Fcγ receptor on effector cells. Defucosylated IgG1 is a more potent activator of NK cells than nondefucosylated IgG1 during ADCC.[22] We surmise that the infusion reactions to KW-0761 were mainly induced by cytokines and related cytotoxic molecules released from highly activated NK cells.

The present study demonstrated that compared with the levels in the controls, KW-0761 led to a significant and lasting decrease in the number of CD4+ CCR4+ but not CD4+ CCR4− or CD4− CD8+ cells in patients with ATL. Consistent with the fact that CCR4 is expressed not only on T-helper type 2 cells but also on regulatory T (Treg) cells,[23-26] KW-0761 treatment also resulted in a significant and lasting decrease in CD4+ CD25+ FOXP3+ cells, including both ATL cells and endogenous non-ATL Treg cells.[27-29] Reduction or suppression of Treg cells is expected to be a potentially promising strategy for boosting antitumor immunity in patients with cancer, as observed in studies with ipilimumab,[30-33] although ipilimumab and KW-0761 have different targets; the former suppresses Treg cell function, and the latter decreases their number. Hence, KW-0761 could also lead to activation of antitumor immunity, which might also contribute to its potent anti-ATL response. Because ipilimumab causes immune-related AEs such as diarrhea and colitis, we were especially vigilant in monitoring for this type of AE. Because CCR4 contributes to lymphocyte skin-specific homing,[34] it was not surprising that skin rashes, which could be an immune-related AE, were frequently observed in the present KW-0761 study. Skin rashes, including the most severe case of Stevens-Johnson syndrome, the causal association of which with concomitant medications other than KW-0761 could not be excluded, proved to be manageable, and patients improved in all cases, although some needed systemic or topical steroid treatment. The observed better responses to KW-0761 in patients with grade 2 or higher skin rashes were highly impressive. However, the underlying mechanisms for this finding are not clear; thus, further detailed investigation is warranted. All of the 14 patients who developed grade 2 or higher skin rashes received five or more KW-0761 infusions according to the protocol, whereas only three of the 12 patients who developed no or grade 1 skin rashes received five or more KW-0761 infusions. This suggests the possibility that skin rashes were associated with the number of KW-0761 infusions. The Cochran-Mantel-Haenszel test stratified by the number of KW-0761 infusions ($\leq$ four $v \geq$ five) indicated a significant association between clinical response and skin rashes (no or grade 1 v grades 2 to 4; $P = .009$). However, the sample size is insufficient to draw such a conclusion.

Following on a phase III study (JCOG9801 [Japan Clinical Oncology Group 9801]) for untreated aggressive ATL,[5] the present promising results for KW-0761 monotherapy prompted us to conduct a subsequent randomized trial of VCAP-AMP-VECP chemotherapy with or without KW-0761 for previously untreated ATL (Clinicaltrials.gov: NCT01173887). CCR4 is also expressed on tumor cells from a subgroup of PTCL other than ATL, which also has an unfavorable prognosis.[2,35,36] Thus, we are currently conducting a phase II study of KW-0761 monotherapy for relapsed CCR4-positive PTCL (Clinicaltrials.gov:NCT01192984). In addition, Duvic et al[37] recently reported a phase I/II study of KW-0761 for refractory cutaneous T-cell lymphoma. They found that KW-0761 was well tolerated at doses of 0.1 to 1.0 mg/kg, and a promising ORR of 39% (15 of 38 patients) was achieved, although expression of CCR4 on lymphoma cells was not included as one of the eligibility criteria (Clinicaltrials.gov: NCT00888927). Furthermore, clinical trials of KW-0761 for patients with Hodgkin's lymphoma may be worth trying, because it has been reported that Hodgkin's lymphoma tumor cells produce CCR4 ligand molecules, and migratory CCR4-expressing Treg cells prevent a host immune attack on tumor cells, thereby creating an immunologically favorable environment for the tumor cells.[38]

Although this phase II study offers a novel promising treatment option (KW-0761) for patients with relapsed ATL, some limitations should be discussed. First, the present phase II study was relatively small, with consequent limitations on drawing definitive conclusions about the efficacy and safety profile of KW-0761. Second, patients received different prior systemic chemotherapy regimens, which could affect the results of the present study. Finally, the enrolled patients all had aggressive ATL, but three clinical subtypes (acute, lymphoma, and unfavorable chronic type) were included. Although there may be no significant differences in susceptibility to conventional chemotherapies between these subtypes, the heterogeneity of the enrolled patients might have affected the results.

In conclusion, this multicenter phase II study demonstrated that KW-0761 monotherapy showed clinically meaningful antitumor activity in patients with relapsed ATL, with an acceptable toxicity profile. Further investigation of KW-0761 for ATL and other T-cell neoplasms is warranted on the basis of the present results.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Tax is a potential molecular target for immunotherapy of adult T-cell leukemia/lymphoma

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We expanded CTL specific for Tax (a human T-lymphotropic virus type-1-encoded gene product) *in vitro* from PBMC of several adult T-cell leukemia/lymphoma (ATL) patients, and document its potential significance as a target for ATL immunotherapy. Tax-specific CTL responses against tumor cells were restricted by Tax-expression and the appropriate human leukocyte antigen (HLA) type. Tax-specific CTL recognized HLA/Tax-peptide complexes on autologous ATL cells, even when their Tax expression was so low that it could only be detected by RT-PCR but not by flow cytometry. Recognition resulted in interferon gamma (IFN-γ) production and target cell lysis. This would be the first report that Tax-specific CTL from ATL patients specifically recognized and killed autologous tumor cells that expressed Tax. The Tax-specific CTL responded to as little as 0.01 pM of the corresponding peptide, indicating that their T-cell receptor avidity was much higher than that of any other CTL recognizing viral or other tumor antigens. This is presumably the reason why the Tax-specific CTL recognized and killed autologous ATL cells despite their very low Tax expression. In addition, cell cycle analyses and experiments with primary ATL cell-bearing mice demonstrated that ATL cells present at the site of active cell proliferation, such as in the tumor masses, expressed substantial amounts of Tax, but it was minimally expressed by the tumor cells in a quiescent state, such as in the blood. The present study not only provides a strong rationale for exploiting Tax as a possible target for ATL immunotherapy but also contributes to our understanding of the immunopathogenesis of ATL. (*Cancer Sci* 2012; 103: 1764–1773)

Adult T-cell leukemia/lymphoma (ATL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1).[1,2] ATL has a long latency period of 50–60 years, so affected individuals have usually been exposed to HTLV-1 early in their lives via agents including infected lymphocytes, mainly from mother's breast milk.[3,4] Only small subpopulations (approximately 5%) of HTLV-1-infected individuals progress to ATL, but there are no clear biomarkers separating those who will develop ATL from those who remain asymptomatic carriers (AC).[2] There are four clinical subtypes of ATL: acute, lymphoma, chronic and smoldering.[5] The two former types have more aggressive clinical courses (aggressive variants), while the latter are less aggressive (indolent variants).

Human T-lymphotropic virus type 1 Tax, a virus-encoded regulatory gene product, is required for the virus to transform cells,[6] and is thought to be indispensable for oncogenesis. Therefore, Tax has been considered as a molecular target for immunotherapy against ATL, and many such investigations have been published.[7–10] However, it has been reported that the level of Tax expression in HTLV-1-infected cells decreases during disease progression, and *Tax* transcripts are detected only in approximately 40% of established ATL cases.[11] Moreover, weak or absent responses to Tax have been observed in ATL patients,[12] leading to controversy as to whether Tax is an appropriate target for immunotherapy of ATL. In the present study, we expanded Tax-specific CTL *in vitro* from PBMC of several ATL patients, and tested their ability to respond to several ATL cell lines, HTLV-1-immortalized lines and to autologous ATL cells. The aim was to clarify the involvement of Tax-specific CTL (Tax-CTL) in the immunopathogenesis of ATL, and to confirm the significance of Tax as a potential immunotherapeutic target in ATL.

Materials and Methods

Primary adult T-cell leukemia/lymphoma cells. Primary ATL cells were separated from PBMC using anti-human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). 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.

Cell lines. TL-Su and TL-Om1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). TCL-Kan was kindly provided by Professor Mari Kannagi (Tokyo Medical and Dental University, Tokyo, Japan).[13] HUT102, ATN-1, MT-2 and MT-1 have been previously described.[14,15] MT-4 was purchased from the Health Science Research Resources Bank (Osaka, Japan). HUT102, ATN-1, MT-1 and TL-Om1 are ATL cell lines, and TL-Su, TCL-Kan, ILT-#37, MT-2 and MT-4 are HTLV-I-immortalized lines. K562 is the chronic myelogenous leukemia blast crisis cell line.[16]

Human leukocyte antigen typing. Genotyping of HLA-A, B and C was performed using an HLA-typing Kit (WAKFlow HLA-typing kit, WAKUNAGA Pharmacy, Hiroshima, Japan).

Expansion of human T-lymphotropic virus type 1 Tax-specific CTL. PBMC from ATL patients or HTLV-1 AC were suspended in RPMI-1640 supplemented with 10% autologous plasma and 0.1 μM of the corresponding Tax epitope peptides (LLFGYPVYV or SFHSLHLLF) at a cell concentration of 2.0×10^6/mL. These two synthetic peptides were purchased from Invitrogen (Carlsbad, CA, USA). The cell suspension was cultured at 37°C in 5%CO_2 for 2 days, and then an equal volume of RPMI-1640 supplemented with 100 IU/mL of IL-2

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was added. After subsequent culture for 5 days, an equal volume of ALyS505N (Cell Science & Technology Institute, Sendai, Japan) supplemented with 100 IU/mL of IL-2 was added, and the cells were cultured with appropriate medium (ALyS505N with 100 IU/mL of IL-2) for 7 days. Cytomegalovirus (CMV)-pp65 specific CTL were expanded in the same manner using peptides such as NLVPMVATV or QYDPVAALF (Invitrogen). Viable cell counts were determined using the trypan blue assay.

Antibodies, tetramers and flow cytometry. Phycoerythrin-conjugated HLA-A*02:01/Tax11–19 (LLFGYPVYV), HLA-A*24:02/Tax301–309 (SFHSLHLLF), HLA-A*02:01/pp65 495–503 (NLVPMVATV) and HLA-A*24:02/pp65 341–349 (QYDPVAALF) tetramers, and phycoerythrin-Cyanin5-conjugated anti-CD8 monoclonal antibody (mAb) were purchased from Medical & Biological Laboratories, Nagoya, Japan. Allophycocyanin-conjugated anti-human CD45 mAb (2D1) and PerCP-conjugated anti-CD4 mAb (SK3) were purchased from BD Biosciences (San Jose, CA, USA). Tax expression was assessed by FITC-conjugated anti-Tax mAb Lt-4.[17] FITC-conjugated anti-interferon gamma (IFN-γ) mAb (45.15) was purchased from Medical & Biological Laboratories. Cell cycle assessments were performed by BrdU Flow Kits (BD Biosciences). Cells were analyzed on a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Ashland, OR, USA).

CTL assay. Cytotoxic activity was determined by a standard 4-h chromium^{51} release assay as previously described.[18] All values given are means of triplicate determinations.

Quantitative RT-PCR. *Tax*, *human CD4* and *β-actin* mRNA were amplified as previously described.[19] The primer set for Tax was as follows: sense, 5′-AAGACCACCAACACCA TGGC-3′; and antisense, 5′-CCAAACACGTAGACTGGGTAT CC-3′.

Animals. NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All of the *in vivo* experiments were approved by the Ethics Committee of the Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences.

Results

Expansion of Tax-specific CTL. Expansion of Tax-CTL was performed by stimulating PBMC from 14 ATL patients and 6 HTLV-1 AC with synthetic peptides. PBMC from patients 1, 2, 3, 6, 8, 9 and 13 were stimulated with Tax11–19, and those from patients 4, 5, 7, 10, 11, 12 and 14 with Tax301–309 (Tables 1 and 2). Patients 1–6 were all in complete remission (CR) at the time of blood sampling. Patient 1 had achieved CR after allogeneic hematopoietic stem cell transplantation (HSCT) 5 years previously, patients 2 and 3 after systemic chemotherapy and anti-CCR4 mAb treatment,[20,21] patient 4 after systemic chemotherapy alone, and patient 5 after allogeneic HSCT 9 months earlier (and was receiving FK506 at the time of sampling). Finally, patient 6 achieved CR after systemic chemotherapy and anti-CCR4 mAb treatment, and was receiving prednisolone at the time of sampling. As shown in Table 1, Tax-CTL could be expanded *in vitro* (fold expansion >10) by stimulation with Tax peptide in 13 of 17 ATL cases. With respect to HTLV-1 AC, we confirmed efficient expansion (fold expansion >10^2) of Tax-CTL from six of six individuals using Tax11–19 or Tax301–309 peptides in the same manner (data not shown), which are consistent with a previous report.[22] Although the degree of expansion of Tax-CTL varied among the ATL patients, there was a trend for higher rates in PBMC from those with indolent variant ATL not on any systemic treatment, or from patients with aggressive ATL in treatment-induced remission, compared to lower or absent expansion in patients initially diagnosed with an aggressive variant. In particular, patient 8 progressed from chronic to acute subtype during the present study. Tax-CTL could be efficiently expanded from this patient during the chronic phase, but no longer after progression to acute subtype. This was despite the finding that the percentage of HLA-A*02:01/Tax11–19 tetramer-positive cells in the PBMC was almost the same as before disease progression (Fig. 1). These observations collectively indicate that insufficient responses to Tax observed in ATL patients, which are also reported by other investigators,[12,23,24] are related to disease progression from indolent to aggressive clinical variants. Subsequently, patient 8 received systemic chemotherapy but failed to achieve CR. He then received allogeneic HSCT with reduced intensity conditioning and entered partial remission. At this time, when he was not receiving immunosuppression after HSCT, his Tax-CTL could again be efficiently expanded from PBMC. This indicates that substantial anti-Tax responses can be restored by appropriate anti-ATL therapies, when the patient is brought from active ATL into remission (Fig. 1). Even though patients were in CR, immunosuppressive agents such as FK506 or prednisolone were likely to have prevented CTL expansions, as observed in patients 5 and 6, consistent with reports that HTLV-1 AC liver transplant recipients developed ATL under immunosuppression.[25,26] In patient 14, the Tax-CTL expansion rate was drastically increased by depletion of CD4+ cells, most of which consisted of the ATL cells themselves. This suggests that Tax-specific immune responses were suppressed by the tumor cells, consistent with our previous report that ATL cells from a subgroup of patients functioned as regulatory T (Treg) cells.[27]

T-cell receptor avidity of the expanded Tax-specific CTL. Specific IFN-γ production following stimulation with serial concentrations (0.01–100 pM) of Tax11–19 or Tax301–309 peptides was used as a readout to measure the T-cell receptor (TCR) avidity of the expanded Tax-CTL. Intracellular IFN-γ was clearly detected specifically even at a peptide concentration of 0.01 pM in both HLA-A*02:01-restricted Tax-CTL from patient 1 (Fig. 2A) and HLA-A*24:02-restricted Tax-CTL from patient 7 (Fig. 2B). We also analyzed the TCR avidity of CMV-pp65-specific CTL expanded from the same patients. Specific IFN-γ production by HLA-A*02:01-restricted pp65-CTL was lower than Tax-CTL at any peptide concentration. Furthermore, no specific IFN-γ production by HLA-A*24:02 pp65-CTL could be detected at all at peptide concentrations of 0.01–1 pM. In general in the literature, peptide concentrations of other viral or tumor antigen epitopes that the corresponding specific CTL recognize and respond to are in the range 1 nM–10 μM, although this varies according to the antigen.[28–32] Collectively, the results presented here indicate that the TCR avidities of these Tax-CTL can be considered to be extremely high.

Expression of human T-lymphotropic virus type 1 Tax in adult T-cell leukemia/lymphoma cells. Given the high TCR avidity of Tax-CTL, we next analyzed whether these CTL could recognize, respond to and kill ATL cells. To this end, Tax expression in ATL cell lines, HTLV-1-immortalized lines, K562 and short-term cultured primary ATL cells was assessed (Fig. 3). Tax expression was detected both by flow cytometry and RT-PCR in TL-Su, TCL-Kan, HUT102, MT-2 and MT-4, but not in K562, MT-1 or TL-Om1 by either technique. No Tax protein was seen in ATN-1 or in short-term cultured primary ATL cells from patients 7, 8 and 14, although *Tax* mRNA was present at levels 1/10–1/100th of those in TL-Su.

Tax-specific CTL responses against autologous adult T-cell leukemia/lymphoma cells. PBMC from patient 7 were stimulated with HLA-A*24:02 restricted Tax301–309 peptide, and the resulting CTL were expanded (Fig. 4A, upper-left panel).

Table 1. Tax-specific CTL expansion in adult T-cell leukemia/lymphoma (ATL) patients

Patient number	Clinical subtype	ATL status at blood sampling	Total cells (number)		Tax tetramer + cells/ lymphocytes (%)		Tax tetramer + cells (number)		Expansion rate†
			Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	
Patient 1	Acute	Complete remission	4.5×10^6	9.5×10^6	0.01	4.51	4.5×10^2	4.28×10^5	951.1
Patient 2	Acute	Complete remission	3.0×10^6	2.8×10^6	<0.01	10.02	$<3.0 \times 10^2$	2.81×10^5	936.7
Patient 3	Chronic	Complete remission	8.6×10^6	1.5×10^7	0.02	9.02	1.72×10^3	1.35×10^6	784.9
Patient 4	Lymphoma	Complete remission	7.5×10^6	1.1×10^7	0.06	10.92	4.5×10^3	1.02×10^6	226.7
Patient 5	Acute	Complete remission	3.0×10^6	1.0×10^7	0.03	0.15	9.0×10^2	1.50×10^4	16.7
Patient 6	Lymphoma	Complete remission	4.3×10^6	3.5×10^6	<0.01	0.62	$<4.3 \times 10^2$	2.17×10^4	>50.5
Patient 7	Chronic	Watchful waiting	2×10^7	1.0×10^8	1.32	12.50	2.64×10^5	1.25×10^7	47.3
Patient 8	Chronic	Watchful waiting	6.5×10^6	9.2×10^6	0.01	7.05	6.5×10^2	6.49×10^5	998.5
Patient 8′‡	Acute	Before treatment	5.26×10^6	5.5×10^6	0.02	0.02	1.05×10^4	1.10×10^4	1.05
Patient 8″‡	Acute	Partial remission	3.5×10^6	6.8×10^6	0.06	26.36	2.1×10^3	1.79×10^6	852.4
Patient 9	Smoldering	Under systemic phototherapy for skin	3.0×10^6	5.8×10^6	0.02	28.78	6.0×10^2	1.67×10^6	2783.3
Patient 10	Lymphoma	Initially diagnosed	7.3×10^6	1.2×10^7	<0.01	0.28	$<7.3 \times 10^2$	3.36×10^4	>46.0
Patient 11	Acute	Initially diagnosed	4.3×10^6	4.1×10^6	<0.01	0.14	$<4.3 \times 10^2$	5.74×10^3	>13.3
Patient 12	Acute	Initially diagnosed	5.2×10^6	ND	ND	ND	ND	ND	ND
Patient 13	Acute	Initially diagnosed	1.0×10^7	ND	ND	ND	ND	ND	ND
Patient 14	Acute	Diagnosed as relapse with	6.0×10^6	7.0×10^6	0.01	0.03	6.0×10^2	2.10×10^3	3.5
Patient 14 (CD4-subset)§		acute type phenotype	6.0×10^6	2.6×10^6	0.01	3.79	6.0×10^2	9.90×10^4	165.0

†Cell numbers of Tax tetramer + cells on day 14 was divided by that of day 0. ‡Patient 8 progressed from chronic to acute subtypes, and then he received allogeneic hematopoietic stem cell transplantation. §CD4+ cells were depleted on day 4. ATL, adult T-cell leukemia/lymphoma; CTL, cytotoxic T lymphocytes; HTLV-1, human T-lymphotropic virus type-1; ND, not detected.

In this culture, HLA-A2-restricted Tax11–19 specific CTL were also expanded (Fig. 4A, middle-left panel), even though the Tax11–19 peptide was not used as a stimulator. We surmised that pre-existing Tax-CTL, including these HLA-A2 Tax11–19 CTL, were stimulated by the ATL cells constitutively expressing HLA-A2/Tax11–19 complexes, contained in the cultured PBMC. These expanded T-cells were co-cultured with ATL cell lines, HTLV-1-immortalized lines or autologous ATL cells, and their responses were evaluated by IFN-γ production. HLA-A*24:02/Tax301–309 tetramer-positive fractions of these expanded CD8-positive cells produced IFN-γ when co-cultured with autologous ATL cells or ATN-1 (Fig. 4A), even though the Tax expression was so low as to be undetectable by flow cytometry, and only detectable by RT-PCR (Fig. 3). These tetramer-positive cells also responded to TL-Su and MT-2, but did not respond to the other ATL cell lines, or HTLV-1-immortalized lines tested. This indicates that only target cells having both HLA-A*24:02 and Tax were recognized (Table 2 and Fig. 3). The HLA-A*24:02/Tax301–309 tetramer-negative fractions of these expanded CD8-positive cells also produced IFN-γ when stimulated with autologous ATL cells. This suggests that they recognize unidentified Tax-derived epitopes, or antigens derived from HTLV-1 components other than Tax, or ATL-related tumor antigens not of viral origin. Finally, the HLA-A*02:01/Tax11–19 tetramer-positive fractions within these expanded CD8-positive cells were also found to produce IFN-γ on challenge with autologous ATL cells and TCL-Kan, but not the other ATL cell lines or HTLV-1-immortalized lines. This indicates that HLA-A2 and Tax expression were both required for recognition. HLA-A*02:01/Tax11–19 tetramer-negative cells also produced IFN-γ when stimulated by TCL-Kan. Because both patient 7 and TCL-Kan share HLA-B*46:01 and HLA-C*01:02 (Table 2), the tetramer-negative cells might be recognizing unidentified Tax-derived epitopes, other HTLV-1 antigens or ATL tumor antigens-derived epitopes presented on a different shared MHC allele. These effector cells did not respond to K562 by IFN-γ production, showing that they had no NK activity.

Next, PBMC from patient 8 at chronic stage were investigated in a similar manner, stimulated with Tax11–19 peptide (Fig. 4B, upper-left panel). HLA-A*02:01/Tax11–19 tetramer-positive cells in these expanded CD8-positive cells also produced IFN-γ (Fig. 4B) when stimulated with Tax RT-PCR-positive but flow cytometry-negative autologous ATL cells

Table 2. Human leukocyte antigen (HLA) information

	HLA-A		HLA-B		HLA-C	
TL-Su	*11:01	*24:02	*15:01	*40:02	*03:04	*04:01
TCL-Kan	*02:06	*02:07	*46:01	*56:01	*01:02	*07:02
K562						
HUT102	*30:02	*66:02				
ATN-1	*11:01	*24:02	*54:01	*67:01	*01:02	*07:02
MT-1	*11:01	*26:01	*39:01	*40:02	*03:04	*07:02
MT-2	*24:02	*24:02	*40:02	*51:01	*03:03	*14:02
MT-4	*11:01	*31:01	*39:02	*67:01	*07:02	*07:02
TL-Om1	*02:01	*02:01	*52:01	*52:01	*12:02	*12:02
Patient 1	*02:01	*02:01	*15:01	*40:02	*03:04	*07:02
Patient 3	*02:01	*31:01				
Patient 4	*24:02	*26:01				
Patient 5	*02:06	*24:02				
Patient 6	*02:06	*31:01				
Patient 7	*02:07	*24:02	*46:01	*52:01	*01:02	*12:02
Patient 8	*02:01	*02:06	*35:01	*55:02	*01:02	*03:03
Patient 9	*02:01	*31:01				
Patient 10	*11:01	*24:02				
Patient 11	*11:01	*24:02				
Patient 12	*02:06	*24:02				
Patient 13	*02:03	*31:01				
Patient 14	*24:02	*31:01	*07:02	*40:01	*03:04	*07:02

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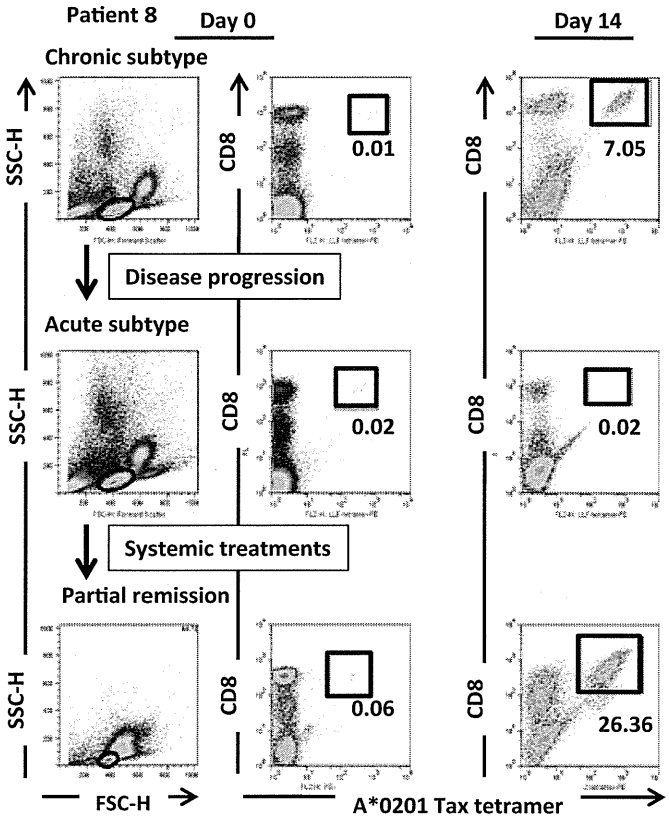

Fig. 1. Expansion of Tax-specific CTL from PBMC of patient 8 at different clinical stages. Flow cytometric analyses of the expanded cells are presented. The lymphocyte population was determined by FSC-H and SSC-H levels (left panels) and the data are plotted to show CD8 and human leukocyte antigen (HLA)-A*02:01/Tax tetramer-positivity (right two panels). Both CD8 and HLA-A*02:01/Tax tetramer-positive cells are gated, and their percentages relative to the entire lymphocyte population are indicated in each panel. Patient 8 progressed from chronic to acute stage disease. His Tax-CTL could be efficiently expanded during the chronic phase (upper panels), but no longer after progression to acute stage (middle panels). Subsequently, he received allogeneic hematopoietic stem cell transplantation, and achieved partial remission. At this time, his Tax-CTL could be efficiently expanded from PBMC once more (lower panels).

(Fig. 3). These tetramer-positive cells responded to TCL-Kan but not to the other ATL cell lines or HTLV-1-immortalized lines. Thus, their recognition was also restricted by the expression of HLA-A2 and Tax (Table 2 and Fig. 3). HLA-A*02:01/Tax11–19 tetramer-negative fractions were also stimulated by autologous ATL cells, again suggesting recognition of unidentified epitopes. HLA-A*02:01/Tax11–19 tetramer-negative cells also produced IFN-γ when stimulated by TCL-Kan. Because patient 8 and TCL-Kan are both HLA-C*01:02-positive (Table 2), these effector cells might be recognizing unidentified epitopes presented on this shared MHC allele. Again, there was no IFN-γ production against K562.

We also repeated these experiments with PBMC from patient 14, and evaluated them in the same manner. In this case as well, the HLA-A*24:02/Tax301–309 tetramer-positive cells responded to autologous ATL cells and ATN-1 (Fig. 4C), again despite the very low level of Tax expression. They also responded to TL-Su, but not the other ATL cell lines or HTLV-1-immortalized lines, showing HLA-A*24:02 and Tax restriction (Table 2 and Fig. 3). Once more, the HLA-A*24:02/Tax301–309 tetramer-negative cells were also stimulated by autologous ATL cells, indicating recognition of unidentified epitopes presented on autologous MHC molecules. HLA-A*24:02/Tax301–309 tetramer-negative cells also produced IFN-γ when stimulated with TL-Su, which shares HLA-C*03:04 with patient 14 (Table 2). Again, no NK activity was detectable.

Lysis of autologous adult T-cell leukemia/lymphoma cells by Tax-specific CTL. Cells from patient 7 expanded by Tax301–309 peptide (Fig. 4A) killed TL-Su, MT-2, ATN-1 and autologous ATL cells in an E/T ratio-dependent manner, but did not lyse MT-1 or HUT102 (Fig. 5, left panel). Lysis depended on the presence of both HLA-A*24:02 and Tax (Table 2 and Fig. 3). Although as mentioned before, the level of Tax expression by these autologous ATL cells and ATN-1 was so low as to be detectable only by RT-PCR and not by flow cytometry, objective lysis of both cells was still observed. The patient 7 Tax-CTL expanded by Tax301–309 peptide stimulation also killed TCL-Kan. HLA-A2-restricted Tax11–19 CTL included in the effector subset presumably contributed to the lyses of TCL-Kan as well as autologous tumor cells (Fig. 4A, middle-left panel). Again, these expanded cells did not possess NK activity. The cells from patient 8 at chronic stage expanded by Tax11–19 peptide (Fig. 4B) killed TCL-Kan and autologous ATL cells, but not TL-Om1 (Fig. 5, middle panel) in an HLA-A2-restricted and Tax-restricted manner (Table 2 and Fig. 3). Again, Tax expression by the autologous ATL cells was extremely low, but the targets were, nonetheless, killed. As with the other patients, there was no NK activity present in the expanded cells.

Finally, cells from patient 14 stimulated by Tax301–309 peptide (Fig. 4C) killed TL-Su and autologous ATL cells, but not MT-1 (Fig. 5, right panel), restricted by HLA-A*24:02 and Tax (Table 2 and Fig. 3), again with no NK activity.

Tax expression in primary adult T-cell leukemia/lymphoma cells induced by short-term culture. It was previously reported that although Tax expression was not detectable in primary ATL cells by flow cytometry in most cases, short-term culture of such cells could induce Tax expression in nearly half of cases.[33] Tax expression and its regulation in primary ATL cells is currently not fully understood. We tested Tax expression of primary ATL cells from patients 7, 8, 13 and 14, as listed in Table 1, and 2 additional patients, 15 and 16 (both chronic type). Tax protein was not present in any primary uncultured ATL cells isolated with anti-human CD4 microbeads from patients' peripheral blood. In all cases, these cells were in a quiescent state, as determined by 7-AAD staining (Fig. 6A). Cells incorporating BrdU (S phase) and those having double DNA content (G2/M phase) first appeared on culture of the primary ATL cells for several days, indicating that they had begun to cycle. At the same time, Tax-expressing cells appeared in three of six cases (patients 7, 8 and 13) (Fig. 6B). These findings indicate that Tax expression was induced in primary ATL cells when they were actively cycling (i.e. cells not in G0 phase). Because most primary ATL cells in the peripheral blood are in a quiescent state (G0 phase), they express little or no Tax.

Tax expression in primary adult T-cell leukemia/lymphoma cell-bearing NOG mice. NOG mice bearing primary ATL cells were established using ATL cells of patients 7, 12 and 13, as previously described.[34] ATL mice from patient 7 presented with large intraperitoneal tumor masses, and tumor cells aggressively infiltrated into liver and spleen, but into the blood only to a lesser extent. Setting the *Tax/human CD4* mRNA level of TL-Su as unity, these values for blood cells, liver, spleen and tumor cell suspensions were 0.00195 ± 0.00065 (standard deviation), 0.023000 ± 0.00312, 0.00626 ± 0.00214 and 0.19533 ± 0.02185, respectively. Because there was little ATL cell infiltration into bone marrow, the *Tax/human CD4* mRNA value of bone marrow cells was under the limit of detection

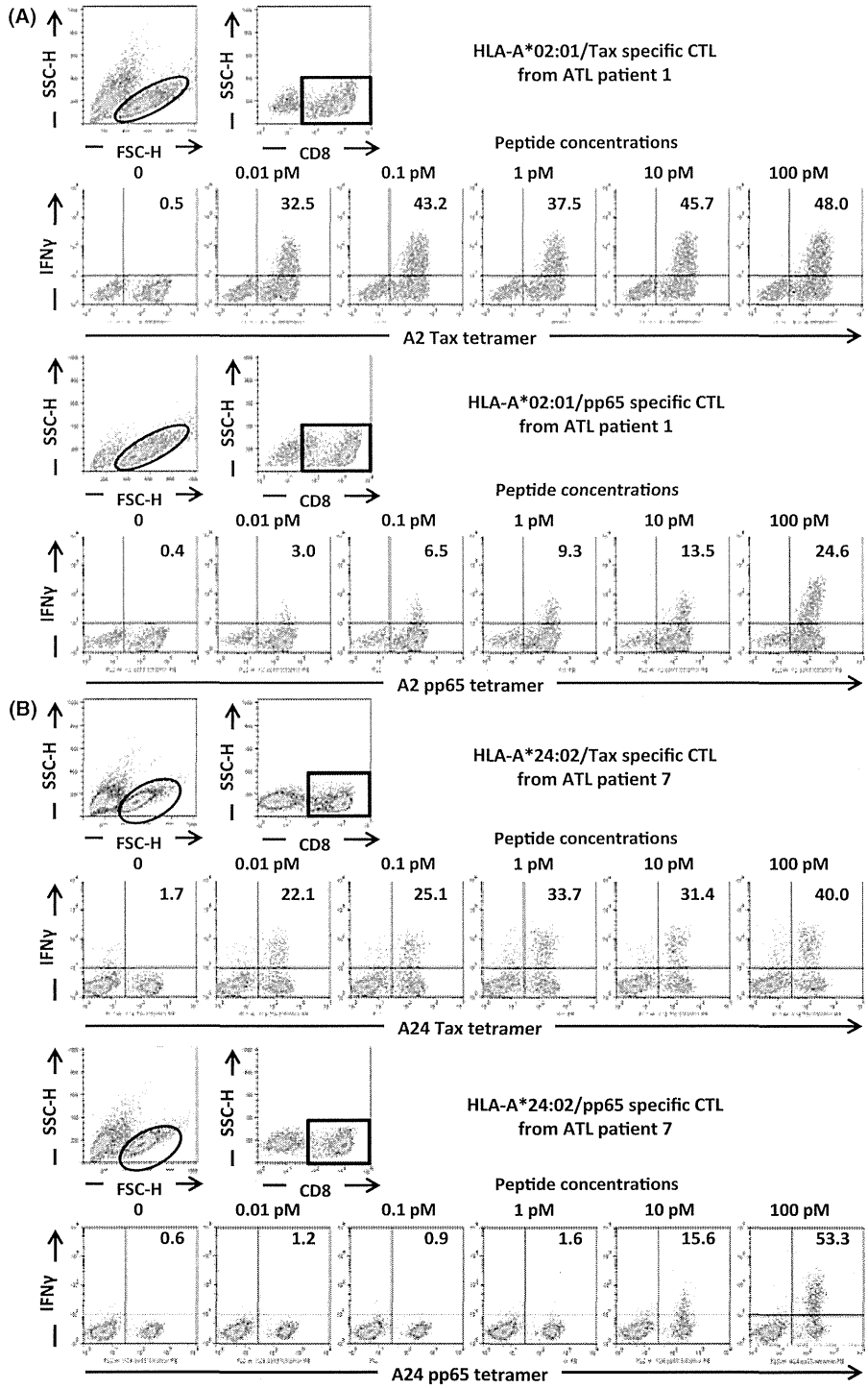

Fig. 2. T-cell receptor avidity of Tax-CTL for Tax epitope peptides. (A) PBMC from adult T-cell leukemia/lymphoma (ATL) patient 1 were stimulated by Tax11–19 peptide, and the expanded cells were then cultured with serial concentration of the cognate peptide. Flow cytometric analyses of those cells are presented. The lymphocyte population was identified by FSC-H and SSC-H levels, and CD8-positive cells gated. These were then plotted according to human leukocyte antigen (HLA)-A*02:01/Tax tetramer-positivity and interferon gamma (IFN-γ) production. The percentages of IFN-γ-producing cells relative to the entire population of HLA-A*02:01/Tax-positive cells are indicated in each panel (upper panels). PBMC from ATL patient 1 were also stimulated by CMV-pp65 495–503 peptide, and then restimulated with the cognate peptide, and flow cytometric analyses of those cells are presented in the same manner as above. The percentages IFN-γ-producing cells relative to the entire population of HLA-A*02:01/CMV-pp65-positive cells are indicated in each panel (lower panels) (B) PBMC from ATL patient 7 were stimulated with Tax301–309 peptide, and then restimulated as above: HLA-A*24:02/Tax301–309 tetramer positivity and IFN-γ production (upper panels). PBMC from ATL patient 7 stimulated with CMV-pp65 495–503 peptide, and treated as above. Each result represents three independent experiments.

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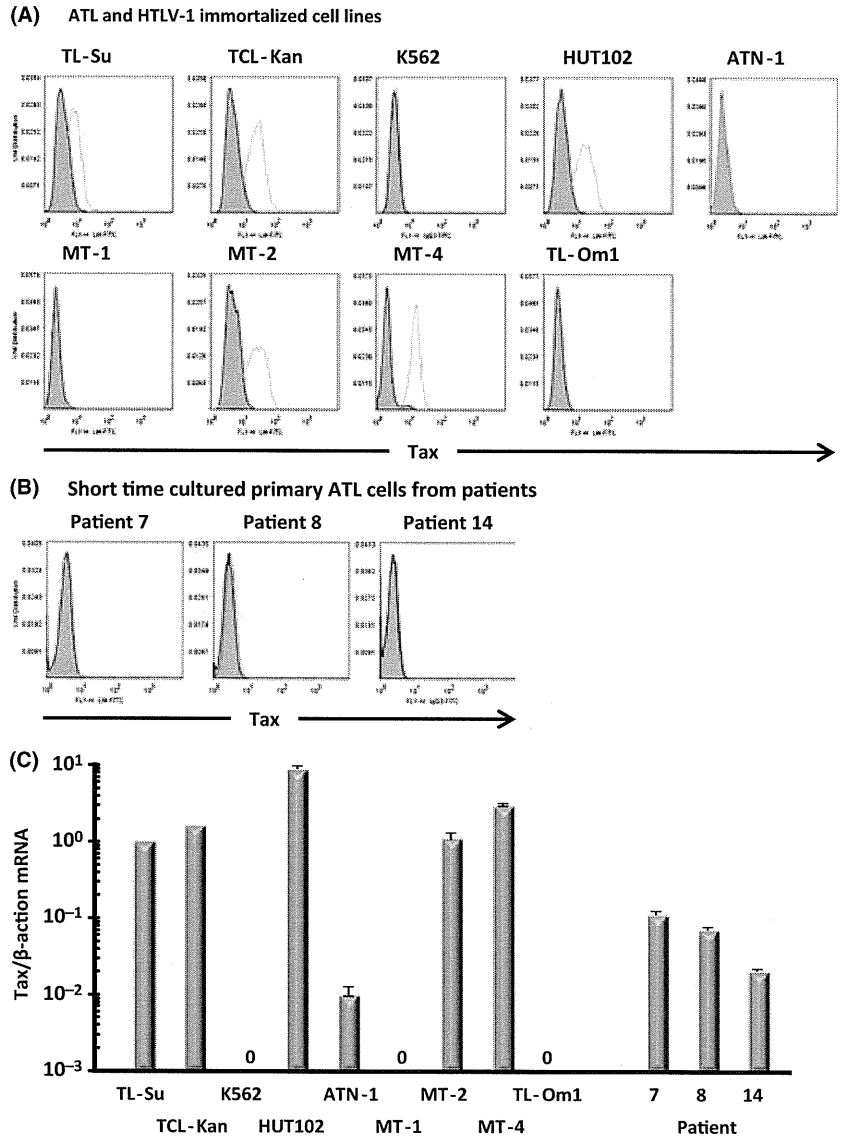

Fig. 3. Expression of human T-lymphotropic virus type 1 (HTLV-1) Tax in adult T-cell leukemia/lymphoma (ATL) cells. (A) Tax expression in ATL cell lines, HTLV-1-immortalized lines and K562 were analyzed by flow cytometry. The cells lines were stained with anti-Tax mAb (blank histograms) or isotype control mAb (filled histograms). (B) Tax expression in short-term cultured ATL cells from patients analyzed by flow cytometry. (C) *Tax* expression in the cell lines and short-term cultured ATL cells from patients analyzed by quantitative RT-PCR by dividing the *Tax* expression level by *β-actin*, resulting in a *Tax/β-actin* mRNA ratio with the expression level in TL-Su set at unity. Columns, mean of triplicate experiments; bars, standard deviation.

(Fig. 7A). *Tax* expression in ATL cells from tumor masses was almost 100-fold higher than in the blood.

ATL mice from patient 12 presented with marked hepatosplenomegaly, but few tumor cells in the blood. *Tax/human CD4* mRNA values of blood cells, liver, and spleen cell suspensions were 0.01337 ± 0.00083, 0.05277 ± 0.00805 and 0.08323 ± 0.00080, respectively. Again, no *Tax/human CD4* mRNA could be detected in bone marrow cells (Fig. 7B).

Adult T-cell leukemia/lymphoma mice from patient 13 also presented with marked hepatosplenomegaly, but also with tumor infiltration into blood and bone marrow. *Tax/human CD4* mRNA values of blood cells, liver, spleen cell suspensions and bone marrow cells were 0.01013 ± 0.00102, 0.12742 ± 0.01524, 0.15411 ± 0.01612 and 0.28881 ± 0.07319, respectively (Fig. 7C).

These observations are consistent with other results from the present study that Tax expression is observed predominantly in actively cycling ATL cells, whereas most primary ATL cells in the peripheral blood are in a quiescent state. Thus, only ATL cells present at the site of active cell proliferation, such as in the tumor masses, liver or spleen, strongly express Tax, but this factor is minimally expressed by the tumor cells in a quiescent state, such as in the blood.

Discussion

The significant findings in the present study are as follows. The efficiency of *in vitro* Tax-CTL expansion was dependent on the stage of disease development following HTLV-1 infection. HTLV-1 Tax-CTL expanded *in vitro* could recognize HLA/Tax-peptide complexes on autologous ATL cells, the Tax expression of which was so low as to be detectable only by RT-PCR and not by flow cytometry. Tax recognition resulted in the production of IFN-γ and killing of the target cells. In an assay of TCR avidity, both HLA-A*02:01-restricted and HLA-A*24:02-restricted Tax-CTL responded to as little as 0.01 pM of the epitope peptide, a concentration much lower than required for recognition of any other viral or tumor antigens. This documents the extremely high TCR avidity of Tax-CTL, which is presumably one of the reasons why these CTL could recognize and kill the autologous ATL cells, despite their very low Tax expression. To the best of our knowledge, this is the first report of Tax-specific CTL from ATL patients specifically recognizing and killing autologous tumor cells that express the Tax antigen. Earlier studies examined the responses of CD8 cells against autologous cells from ATL, HTLV-1-associated myelopathy/tropical spastic paraparesis patients or HTLV-1

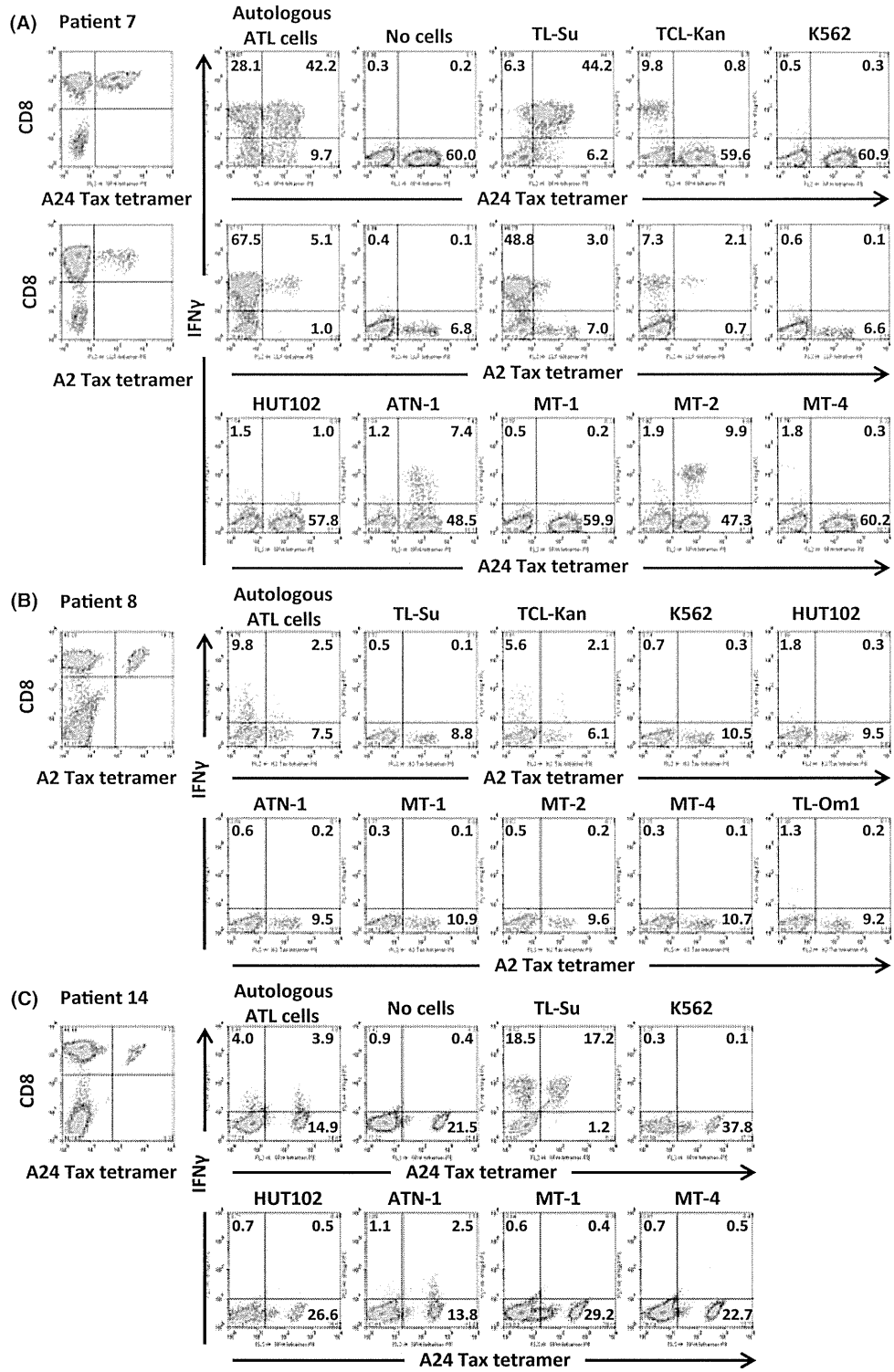

Fig. 4. Tax-specific CTL responses against autologous adult T-cell leukemia/lymphoma (ATL) cells. (A) PBMC from patient 7 were stimulated with human leukocyte antigen (HLA)-A*24:02 restricted Tax301–309 peptide, and the resulting CTL were expanded (upper-left panel). In this culture, HLA-A2-restricted Tax11–19 specific CTL were also expanded (middle-left panel). The expanded cells were co-cultured with autologous ATL cells, ATL cell lines, human T-lymphotropic virus type 1 (HTLV-1)-immortalized lines and K562 (all CD8-negative) for 4 h. CD8-positive cells are plotted according to HLA-A*24:02/Tax301–309 or HLA-A*02:01/zax11–19 tetramer-positivity and interferon gamma (IFN-γ) production, and the percentages in each quadrant are presented in the panels. (B) PBMC from ATL patient 8 at chronic stage were stimulated by Tax11–19 peptide, and the expanded cells co-cultured with the same range of cells as in (A). CD8-positive cells are plotted by HLA-A*0201/Tax11–19 tetramer positivity and IFN-γ production. The HLA-A*02:01/Tax11–19 tetramer recognized HLA-A*02:07-restricted Tax11–19 specific CTL. (C) PBMC from ATL patient 14 were stimulated with Tax301–309 peptide, and treated as in (A, B) above. Each result represents three independent experiments.

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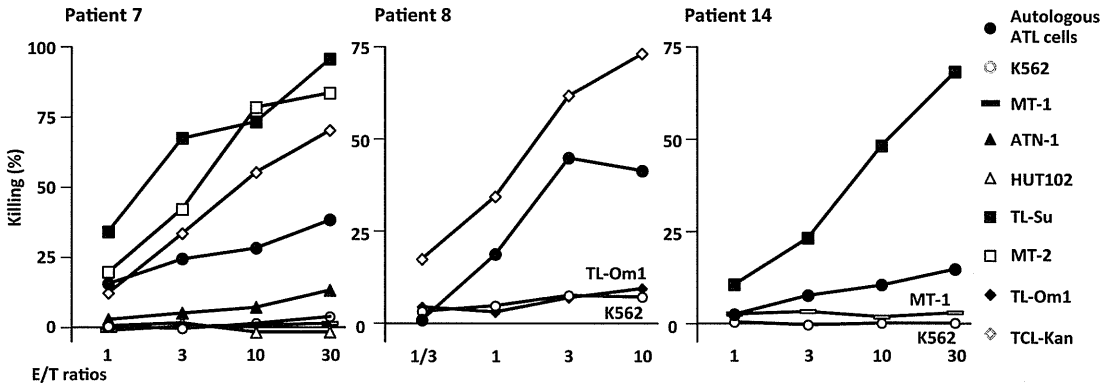

Fig. 5. Lysis of autologous adult T-cell leukemia/lymphoma (ATL) cells by Tax-specific CTL. Tax301–309 peptide-expanded cells from ATL patient 7 (left panel), Tax11–19 peptide-expanded cells from patient 8 (middle panel) and Tax301–309-stimulated patient 14 (right panel) were evaluated for cytotoxicity 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

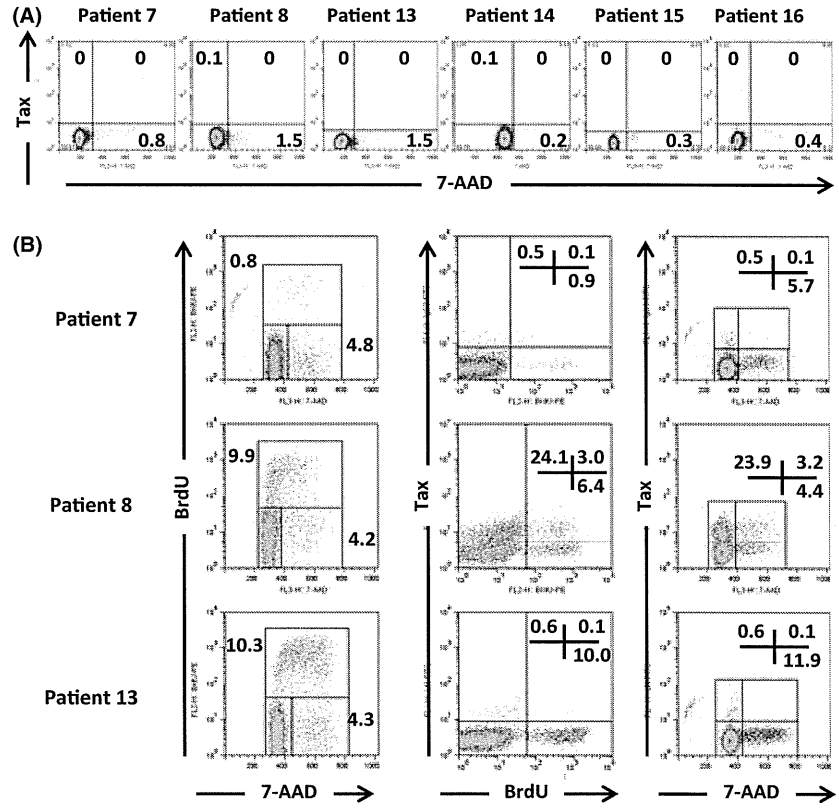

Fig. 6. Tax expression in adult T-cell leukemia/lymphoma (ATL) cells induced by short-term culture. (A) Lack of Tax expression in primary ATL cells from peripheral blood of patients 7, 8, 13, 14, 15 and 16. Cells were in a quiescent state as determined by 7-ADD staining. (B) Cell cycle status and Tax expression of short-term cultured primary ATL cells. Tax expression was induced when cells were actively cycling. Each result represents three independent experiments.

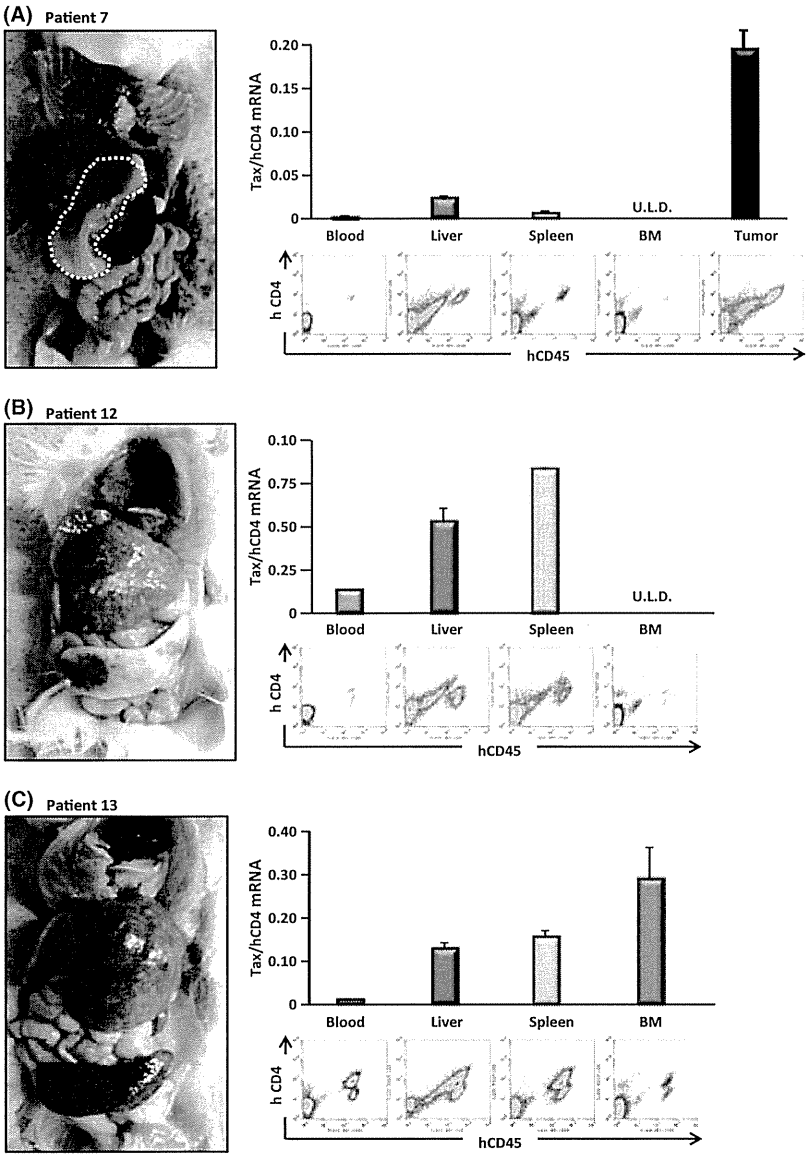

Fig. 7. Tax expression in primary adult T-cell leukemia/lymphoma (ATL) cell-bearing NOG mice. Tax expression of ATL cells in each affected organ of NOG mice bearing primary ATL cells from patient 7 (A), 12 (B) and 13 (C) were evaluated. NOG mice with cells from patient 7 presented with large intraperitoneal 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.

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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-2Rγ^{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.

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Keywords: bevacizumab; NOD/Shi-*scid*; IL-2Rγ^{null} (NOG) mouse; lymphoma; tumor microenvironment

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-2Rγ^{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 ⩾50%, 2+ positive if <50 ⩾30%, 1+ positive if <30 ⩾10% and negative if <10% of the DLBCL tumor cells were stained with the corresponding antibody. Nine ×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 ×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 1.0×10^7 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 1.0×10^7 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 (%)

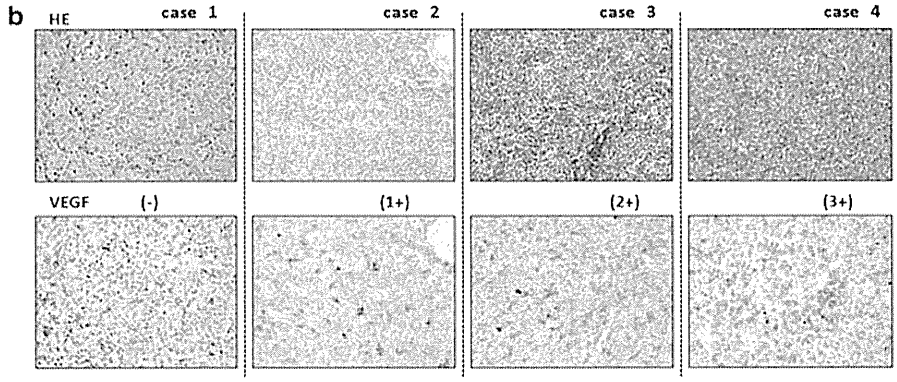

Figure 1. VEGF-A expression in DLBCL. (**a**) VEGF-A expression of DLBCL cells in the lymph node lesions according to GCB or non-GCB phenotypes. VEGF-A expression was categorized based on the percentage of DLBCL cells stained as follows: ⩾50%, 3+ positive; 30–49%, 2+ positive; 10–29%, 1+ positive; <10%, 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 immunopathological 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

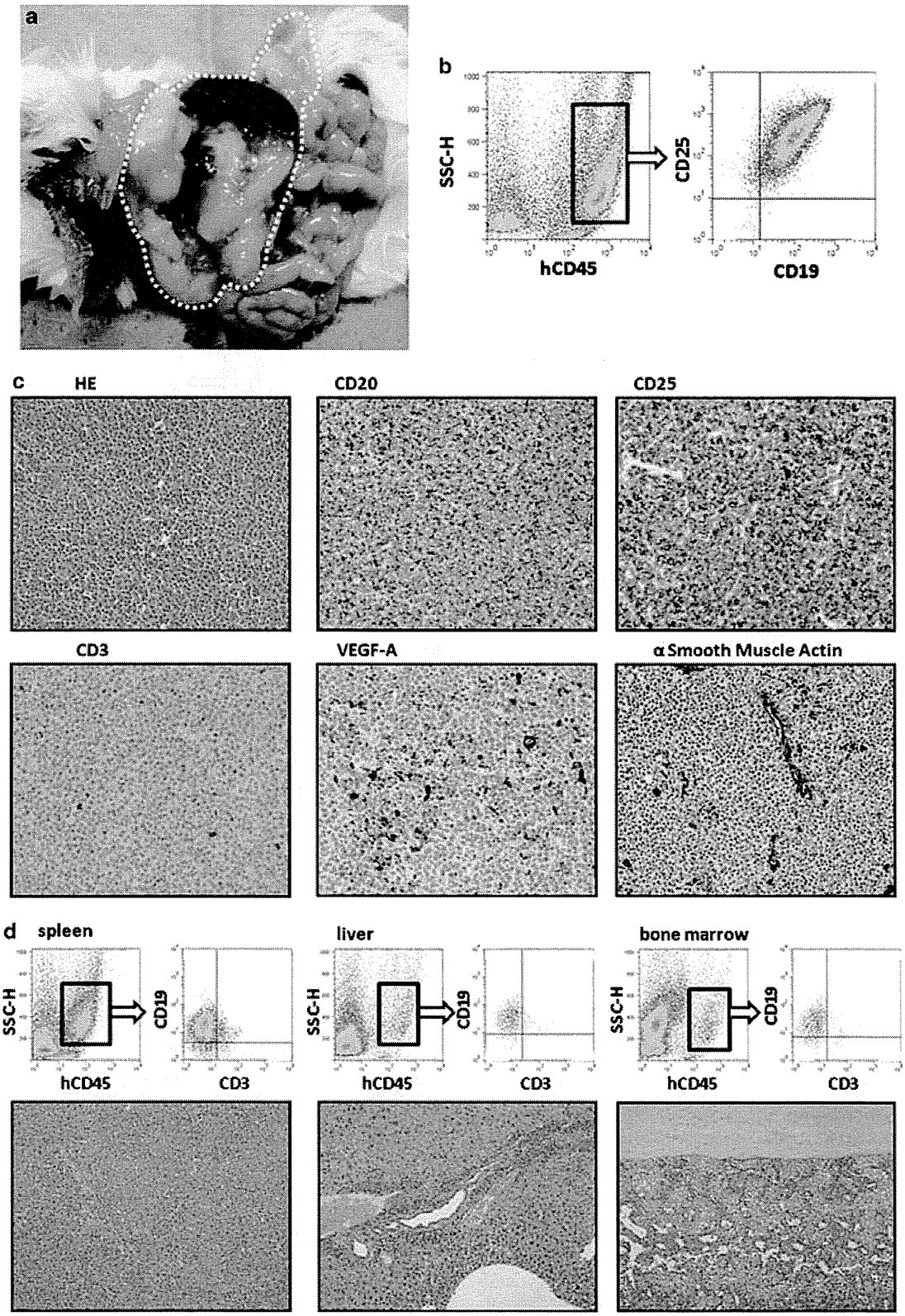

Figure 2. Primary DLBCL cell-bearing NOG mouse model. (**a**) Macroscopic appearance of a primary DLBCL cell-bearing NOG mouse. The intraperitoneal mass is demarcated by a thin white dotted line. (**b**) Human CD45 + cells in the mass determined by human CD19 and CD25 expression. (**c**) Immunohistochemical 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.

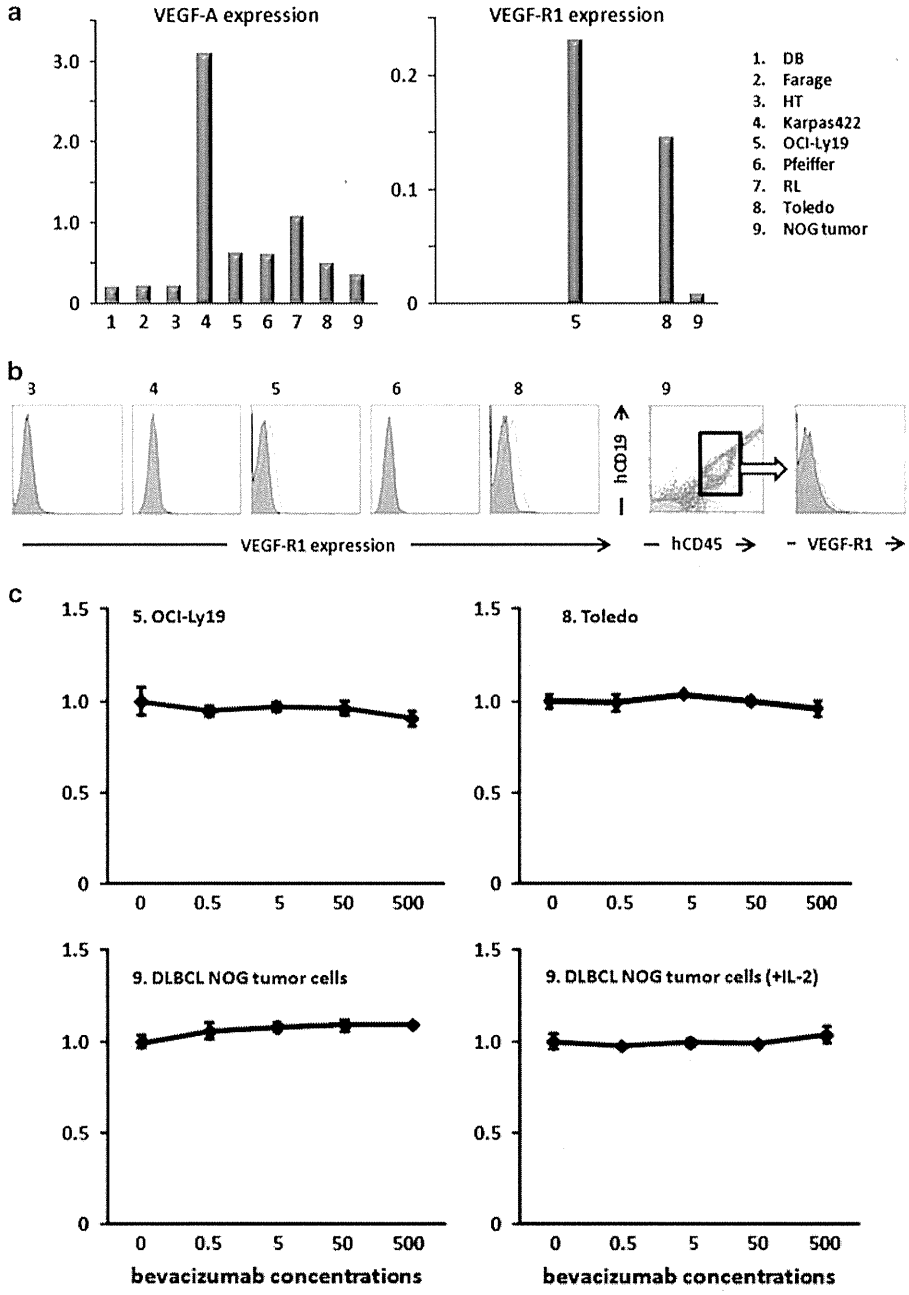

Figure 3. VEGF-A, VEGF-R1 and VEGF-R2 expression in DLBCL cell lines. (**a**) Quantitative reverse transcription (RT)-PCR analysis for VEGF-A and VEGF-R1 in eight DLBCL cell lines, and NOG DLBCL cells from the intraperitoneal mass. (**b**) Flow cytometry for VEGF-R1 in DLBCL cell lines, and NOG DLBCL 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 $\times 10^3$ pg/ml), compared with CHOP alone (83.5, 78.1, 49.5–119.3 $\times 10^3$ 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).

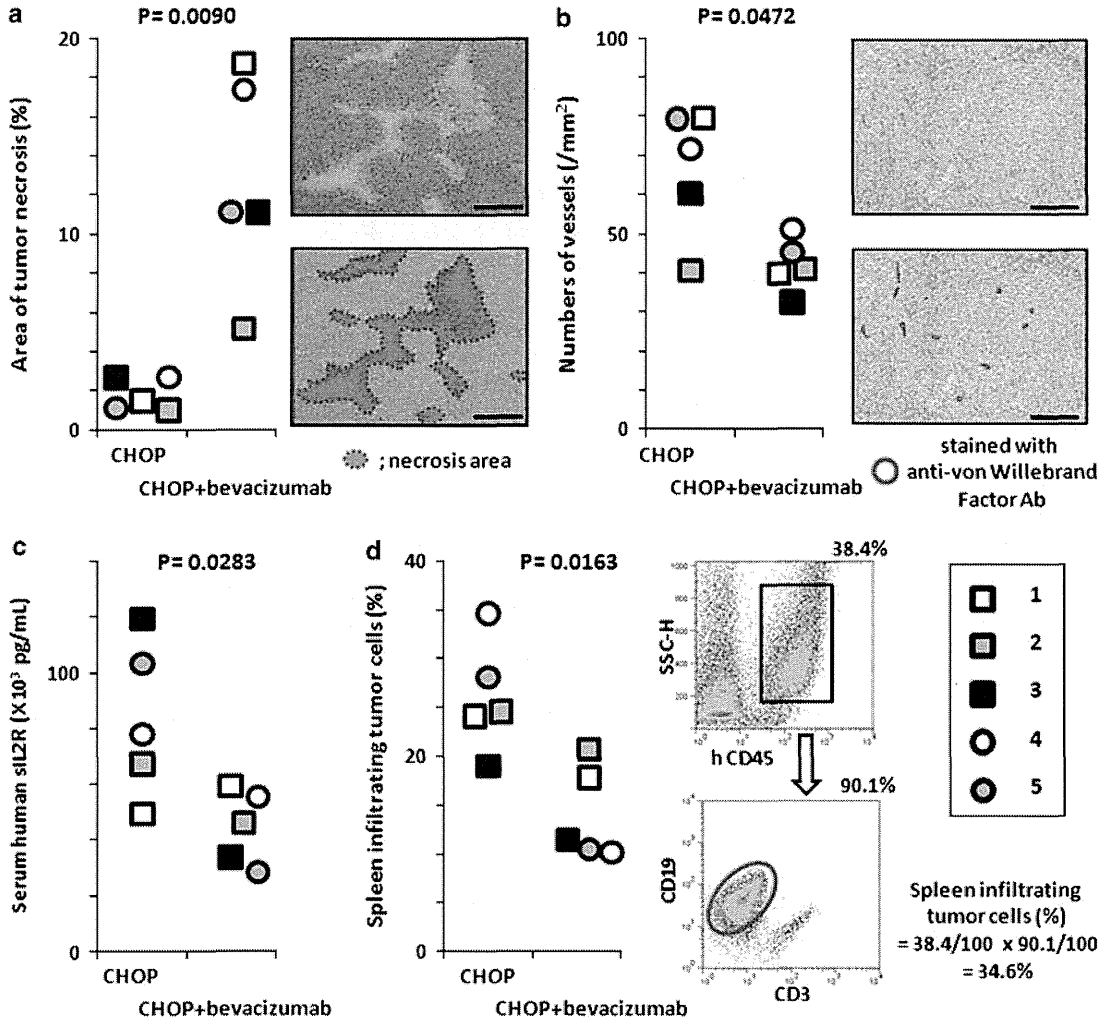

Figure 4. CHOP + bevacizumab has greater therapeutic efficacy than CHOP alone. (**a**) Area of tumor necrosis (%) of each primary DLBCL-bearing NOG mouse. The CHOP + bevacizumab-treated mice had significantly greater tumor necrosis than CHOP-treated mice (left panel). An example of a calculation for 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 $\times 10^3$ pg/ml), compared with controls (459.6, 482.8, 201.5–689.5 $\times 10^3$ 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

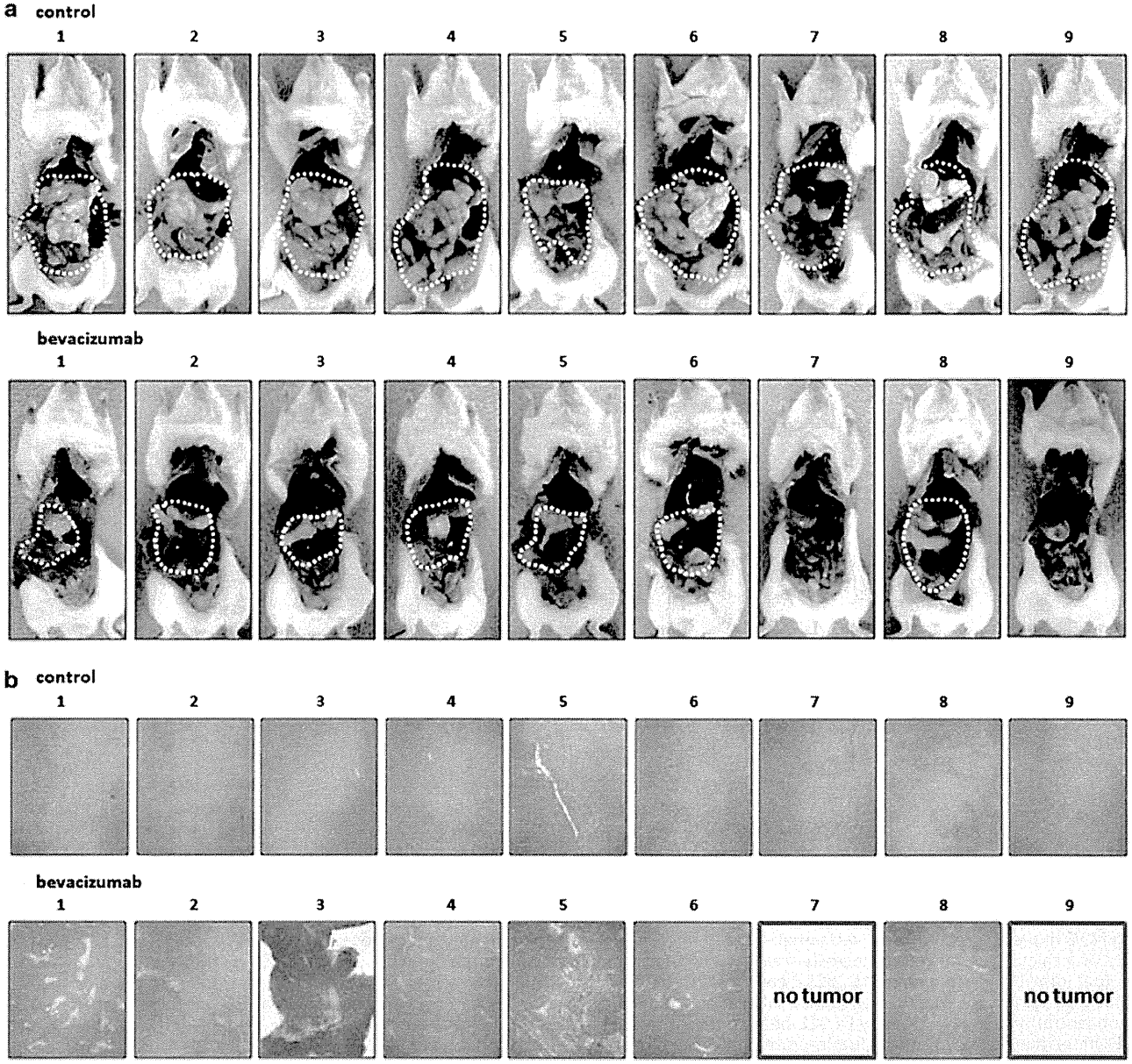

Figure 5. Macroscopic and microscopic findings of mice with or without bevacizumab therapy. (**a**) Macroscopic appearance of mice treated with saline (control; upper panels) or bevacizumab (lower panels). Tumor masses are demarcated by thin white dotted lines. (**b**) Photomicrographs with hematoxylin 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

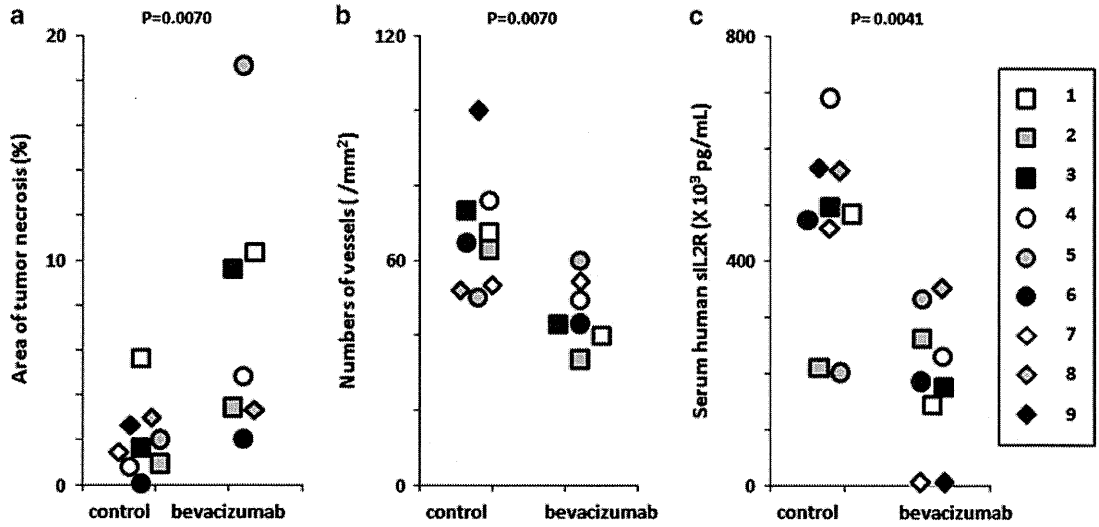

Figure 6. Bevacizumab therapy has significant therapeutic efficacy in the DLBCL mice. (**a**) Area of tumor necrosis (%) of each primary DLBCL-bearing NOG mouse. The bevacizumab-treated mice had significantly more tumor necrosis than controls. (**b**) Vessel numbers (per mm^2) of each primary DLBCL-bearing 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.

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