The reason for the difficulty in producing MHC class II tetramers has generally been considered to be due to inappropriate accommodation of the peptide in the groove of the MHC class II molecule, resulting in unnatural conformation. One of the constraints for MHC class II tetramer production is derived from the ambiguity of determining epitopes for CD4 T-cells. Peptides with the addition of various lengths of N- and C-terminal ends to the minimal core sequence are recognized by CD4 T-cells. Moreover, it is difficult to determine whether the minimal peptide is a naturally presenting epitope or not [18,19]. Lack of accurate information about natural HLA class II epitopes appears to be one of the reasons for the difficulty in HLA class II tetramer production.

Moreover, low binding affinity/avidity of the peptide to MHC class II molecules may also be involved. In this study, we confirmed successful tetramer production with differential retention time by HPLC. For example, the prolongation of the retention time was 0.554 min with the addition of the 12-mer NY-ESO-1 123–134 peptide (LKEFTVSGNILT) to the DRB1*08:03 monomer, but was 0.039 min with the addition of a negative control peptide to DRB1*08:03. The prolongation of the retention time was 0.246 min with the positive control 15-mer CLIP peptide (PVSKMRMATPLLMQA). However, the possibilities discussed above were also considered for the failure to produce a tetramer using the minimal epitope peptides. First, the use of an inappropriate epitope may have been involved. Defining the precise length of natural epitopes bound to class II molecules is extremely difficult as described above. Second, the epitope peptide may have weak binding affinity for the MHC class II molecules used for tetramer production (see below). With the core 9-mer peptides bound to HLA-DRB1*08:03, hydrophobic residues at P1 as phenylalanine (F) or tyrosine (Y) and residues at P6 as proline (P), serine (S), arginine (R) or asparagine (N) are relevant as anchor residues [20,21]. F at position 126 and N at position 131 in NY-ESO-1 121–138 may contribute to binding. Addition of isoleucine (I) at position 135 strongly stabilized tetramer production. Third, binding instability of the peptide to class II molecules may also be involved.

In addition to the failure to produce MHC class II tetramers using the epitope peptides, this study showed unexpected binding of the tetramer with a peptide not recognized by CD4 T-cells. The clone Mz-1B7 did not recognize the free peptide 122–135 on autologous EBV-B cells as APC, but the peptide 122–135/DRB1*08:03 tetramer bound to the TCR on those cells. The possibility of a lack of binding of the free peptide 122–135 to the DRB1*08:03 molecule on autologous APC is unlikely because clone Ue-21 recognized it efficiently. Rather, the tetramer binding could be due to a subtly modified structure of the 122–135 peptide/DRB1*08:03 tetramer from the structure of the free 122–135 peptide/DRB1*08:03 molecule. This could result from structural modification of either the peptide or the DR molecule, or both, during preparation of the peptide/DR tetramer, or simply be due to a subtle conformational change in the DR molecule itself due to fusion of the leucine zipper motif [8]. In the latter, it is possible that association of DRα and DRβ chains by the leucine zipper motif on each chain caused a subtle difference in the conformation of the natural DR molecule, although there was no convincing evidence to support this idea in this study.

Here, we also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from a prostate cancer patient P-3 and an esophageal cancer patient E-1 after CHP-NY-ESO-1 vaccination, and a lung cancer patient TK-OLP-01 after NY-ESO-1 OLP vaccination. These patients possessed the DRB1*08:03 allele. Patient P-3 was positive for the NY-ESO-1 antibody before vaccination (sero-positive) and patients E-1 and TK-OLP-01 were sero-negative [15]. In these patients, tetramer-positive CD4 T-cells were detected after vaccination.

Based on the discussion above, a possible difference in CD4 T-cell clones recognizing the epitope peptides from those detected by the respective peptide/HLA class II tetramer should be taken into consideration in HLA class II tetramer analysis.

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Conflict of interest: There is no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.12.042.

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Vaccination With NY-ESO-1 Overlapping Peptides Mixed With Picibanil OK-432 and Montanide ISA-51 in Patients With Cancers Expressing the NY-ESO-1 Antigen

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Summary: We conducted a clinical trial of an NY-ESO-1 cancer vaccine using 4 synthetic overlapping long peptides (OLP; peptides #1, 79–108; #2, 100–129; #3, 121–150; and #4, 142–173) that include a highly immunogenic region of the NY-ESO-1 molecule. Nine patients were immunized with 0.25 mg each of three 30-mer and a 32-mer long NY-ESO-1 OLP mixed with 0.2 KE Picibanil OK-432 and 1.25 mL Montanide ISA-51. The primary endpoints of this study were safety and NY-ESO-1 immune responses. Five to 18 injections of the NY-ESO-1 OLP vaccine were well tolerated. Vaccine-related adverse events observed were fever and injection site reaction (grade 1 and 2). Two patients showed stable disease after vaccination. An NY-ESO-1-specific humoral immune response was observed in all patients and an antibody against peptide #3 (121–150) was detected firstly and strongly after vaccination. NY-ESO-1 CD4 and CD8 T-cell responses were elicited in these patients and their epitopes were identified. Using a multifunctional cytokine assay, the number of single or double cytokine-producing cells was increased in NY-ESO-1-specific CD4 and CD8 T cells after vaccination. Multiple cytokine-producing cells were observed in PD-1 (−) and PD-1 (+) CD4 T cells. In conclusion, our study indicated that the NY-ESO-1 OLP vaccine mixed with Picibanil OK-432 and Montanide ISA-51 was well tolerated and elicited NY-ESO-1-specific humoral and CD4 and CD8 T-cell responses in immunized patients.

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The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001857) on April 7, 2009 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

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The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1] NY-ESO-1 expression is observed in a wide range of human malignancies, but the expression is restricted to germ cells in the testis in normal adult tissues.[2] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.

Numerous cancer vaccine strategies are under development.[3,4] For patients with hormone-resistant prostate cancer, a dendritic cell (DC) vaccine has recently been approved.[5] The therapy is based on loading autologous DCs ex vivo with a lysate of a cultured prostate cancer cell line transfected with the genes of acid phosphatase and GM-CSF, with subsequent administration to patients to induce specific T-cell responses. However, its clinical efficacy seems to be limited.[6,7] Cancer vaccines using recombinant proteins and peptides are thought to involve DCs in vivo and have advantages in that materials are easy to secure, there is little toxicity, and there are no complex regulatory matters when compared with cell therapy.[8] As cancer vaccines using short peptides showed only limited efficacy, cancer vaccines using synthetic long peptides have been introduced.[4,9–11] Synthetic peptides of 25–50 amino acids are internalized and processed by DCs efficiently, and presented the antigens on MHC class I and II for T cells.[12,13] Maturation of DCs is associated with upregulation of costimulatory molecules on their surfaces and is crucial for efficient induction of T-cell responses. Adjuvants such as TLR ligands induce DC maturation and strongly augment the immunogenicity of cancer vaccines.[3,14]

Detection of pathogen-associated molecular patterns by the pattern recognition receptors on DCs and activation of subsequent signaling induce specific CD4 and CD8 T-cell responses.[15,16] Thus, the stimulatory effect of innate immunity on adaptive immune responses is useful for cancer vaccines. TLRs, nucleotide-binding oligomerization domain–like receptors, the retinoic acid-inducible gene-I-like or RIG-like receptors, and the C-type lectin receptors are the 4 known families of pattern recognition receptors.[17] In this study, we immunized patients with advanced cancers expressing the NY-ESO-1 antigen with 30–32-mer NY-ESO-1 overlapping long peptides (OLP), and Picibanil

OK-432 and Montanide ISA-51 as immunomodulators. OK-432 is a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes*.[9,18,19] It was developed as a nonspecific immune stimulant, TLR2, 4, and/or 9 agonist. OK-432 induces various cytokines including tumor necrosis factor-α (TNF-α), interferon (IFN), interleukin (IL)-2, and IL-6.[20] Recently, it was shown that in vivo administration of OK-432 overcame regulatory T-cell suppression in mice.[21] The vaccine also induced efficient NY-ESO-1 immunity in patients.

MATERIALS AND METHODS

NY-ESO-1 OLP Vaccine

NY-ESO-1 OLPs [peptide #1: NY-ESO-1 79–108 (GARGPESRLLEFYLAMPFATPMEAELARRS), peptide #2: NY-ESO-1 100–129 (MEAELARRSLAQDAPPLP VPGVLLKEFTVS), peptide #3: NY-ESO-1 121–150 (VLLKEFTVSGNILTIRLTAADHRQLQLSIS), and peptide #4: NY-ESO-1 142–173 (HRQLQLSISSCLQQL SLLMWITQCFLPVFLAQ)] were synthesized by Multiple Peptide Systems (San Diego, CA). The vaccine, consisting of 1 mg of NY-ESO-1 OLP including 0.25 mg each of the 4 peptides, 0.2 KE OK-432 (Picibanil; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and 1.25 mL ISA-51(Montanide; Seppic, Paris, France), was emulsified under sterile conditions. Synthesis, production, formulation, and packaging of the investigational agent were in accordance with current Good Manufacturing Practices and met the applicable criteria for use in humans.

Study Design

A pilot, open-label, multi-institutional clinical trial of the NY-ESO-1 OLP vaccine was designed to evaluate the safety, immune response, and clinical response. Patients eligible for entry were those who had advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC), a performance status of 0, 1, or 2, were 20 years old or above, had a life expectancy of 4 months or more, and did not have impaired organ function. Patients ineligible were those who were positive for HIV antibodies, had multiple cancers, autoimmune disease, serious allergy history, or active brain metastasis, or received chemotherapy, systemic steroid, or immunosuppressive therapy in the last 4 weeks. Nine patients, including 7 patients with esophageal cancer, a patient with lung cancer, and a patient with malignant melanoma, were enrolled in a washout period after surgery, chemotherapy or radiation therapy. The vaccine was administered subcutaneously once every 2 (esophageal cancer patients) or 3 (lung cancer and malignant melanoma patients) weeks to achieve better performance status in esophageal cancer patients to complete a cycle of 6 vaccinations. Four weeks after the last administration, the safety, immune response, and clinical response were evaluated. Thereafter, the vaccine was administered additionally. The 9 patients received 5–18 immunizations. Clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1)[22] and the immune-related response criteria (irRC).[23] Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) (http://ctep.cancer.gov/). The protocol was approved by the Ethics Committee of Osaka, Tokyo and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrollment in the study. The study was conducted in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (unique trial number: UMIN000001857) on April 7, 2009 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

Blood Samples

Peripheral blood was drawn from the patients at baseline, at each time point of immunization, and 4 weeks after the last immunization. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density gradient centrifugation using lymphoprep (AXIS-SHIELD Poc AS, Oslo, Norway). A CD8 T-cell–enriched population was obtained from PBMCs using CD8 microbeads with a large-scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). A CD4 T-cell–enriched population was then obtained from the residual cells using CD4 microbeads. The final residual cells were used as a CD4-depleted and CD8-depleted population. The 3 populations were stored in liquid N_2 until use. HLA typing of PBMCs was performed by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures.

NY-ESO-1 18-Mer Series Peptides

The following series of 28 overlapping NY-ESO-1 18-mer peptides spanning the protein were synthesized: 1–18, 7–24, 13–30, 19–36, 25–42, 31–48, 37–54, 43–60, 49–66, 55–72, 61–78, 67–84, 73–90, 79–96, 85–102, 91–108, 97–114, 103–120, 109–126, 115–132, 121–138, 127–144, 133–150, 139–156, 145–162, 149–166, 153–170, and 156–173. A 30-mer peptide, 151–180, was also synthesized. These 29 peptides (NY-ESO-1 18-mer series peptides) were synthesized using standard solid-phase methods based on N-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on a Multiple Peptide Synthesizer (AMS422; ABIMED, Langenfeld, Germany) at Okayama University.

ELISA

Recombinant NY-ESO-1 protein was prepared as described previously.[24] Recombinant protein (1 μg/mL) or peptide (10 μg/mL) in a coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) was adsorbed onto 96-well Polysorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 200 μL/well of 5% FCS/PBS for 1 hour at room temperature. After washing, 100 μL of serially diluted plasma was added to each well and incubated for 2 hours at room temperature. After extensive washing, horseradish peroxidase-conjugated goat anti-human pan-IgG, IgG_1, IgG_2, IgG_3, IgG_4, or IgM (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells, and the plates were incubated for 1 hour at room temperature. After washing and development, absorbance at 490 nm was read. Recombinant murine Akt protein[24] and ovalbumin (albumin from chicken egg white; Sigma, St. Louis, MO) were used as control proteins.

In Vitro Stimulation of CD4 and CD8 T Cells

Frozen cells were thawed and resuspended in AIM-V (Invitrogen, Carlsbad, CA) medium supplemented with 5% heat-inactivated pooled human serum (CM), and kept at room temperature for 2 hours. CD4-enriched and CD8-enriched populations (2×10^6) were cultured with irradiated

(30 Gy), autologous CD4-depleted and CD8-depleted PBMCs (2×10^6) in the presence of 29 NY-ESO-1 18-mer series peptides in 2 mL of CM supplemented with 10 U/mL rIL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/mL rIL-7 (Peprotech, London, UK) in a 24-well culture plate at 37°C in a 5% CO_2 atmosphere for 12 days. For the second stimulation, 1×10^6 instead of 2×10^6 responder cells were used in the culture described above. For nonspecific immune activation, T cells were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin with GolgiStop (BD Biosciences, Franklin Lakes, NJ) for 6 hours at 37°C.

IFN-γ Catch Assay

Responder CD4 or CD8 T cells (5×10^4) from the stimulation culture were cultured for 4 hours with autologous EBV-B cells (5×10^4) pulsed with mixed, or one of the 29 NY-ESO-1 18-mer series peptides. The cells were then treated with a bispecific CD45 and IFN-γ antibody (IFN-γ catch reagent; 2 μL) for 5 minutes on ice. The cells were diluted in AIM-V medium (3 mL) and placed on a slow rotating device (Miltenyi Biotec, Bergisch Gladbach, Germany) to allow IFN-γ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 1 hour, the cells were washed with cold buffer and treated with PE-conjugated anti-IFN-γ (detection reagent), and FITC-conjugated anti-CD4 or anti-CD8 mAb. After incubation for 10 minutes at 4°C, the cells were washed and analyzed by FACS Canto II (BD Biosciences).

IHC

IHC was performed as described previously.[9] E978[25] and EMR8-5 (Funakoshi, Tokyo, Japan) mAbs were used to analyze NY-ESO-1 and HLA class I expression, respectively. The reaction was evaluated as + + + (> 50% stained cells), + + (50%–25% stained cells), + (25%–5% stained cells), and − (< 5% stained cells).

Intracellular Staining (ICS) of Cytokines

CD4 or CD8 T cells were washed and treated with Cytofix and Perm Wash (BD Biosciences) according to the manufacturer instructions to stain them with IFN-γ-APC (BD Biosciences), IL-2-APC (BD Biosciences), IL-5-PE (BD Biosciences), IL-10-APC (BioLegend, San Diego, CA), IL-17 A-PerCP-Cy5.5 (eBioscience, San Diego, CA), and/or TNF-α-FITC (eBioscience) intracellularly. For Foxp3 staining, a human Foxp3 staining kit, Alexa 488 (BD Biosciences), was used. Cells were analyzed using a FACS Canto II (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

To amplify the *NY-ESO-1* cDNA segment, primers specific for *NY-ESO-1* were designed as described previously.[26] Primers for RT-PCR were: *NY-ESO-1 5′-AGT TCTACCTCGCCATGCCT-3′* (forward), *5′-TCCTCCT CCAGCGACAAACAA-3′* (reverse), *GAPDH 5′-ACCACA GTCCATGCCATCAC-3′* (forward), *5′-TCCACCACC CTGTTGCTGTA-3′* (reverse). The amplification program for *NY-ESO-1* was 1 minute at 94°C, 1 minute at 60°C, and 1.5 minutes at 72°C for 35 cycles after denaturing at 94°C for 1 minute. These cycles were followed by a 10-minute elongation step at 72°C. The amplification program for *GAPDH* was 1 minute at 94°C, 1 minute at 66°C, and 1.5 minutes at 72°C for 30 cycles after denaturing at 94°C for 1 minute. These cycles were followed by a 10-minute elongation step at 72°C. The PCR products were analyzed on a 1.3% agarose gel.

RESULTS

Patient Characteristics

Nine patients with advanced cancers expressing the NY-ESO-1 antigen were enrolled (Table 1). Seven were esophageal cancer patients. One was a malignant melanoma patient and 1 was a lung cancer patient. Eight patients completed a cycle of 6 vaccinations. One esophageal cancer patient, P-5, did not complete a cycle of vaccination due to disease progression and was given only 5 vaccinations, but was included in the analysis. Two patients, P-6 and P-7, were given additional vaccinations because of stable disease (SD) during treatment (Table 2).

Safety

Injection site reactions were observed in all patients (Table 1). The reaction was grade 1 in 7 patients and grade 2 with induration in 2 patients, but resolved without any treatment several months after vaccination. Fever (grade 1) was observed in 3 patients. No other adverse events either related or nonrelated to the vaccine were observed. The vaccine was well tolerated.

Monitoring of Humoral Immune Response

Serum antibodies against full-length NY-ESO-1 protein and also against 4 individual NY-ESO-1 OLP (peptides #1, 79–108; #2, 100–129; #3, 121–150; and #4, 142–173) used for the vaccine were investigated (Fig. 1). Three patients, P-2, P-3, and P-4, had antibodies against the NY-ESO-1 protein at the baseline (seropositive). Of those, P-2 and P-3 showed no antibodies against the 4 peptides, whereas P-4 showed antibodies against peptide #1 (79–108),

TABLE 1. Patient Characteristics

Patients	Age/Sex	Cancer	Vaccination	Vaccine-related Toxicity
P-1	69/M	Malignant melanoma	6	Injection site reaction (grade 1)
P-2	55/M	Lung cancer (adenocarcinoma)	6	Fever (grade 1), injection site reaction (grade 1)
P-3	66/M	Esophageal cancer (squamous cell carcinoma)	6	Injection site reaction (grade 1)
P-4	70/M	Esophageal cancer (squamous cell carcinoma)	6	Injection site reaction (grade 1)
P-5	58/M	Esophageal cancer (squamous cell carcinoma)	5	Injection site reaction (grade 1)
P-6	67/M	Esophageal cancer (squamous cell carcinoma)	18	Injection site reaction (grade 1)
P-7	74/M	Esophageal cancer (squamous cell carcinoma)	8	Injection site reaction (grade 1)
P-8	69/M	Esophageal cancer (squamous cell carcinoma)	6	Fever (grade 1), injection site reaction (grade 2)
P-9	70/F	Esophageal cancer (squamous cell carcinoma)	6	Fever (grade 1), injection site reaction (grade 2)

TABLE 2. Immune Responses and Tumor Responses After Vaccination With the NY-ESO-1 OLP Peptide

Patients	IHC*		Antibody‡		CD4§		CD8§		Target Tumor		Clinical Response‖ (Duration)¶
	MHC	NY-ESO-1	Pre	Post	Pre	Post	Pre	Post	Region	Total Diameter (mm)	
P-1	ND	+†	−	+	−	+	−	+	abLN + axLN	48	PD
P-2	+	+	−	+	−	+	−	+	meLN	24	PD
P-3	+	+	−	+	−	+	−	+	abLN	32	PD
P-4	+	+	+	+	ND	ND	ND	ND	liver	20	PD
P-5	+	+	−	+	−	−	−	−	pleura	28	PD
P-6	+	+	−	+	−	+	−	−	abLN + liver	33	SD (7 mo)
P-7	+	+	−	+	−	+	−	+	neLN	18	SD (4 mo)
P-8	+	+	−	+	−	+	−	+	pleura	30	PD
P-9	+	+	−	+	ND	ND	ND	ND	pleura	25	PD

*IHC was performed using EMR8-5 mAb for MHC class I (MHC) and E957 mAb for NY-ESO-1. IHC-positive cells: + + + > 50%; 50% ≥ + + > 25%; 25% ≥ + > 5%; 5% ≥ −.
†NY-ESO-1 expression of P-1 was determined by RT-PCR.
‡Antibody response was determined by ELISA (see Materials and methods section) using OD values at 25× dilution for each OLP peptide and at 100× or 1600× dilution for NY-ESO-1 protein. Antibody response shown here represents OD for NY-ESO-1 OLP: + + + > 2; 2 ≥ + + > 0.5; 0.5 ≥ + > 0.1; 0.1 ≥ −.
§CD4 and CD8 T-cell responses were determined by an IFN-γ catch assay with the cells stimulated in vitro once (1°IVS). IFN-γ-positive cells: + + + > 10%; 10% ≥ + + > 5%; 5% ≥ + > 1%; 1% ≥ −. The response was confirmed by cells stimulated in vitro twice (2°IVS).
‖Clinical response was evaluated by RECIST and irRC.
¶SD duration was measured from the start of the immunization until the patients were defined as PD by RECIST (P-6) or removed due to disease progression (P-7).
Ab indicates abdomen; ax, axilla; LN, lymph node; me, mediastinum; ne, neck; ND, not done; PD, progressive disease; SD, stable disease.

but not against the others. After vaccination, antibodies against peptide #3 (121–150) were detected firstly and strongly in all patients. Antibodies against peptide #4 (142–173) were also detected in 7 of 9 patients. In contrast, the antibody responses against peptides #1 (79–108) and #2 (100–129) were relatively weak. The antibody responses against the NY-ESO-1 protein also increased in parallel, or with a delay, compared with antibody responses against the peptides in all patients. Notably, in patients P-6 and P-7 who showed SD, strong antibody responses against the peptides and the protein were observed. The dominant Ig subtypes were IgG_1 and IgG_3. An increased IgM response against peptide #3 after vaccination was observed in 6 patients.

Monitoring of CD4 and CD8 T-Cell Responses

CD4 and CD8 T cells purified from PBMCs using antibody-coated magnetic beads were cultured for 12 days with a mixture of 29 NY-ESO-1 18-mer series peptides spanning the entire NY-ESO-1 protein [stimulated in vitro once (1°IVS)] and assayed for IFN-γ production by an IFN-γ catch assay after stimulation with a mixture of 29 NY-ESO-1 18-mer series peptides for 4 hours. CD4 and CD8 T cells harvested from 1°IVS were again cultured in a similar way to 1°IVS, except using 1×10^5 instead of 1×10^6 responder cells [stimulated in vitro twice (2°IVS)] and assayed by an IFN-γ catch assay to confirm weak response. As shown in Figure 2, an increase in the CD4 T-cell response was observed in all 7 patients investigated in 1°IVS. In contrast, an increase in the CD8 T-cell response was observed in 5 of 7 patients in 1°IVS. A CD8 T-cell response was detected after 2°IVS in 2 patients (P-5 and P-6), who showed only a marginal response in 1°IVS. The Supplementary Figure (Supplemental Digital Content 1, http://links.lww.com/JIT/A313) shows the representative results of the IFN-γ catch assay for P-3 and P-7.

Next, the peptides recognized by CD4 and CD8 T cells were investigated by an IFN-γ catch assay using cells stimulated in 2°IVS and testing against individual 29 NY-ESO-1 18-mer series peptides. As shown in Figure 3, CD4 T cells dominantly recognized 18-mer peptides #15 and #21 and their adjacent peptides. A CD8 T-cell response was observed against various peptides, including 18-mer peptides #15 and #21, which were recognized relatively dominantly. In the case of CD8, a response against peptides (1–78) not included in the vaccine preparation was also observed. The Supplementary Table (Supplemental Digital Content 2, http://links.lww.com/JIT/A314) shows patient HLA.

$Foxp3^+$ CD4 cells were also examined by ICS. As shown in Figure 2, a decrease after vaccination was observed in 4 of 6 patients investigated. In contrast, a slight increase followed by a decrease was observed in 2 patients.

Clinical Observation

As shown in Table 2, patients P-6 and P-7 showed SD during vaccinations and were given additional 12 and 2 vaccinations, respectively. The other 7 patients showed PD during vaccinations. There was no discrepancy in evaluation between RECIST and irRC and no evidence of clinical benefit after immunizations. The results of immunomonitoring and clinical responses are summarized in Table 2. As previously described, in patients P-6 and P-7 who showed SD, strong antibody responses against the peptides and the protein were observed.

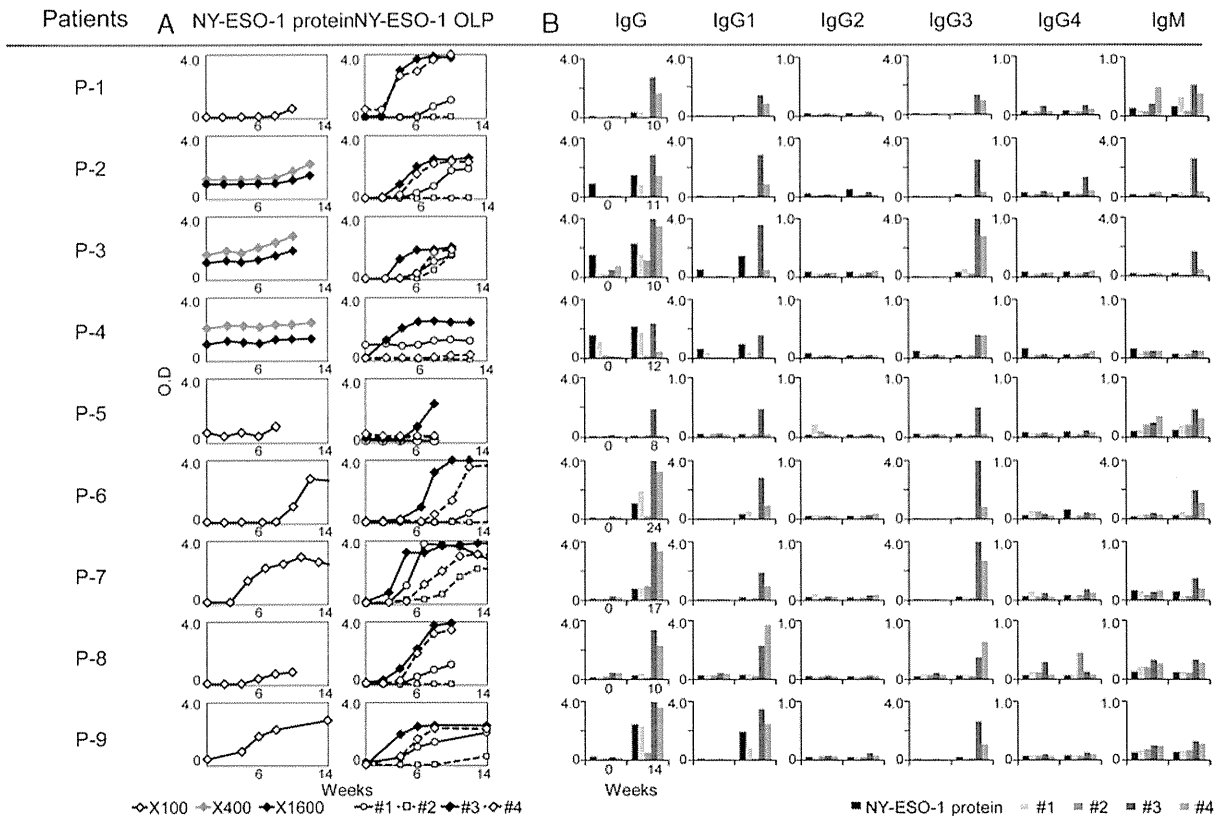

FIGURE 1. Immunomonitoring of humoral immune responses after NY-ESO-1 OLP vaccination. NY-ESO-1 antibody responses against recombinant NY-ESO-1 protein for each of the NY-ESO-1 OLP peptides were evaluated using plasma obtained before and after vaccination with NY-ESO-1 OLP from the 9 patients. A, Whole IgG antibody responses against recombinant NY-ESO-1 protein or each peptide included in the NY-ESO-1 OLP vaccine at a dilution of 1:100 (open diamond) or 1:1600 (closed diamond) for the protein and at a dilution of 1:25 for the peptides at the baseline (week 0) and at each vaccination. B, Whole IgG, IgG_1, IgG_2, IgG_3, IgG_4, and IgM antibody responses against the protein or each peptide at a dilution of 1:25 at the baseline and at the indicated week after vaccination.

Multiple Cytokine Production by CD4 and CD8 T Cells After Stimulation With NY-ESO-1 OLP

CD4 and CD8 T cells from patient P-3 and P-7 PBMCs purified by antibody-coated magnetic beads were stimulated in vitro with a mixture of 29 NY-ESO-1 18-mer series peptides for 12 days as previously shown and assayed for IL-2, TNF-α, and IFN-γ-producing cells by ICS using FACS. As shown in Figure 4, an increase in the number of single cytokine-producing cells was observed in CD4 and CD8 T cells after vaccination. A slight increase in double cytokine-producing cells was also observed. No triple cytokine-producing cells were observed. In addition, an increase in CD4 T cells producing IL-5 or IL-17, but not IL-10, was observed after vaccination, suggesting activation of Th2 and Th17, as well as Th1 (Fig. 4B).

Nonspecific Immune Activation by the Vaccine

In addition, nonspecific immune activation by the vaccine preparation was investigated using CD4 T cells from patient P-7 obtained before and after vaccination, and stimulated with PMA/ionomycin by ICS. As shown in Figures 4C and D, while no triple cytokine-producing cells were observed in either PD-1 (–) or PD-1 (+) CD4 T cells at day 0, these cells appeared even after a single vaccination (day 15). No further increase in these cells was observed in CD4 T cells obtained after the 10th vaccination (day 78). No significant change in PD-1 (–) or PD-1 (+) CD4 T-cell populations was observed during the vaccination period.

DISCUSSION

In this study, we showed that an NY-ESO-1 OLP vaccine with Picibanil OK-432 and Montanide ISA-51 was safe and induced NY-ESO-1 humoral and cellular immune responses in all patients. In our previous study on cancer vaccines with NY-ESO-1 protein[24–29] and the NY-ESO-1f peptide,[9] NY-ESO-1-specific humoral immune responses appeared to be useful as an immunological marker to predict the clinical responses of the patients vaccinated. This study showed that of the 4 peptides used, the antibody response against peptide #3 (121–150) was the most useful for monitoring than the responses against other peptides. The dominant epitope recognized by CD4 T cells (18-mer peptide #21, NY-ESO-1 121–138) in Figure 3 is included in the sequence of peptide #3. It is interesting to note that, the epitope peptide recognized by the antibody induced spontaneously in patients with NY-ESO-1-expressing tumors or in patients vaccinated with the NY-ESO-1 protein was NY-ESO-1 91–108, and was not included in peptide #3.[23] A mixture of four 30–32-mer long peptides used for the vaccine included a hydrophobic sequence located in a region approximately 121–170 amino acid, which is normally buried in the molecule[23] and therefore altered the immunological dominance of the antibody response to NY-ESO-1.

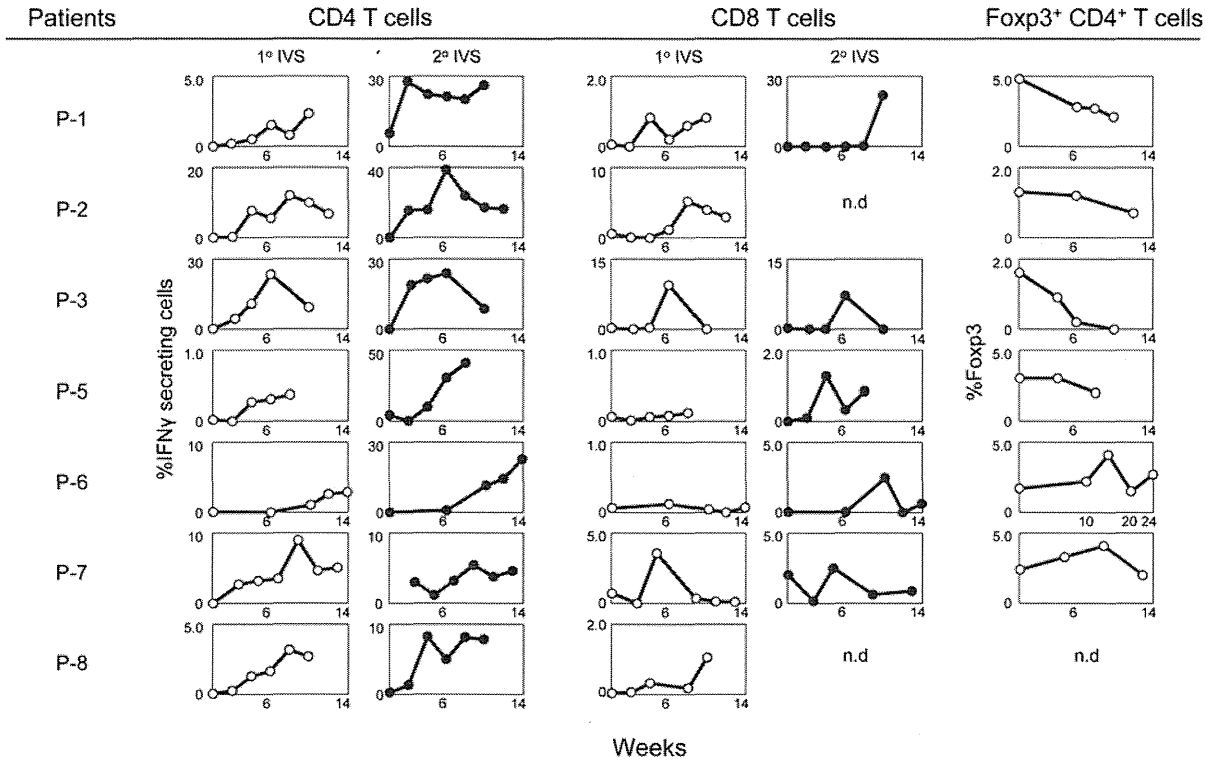

FIGURE 2. Immunomonitoring of CD4 and CD8 T-cell responses and Treg cells. CD4 and CD8 T cells were stimulated with 29 NY-ESO-1 18-mer series peptides once (1°IVS) or twice (2° IVS) and the net percentage of interferon (IFN)-γ-secreting cells was evaluated by an IFN-γ catch assay using bispecific CD45 and an IFN-γ antibody. Foxp3+ CD4 Tregs were evaluated by ICS. The data are plotted with time. The analysis was conducted by FACS Canto II. ND indicates not done.

We used an IFN-γ catch assay to detect antigen-specific cellular responses using PBMCs in immunomonitoring. The assay can potentially give rise to false-positive reactions due to neighboring cells picking up IFN-γ by the bystander effect. However, it could be avoided by carefully performing the assay using a larger volume in a limited

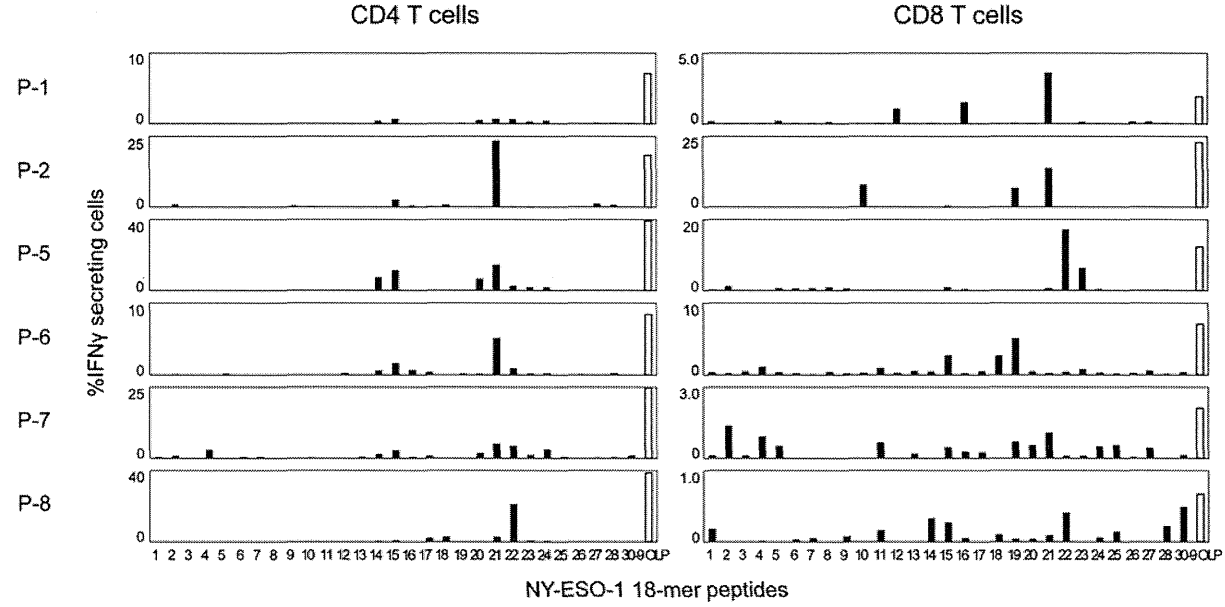

FIGURE 3. Epitope peptides recognized by CD4 and CD8 T cells. Using CD4 and CD8 T cells stimulated twice for 26 days with irradiated (30 Gy) autologous CD4-depleted and CD8-depleted PBMCs in the presence of 29 NY-ESO-1 18-mer series peptides, the epitope peptides recognized were determined by an interferon (IFN)-γ catch assay. In the assay, the cells were stimulated with autologous EBV-B cells pulsed with each 29 NY-ESO-1 18-mer peptide individually. The analysis was conducted by FACS Canto II.

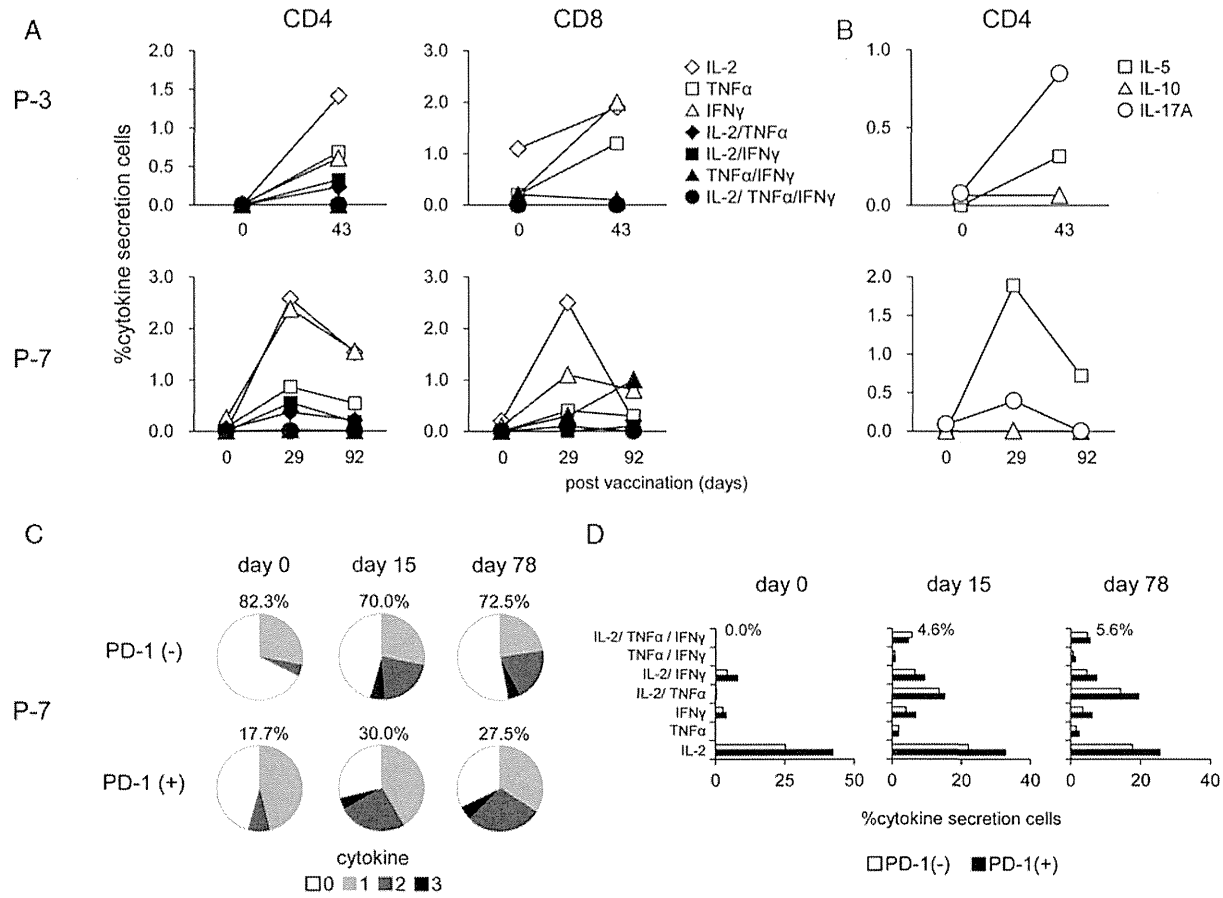

FIGURE 4. Multiple cytokine production by CD4 and CD8 T cells and nonspecific immune activation after NY-ESO-1 OLP vaccination. Multiple cytokine production by CD4 and CD8 T cells stimulated with a mixture of 29 NY-ESO-1 18-mer series peptides (A, B) or PMA/ionomycin (C, D) was analyzed. The cells from patients P-3 (A, B) and P-7 (A, B and C, D) obtained before and after vaccination were assayed for IL-2, TNF-α, and interferon (IFN)-γ-producing cells (A, C, D) or IL-5, IL-10, and IL-17A-producing cells (B) by intracellular staining. Frequency of single, double, and triple cytokine-producing cells in CD4 and CD8 T cells (A, B) or in PD-1 (–) or (+) CD4 T cells (C, D) before and after vaccination are depicted.

concentration of IFN-γ. The assay was highly reproducible and sensitive compared with intracytoplasmic staining or an ELISPOT assay. In the ELISPOT assay, it should be noted that the response resulting from a minor CD4 T-cell population contaminated in a purified CD8 population can sometimes make the interpretation of results difficult, especially after stimulation with longer peptides. Induction of CD4 T-cell responses was observed in all patients vaccinated and their increase during the vaccination period was consistent with the results in our previous study on a cancer vaccine with NY-ESO-1 protein and NY-ESO-1f peptide. For induction of NY-ESO-1-specific CD4 T cells, overlapping peptides appeared to be much more efficient than protein.[10] In contrast, induction of CD8 T-cell responses was similarly observed in all patients vaccinated with NY-ESO-1 OLP. However, the responses were relatively weak and fluctuated a lot during vaccination compared with the CD4 T-cell response. In our previous study with NY-ESO-1 protein, even a patient with a tumor that almost completely disappeared showed only a marginal CD8 T-cell response in PBMCs.[25,29] We observed some Foxp3$^+$ CD4 T cells infiltrating tumor tissue from this patient. In this study, we detected Foxp3$^+$ CD4 T cells in PBMCs from all patients analyzed during vaccination with NY-ESO-1 OLP. No increase in regulatory T cells was observed during vaccination in PBMCs. It is possible that those cells suppress CD8 T-cell responses in tumor microenvironments. Future studies combining cancer vaccines and inhibition of regulatory T-cell function will be intriguing.

TLRs are expressed either on the cell surface (TLR1, 2, 4, 5, 6, and 10) or on the membrane of intracellular organelles such as endosomes (TLR3, 7, 8, and 9). The bacterial CpG motif is the ligand for TLR9. Viral single-stranded RNAs are ligands for TLR7 and 8, and double-stranded viral RNAs such as PolyI:C are ligands of TLR3. Molecular patterns of extracellular microbes are recognized by the cell surface-expressed TLR1, 2, 4, 5, 6, and 10.[30] OK-432 is a ligand for TLR2, 4, and/or 9.[9] Triggering of TLR signaling leads to the activation of nuclear factor κB, activating protein-1, and/or IRF3, which results in secretion of type 1 IFNs and/or proinflammatory cytokines such as IL-1β, IL-12, and TNF-α.[17] We used OK-432 as an immunemodulator for the NY-ESO-1f peptide, CHP-NY-ESO-1 and CHP-HER2 cancer vaccine and observed efficient induction of tumor antigen-specific immune responses.[9,18,31] Sabbatini et al[32] used an NY-ESO-1 OLP vaccine with or without poly-ICLC in ovarian cancer patients. They observed that an efficient antibody response against NY-ESO-1 OLP was

induced with its use in combination with poly-ICLC and Montanide, but not without poly-ICLC. In this regard, OK-432 could be a feasible immune-modulator for a cancer vaccine with tumor antigens.

Two out of 9 vaccinated patients showed SD in the clinical response. Although strong induction of the NY-ESO-1 antibody against both the peptides used for the vaccine and the NY-ESO-1 protein was observed in these patients, there is no convincing evidence as to whether the strong antibody response is related to the clinical response.

Recently, it was shown that antibodies against immune checkpoint molecules had a significant antitumor effect, and a combination of different antibodies augmented this effect. With the proviso of control of immunosuppression in the tumor microenvironment, the use of immunogenic vaccines will be relevant. Thus, the use of both reagents controlling immunosuppression and immunogenic vaccines will be important in the future. We are planning combination therapies of immune checkpoint modulators with NY-ESO-1 vaccine.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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All authors have declared there are no financial conflicts of interest with regard to this work.

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Hepatology Research

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Case Report

Reactivation of hepatitis B virus in a patient with adult T-cell leukemia–lymphoma receiving the anti-CC chemokine receptor 4 antibody mogamulizumab

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The introduction of molecularly targeted drugs has increased the risk of reactivation of hepatitis B virus (HBV), which is a potentially fatal complication following anticancer chemotherapy even in patients who have previously resolved their HBV infection. CC chemokine receptor 4 (CCR4) has been identified as a novel molecular target in antibody therapy for patients with adult T-cell leukemia–lymphoma (ATL) and peripheral T-cell lymphoma, and the humanized anti-CCR4 monoclonal antibody mogamulizumab has been developed. We reported HBV reactivation of an ATL patient with previously resolved HBV infection after mogamulizumab treatment in a dose-finding study for this antibody. Our retrospective analysis using preserved samples also revealed the detailed kinetics of HBV DNA levels before and just after HBV reactivation.

Key words: CC chemokine receptor 4, hepatitis B virus, mogamulizumab, reactivation

INTRODUCTION

REACTIVATION OF HEPATITIS B virus (HBV) following anticancer chemotherapy and immunosuppressive therapy is a potentially fatal complication that needs to be followed up carefully.[1] The advent of molecularly targeted drugs, which have immunosuppressive or immunomodulating actions, has increased the risk of HBV reactivation. The anti-CD20 monoclonal antibody rituximab, which forms part of the standard regimen for B-cell non-Hodgkin's lymphoma, has the potential to cause HBV reactivation, even in patients who have previously resolved their HBV infection and are hepatitis B surface antigen (HBsAg) negative at baseline.[2–6] CC chemokine receptor 4 (CCR4) has been identified as a novel molecular target in antibody therapy for patients with adult T-cell leukemia–lymphoma (ATL) and peripheral T-cell lymphoma, and the humanized anti-CCR4 monoclonal antibody mogamulizumab, the Fc region of which is de-fucosylated to enhance antibody-dependent cellular cytotoxicity, has been developed.[7–10] We herein report HBV reactivation of an ATL patient with previously resolved HBV infection after mogamulizumab treatment in a dose-finding study for this antibody.

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Conflict of interest: S. K., research funding from Kyowa Hakko Kirin and honoraria from Bristol-Myers Squibb; Y. T., research funding and honoraria from Bristol-Myers Squibb; T. I., research funding and honoraria from Kyowa Hakko Kirin; S. A., employment from Kyowa Hakko Kirin; R. U., research funding from Kyowa Hakko Kirin; and A. U., advisory role and honoraria from Kyowa Hakko Kirin.
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CASE REPORT

A 65-YEAR-OLD JAPANESE woman complained of persistent fatigue and weight loss of 8 kg in 2 weeks. The laboratory findings showed that her white blood cell count was elevated to 16 800/μL, of which abnormal lymphocytes accounted for 18%, and seropositivity for human T-cell leukemia virus type-1 (HTLV-1). Monoclonal integration of HTLV-1 was revealed by Southern blotting of DNA from peripheral blood. She was diagnosed as ATL, chronic type, in April 2004. Since then, she had experienced repeating infectious episodes and systemic lymph node swelling. On April 2005, she began to receive systemic chemotherapy composed of sobuzoxane (400 mg/day), etoposide (25 mg/day) and prednisolone (10 mg/day) p.o. twice a week because of disease progression to acute type which was accompanied by new ATL involvement in her right breast region and right axilla lymphadenopathy. As her disease was refractory to this regimen, she received four cycles of THP-COP regimen (cyclophosphamide, pirarubicin, vincristine and prednisolone) from August 2005 through October 2005 (Fig. 1a). She achieved a partial response and was followed up without subsequent chemotherapy including steroids for 1.4 years, but her disease progressed with markedly increased ATL cells

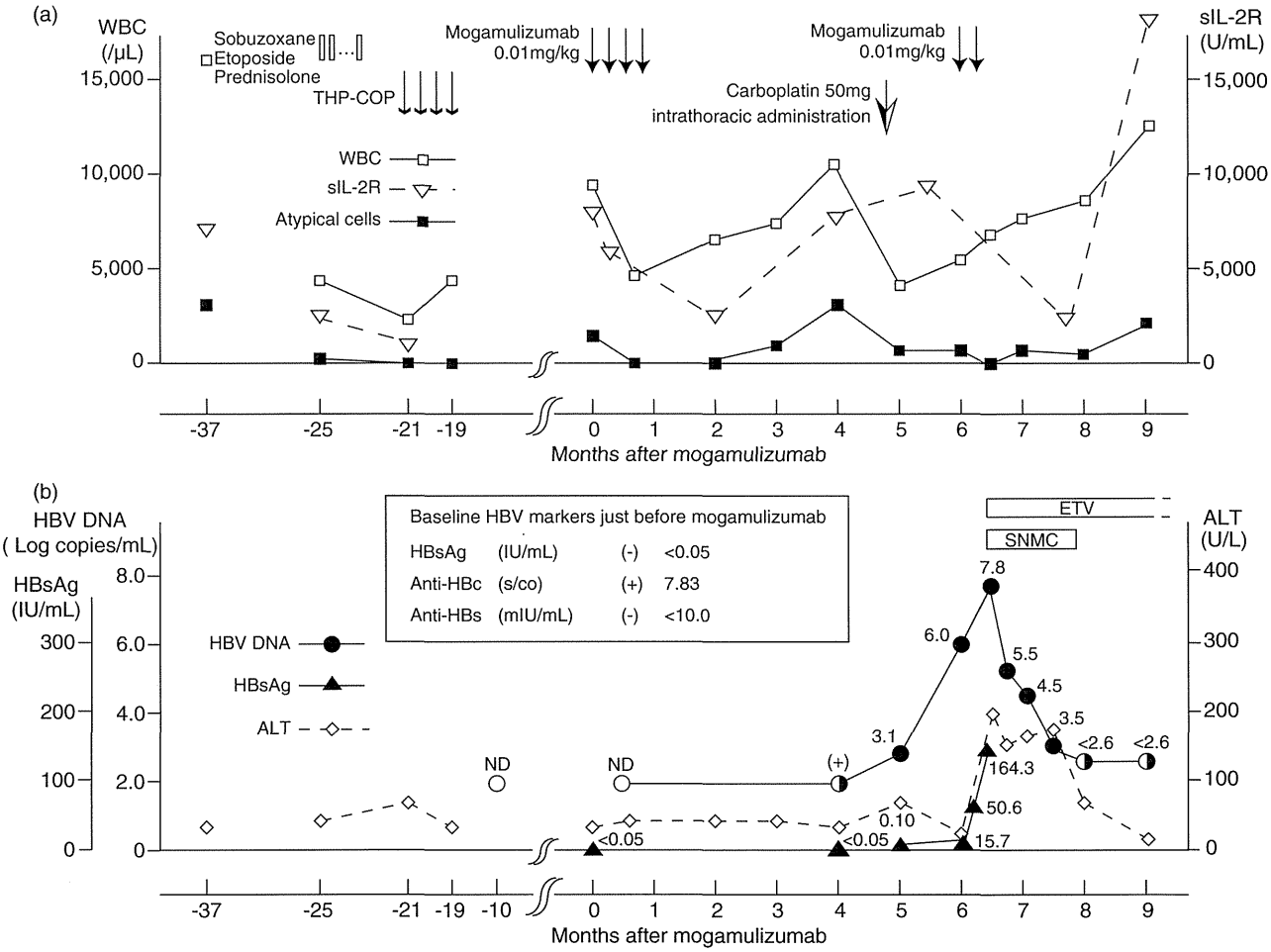

Figure 1 Clinical course and kinetics of HBV markers in a patient with adult T-cell leukemia–lymphoma before and after the anti-CC chemokine receptor 4 monoclonal antibody mogamulizumab treatment. ALT, alanine aminotransferase; anti-HBc, antibody against hepatitis core antigen; anti-HBs, antibody against hepatitis surface antigen; ETV, entecavir; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ND, not detectable; sIL-2R, soluble interleukin-2 receptor; SNMC, Stronger Neo-Minophagen C; THP-COP, cyclophosphamide, pirarubicin, vincristine and prednisolone; WBC, white blood cells.

and an elevated lactate dehydrogenase value in peripheral blood in March 2007. She was enrolled into a phase 1 study for dose-finding of the anti-CCR4 antibody, mogamulizumab,[9] and received this antibody at 0.01 mg/kg by i.v. infusion once a week for 4 weeks (Fig. 1a, thin arrows). No combination of other anticancer chemotherapy was performed and no steroids were given, except for allergic prophylaxis. She was HBsAg negative at baseline on enrollment in the phase 1 study. Retrospective analysis using preserved samples revealed that she was anti-hepatitis B core positive, anti-hepatitis B surface negative, and HBV DNA was undetectable at baseline, attributed to previously resolved HBV infection (Fig. 1b). After mogamulizumab, ATL cells disappeared immediately from the peripheral blood, the nodal disease partially improved and no severe adverse event was observed. However, at 9 weeks after the end of mogamulizumab, the ATL cells reappeared in the peripheral blood. Furthermore, she received intrathoracic administration of carboplatin for involvement of ATL (right pleural effusion) in August 2007. During the next month, her cervical lymph nodes enlarged rapidly and we decided to re-treat with mogamulizumab because of the previous efficacy and safety of this antibody. After two doses of mogamulizumab, she was hospitalized in emergency due to ALT flare on October 2007 (Fig. 1b, 6.5 months after mogamulizumab). The laboratory findings showed that HBsAg had become positive and her HBV DNA levels increased to 7.8 log copies/mL, suggesting that the liver damage was caused by HBV reactivation. Entecavir (0.5 mg/day) and Stronger Neo-Minophagen C (40 mg/day) were given immediately and hepatitis B improved gradually (with ALT peaking at 205 U/L) for approximately 2 months. Entecavir was effective in controlling hepatitis B, and was continued for 1.5 years without any severe adverse events.

DISCUSSION

THE PRESENTED CASE is the first report of HBV reactivation in a HBsAg negative patient receiving mogamulizumab. We analyzed preserved samples retrospectively and showed that her liver damage was attributable to HBV reactivation. Also, those analyses showed the following important findings regarding the kinetics of HBV DNA during reactivation: First, HBV DNA was undetectable at baseline, before administration of mogamulizumab. Elevated HBV DNA levels were detectable, in which polymerase chain reaction (PCR) signals were only detected 10 weeks prior to the development of hepatitis and 13 weeks after the end of this antibody treatment. HBV DNA levels, measured by PCR-based assay, increased rapidly from 3.1 to 6.0 log copies/mL for 1 month and, finally, up to 7.8 log copies/mL. Second, the elevated HBV DNA levels preceded the detection of HBsAg (Architect Assay; Abbott Laboratories, North Chicago, IL, USA) by 1 month. Third, the patient was infected with HBV genotype C with a point mutation in the precore regions (G1896A) which might have been associated with the rapidly increasing kinetics of HBV DNA levels in this case.

How was the anti-CCR4 antibody mogamulizumab involved in the HBV reactivation? CCR4 is a chemokine receptor expressed on T-helper type 2 and regulatory T cells, and is thought to carry an important role in maintaining the balance of the human immune system.[7–9] It is difficult to demonstrate how mogamulizumab caused HBV reactivation in this case; the reduction of CCR4-expressing cells following this antibody treatment might have been associated with imbalance of antiviral immunity, resulting in the development of hepatitis due to HBV reactivation. Other than mogamulizumab, the intrathoracic administration of carboplatin and the ATL disease progression are considered to be factors potentially influencing HBV reactivation. However, retrospective analysis showed that HBV DNA levels were detectable in the peripheral blood before administration of carboplatin, suggesting that carboplatin is unlikely to have been mainly involved in the HBV reactivation. ATL is often diagnosed with a compromised immune system, and the disease progression might have been associated with reactivation of the virus. Interestingly, the timing of the rapid increase in ATL cells in the peripheral blood coincided with that of HBV replication in this case. However, disease progression of ATL alone is very unlikely to have caused the HBV reactivation because reactivation did not occur during the previous ATL progression.

To prevent hepatitis due to HBV reactivation, what lesson can we learn from this case? HBV reactivation following immunosuppressive therapy may lead to acute liver failure or fulminant hepatitis, and the patients have poor prognosis regardless of intensive antiviral treatment.[11,12] For preventing HBV reactivation in patients with previously resolved HBV infection, monitoring of HBV DNA-guided preemptive antiviral therapy is recommended in some guidelines,[13,14] however, the evidence of optimal interval of HBV DNA monitoring is limited. Most recently, monthly monitoring of HBV DNA was shown to effectively prevent HBV reactivation in patients with previously resolved HBV

infection who received rituximab plus steroids containing chemotherapy.[15] The kinetics of HBV reactivation in this case strongly suggested that monthly monitoring of HBV DNA could prevent hepatitis even in such a highly replicative clone with a precore mutation.

In summary, we first reported HBV reactivation following treatment with the anti-CCR4 antibody mogamulizumab and revealed the detailed kinetics of HBV replication during reactivation. Further well-designed studies are warranted to address the mechanisms of HBV reactivation and to establish standard management for reactivation in patients with previously resolved HBV infection, following anticancer chemotherapy and immunosuppressive therapy.

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ORIGINAL ARTICLE

Antitumor effects of bevacizumab in a microenvironment-dependent human adult T-cell leukemia/lymphoma mouse model

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Abstract

Objective: The objective of this study was to evaluate the therapeutic potential of bevacizumab with or without systemic chemotherapy for adult T-cell leukemia/lymphoma (ATL) and clarify the significance of angiogenesis for ATL pathogenesis. *Methods:* NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice were used as recipients of tumor cells from a patient with ATL, which engraft and proliferate in a microenvironment-dependent manner. The ATL cells could be serially transplanted in NOG mice, but could not be maintained in *in vitro* cultures. *Results:* Injection of bevacizumab alone significantly increased necrosis and decreased vascularization in the tumor tissue. Levels of human soluble interleukin two receptor in the serum (reflecting the ATL tumor burden) of bevacizumab-treated mice were significantly lower than in untreated mice. Although bevacizumab monotherapy showed these clear anti-angiogenesis effects, it did not prolong survival. In contrast, injection of bevacizumab together with cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP) led to a significant prolongation of survival of the ATL mice relative to CHOP alone. *Conclusions:* This is the first report to evaluate the efficacy of bevacizumab for ATL in a tumor microenvironment-dependent model. Bevacizumab therapy combined with chemotherapy could be a valuable treatment strategy for that subgroup of ATL probably depending to a large extent on angiogenesis via vascular endothelial growth factor.

Key words Adult T-cell leukemia-lymphoma; Bevacizumab; tumor microenvironment

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Adult T-cell leukemia-lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus type 1 (HTLV-1). The disease is resistant to conventional chemotherapeutic agents, and currently there are only limited treatment options; thus, it has a very poor prognosis (1–4). Over the past decade, allogeneic hematopoietic stem-cell transplantation has evolved into a potential approach to treating patients with ATL. However, only a small fraction of patients have the opportunity to benefit from transplantation, such as those who are younger, have achieved sufficient disease control, and have an appropriate stem-cell source (5, 6). Therefore, the development of alternative treatment strategies for patients with ATL is an urgent issue.

Bevacizumab is a humanized monoclonal antibody against the vascular endothelial growth factor A (VEGF-A), a key

factor inducing the formation of blood vessels (angiogenesis) in tumors (7). Bevacizumab is currently approved worldwide for the treatment of several types of cancer such as metastatic colorectal cancer, metastatic non-small-cell lung cancer, renal cell carcinoma, and advanced ovarian cancer, in combination with chemotherapy or interferon (8–14). Bevacizumab is also approved as a single agent for recurrent glioblastoma in the USA (15). In this context, many aspects of pathological angiogenesis have been extensively studied in many types of cancer. On the other hand, the precise role of these processes in pathogenesis of hematological malignancies including ATL is still under active investigation (16–19). Thus far, bevacizumab has not been approved for the treatment of any hematological malignancy in the USA, Europe, or Japan. The aim of the present study was to evaluate the therapeutic potential of bevacizumab with or without systemic chemotherapy for ATL and clarify the significance of angiogenesis for ATL pathogenesis, using a microenvironment-dependent murine ATL model.

Methods

Animals

NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice (20) were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan) and used at 6–8 wk 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 23 patients with ATL by immunopathology. The patients provided written informed consent in accordance with the Declaration of Helsinki, and this present study was approved by the institutional Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Hematoxylin and eosin (HE) staining and immunostaining using anti-human CD4 (4B12; Novocastra, Wetzlar, Germany), CD25 (4C9; Novocastra), CD20 (L26; DAKO, Glostrup, Denmark), VEGF-A (sc-152, rabbit polyclonal; Santa Cruz, Heidelberg, Germany), Alpha-Smooth Muscle Actin (α-SMA) (1A4; DAKO), CD31 (JC70A; DAKO), and von Willebrand Factor (Rabbit polyclonal; DAKO) were performed on formalin-fixed, paraffin-embedded sections. 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 ATL tumor cells were stained with the corresponding antibody. Nine 100× high-power fields (HPF) of HE tumor specimens were randomly selected, and the area of tumor necrosis (%) was calculated by Image J software (21), and then averaged. Nine 100× HPF of von Willebrand Factor-stained tumor specimens were randomly selected, and numbers of vessels (per mm^2) were calculated by Image J software and then averaged.

ATL mouse model

A leukemic cell clone from a patient with ATL, which could be serially transplanted into SCID mice, designated S-YU as reported previously (22), was injected intraperitoneally (i.p.) into NOG mice. Three to 4 wk after i.p. injection, NOG mice were presented with intraperitoneal masses along the mesentery. Cells from these intraperitoneal masses were suspended in RPMI-1640 and inoculated i.p. into healthy NOG mice, which then presented with features identical to those of the original mice.

Cell lines

ATN-1, MT-1, and TL-Om1 are ATL cell lines, whereas MT-2, MT-4, and TL-Su are HTLV-1-immortalized lines, as previously described (23).

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated with RNeasy Mini Kits (QIAGEN, Tokyo, Japan). Reverse transcription from the RNA to first strand cDNA was carried out using High Capacity RNA-to-cDNA Kits (Applied Biosystems Inc, Foster City, CA, USA). *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™. 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: APC-conjugated anti-human CD45 mAb (2D1; BD Biosciences, San Jose, CA, USA), PerCP-conjugated anti-CD4 mAb (SK3; 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 was treated with BD FACS lysing solution (BD Biosciences) to remove RBC. Stained cells were analyzed on a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Inc. Ashland, OR, USA).

Cell proliferation assay

Proliferation of S-YU and HTLV-I-immortalized lines expressing both VEGF-A and VEGF-R1 in the presence of different concentrations of bevacizumab for 48 h was assessed using CellTiter 96 Aqueous One Solution cell proliferation assay kits (Promega Corporation, Madison, WI, USA). Bevacizumab was purchased from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

ATL cell-bearing mice treated with bevacizumab

ATL tumor cells (S-YU) from the intraperitoneal masses were suspended in RPMI-1640, and 1.0×10^7 was inoculated i.p. into each of 14 NOG mice. The animals were divided into two groups of seven each for treatment with bevacizumab or to serve as controls. Bevacizumab (10 mg/kg) or vehicle (saline) was i.p. injected into the mice 3, 10, and 17 d after tumor cell inoculations. Therapeutic efficacies were evaluated for area of tumor necrosis, number of vessels, and serum human sIL2R levels 22 d after tumor inoculation. The concentration of human sIL2R in the serum was measured by ELISA using human sIL2R immunoassay kits (R&D Systems, Inc.).

ATL cells from the intraperitoneal masses suspended in RPMI-1640 were also inoculated i.p. into another 10 NOG mice at 1.0×10^7 per mouse. These animals were randomly divided into two groups of five each for treatment with bevacizumab or as controls. Bevacizumab (10 mg/kg) or saline was injected i.p. into the mice 2, 9, 16, and 23 d after tumor cell inoculation. Therapeutic efficacy of bevacizumab was evaluated by survival times.

A further 16 NOG mice that had also received 1.0×10^7 ATL cells from intraperitoneal masses were randomly divided into two groups of eight each for treatment with bevacizumab + cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP) or CHOP alone. Bevacizumab (10 mg/kg) or saline was i.p. injected into the mice 2, 9, 16, 23, 30, and 37 d after tumor cell inoculations. CHOP was given i.p. 17 d after tumor inoculation at the following doses: cyclophosphamide, 40 mg/kg; doxorubicin, 3.3 mg/kg; vincristine, 0.5 mg/kg; and prednisolone, 0.2 mg/kg (24, 25). Therapeutic efficacy of bevacizumab was evaluated by survival time. 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.

Statistical analysis

The differences between groups regarding the tumor necrosis area, vascular number, and human sIL2R concentrations in serum were analyzed by the Mann–Whitney U test. In this study, $P < 0.05$ was considered significant.

Results

VEGF-A expression in ATL

VEGF-A expression by ATL cells in the lymph node lesions is shown in Fig. 1A. Immunopathological features of four cases from each group stratified by VEGF-A expression are shown in Fig. 1B. Most of the ATL cases (96%) were positive for VEGF-A.

ATL cell-bearing NOG mice

In earlier studies, S-YU ATL tumor cells, which were serially transplanted into SCID mice (22), manifested multiple enlarged mesenteric lymph nodes. In the present study, in which NOG mice rather than SCID mice were the S-YU recipients, larger tumor masses formed along the intestinal tract. Figure 2A shows the intraperitoneal masses and intestinal tract adhering tightly to one another in a NOG mouse (demarcated by thin white dotted lines in the figure). Flow cytometric analysis demonstrated that the mass mainly consisted of human cells expressing CD4 and CD25 (Fig. 2B). Immunopathological analysis revealed large atypical cells with irregular and pleomorphic nuclei, and blood vessels. The cells were CD4-positive, CD25-positive, but CD20-negative (Fig. 2C). These findings are consistent with an ATL cell phenotype in humans, and with earlier studies in the SCID/S-YU model. The S-YU tumor cells in the NOG mice were classed as VEGF-A 1+ positive (Fig. 2C). Blood vessels in the tumor tissue were stained by anti-α-SMA Ab (Fig. 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). Together, these results show that the blood vessels in the tumor tissue originated from the mouse, because anti-α-SMA and von Willebrand Factor Ab used in this study recognized the corresponding protein derived from both human and mouse, whereas the anti-CD31 mAb recognized the corresponding human but not murine protein (data not shown). CD4-positive CD25-positive ATL cell mild infiltration into spleen, liver, and bone marrow was seen by flow cytometry (Fig. 2D).

VEGF-A, VEGF-R1, and -R2 expression in ATL and HTLV-1-immortalized lines

VEGF-A mRNA expression was detected in all 7 ATL and HTLV-1-immortalized lines tested, and in S-YU cells

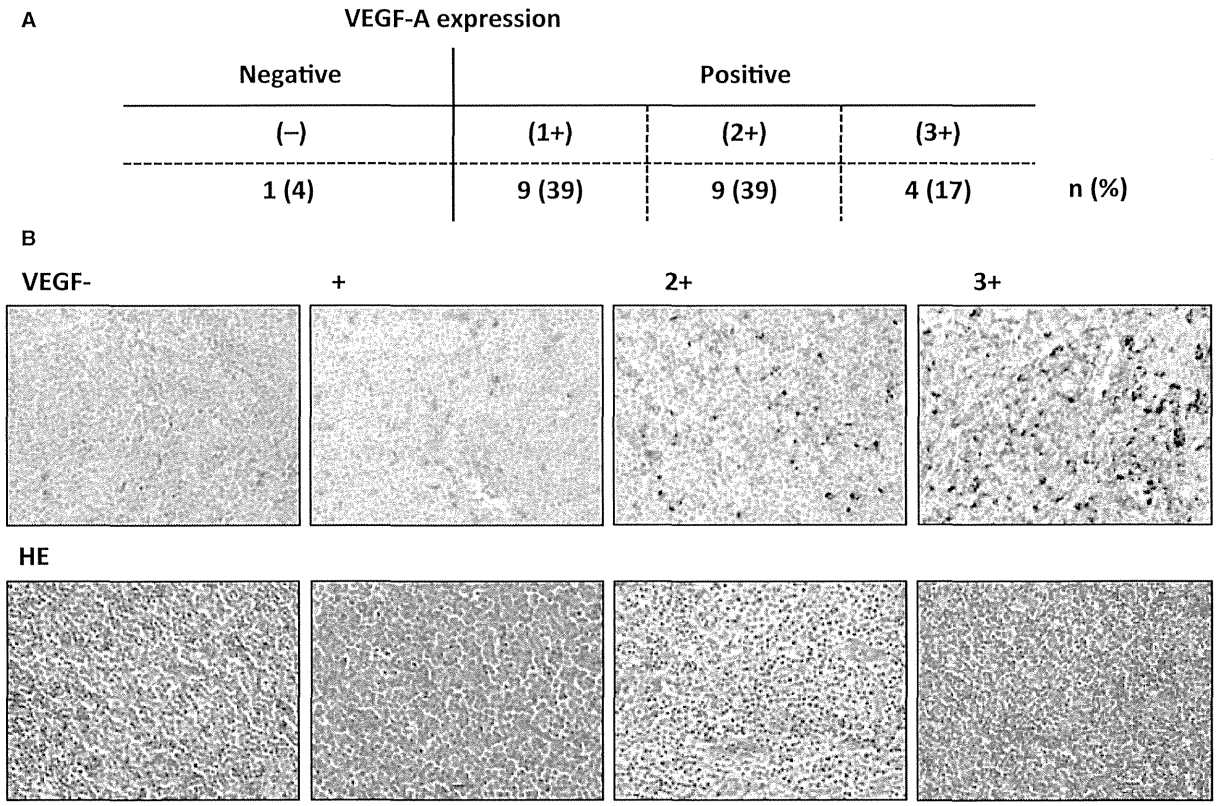

Figure 1 Vascular endothelial growth factor A (VEGF-A) expression in ATL. (A) VEGF-A expression of ATL cells in the lymph node lesion. VEGF-A expression was categorized based on the percentage of ATL cells stained as follows: ≥50%, 3+ positive; 30–49%, 2+ positive; 10–29%, 1+ positive; <10%, negative. (B) Cases 1, 2, 3, and 4 are representative of VEGF-A-negative, 1+, 2+, and 3+ positive categories, respectively. Photomicrographs with VEGF-A (upper panels) and hematoxylin and eosin staining (lower panels) are shown.

from intraperitoneal masses (Fig. 3A, upper left panel). *VEGF-R1 mRNA* expression was not present in ATL and in only two HTLV-1-immortalized lines (MT-2 and TL-Su) but was present in S-YU cells (Fig. 3A, upper right panel). No *VEGF-R2 mRNA* expression was detected in any of the 7 ATL and HTLV-1-immortalized lines tested, or in S-YU cells (data not shown). Flow cytometry demonstrated that VEGF-R1 protein was also expressed in MT-2 and TL-Su, and very weakly in NOG S-YU cells (Fig. 3A, lower panels), consistent with the RT-PCR results. Flow cytometry demonstrated that VEGF-R2 was not expressed at all in any of the ATL and HTLV-1-immortalized lines tested, or in S-YU cells (data not shown), which was also consistent with the RT-PCR results.

VEGF-R1 and VEGF-R2 expression in primary ATL cells

CD4-positive CD25-positive primary ATL cells in PBMC obtained from nine individual patients with ATL (i–ix) were evaluated for VEGF-R1 and -R2 expression. VEGF-R1 protein was expressed in only one patient (patient v) and VEGF-R2 was not expressed in any of the patients (Fig. 4B).

No Bevacizumab-mediated anti-proliferative activity against HTLV-1-immortalized lines and S-YU *in vitro*

Bevacizumab did not directly block the proliferation of MT-2 and TL-Su cells *in vitro*, despite their expression of both VEGF-A and VEGF-R1. Neither did it inhibit S-YU cells (Fig. 3C).

Therapeutic efficacy of bevacizumab monotherapy in S-YU cell-bearing NOG mice

Photomicrographs of tumor tissue from each mouse are shown (Fig. 4A). Treatment with bevacizumab resulted in an increased percentage of tumor necrosis in the NOG/S-YU mice (mean 25.3%, median 24.1%, range 19.2–33.6%), compared to control mice (mean 15.9%, median 15.4%, range 11.7–21.0%, $P = 0.0060$) (Fig. 4B, left panel). An example of calculating the percentage necrotic area is presented in Fig. 4B, right-hand panels. Bevacizumab treatment resulted in decreased vascular number in the tumor tissues [3.1, 2.6,

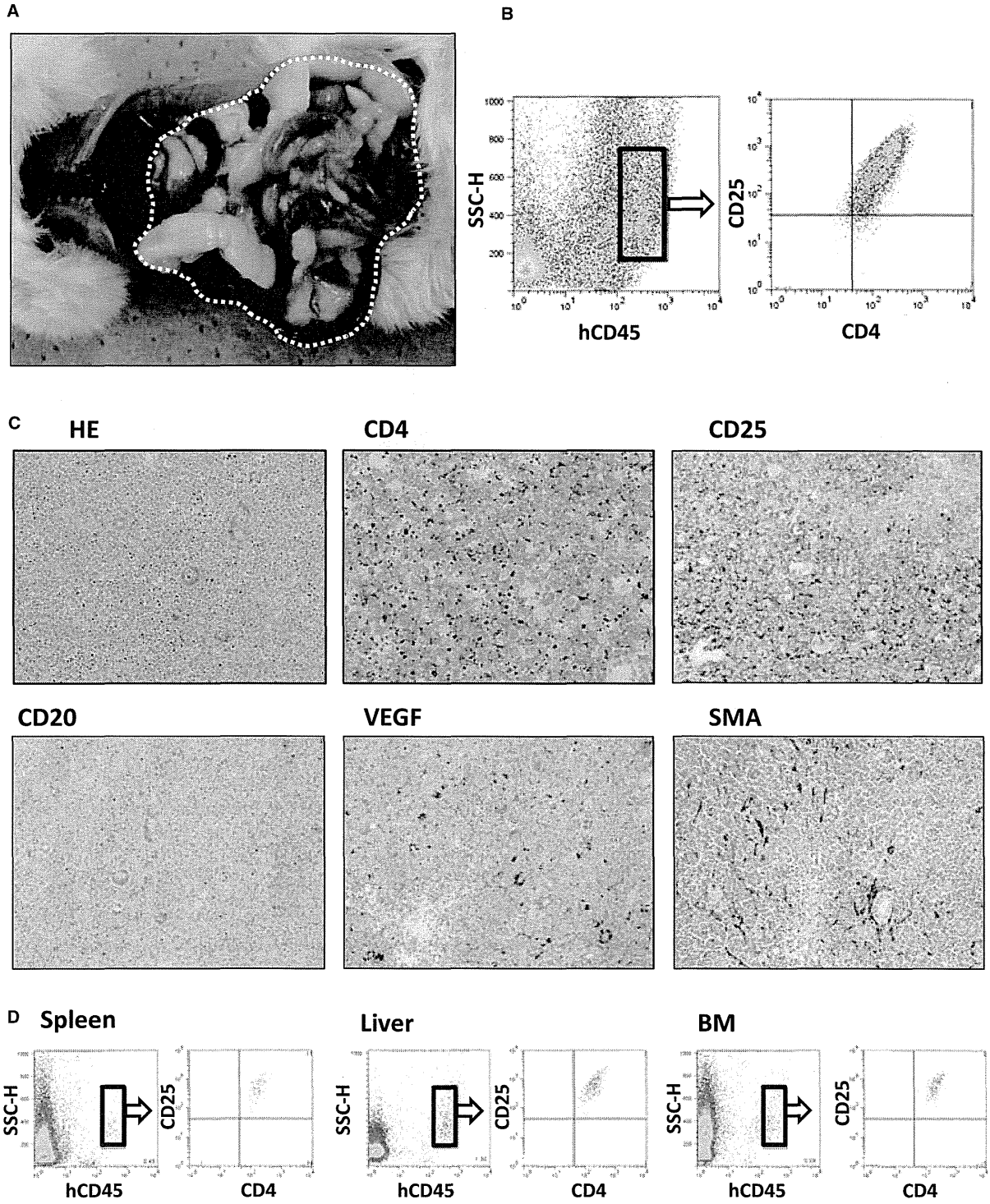

Figure 2 ATL cell-bearing NOG mouse model. (A) Macroscopic appearance of a primary DLBCL cell-bearing ATL mouse. The intraperitoneal mass is demarcated by a thin white dotted line. (B) Human CD45-positive cells in the mass determined by human CD4 and CD25 expression. (C) Immunohistochemical images of the intraperitoneal mass. (D) Human CD45-positive cells of each organ determined by human CD4 and CD25 expression.

0.0–8.3/mm^2; (mean, median, range)], compared to controls (12.8, 15.6, 1.6–19.3/mm^2, $P = 0.0127$) (Fig. 4C, left panel). An example of this calculation is presented in Fig. 4C, right-hand panels. Because sIL2R appears in the serum, concomitant with its increased expression on cells, we measured human sIL2R concentrations as a surrogate marker reflecting