human cancers. However, detailed analysis of CT antigen expression in hematologic disorders has been limited.[21,22] In the present study, we have investigated possible expression of CT antigens by ATLL cells and possible humoral and cellular immune responses against CT antigens in ATLL patients.

## Methods

### Primary ATLL cells and peripheral blood

Blood or lymph node samples were obtained from ATLL patients, and mononuclear cells were isolated with Ficoll-Paque. Diagnosis and classification of clinical subtypes of ATLL were according to the criteria proposed by the Japan Lymphoma Study Group.[23] All donors provided informed written consent before sampling according to the Declaration of Helsinki, and the present study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences, Osaka University, and Imamura Bun-in Hospital.

### ATLL cell lines

ATN-1, ATL102, HUT102, MT-2, and MT-1 were previously described.[24,25] TL-Om1 and TL-Su were kindly provided by the Cell Resource Center for

Biomedical Research, Tohoku University (Sendai, Japan). MT-4 was purchased from the Health Science Research Resources Bank (Osaka, Japan). MJ was purchased from ATCC. TCL-Kan was kindly provided by Professor M. Kannagi (Tokyo Medical and Dental University, Tokyo, Japan).

### Reagents

Phycoerythrin-conjugated anti–IFN-γ (4S.B3) mAb and phycoerythrin-cyanine7–conjugated anti–TNF-α (MAb11) mAb were purchased from eBioscience. Fluorescein isothiocyanate-conjugated anti–IL-2 (MQ1-17H12) mAb and allophycocyanin-cyanine7–conjugated anti-CD8 (SK1) mAb were purchased from BD Biosciences. Anti–NY-ESO-1 mAb (E978, mouse IgG1)[26] and pan-MAGE mAb (57B, mouse IgG1)[27] were purified from hybridoma supernatant by protein G affinity chromatography. Synthetic peptides of NY-ESO-1$_{1-20}$ (MQAEGRGTGGSTGDADGPGG), NY-ESO-1$_{11-30}$ (STGDADGPGGPGIPDGPGGN), NY-ESO-1$_{21-40}$ (PGIPDGPG-GNAGGPGEAGAT), NY-ESO-1$_{31-50}$ (AGGPGEAGATGGRGPRGAGA), NY-ESO-1$_{41-60}$ (GGRGPRGAGAARASGPGGGA), NY-ESO-1$_{51-70}$ (ARASGPGGGAPRGPHGGAAS), NY-ESO-1$_{61-80}$ (PRGPHGGAASGL-NGCCRCGA), NY-ESO-1$_{71-90}$ (GLNGCCRCGARGPESRLLEF), NY-ESO-1$_{81-100}$ (RGPESRLLEFYLAMPFATPM), NY-ESO-1$_{91-110}$ (YLAMP-FATPMEAELARRSLA), NY-ESO-1$_{101-120}$ (EAELARRSLAQDAPPLP-VPG), NY-ESO-1$_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-1$_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-1$_{131-150}$ (NILTIRLTA-ADHRQLQLSIS), NY-ESO-1$_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-1$_{151-170}$ (SCLQQLSLLMWITQCFLPVF), and NY-ESO-1$_{161-180}$ (WITQCFLPVFLAQPPSGQRR) were obtained from Invitrogen.

### RT-PCR

Total RNA was isolated with RNeasy Mini Kit (QIAGEN). cDNA was synthesized from 0.1 μg of total RNA using SuperScript III reverse

**Table 1. Summary of CT antigen expression in ATLL cells from primary ATLL patients**

| | No. of patients | NY-ESO-1 | MAGE | |
| --- | --- | --- | --- | --- |
| | | | A3 | A4 |
| **Age (mean, 60.6 y)** | | | | |
| ≥ 60 y | 29 | 20 | 12 | 20 |
| < 60 y | 28 | 15 | 6 | 15 |
| **Sex** | | | | |
| Male | 30 | 17 | 8 | 17 |
| Female | 27 | 18 | 10 | 18 |
| **Disease type** | | | | |
| Aggressive | 40 | 25 (62.5%) | 14 (35.0%) | 26 (65.0%) |
| Acute | 37 | 22 | 13 | 24 |
| Lymphoma | 3 | 3 | 1 | 2 |
| Indolent | 17 | 9 (52.9%) | 4 (23.5%) | 9 (52.9%) |
| Chronic | 11 | 7 | 2 | 8 |
| Smoldering | 6 | 3 | 2 | 1 |

mRNA expression of NY-ESO-1, MAGE-A3, and MAGE-A4 in ATLL cells from primary ATLL patients was analyzed with RT-PCR. CT antigen expression was summarized based on several clinical parameters.

transcriptase kit (Invitrogen) and the Oligo (dT) primer in a total volume of 20 μL. cDNA was amplified in a final volume of 20 μL containing 10μM of each CT antigen primer as reported,[21,28] except NY-ESO-1 (sense, 5′-AGT TCT ACC TCG CCA TGC CT-3′; antisense, 5′- TCC TCC TCC AGC GAC AAA CAA-3′) and 0.2 μL of Ex-Taq polymerase (Takara Bio) according to the instructions provided by the manufacturer.

### ELISA

Patients' sera were analyzed by ELISA for seroreactivity to bacterially produced recombinant proteins NY-ESO-1/CTAG1B, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT7/MAGE-C1, CT10/MAGE-C2, SSX1, SSX2, and SSX4.[29] Sera were diluted serially from 1/100 to 1/100 000 and added to low-volume 96-well plates (Corning Life Sciences) coated overnight at 4°C with 1 μg/mL antigen in 25 μL volume and blocked for 2 hours at room temperature with PBS containing 5% nonfat milk. After overnight incubation, plates were extensively washed and rinsed with PBS. Antigen-specific IgG was detected with specific mAb conjugated with alkaline phosphatase (Southern Biotechnology). After addition of AT-TOPHOS substrate (Fisher Scientific), absorbance was measured using a fluorescence reader Cytofluor Series 4000 (PerSeptive Biosystems). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. This value was extrapolated by determining the intersection of a linear trend regression with a cutoff value. The cutoff was defined as 10 times the average of optical density (OD) values from the first 4 dilutions of a negative control pool made of 5 healthy donor sera. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls.

### Immunohistochemistry

All tissue specimens were fixed with formalin and embedded in paraffin. Tissue sections of 3 μm thickness on charged glass slides were deparaffinized and rehydrated. Antigen retrieval was performed by autoclave (105°C for 20 minutes) using Tris-EDTA buffer (pH 9.0) as heating solution. Primary mAb for NY-ESO-1 (E978) and pan-MAGE protein (57B) were used at concentrations of 5 μg/mL and 2 μg/mL, respectively. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and 1% sodium azide. Simple stain max-PO (Multi; Nichirei) was used for secondary detection; 3,3′-diamino-benzidine was used as chromogen. Human testicular tissue served as positive control. All histological pictures were captured by NIKON Eclipse microscope and NIKON Image software system (Version 3.22).

### In vitro sensitization

NY-ESO-1–specific CD8+ and CD4+ T cells were presensitized as described previously.[30,31] Briefly, CD8+ T cells and CD4+ T cells were

isolated from peripheral blood mononuclear cells (PBMCs) using a CD8 Microbeads and a CD4+ T cell Isolation Kit, respectively (Miltenyi Biotec). The purity of isolated populations was confirmed to be more than 90%. The non-CD8+/CD4+ cell population was pulsed with 10μM of pooled peptides overnight and was used as antigen-presenting cells (APCs). After irradiation, 5 to 10 × 10⁵ APCs were added to round-bottom 96-well plates (Corning Life Sciences) containing 1 to 5 × 10⁵ CD8+ or CD4+ T cells and were fed with IL-2 (10 U/mL; Roche Diagnostics) and IL-7 (20 ng/mL; R&D Systems). Subsequently, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/mL) and IL-7 (40 ng/mL) twice per week.

### Intracellular cytokine staining

CD8+ T cells from PBMCs from ATLL patients were presensitized for 10 to 18 days. These presensitized CD8+ T cells were restimulated for 6 hours with peptide-pulsed T-APCs,[31] and GolgiStop reagent (BD Biosciences) was added 1 hour later. Cells were stained for cell surface markers and for intracellular cytokines, such as IFN-γ and TNF-α after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences) and FlowJo Version 7.6.5 software for Macintosh (TreeStar).

### Tetramer assay

Tetramer staining was performed as previously described.[31] Briefly, presensitized CD8+ T cells were stained with phycoerythrin-labeled tetramers (prepared by Drs P. Guillaume and I. Luescher at the Ludwig Institute Core Facility, Lausanne, Switzerland) for 15 minutes at 37°C before additional staining with allophycocyanin-cyanine7–conjugated anti-CD8 mAb for 15 minutes at 4°C. After washing, results were analyzed by FACSCanto and FlowJo Version 7.6.5 software.

### Statistical analysis

The significance of the difference in each CT antigen expression between 2 groups was assessed by Fisher exact test. P values less than .05 were considered significant.

## Results

### A subset of CT antigens is highly expressed in ATLL cell lines

To examine possible expression of 11 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4, and SCP-1) by ATLL cells, we assessed mRNA expression by RT-PCR in 10 ATLL cell lines (MT-1, MT-2, MT-4, MJ, ATL102, ATN-1, TL-Om-1, TL-Su, TCL-kan, and HUT102). In sharp contrast to a previous report showing that the majority of T-cell lymphomas did not express CT antigens except SCP-1,[21] a high percentage of ATLL cell lines expressed NY-ESO-1, MAGE-A3, and MAGE-A4 (90%, 50% and 70%, respectively) in the present study (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). SCP-1, another CT antigen, was also detected in 40% of ATLL cell lines, as in other T-cell lymphomas (supplemental Table 1).

### NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed in primary ATLL cells

Given the high percentage of CT antigen mRNA expression in ATLL cell lines, we next examined the expression of 11 CT antigens in primary tumor cells from 57 individual ATLL patients. As shown in Figure 1A, Table 1, and supplemental Table 2, NY-ESO-1, MAGE-A3, and MAGE-A4 mRNA expression was detected in 61.4% (35 of 57), 31.6% (18 of 57), and 61.4% (35 of 57), respectively, of primary ATLL patients. SCP-1 expression was
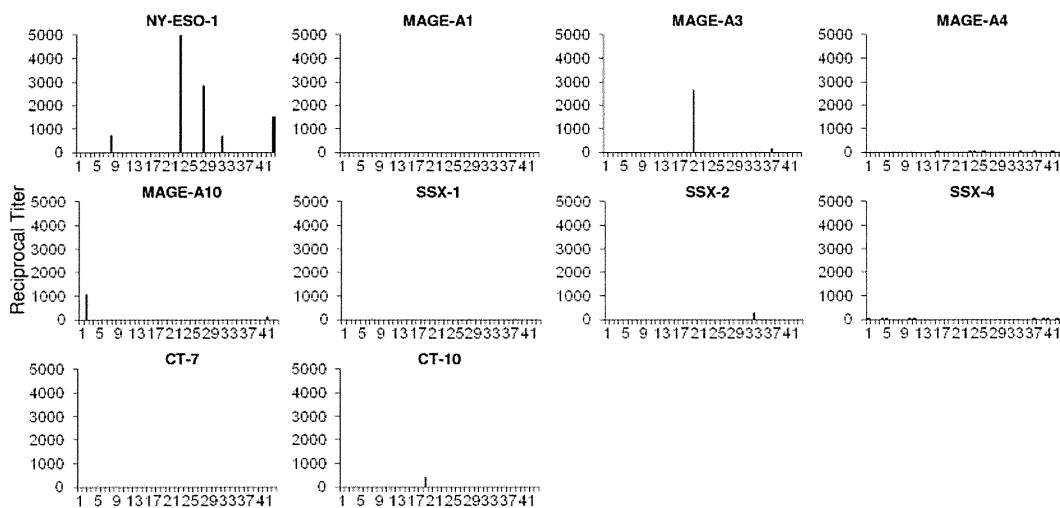
**Figure 2. Humoral immune responses against NY-ESO-1 are detected in a subset of patients with ATLL.** Sera were collected from 43 primary ATLL patients, and antibody responses against 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were analyzed by ELISA as described in "ELISA." This experiment was performed at least twice with similar results.

also detected in 23.2% (13 of 56). Among 4 subtypes (acute, lymphoma, chronic, and smoldering) of ATLL, the acute and lymphoma types show aggressive clinical courses (aggressive types), whereas the chronic and smoldering types progress more indolently (indolent types).[2,23] There were no significant differences between the ATLL patients with aggressive and indolent types in the expression of NY-ESO-1, MAGE-A3, and MAGE-A4; yet there was a trend for more frequent expression of CT antigens in aggressive ATLL types (Table 1). Immunohistochemical analysis confirmed the expression of NY-ESO-1 and pan-MAGE at the protein level in samples available for pathologic analysis (Figure 1B). By contrast, expression of other CT antigens was limited: MAGE-A1, 8.8% (3 of 34); MAGE-A10, 0% (0 of 33); CT-7, 0% (0 of 33); CT-10, 0% (0 of 33); SSX-1, 0% (0 of 33); SSX-2, 0% (0 of 33); and SSX-4, 0% (0 of 33) by RT-PCR.

Taken together, CT antigens, such as NY-ESO-1, MAGE-A3 and MAGE-A4, are expressed in a significant fraction of primary ATLL cases, and 87.7% (50 of 57) of ATLL patients expressed at least one of these 3 CT antigens.

### Humoral immune responses against NY-ESO-1 are detected in a subset of ATLL patients

We next asked whether ATLL patients spontaneously developed humoral and cellular immune responses specific for CT antigens. Serum samples from 43 primary ATLL patients were assessed by ELISA for the reactivity to 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4). Significant antibody titers were detected against NY-ESO-1 in 5 of 43 (11.6%) patients and against MAGE-A1, MAGE-A3, and MAGE-A4 in 0% (0 of 43), 2.3% (1 of 43), and 0% (0 of 43), respectively (Figure 2). Humoral immune responses against CT antigens whose expression was not detected by RT-PCR (MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were limited (Figure 2). These data collectively indicate that NY-ESO-1 expressed in primary ATLL cells elicits spontaneous antibody responses in ATLL patients as in patients with NY-ESO-1 expressing solid tumors.[19]

### Cellular immune responses against NY-ESO-1 in ATLL patients

We then assessed CT antigen-specific cellular immune responses in ATLL patients. As the majority of ATLL patients are in a severe

anemic state as a characteristic of the disease and also because of intensive chemotherapy, we collected PBMCs from patients in partial or complete remission, except patient 8 with an indolent ATLL type. Considering this limitation of available sample sizes and predominant humoral immune responses against NY-ESO-1, we focused on NY-ESO-1 as a parameter of cellular immune responses. With sufficient amounts of PBMCs from 9 ATLL patients (patients 1, 2, 4, 8, 13, 14, 19, 27, and 43), NY-ESO-1 expression by ATLL cells was confirmed by RT-PCR in each patient, except for patient 13 (no detection) and patient 43 (unavailability of sample; supplemental Table 2). CD8$^+$ T cells were presensitized with autologous CD4$^-$CD8$^-$ PBMCs pulsed with a pool of NY-ESO-1 peptides, and antigen-specific CD8$^+$ T cells were analyzed with NY-ESO-1/HLA tetramers corresponding to the HLA allele of each patient. NY-ESO-1–specific CD8$^+$ T cells were detected in ATLL patients 2, 4, 14, and 43 (Figure 3A). The results indicate that NY-ESO-1–specific CD8$^+$ T-cell responses spontaneously develop in a subset of patients harboring NY-ESO-1 expressing ATLL.

### NY-ESO-1–specific CD8$^+$ T cells produce cytokines and recognize an autologous ATLL cell line

With the presence of NY-ESO-1–specific CD8$^+$ T cells in ATLL patients, we further analyzed their cytokine production in response to a pool of NY-ESO-1 peptides or autologous tumor cells. Sufficient amounts of PBMCs for the cytokine analysis were available from 3 ATLL patients (patients 4, 14, and 19), in which NY-ESO-1 expression was confirmed by RT-RCR (supplemental Table 2). NY-ESO-1–specific CD8$^+$ T cells were detected with NY-ESO-1/MHC tetramers in 2 of them (patients 4 and 14), but relevant tetramers were not available for another patient (patient 19). NY-ESO-1–specific CD8$^+$ T cells prepared from these 3 patients by presensitization with NY-ESO-1 peptide-pulsed CD4$^-$CD8$^-$ PBMCs produced IFN-$\gamma$ and/or TNF-$\alpha$ by intracellular cytokine staining (Figure 3B). In patient 14, the frequency of NY-ESO-1–specific CD8$^+$ T cells producing IFN-$\gamma$ was much higher than NY-ESO-1–specific CD8$^+$ T cells detected by NY-ESO-1/HLA-Cw*0304 tetramer, suggesting that this patient may have CD8$^+$ T cells recognizing other epitopes of NY-ESO-1 (Figure 3A-B). Taken together, NY-ESO-1–specific CD8$^+$ T cells were detected in 5 of 9 (55.6%) ATLL patients.
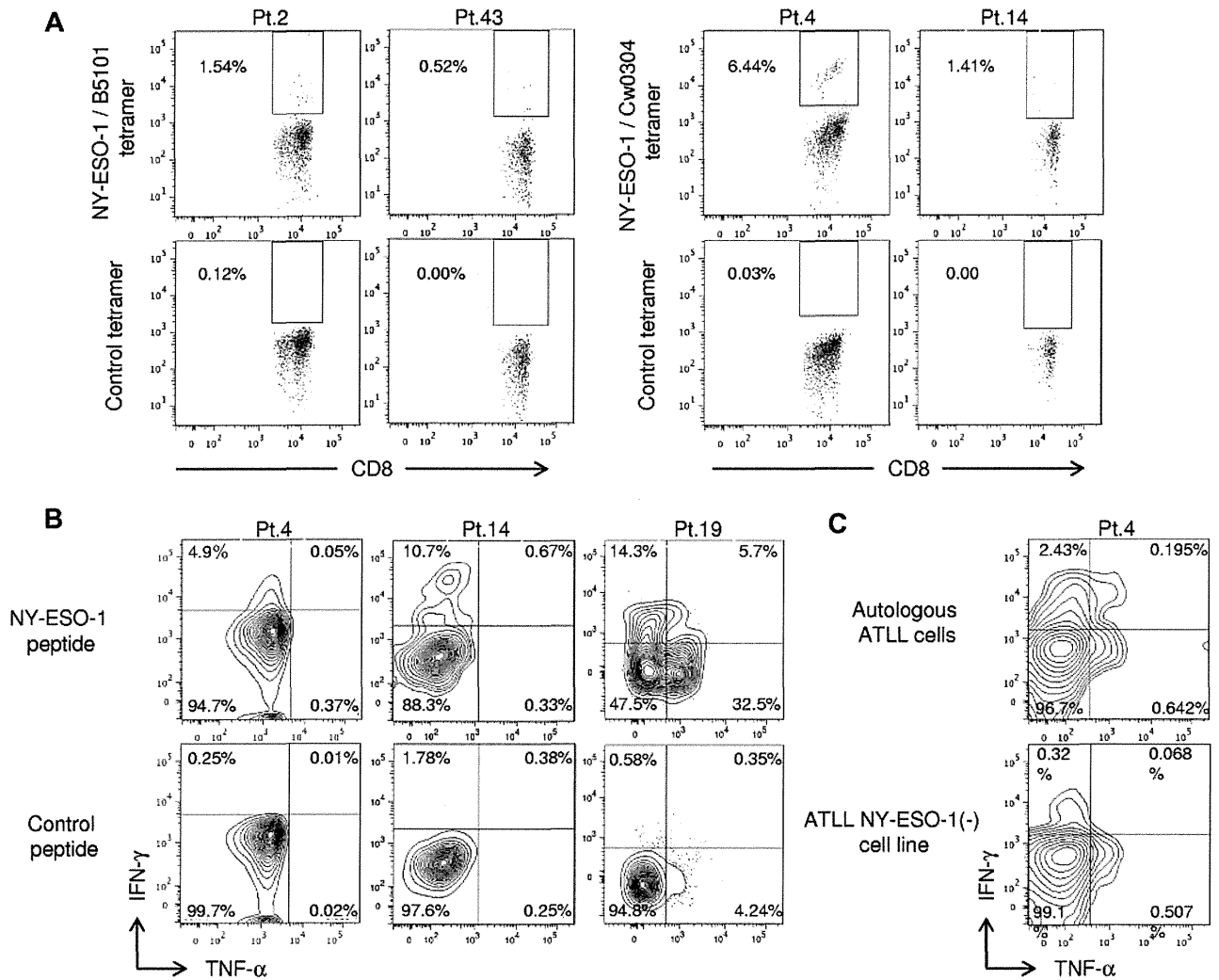
**Figure 3. NY-ESO-1–specific CD8+ T cells are detected in ATLL patients.** CD8+ T cells derived from PBMCs of patients 1, 2, 4, 8, 13, 14, 19, 27, and 43 were presensitized by CD4− CD8− PBMCs pulsed with NY-ESO-1 peptides covering the entire sequence of NY-ESO-1 as described in "In vitro sensitization." (A) Induction of specific CD8+ T cells was analyzed by staining with NY-ESO-1/HLA tetramers indicated. Cytokine (IFN-γ and TNF-α) secreting capacity of NY-ESO-1–specific CD8+ T cells was analyzed by intracellular cytokine staining for recognition of (B) autologous activated T-cell APCs pulsed with NY-ESO-1 peptides or (C) autologous ATLL cells. These experiments were performed independently at least twice with similar results.

We also examined whether NY-ESO-1–specific CD8+ T cells from patient 4 recognized autologous ATLL cells. They produced IFN-γ and TNF-α against autologous ATLL cells expressing NY-ESO-1 but not against a control HLA-matched ATLL cell line (ATL-102) without NY-ESO-1 expression (Figure 3C). Collectively, these data indicate that NY-ESO-1–specific CD8+ T cells are present in ATLL patients and are able to recognize and kill autologous leukemic cells.

### CD4+ T-cell responses against NY-ESO-1 in ATLL patients

To determine whether NY-ESO-1–specific CD4+ T cells were present in these ATLL patients, CD4+ T cells derived from PBMCs obtained from ATLL patients (patients 1, 2, 4, 8, 13, 14, 19, 27, and 43) were presensitized by CD4−CD8− PBMCs pulsed with a pool of NY-ESO-1 peptides, and assessed for cytokine production by intracellular cytokine staining. Of 9 patients, NY-ESO-1–specific CD4+ T cells were detected only in one patient (patient 19), who was in complete remission after receiving allogeneic hematopoietic stem cell transplantation (Figure 4). Thus, NY-ESO-1–specific CD4+ T cells are present in a subset of ATLL patients, but a much lower frequency than CD8+ T-cell responses, partly because the

presence of ATLL cells, which are CD4+, may make the detection difficult and possibly because CD25+CD4+ Tregs are present in the CD4+ T-cell fraction.

### Discussion

Since the initial description of ATLL as a unique type of T-cell leukemia/lymphoma,[1] various therapeutic attempts have been made. Yet, the prognosis of ATLL is still poor despite advances in our knowledge regarding the oncogenic process of the disease.[10,23] Here, we have examined CT antigen expression and its immunogenicity in ATLL patients to explore the potential for immunotherapy of ATLL by targeting CT antigens. We found that CT antigens, such as NY-ESO-1, MAGE-A3, and MAGE-A4, were highly expressed in ATLL. In particular, the frequencies of NY-ESO-1 and MAGE-A4 expression (61.4% and 61.4%, respectively) at the mRNA level were higher than or comparable to those in other malignancies. For example, the frequency of NY-ESO-1 and MAGE-A4 expression was 32% to 45% and 28%, respectively, in malignant melanoma, 24% to 33% and 63% to 90.2% in esophageal cancer, 30% to 43%
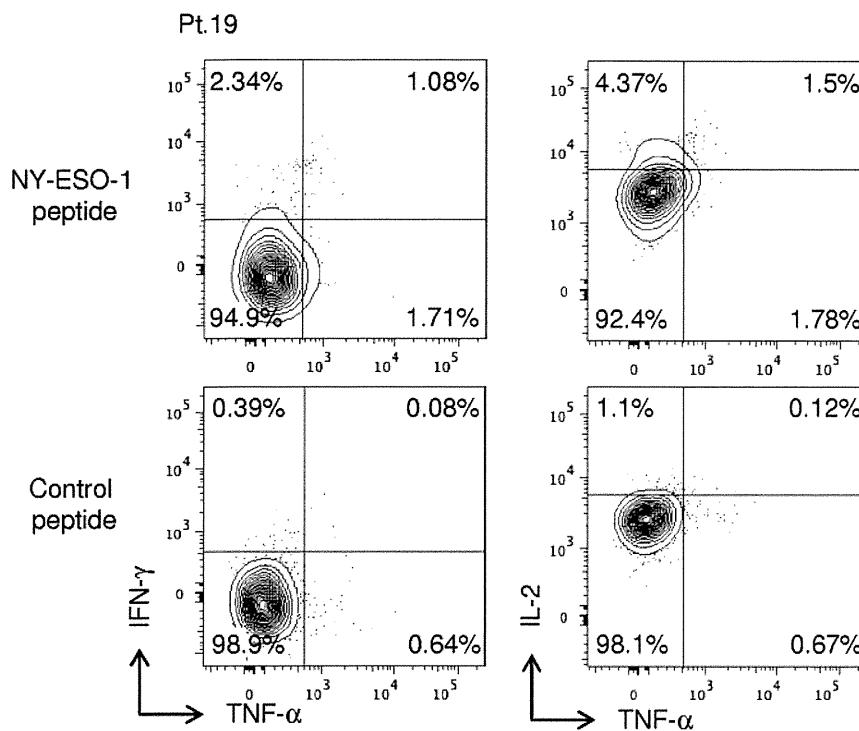
Figure 4. NY-ESO-1–specific CD4⁺ T cells are present in an ATLL patient (patient 19) receiving an allogeneic hematopoietic stem cell transplantation. CD4⁺ T cells were presensitized by CD4⁻ CD8⁻ PBMCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1 as described in "In vitro sensitization." Induction of NY-ESO-1–specific CD4⁺ T cells was analyzed by intracellular cytokine staining using autologous activated T-cell APCs pulsed with NY-ESO-1 peptides. These experiments were performed independently twice with similar results.

and 57% in ovarian cancer, and 18% to 35% and 33% in bladder cancer.[19,32,33] In addition, we have revealed that NY-ESO-1 was immunogenic in ATLL patients and elicited specific humoral and cellular immune responses in a subset of patients. These data strongly support CT antigens as novel targets for ATLL immunotherapy.

The frequency of humoral immune responses in ATLL patients was much higher against NY-ESO-1 compared with other CT antigens, such as MAGE-A3 and MAGE-A4. Although NY-ESO-1 expression by ATLL cells is presumably required for antibody induction, the level of NY-ESO-1 mRNA expression did not reflect the induction of humoral immune responses (supplemental Table 2). This lack of correlation appears in part to be the result of immunologic properties of NY-ESO-1 antigen itself.[19,26] It has been shown that polymeric structures of NY-ESO-1 through disulfide bonds and their interaction with calreticulin-TLR4 on immature dendritic cell surface are required to induce phagocytosis of NY-ESO-1 protein and deliver danger signals for making the protein immunogenic.[34] In addition, some non-MAGE-A members of the MAGE family are ubiquitously expressed and therefore possibly in more stable tolerance compared with NY-ESO-1. These properties of NY-ESO-1 and MAGE proteins might make the former more immunogenic than the latter.

Despite high immunogenicity of NY-ESO-1, the frequency (13.6%, 3 of 22) of primary ATLL patients who spontaneously developed NY-ESO-1 antibody responses against NY-ESO-1 expressing leukemic cells was lower compared with the frequencies in patients with malignant melanoma or non-small-cell lung cancers expressing NY-ESO-1 (~ 50%).[19,26] This may reflect the feature of ATLL that tumor cells from the majority of ATLL patients express Foxp3, a key transcription factor for CD25⁺CD4⁺ Tregs.[6,9,35-37] ATLL cells from a subset of patients indeed appear to function as Tregs and contribute to profound immunosuppression that hampers the host's immune responses.[6,36] Alternatively or additionally, the chemokine CCL22 produced by ATLL cells might enhance the migration of CCR4-expressing CD25⁺CD4⁺ Tregs to

tumor sites.[38] It remains to be determined whether Tregs in ATLL patients or ATLL cells themselves suppress NY-ESO-1–specific immune responses. Yet, it has been shown that helper T-cell responses against NY-ESO-1 are subject to active suppression by Tregs in patients with solid tumors and healthy persons.[30,37] Further studies are required to understand immunosuppressive property of ATLL to enhance immune responses against CT antigens in ATLL patients.

NY-ESO-1–specific cellular immune responses were detected in a significant number (5 of 9, 55.6%) of ATLL patients in partial or complete remission. This indicates that reducing the number of ATLL cells before CT antigen-targeted immunotherapy may be a crucial component to successfully induce/augment antigen–specific CD8⁺ T cells. We have recently reported that humanized anti-CCR4 mAb, KW-0761, showed clinically significant antitumor activity as a salvage therapy for patients with relapsed ATLL.[8,39] Patients 2, 4, and 14, from whom we detected NY-ESO-1–specific CD8⁺ T cells, were in complete or partial remission after anti-CCR4 mAb treatment.[8,39] As CCR4 is expressed on ATLL cells as well as CD25⁺CD4⁺FOXP3⁺ Tregs, in addition to T-helper type 2 cells,[8,40,41] anti-CCR4 mAb treatment can reduce not only ATLL cells but also endogenous CD25⁺CD4⁺FOXP3⁺ cells,[42] thereby contributing to evoking NY-ESO-1–specific CD8⁺ T-cell responses. Indeed, the high frequency of NY-ESO-1–specific CD8⁺ T cells detectable in vitro in ATLL patients could be the result in part of the absence of Tregs. Thus, combining CT antigen-targeted immunotherapy after reduction of endogenous Tregs as well as ATLL cells by anti-CCR4 mAb treatment would be an ideal strategy for ATLL immunotherapy.

NY-ESO-1–specific CD4⁺ and CD8⁺ T-cell responses observed in patient 19 who was in a complete remission after allogeneic hematopoietic stem cell transplantation suggest an association of immune responses against CT antigens with a graft-versus-ATLL effect. This indicates that CT antigen-targeted immunotherapy combined with allogeneic stem cell transplantation may augment the efficacy of the current allogeneic hematopoietic stem cell

transplantation for treating ATLL. In addition, as HTLV-1 Tax-specific CD8^{+} T cells reportedly contribute to graft-versus-ATLL effects,[43] a combination immunotherapy with CT antigen and Tax after stem cell transplantation might also be therapeutically effective. Further, considering the reported high efficacy of NY-ESO-1–targeted adoptive T-cell therapy against malignant melanoma and synovial cell sarcoma,[44,45] similar adoptive T-cell therapy for ATLL could also be effective.

ATLL is, to our knowledge, the first example of high expression of CT antigens in lymphomas or leukemia, as detailed analyses of CT antigen expression in other hematologic disorders have been limited.[21,22] It contrasted with a previous report showing that T-cell lymphoma lacks expression of CT antigens, except SCP-1. As a probable mechanism for transcriptional activation of the CT antigen genes in malignant cells, it has been suggested that the expression of CT antigen is induced by CpG island hypomethylation at the promoter regions.[18] An inflammatory environment created by infection may also trigger NY-ESO-1 expression. It cannot be excluded that viral components may promote this hypomethylation at the promoter regions or elicit inflammatory environment, thereby inducing CT antigen expression. Yet, we failed to observe any correlation between viral gene expression, such as Tax and HBZ and CT antigen expression (supplemental Table 2). In addition, high titers of HTLV-1 Gag/Env antibody responses were not associated with the induction of humoral immune responses against CT antigens (supplemental Table 2). Peripheral T-cell lymphomas, not otherwise specified (PTCL-NOS) are particularly heterogeneous; yet, the CCR4-positive subset of PTCL-NOS might be a distinct disease entity whose clinicopathologic features and genomic profiles were reportedly very similar to the lymphoma type of ATLL.[24,46,47] PTCL-NOS, especially the CCR4-positive subset, have a very poor prognosis like ATLL, and no standard treatment strategies are available.[48] Notably, we have found that CCR4-positive PTCL-NOS also expressed NY-ESO-1 at a high frequency (H.N., T.I., R.U. and S.S., unpublished data, August 2011). These data, when taken together, indicate that the frequent expression of CT antigens in ATLL may not be induced by HTLV-1 infection itself, but rather it may constitute a novel subtype of T-cell leukemia/lymphoma that expresses CCR4 and CT antigens regardless of HTLV-1 infection.

A small population (~ 5%) of HTLV-1–infected persons progresses to ATLL after a long latency period of approximately 50 to 70 years.[2] Although the detailed mechanisms of this leukemogenesis from HTLV-1 infection to ATLL have not yet been well elucidated, host immune responses against HTLV-1–infected cells have been suspected to play an important role.[8,16,49] Interestingly, some of HTLV-1–infected asymptomatic HTLV-1 carriers harbored significant titers of antibody against NY-ESO-1, MAGE-A3, and MAGE-A4, despite that CT antigens, including NY-ESO-1, MAGE-A3, and MAGE-A4, were not detected at the mRNA level in their PBMCs (supplemental Figure 1). This discrepancy raises several possibilities; for example, the number or the frequency of HTLV-1–infected cells expressing CT antigens in circulating PBMCs may be too low to detect the antigen. Alternatively, it is possible that HTLV-1–infected cells expressing CT antigens may be eliminated in HTLV-1–infected asymptomatic carriers by anti-CT antigen-specific immune responses as tumor immunosurveillance,[50] whereas

antibody responses may linger on. Future studies with a large cohort of HTLV-1–infected asymptomatic carriers need to address the kinetics of their CT antigen expression during a long latency period and their immune responses against the antigen in the course of ATLL development. The studies will not only provide a rationale for including CT antigens as possible targets of ATLL immunotherapy but also contribute to our understanding of the multistep oncogenesis of ATLL and devising preventive strategies for ATLL by targeting CT antigens.

## Authorship

Contribution: H.N., T.I., S.G., L.J.O., R.U., and S.S. designed the research; H.N., Y.M., T.I., S.G., E.S., F.M., D.S., A.I., and Y.F. performed experiments; T.I., F.M., A.I, A.U., H.I., and R.U. collected samples and obtained clinical data; H.N., Y.M., T.I., S.G., E.S., Y.F., R.U., and S.S. analyzed data; and H.N., Y.M., T.I., S.G., E.S., R.U., and S.S. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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## References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood.* 1977;50(3):481-492.
2. Matsuoka M, Jeang KT. Human T-cell leukaemia

virus type 1 (HTLV-1) infectivity and cellular transformation. *Nat Rev Cancer.* 2007;7(4):270-280.
3. Goncalves DU, Proietti FA, Ribas JG, et al. Epidemiology, treatment, and prevention of human

T-cell leukemia virus type 1-associated diseases. *Clin Microbiol Rev.* 2010;23(3):577-589.
4. Uchiyama T, Sagawa K, Takatsuki K, Uchino H. Effect of adult T-cell leukemia cells on pokeweed

mitogen-induced normal B-cell differentiation. *Clin Immunol Immunopathol.* 1978;10(1):24-34.

5. Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol.* 2004;16(11):1643-1656.

6. Matsubara Y, Hori T, Morita R, Sakaguchi S, Uchiyama T. Phenotypic and functional relationship between adult T-cell leukemia cells and regulatory T cells. *Leukemia.* 2005;19(3):482-483.

7. Chen S, Ishii N, Ine S, et al. Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells. *Int Immunol.* 2006;18(2):269-277.

8. Ishida T, Ueda R. Immunopathogenesis of lymphoma: focus on CCR4. *Cancer Sci.* 2011;102(1):44-50.

9. Satou Y, Yasunaga J, Zhao T, et al. HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. *PLoS Pathog.* 2011;7(2):e1001274.

10. Tsukasaki K, Utsunomiya A, Fukuda H, et al. VCAP-AMP-VECP compared with biweekly CHOP for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study JCOG9801. *J Clin Oncol.* 2007;25(34):5458-5464.

11. Tsukasaki K, Hermine O, Bazarbachi A, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an International Consensus Meeting. *J Clin Oncol.* 2009;27(3):453-459.

12. Hishizawa M, Kanda J, Utsunomiya A, et al. Transplantation of allogeneic hematopoietic stem cells for adult T-cell leukemia: a nationwide retrospective study. *Blood.* 2010;116(8):1369-1376.

13. Takeda S, Maeda M, Morikawa S, et al. Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int J Cancer.* 2004;109(4):559-567.

14. Suemori K, Fujiwara H, Ochi T, et al. HBZ is an immunogenic protein, but not a target antigen, for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes. *J Gen Virol.* 2009;90(8):1806-1811.

15. Verhasselt V, Milcent V, Cazareth J, et al. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat Med.* 2008;14(2):170-175.

16. Shimizu Y, Takamori A, Utsunomiya A, et al. Impaired Tax-specific T-cell responses with insufficient control of HTLV-1 in a subgroup of individuals at asymptomatic and smoldering stages. *Cancer Sci.* 2009;100(3):481-489.

17. Simpson AJ, Caballero OL, Jungbluth A, Chen Y-T, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer.* 2005;5(8):615-625.

18. Caballero OL, Chen Y-T. Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci.* 2009;100(11):2014-2021.

19. Gnjatic S, Nishikawa H, Jungbluth AA, et al. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res.* 2006;95:1-30.

20. Dougan M, Dranoff G. Immune therapy for cancer. *Annu Rev Immunol.* 2009;27:83-117.

21. Xie X, Wacker HH, Huang S, et al. Differential expression of cancer testis genes in histological subtypes of non-Hodgkin's lymphomas. *Clin Cancer Res.* 2003;9(1):167-173.

22. Greiner J, Ringhoffer M, Taniguchi M, et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. *Int J Cancer.* 2004;108(5):704-711.

23. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukemia-lymphoma: a report from the Lymphoma Study Group (1984-87). *Br J Haematol.* 1991;79(3):428-437.

24. Ishida T, Utsunomiya A, Iida S, et al. Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin Cancer Res.* 2003;9(10):3625-3634.

25. Ri M, Iida S, Ishida T, et al. Bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on upregulation of Noxa and functional repression of Mcl-1. *Cancer Sci.* 2009;100(2):341-348.

26. Stockert E, Jager E, Chen Y-T, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med.* 1998;187(8):1349-1354.

27. Rimoldi D, Salvi S, Schultz-Thater E, Spagnoli GC, Cerottini JC. Anti-MAGE-3 antibody 57B and anti-MAGE-1 antibody 6C1 can be used to study different proteins of the MAGE-A family. *Int J Cancer.* 2000;86(5):749-751.

28. Gure AO, Chua R, Williamson B, et al. Cancer-testis genes are coordinately expressed and are markers of poor outcome in non-small cell lung cancer. *Clin Cancer Res.* 2005;11(22):8055-8062.

29. Gnjatic S, Ritter E, Buchler MW, et al. Seromic profiling of ovarian and pancreatic cancer. *Proc Natl Acad of Sci U S A.* 2010;107(11):5088-5093.

30. Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. CD4+CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T-cell responses in cancer patients. *Blood.* 2005;106(3):1008-1011.

31. Nishikawa H, Sato E, Briones G, et al. In vivo antigen delivery by a Salmonella typhimurium type III secretion system for therapeutic cancer vaccines. *J Clin Invest.* 2006;116(7):1946-1954.

32. Duffour MT, Chaux P, Lurquin C, Cornelis G, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. *Eur J Immunol.* 1999;29(10):3329-3337.

33. Barrow C, Browning J, MacGregor D, et al. Tumor antigen expression in melanoma varies according to antigen and stage. *Clin Cancer Res.* 2006;12(3):764-771.

34. Liu YN, Tian XL, Leitner WW, et al. Polymeric structure and host Toll-like receptor 4 dictate immunogenicity of NY-ESO-1 antigen in vivo. *J Biol Chem.* 2011;286(43):37077-37084.

35. Karube K, Ohshima K, Tsuchiya T, et al. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. *Br J Haematol.* 2004;126(1):81-84.

36. Yano H, Ishida T, Inagaki A, et al. Regulatory T-cell function of adult T-cell leukemia/lymphoma cells. *Int J Cancer.* 2007;120(9):2052-2057.

37. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. *Int J Cancer.* 2010;127(4):759-767.

38. Toulza F, Nosaka K, Tanaka Y, et al. Human T-lymphotropic virus type 1-induced CC chemokine ligand 22 maintains a high frequency of functional FoxP3+ regulatory T cells. *J Immunol.* 2010;185(1):183-189.

39. Yamamoto K, Utsunomiya A, Tobinai K, et al. Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma. *J Clin Oncol.* 2010;28(9):1591-1598.

40. Iellem A, Mariani M, Lang R, et al. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4+CD25+ regulatory T cells. *J Exp Med.* 2001;194(6):847-853.

41. Ishida T, Iida S, Akatsuka Y, et al. The CC chemokine receptor 4 as a novel specific molecular target for immunotherapy in adult T-cell leukemia/lymphoma. *Clin Cancer Res.* 2004;10(22):7529-7539.

42. Ishida T, Joh T, Uike U, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study [published online ahead of print February 6, 2012]. *J Clin Oncol.* doi:10.1200/JCO.2011.37.3472.

43. Harashima N, Kurihara K, Utsunomiya A, et al. Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation. *Cancer Res.* 2004;64(1):391-399.

44. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+T cells against NY-ESO-1. *N Engl J Med.* 2008;358(25):2698-2703.

45. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol.* 2011;29(7):917-924.

46. Ishida T, Inagaki H, Utsunomiya A, et al. CXC chemokine receptor 3 and CC chemokine receptor 4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. *Clin Cancer Res.* 2004;10(16):5494-5500.

47. Nakagawa M, Nakagawa-Oshiro A, Karnan S, et al. Array comparative genomic hybridization analysis of PTCL-U reveals a distinct subgroup with genetic alterations similar to lymphoma-type adult T-cell leukemia/lymphoma. *Clin Cancer Res.* 2009;15(1):30-38.

48. Weisenburger DD, Savage KJ, Harris NL, et al. Peripheral T-cell lymphoma, not otherwise specified: a report of 340 cases from the International Peripheral T-cell Lymphoma Project. *Blood.* 2011;117(12):3402-3408.

49. Kannagi M, Harashima N, Kurihara K, et al. Tumor immunity against adult T-cell leukemia. *Cancer Sci.* 2005;96(5):249-255.

50. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science.* 2011;331(6024):1565-1570.