

Production of NY-ESO-1 peptide/DRB1*08:03 tetramers and ex vivo detection of CD4 T-cell responses in vaccinated cancer patients



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ABSTRACT

We established CD4 T-cell clones, Mz-1B7, and Ue-21, which recognized the NY-ESO-1 121–138 peptide from peripheral blood mononuclear cells (PBMCs) of an esophageal cancer patient, E-2, immunized with an NY-ESO-1 protein and determined the NY-ESO-1 minimal epitopes. Minimal peptides recognized by Mz-1B7 and Ue-21 were NY-ESO-1 125–134 and 124–134, respectively, both in restriction to DRB1*08:03. Using a longer peptide, 122–135, and five other related peptides, including either of the minimal epitopes recognized by the CD4 T-cell clones, we investigated the free peptide/DR recognition on autologous EBV-B cells as APC and peptide/DR tetramer binding. The results showed a discrepancy between them. The tetramers with several peptides recognized by either Mz-1B7 or the Ue-21 CD4 T-cell clone did not bind to the respective clone. On the other hand, unexpected binding of the tetramer with the peptide not recognized by CD4 T-cells was observed. The clone Mz-1B7 did not recognize the free peptide 122–135 on APC, but the peptide 122–135/DRB1*08:03 tetramer bound to the TCR on those cells. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

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1. Introduction

To analyze T-cell immunomonitoring after vaccination, peptide/MHC tetramers have become widely used [1]. Peptide/MHC tetramers identified and visualized antigen specific T-cells. MHC class I tetramers were originally developed by Altman and Davis [2], and used for various antigens including those of viral or tumor origin [3,4]. However, MHC class II tetramers have been used in only a few studies because of the difficulty in preparation [5]. The soluble form of MHC class II molecules is necessary to produce tetramers. However, production of such molecules

using extracellular domains of MHC class II α and β chains is generally difficult because of a lack of assembly or aggregation [6]. These findings indicate the necessity of transmembrane regions for the proper assembly of the molecules. Kalandadze et al. [7] found that replacement of the hydrophobic transmembrane regions by the Fos and Jun leucine zipper dimerization motifs resulted in the assembly and secretion of DR $\alpha\beta$ heterodimers in yeast. Novak et al. [8] developed MHC class II tetramers using DR molecules incorporating leucine zipper motifs to stabilize the DR α and β heterodimer. The procedure has been widely used, but successful production of MHC class II tetramers is still limited [9–13].

We recently analyzed CD4 T-cell responses against NY-ESO-1 in PBMCs from patients who were vaccinated with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1) in our clinical trial and determined three novel NY-ESO-1 CD4 T-cell epitopes: NY-ESO-1 87–100 bound to DRB1*09:01, NY-ESO-1 95–107 bound to DQB1*04:01, and NY-ESO-1 124–134 bound to DRB1*08:03 [14]. CD4 T-cells that

Abbreviations: APC, antigen-presenting cell; CHP-NY-ESO-1, complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 whole protein; Fmoc, N-(9-fluorenyl)-methoxycarbonyl; HD, healthy donor; MFI, mean fluorescence intensity; OLP, overlapping peptide; PBMC, peripheral blood mononuclear cell.

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recognized these epitope peptides also recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or an NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. These CD4 T-cells had a cytokine profile with Th1 characteristics.

In this study, we showed that tetramers with several peptides recognized by the CD4 T-cell clones did not bind to the same clones. On the other hand, unexpected binding of the tetramer with a peptide not recognized by CD4 T-cells was observed. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected *ex vivo* CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

2. Materials and methods

2.1. Patients and blood samples

Peripheral blood samples were drawn from esophageal cancer patients E-1 and E-2, and a prostate cancer patient P-3, who were vaccinated with CHP-NY-ESO-1, and a lung cancer patient TK-OLP-01, who was vaccinated with NY-ESO-1 OLP in our clinical trials [15,16] after obtaining written informed consent. PBMCs were isolated by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO). CD4 T-cells and CD19⁺ cells were purified from PBMCs using CD4 and CD19 microbeads, respectively, using a large scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). The cells were stored in liquid N₂ until use. HLA typing was done using PBMCs with a sequence-specific oligo-nucleotide probe and sequence-specific priming of genomic DNA using standard procedures. Patient E-2 was found to possess homozygous alleles.

2.2. Peptides

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 (Okayama, Japan).

2.3. Cell lines

E-2 bulk CD4 T-cells were stimulated *in vitro* twice as described previously [14]. Clones were then established by limiting dilution. EBV-B cells were generated from CD19⁺ peripheral blood B cells using the culture supernatant from EBV-producing B95-8 cells.

2.4. Generation of HLA-DRB1*08:03 tetramers

HLA-DR tetramers were prepared as described previously [5]. The cDNA coding for the extracellular domains of the HLA-DR α chain was inserted by fusion PCR in a basic leucine zipper and His tag. The HLA-DR β chain was fused with an acidic leucine zipper and the BirA substrate peptide for BirA enzyme-dependent biotinylation. The HLA-DR α and HLA-DR β chimeric cDNA were cloned into the pcDNA3.1 vector, respectively. The expression vectors containing the HLA-DR α and HLA-DR β chains were co-transfected into CHO cells.

2.5. ELISA

Supernatants (100 μ l) from cultures of CD4 T-cells (5×10^3) stimulated for 18 h with autologous EBV-B cells (5×10^3)

pre-pulsed for 30 min with peptide in a 96-well round bottomed culture plate, or with solid-phase peptide/HLA-DRB1*08:03 tetramers in a 96-well flat bottomed culture plate, were collected and the amounts of IFN γ were estimated by sandwich ELISA [14]. TNF α , IL-4, IL-10 and IL-17A in the culture supernatants were estimated by DuoSet Sandwich ELISAs (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.6. Flow cytometry

FITC-conjugated anti-human TCR $\alpha\beta$ mAb (BD), PerCP Cy5.5-conjugated anti-human CD3 mAb and APC-conjugated anti-human CD4 mAb (eBioscience, San Diego, CA) were used for T-cell surface staining. The stained cells were detected by FACS Canto II (BD). Flow cytometry results were analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Tetramer staining

CD4 T-cells were incubated with tetramers for 1 h at 37 °C in a 5% CO₂ atmosphere. FITC-conjugated anti-human CD4 mAb (Miltenyi Biotec) was added at the end of tetramer staining and incubated for an additional 20 min at 4 °C.

2.8. IFN γ capture assay

The method has been described previously [14].

2.9. TCR V β and CDR3 sequence analysis

For TCR V β analysis, the iOTest Beta Mark kit (Beckman Coulter, Brea, CA) was used. The CDR3 sequence was determined by PCR as described previously [17].

3. Results

3.1. Determination of NY-ESO-1 minimal epitopes recognized by CD4 T-cell clones Mz-1B7 and Ue-21 established from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1

We established CD4 T-cell clones from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1 which recognized the 18-mer NY-ESO-1 121–138 peptide. The CD4 T-cell clones Mz-1B7 and Ue-21 produced IFN γ , TNF α , but not IL-4, IL-10 or IL-17A (Supplementary Fig. 1), indicating that they have Th1 characteristics. We determined restriction molecules by antibody blocking and minimal epitopes using various N- and C-terminal truncated peptides. Assays were done by ELISA examining IFN γ in the culture supernatant from responding T-cells using autologous EBV-B cells as antigen-presenting cells (APC). As shown in Fig. 1A, recognition of the 18-mer NY-ESO-1 121–138 by CD4 T-cell clones Mz-1B7 and Ue-21 was inhibited by addition of anti-HLA-DR mAb, but not anti-HLA-DQ mAb. Since patient E-2 possessed homozygous haplotypes (DRB1*08:03, DQA1*01:03, DQB1*06:01, DPB1*05:01) according to genetic analysis (see Section 2), the two clones Mz-1B7 and Ue-21 recognized the NY-ESO-1 peptide 121–138 in restriction to DRB1*08:03.

We then investigated recognition of various N- and C-terminal truncated peptides and found that a core peptide region recognized by either clone Mz-1B7 or clone Ue-21 was made up of amino acids 125–134 (Fig. 1B). Further analysis revealed that a minimal peptide recognized by clone Mz-1B7 was peptide 125–134 (10-mer) and that recognized by clone Ue-21 was peptide 124–134 (11-mer) (Fig. 1C). Thus, clones Mz-1B7 and Ue-21 recognized

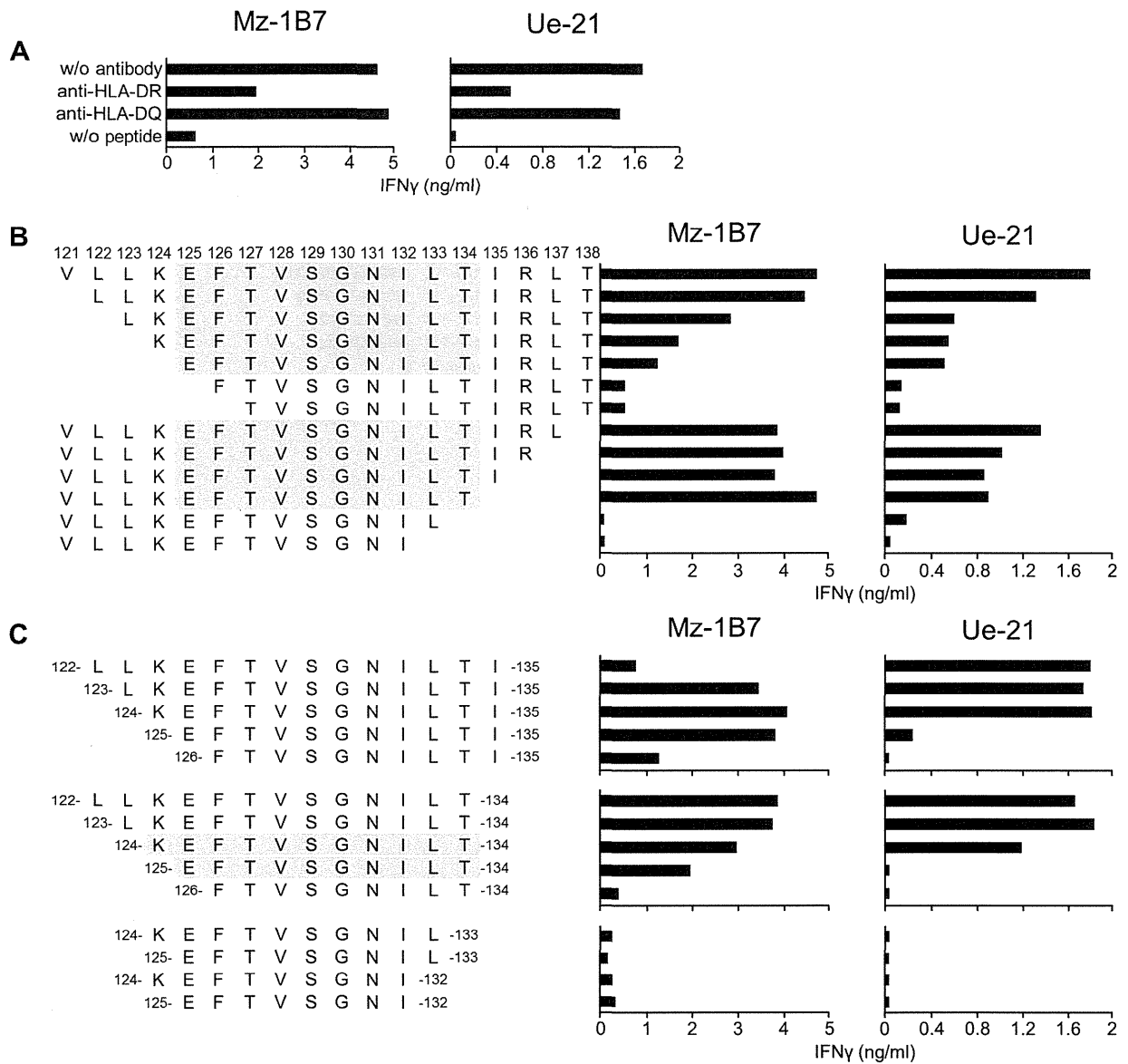


Fig. 1. Antibody blocking (A) and determination of NY-ESO-1 minimal epitopes ((B) and (C)) recognized by E-2 CD4 T-cell clones Mz-1B7 and Ue-21. In (A), CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) in the presence of NY-ESO-1 121–138 (VLLKEFTVSGNLTIRLT) peptide (100 nM), and anti-HLA-DR or anti-HLA-DQ mAb (5 μ g/ml) in the culture. IFN γ in the culture supernatants was determined by ELISA. In B and C, CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) in the presence of truncated NY-ESO-1 121–138 peptides (100 nM). The core peptide region and each minimal epitopes recognized by CD4 T-cell clones are shown in gray boxes. IFN γ in the culture supernatants was determined by ELISA.

closely related, but different, minimal NY-ESO-1 peptides in restriction to the same DRB1*08:03. Recognition of closely related, but different, peptides by these CD4 T-cell clones was further confirmed with responses to other peptides. Peptide 122–135 was recognized by Ue-21, but not Mz-1B7. On the other hand, peptide 125–135 and peptide 126–135 were recognized by Mz-1B7, but not Ue-21.

3.2. Differential recognition by clone Mz-1B7 and clone Ue-21 of the longer peptide 122–135, including minimal epitopes recognized by either clone

To confirm that the longer peptide 122–135 was recognized by only clone Ue-21, but not clone Mz-1B7, irrespective of including epitopes recognized by either clone, an IFN γ capture assay together with ELISA was performed examining IFN γ in the same culture stimulated with peptide 122–135 and five other related

peptides using autologous EBV-B cells as APC as above. As shown in Fig. 2A, a response of clone Mz-1B7 was observed against the peptides 123–135, 124–135, 122–134, 123–134 and 124–134, but not 122–135 in either the IFN γ capture assay or ELISA. No response against peptide 122–135 was observed up to a peptide concentration of 100 nM in ELISA. On the other hand, a response of clone Ue-21 was observed against all of the peptides used. These results were consistent with the results shown in Fig. 1.

3.3. Tetramer binding

We produced tetramers using the longer peptide 122–135, and five other related peptides 123–135, 124–135, 122–134, 123–134 and 124–134. The DR molecule was constructed by combining the DRA*01:01 and DRB1*08:03 chains that fused the leucine zipper motif at the C-terminal ends [8]. In the DRA locus, seven alleles DRA*01:01:01:01, DRA*01:01:01:02,

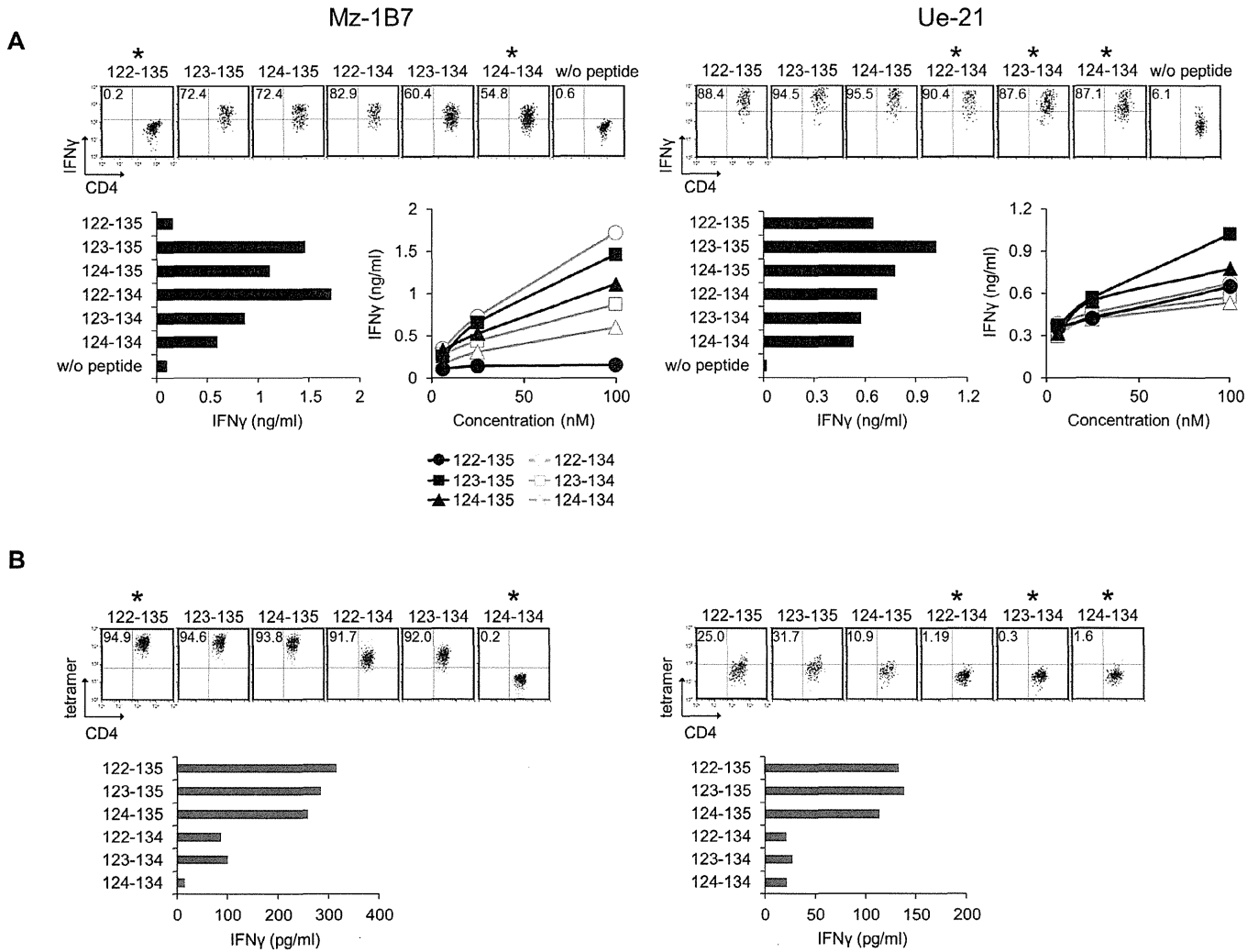


Fig. 2. Discrepancy between peptide recognition (A) and tetramer binding (B) in E-2 CD4 T-cell clones Mz-1B7 and Ue-21. In A top, CD4 T-cell clones (1×10^4) were stimulated for 4 h with the indicated peptides ($1 \mu\text{M}$) using autologous EBV-B cells (1×10^4) as APC. IFN γ -secreting CD4 T-cells were determined by an IFN γ capture assay using FACS Canto II. In A bottom, CD4 T-cell clones (5×10^3) were stimulated for 18 h with autologous EBV-B cells (5×10^3) pre-pulsed for 30 min with the indicated peptides (100 nM) (left) or with graded concentrations (6.25, 25 or 100 nM) of the indicated peptides (right). IFN γ in the culture supernatant was determined by ELISA. In B top, CD4 T-cell clones were stained with the indicated peptide/HLA-DRB1*08:03 tetramers ($5 \mu\text{g/ml}$) at 37°C for 1 h followed by staining with an anti-CD4 mAb, and analyzed using FACS Canto II. In B bottom, CD4 T-cell clones (5×10^3) were stimulated for 18 h with the indicated peptide/HLA-DRB1*08:03 tetramers coated on wells in microculture plates. IFN γ in the culture supernatant was determined by ELISA. The peptides that show a discrepancy between recognition (A) and tetramer binding (B) are marked by *.

DRA*01:01:01:03, DRA*01:01:02, DRA*01:02:01, DRA*01:02:02 and DRA*01:02:03 have been identified. These alleles differ only at amino acid 217 in the cytoplasmic domain, which is included in the region replaced by a leucine zipper motif from amino acid residue 152 in the $\alpha 2$ domain. Therefore, any DRA allele can be used for tetramer production.

With these six peptide/DR tetramers, we examined binding to clones Mz-1B7 and Ue-21. As shown in Fig. 2B, to clone Mz-1B7, binding of tetramers with peptide 122–135, 123–135, 124–135, 122–134 and 123–134, but not 124–134, was observed. The peptide 122–135 including the minimal epitope 125–134 was not recognized by Mz-1B7, but a tetramer constructed using the same peptide bound to Mz-1B7. Furthermore, peptide 124–134 that also included the minimal epitope 125–134 was recognized by Mz-1B7, but a tetramer constructed using the same peptide did not bind to the same clone.

On the other hand, to clone Ue-21, weak binding of tetramers with peptides 122–135, 123–135 and 124–135, but only marginal binding of tetramers with 122–134, 123–134 or 124–134, was observed. The peptides 122–134 and 123–134, including the minimal epitope 124–134 and the peptide 124–134 itself, were

recognized by Ue-21, but the tetramers constructed using the same peptides bound to the same clone only marginally. IFN γ production by CD4 T-cell clones in stimulation with the tetramers was consistent with tetramer binding (Fig. 2B bottom).

We further examined the only marginal binding of a tetramer constructed using the peptide 124–134 to Mz-1B7 and Ue-21 under different culture conditions. As shown in Fig. 3A and B, efficient binding of the tetramer constructed using the peptide 123–135 to clone Mz-1B7 was observed at $25\text{--}37^\circ\text{C}$ after incubation for 10–120 min. However, only marginal binding was observed with the tetramer constructed using the peptide 124–134, even at 37°C after incubation for 120 min. Only marginal binding of the tetramer with the peptide 124–134 to Mz-1B7 or Ue-21 was observed up to a concentration of $10 \mu\text{g/ml}$ (Fig. 3C and D).

3.4. Expression of CD4 and TCR on CD4 T-cell clones

Expression of CD4, CD3 and TCR $\alpha\beta$ was analyzed by FACS. As shown in Fig. 4A, expression of CD4 was observed similarly on clones Mz-1B7 and Ue-21. On the other hand, expression of CD3 and TCR $\alpha\beta$ was observed on Ue-21 strongly, but on Mz-1B7

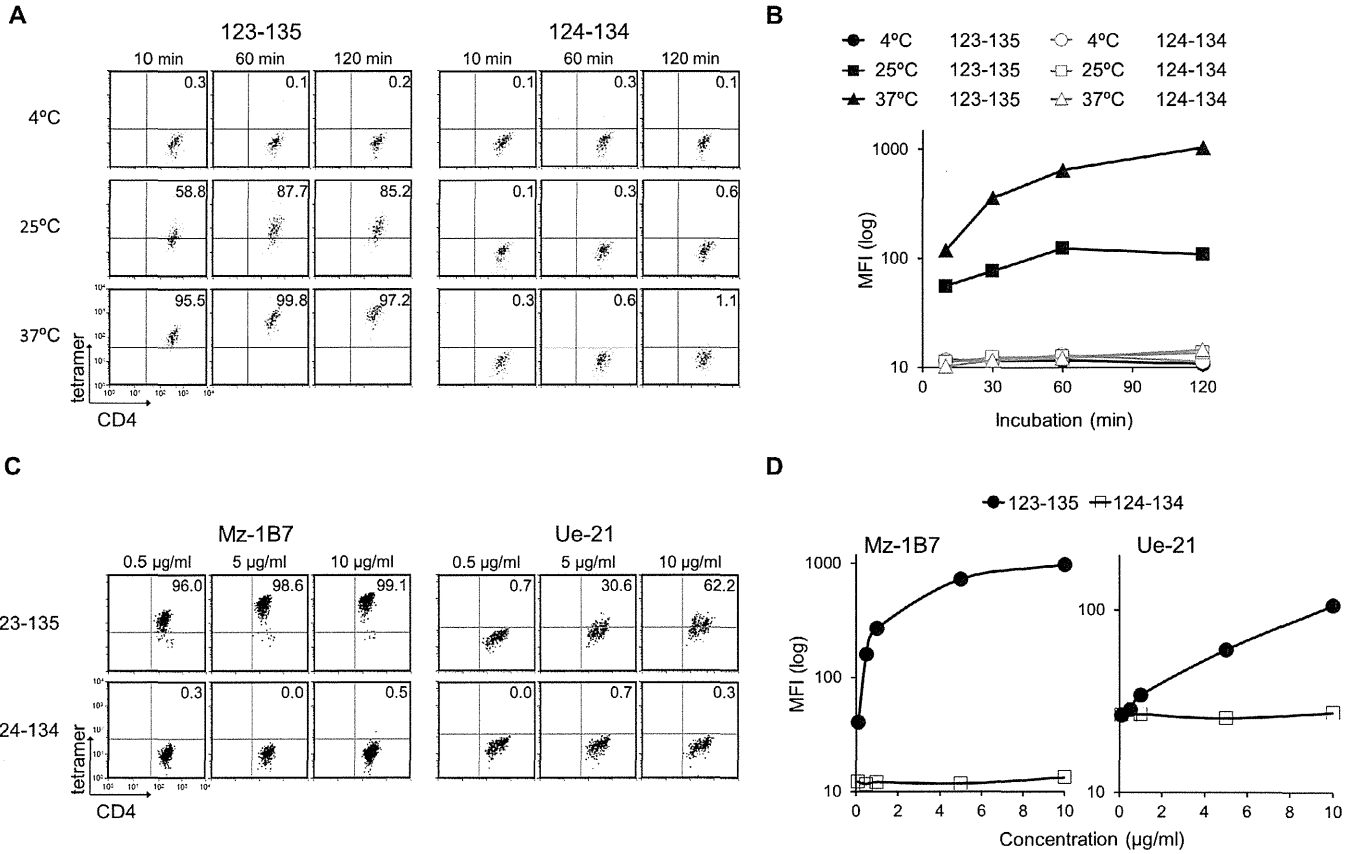


Fig. 3. Effect of temperature, incubation time and dose in tetramer staining. In (A) and (B), the E-2 CD4 T-cell clone Mz-1B7 was stained with NY-ESO-1 123–135 (LKEFTVSGNILT) or NY-ESO-1 124–134 (KEFTVSGNILT) peptide/HLA-DRB1*08:03 tetramers (5 µg/ml) at 4, 25 or 37°C for 10, 30, 60 or 120 min followed by staining with anti-CD4 mAb. In C and D, E-2 CD4 T-cell clones Mz-1B7 and Ue-21 were stained with NY-ESO-1 123–135 (LKEFTVSGNILT) or NY-ESO-1 124–134 (KEFTVSGNILT) peptide/HLA-DRB1*08:03 tetramers (0.5, 1, 5 or 10 µg/ml) at 37°C for 1 h followed by staining with an anti-CD4 mAb. Analysis was done using FACS Canto II. Dot plots (A and C) and the mean fluorescence intensity (MFI) (B and D) of tetramer staining are shown.

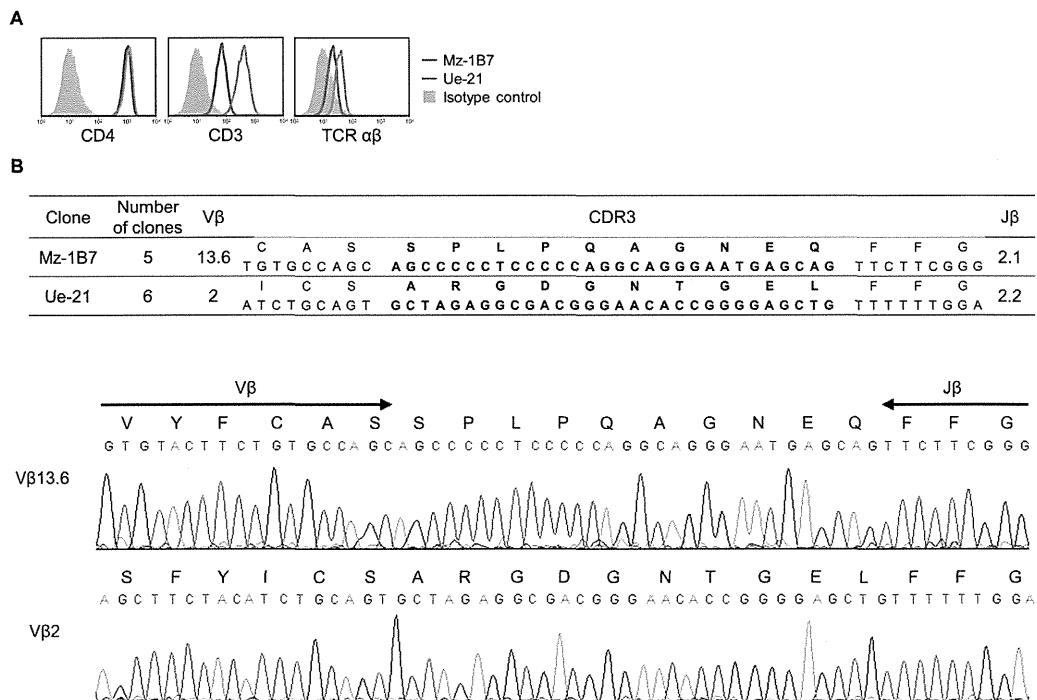


Fig. 4. Surface expression of the molecules on CD4 T-cell clones (A) and analysis of CDR3 sequences (B). In A, CD4 T-cell clones Mz-1B7 and Ue-21 stained with anti-CD4, CD3 and TCRαβ mAb were analyzed using FACS Canto II. In B, the nucleotide sequence and deduced amino acid sequences of the V–D–J junctional region of TCR β chain from the E-2 CD4 T-cell clones are shown.

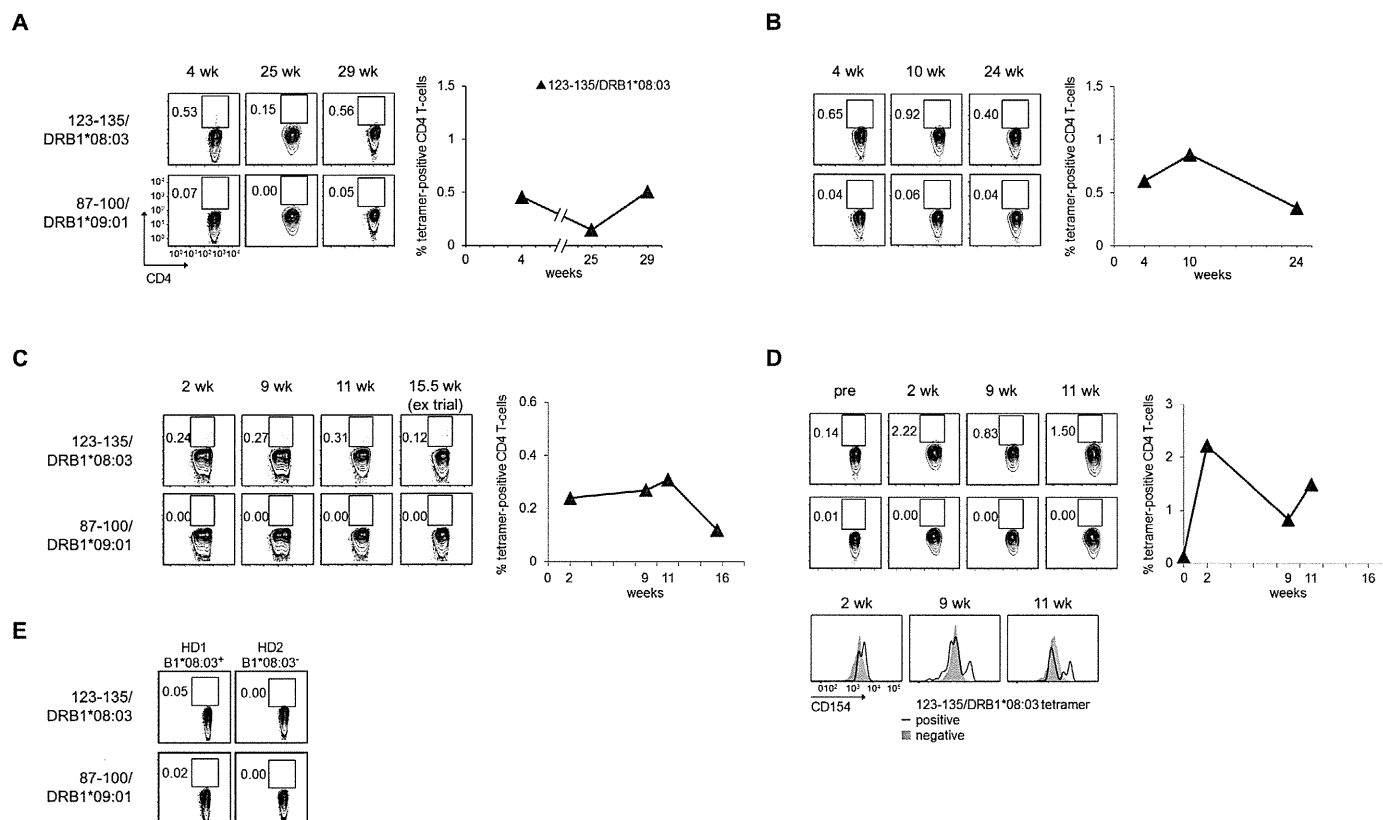


Fig. 5. Immunomonitoring of CD4 T-cell responses by the tetramer in cancer patients immunized with NY-ESO-1. CD4 T-cells from prostate cancer patient P-3 (A) and esophageal cancer patient E-1 (B) who were immunized with CHP-NY-ESO-1, and a lung cancer patient TK-OLP-01 (C) who was immunized with NY-ESO-1 OLP were stained ex vivo with the NY-ESO-1 123–135/HLA-DRB1*08:03 tetramer or a control NY-ESO-1 87–100/HLA-DRB1*09:01 tetramer (5 μ g/ml) at 37 $^{\circ}$ C for 1 h followed by staining with an anti-CD4 mAb. In D, TK-OLP01 CD4 T-cells after in vitro stimulation twice were stained with the NY-ESO-1 123–135/HLA-DRB1*08:03 tetramer or a control NY-ESO-1 87–100/HLA-DRB1*09:01 tetramer and anti-CD154 mAb at 37 $^{\circ}$ C for 2 h followed by staining with an anti-CD4 mAb. The histogram shows CD154 expression on NY-ESO-1 123–135/DRB1*08:03 tetramer-positive (open) and negative (filled) CD4 T-cells. CD4 T-cells from two HDs were stained with tetramers as a negative control (E). HD1 and HD2 are DRB1*08:03-positive and -negative individuals, respectively. Analysis was done using FACS Canto II.

moderately. As shown in Fig. 4B, analysis of CDR3 sequences revealed that clone Mz-1B7 utilizes the V β 13.6, SPLPQAGNEQ sequence for CDR3 and J β 2.1. On the other hand, clone Ue-21 utilizes the V β 2, ARGDGNTGEL sequence for CDR3 and J β 2.2.

By cloning bulk CD4 T-cells from the E-2 patient, we obtained 58 DRB1*08:03-restricted clones. Within these, 5 clones utilized V β 13.6 and 53 clones V β 2. 5 clones with V β 13.6 and 6 clones with V β 2 were sequenced for CDR3. A combination of the same CDR3 sequence and J β was utilized by clones with each V β , respectively.

3.5. Monitoring of CD4 T-cell response by a tetramer constructed using the peptide 123–135 in cancer patients immunized with NY-ESO-1

Tetramers constructed using the peptide 123–135 (NY-ESO-1 123–135/DRB1*08:03) were used to monitor CD4 T-cell responses in DRB1*08:03-expressing cancer patients immunized with CHP-NY-ESO-1, or a mixture of NY-ESO-1 OLPs (NY-ESO-1 79–108, 100–129, 121–150 and 142–173) with Picibanil and Montanide. As shown in Fig. 5, the tetramer detected positive cells ex vivo in CD4 T-cells from PBMCs of a prostate cancer patient (P-3) (Fig. 5A) and an esophageal cancer patient (E-1) (Fig. 5B) who expressed DRB1*08:03 after immunization with CHP-NY-ESO-1. The tetramer also detected positive cells in CD4 T-cells from PBMCs of a lung cancer patient (TK-OLP-01) immunized with NY-ESO-1 OLP ex vivo (Fig. 5C) and after in vitro stimulation (Fig. 5D). Predominant detection of tetramer NY-ESO-1 123–135/DRB1*08:03-positive

cells was observed after in vitro stimulation. Induction of CD154 (CD40L) expression on tetramer-positive cells was examined. At 9 and 11 weeks (3 and 4 vaccinations) after immunization, CD154 (CD40L)-positive cells were detected in tetramer NY-ESO-1 123–135/DRB1*08:03-positive, but not negative, cells suggesting their activation. No tetramer-positive cells were detected in CD4 T-cells from DRB1*08:03-positive or negative healthy donors (HD) (Fig. 5E). No clonal analysis of CD4 T-cells was possible because PBMCs from these patients were not available for further study.

4. Discussion

In this study, we demonstrated that HLA class II tetramers produced using minimal epitope peptides efficiently recognized by CD4 T-cell clones did not bind to cognate CD4 T-cell clones. Furthermore, we showed that a tetramer produced using a peptide which included the epitope sequence, but was not recognized by the cognate CD4 T-cell clone, could bind to the same CD4 T-cell clone.

It has long been observed that production of HLA class II tetramers is extremely difficult when compared to the production of MHC class I tetramers [5,6]. HLA class II tetramers produced using minimal epitope peptides and HLA class II molecules dimerized by a leucine zipper motif incorporated in the molecule generally failed to bind cognate CD4 T-cell clones. There have been only a few reports of successful binding of MHC class II tetramers to CD4 T-cells in which long peptides which were recognized by those T-cells were used for tetramer production [9–11].

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 (PVSKM-RMATPLLMQA). 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 DR α and DR β 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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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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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/ μ L, 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

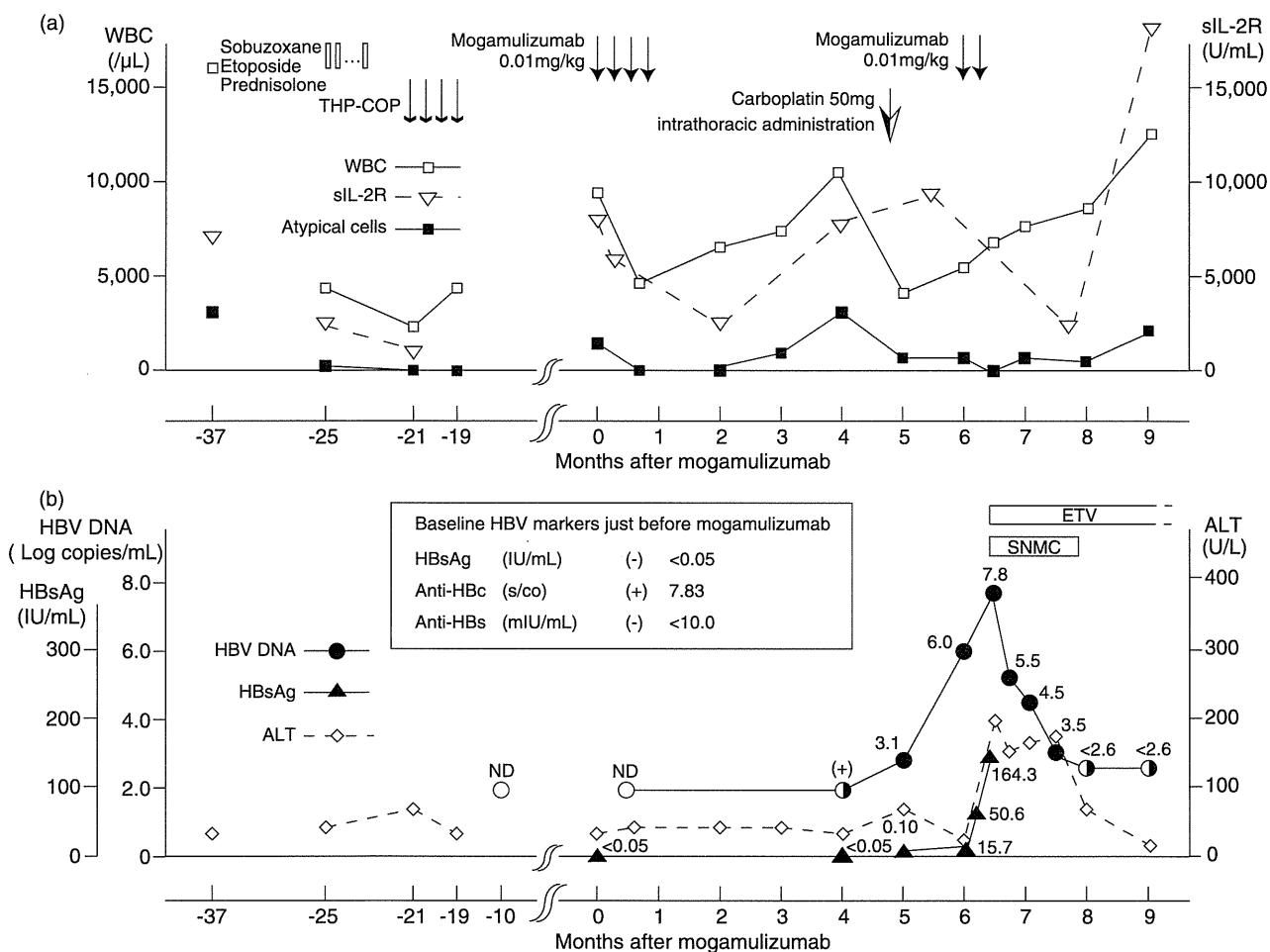


Figure 1 Clinical course and kinetics of HBV markers in a patient with adult T-cell leukemia–lymphoma before and after the anti-CC chemokine receptor 4 monoclonal antibody mogamulizumab treatment. ALT, alanine aminotransferase; anti-HBc, antibody against hepatitis core antigen; anti-HBs, antibody against 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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Regulatory T cells in cancer immunotherapy

Hiroyoshi Nishikawa and Shimon Sakaguchi

FOXP3⁺CD25⁺CD4⁺ regulatory T (Treg) cells, crucial for the maintenance of immunological self-tolerance, are abundant in tumors. Most of them are chemo-attracted to tumor tissues, expanding locally and differentiating into a Treg-cell subpopulation that strongly suppresses the activation and expansion of tumor-antigen-specific effector T cells. Several cancer immunotherapies targeting FOXP3⁺CD4⁺ Treg cells, including depletion of Treg cells, are currently being tested in the clinic. In addition, clinical benefit of immune-checkpoint blockade, such as anti-CTLA-4 monoclonal antibody therapy, could be attributed at least in part to depletion of FOXP3⁺CD4⁺ Treg cells from tumor tissues. Thus, optimal strategies need to be established for reducing Treg cells or attenuating their suppressive activity in tumor tissues, together with activating and expanding tumor-specific effector T cells.

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Introduction

Since the molecular characterization of tumor antigens that are recognized by tumor-reactive antibodies (Ab) and cytotoxic T-lymphocytes (CTLs) in cancer patients, therapeutic vaccination with the tumor antigens has been explored in the clinic as an antigen-specific cancer immunotherapy [1–4]. However, only a minor fraction of patients have exhibited tumor regression after multiple vaccinations despite their development of measurable humoral and cellular immune responses against tumor antigens [5,6,7]. To improve the efficacy of cancer vaccine, efforts have been made in these two decades to discover more immunogenic tumor-associated antigens and devise more effective ways of immunization, for example, by the use of various adjuvants, tumor antigen-expressing vectors and antigen-pulsed dendritic cells. In addition, it has become evident that the immunosuppressive elements present in cancer patients are critical impediments to the success of cancer immunotherapy [7–10]. One of the obstacles is CD25⁺CD4⁺

regulatory T (Treg) cells expressing the transcription factor FOXP3, which are physiologically present in the immune system and actively engaged in the maintenance of immunological self-tolerance by suppressing self-reactive T cells [11]. Considering that most tumor-associated antigens identified to date are antigenically normal self-constituents, it is likely that naturally occurring FOXP3⁺ Treg cells also hamper effective anti-tumor immune responses in cancer patients and that they can be one of the cellular targets to evoke and augment anti-tumor immunity [2–4,9,12].

FOXP3⁺ T cells in humans are heterogeneous in phenotype and function, including suppressive and non-suppressive subpopulations [13]. For example, naive CD4⁺ T cells transiently express FOXP3 at a low level upon *in vitro* T-cell receptor (TCR) stimulation; yet they are hardly suppressive [13,14^{**}]. The attempts to delineate suppressive or non-suppressive FOXP3⁺CD4⁺ T cells present in the peripheral blood have shown that FOXP3⁺CD4⁺ T cells can be dissected into three subpopulations by the expression levels of FOXP3 and the cell surface molecules CD45RA and CD25 (Figure 1): (i) FOXP3^{lo}CD45RA⁺CD25^{lo} cells (Fraction [Fr.] I), designated naive or resting Treg cells, which differentiate into FOXP3^{hi}CD45RA⁻CD25^{hi} cells (Fr. 2) upon antigenic stimulation; (ii) FOXP3^{hi}CD45RA⁻CD25^{hi} cells (Fr.2), designated eTreg cells, which are terminally differentiated and highly suppressive; and (iii) FOXP3^{lo}CD45RA⁻CD25^{lo} non-Treg cells (Fr. III), which do not possess suppressive activity but can secrete pro-inflammatory cytokines [14^{**}]. This classification of FOXP3⁺CD4⁺ T cells is instrumental in defining suppressive or non-suppressive FOXP3⁺ subpopulations, delineating developmental stages of Treg cells, and assessing their adaptive processes in physiological and pathological immune responses.

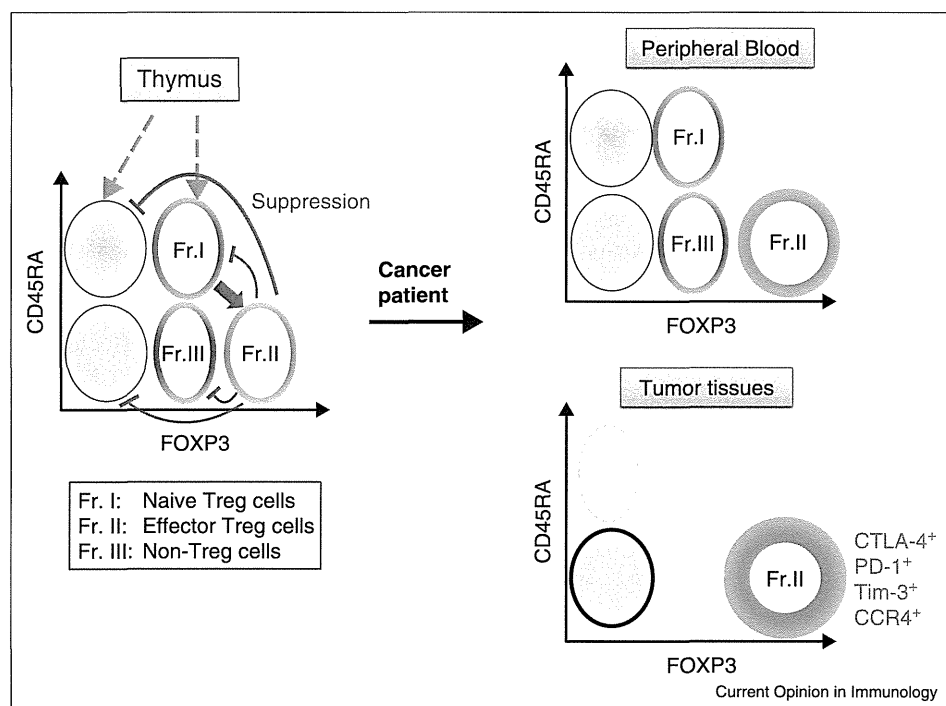
This review focuses on our current understanding of the roles of FOXP3⁺ Treg cells in tumor immunity in humans and discusses a perspective for numerical or functional manipulation of Treg cells as a key strategy in cancer immunotherapy.

Roles of Treg cells in tumor immunity

Treg-cell infiltration is associated with tumor progression

Treg cells are found at high frequencies in tumor tissues of various types of cancers such as breast, lung, liver, pancreatic and gastrointestinal cancers and malignant melanoma (reviewed in [9]). The presence of large proportions of CD4⁺ Treg cells among tumor-infiltrating

Figure 1



(Left) FOXP3⁺CD4⁺ T cells are dissected into three subpopulations by the expression levels of FOXP3 and the cell surface molecule CD45RA: FOXP3^{lo}CD45RA⁺ cells (Fr. I), designated naive or resting Treg cells, which differentiate into FOXP3^{hi}CD45RA⁻ cells (Fr. II), designated eTreg cells. FOXP3^{lo}CD45RA⁻ non-Treg cells (Fr. III) are not suppressive. eTreg cells (Fr. II) are suppressive on other FOXP3⁺ or FOXP3⁻ T cells, in particular, on CD45RA^{hi} naive CD4⁺ T cells. (Right) eTreg cells (Fr. II) are dominant in tumor tissues but not in the peripheral blood. These eTreg cells express CTLA-4, PD-1, CCR4 and Tim-3.

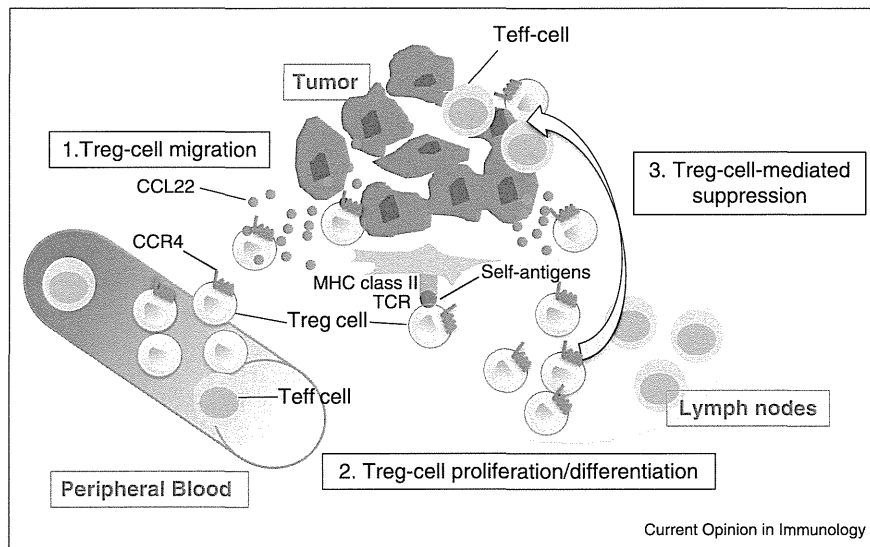
lymphocytes (TILs) [15[°]], in particular, decreased ratios of CD8⁺ T cells to FOXP3⁺CD25⁺CD4⁺ Treg cells among TILs [16[°]], is associated with poor prognosis in ovarian, breast, and gastric cancers (reviewed in [9]). These findings suggest that tumor-reactive CD8⁺ CTLs are suppressed by FOXP3⁺ Treg cells in tumor tissues. In contrast, there are some reports that high infiltration of FOXP3⁺ Treg cells is associated with better prognosis in colon and head/neck cancers and Hodgkin lymphoma [17–19]. This apparent inconsistency can be, at least in part, attributed to different compositions of FOXP3⁺ T-cell subpopulations in tumor tissues (Figure 1 and [14^{°°}]). Melanoma-infiltrating TILs predominantly contained eTreg cells (Fr. II), with very low frequencies of naive Treg cells (Fr. I) and FOXP3⁺ non-Treg cells (Fr. III), compared with the composition of the corresponding FOXP3⁺ subpopulations in the peripheral blood [20^{°°}]. In contrast, FOXP3⁺ T cells infiltrating into colon cancers contained higher frequencies of non-Treg cells (Fr. III) as well as eTreg cells (Fr. II) (H.N. and S.S., unpublished data). The result indicates that the increased FOXP3⁺ non-Treg cells, which are capable of secreting proinflammatory cytokines, could contribute to the better prognosis of some colon cancer patients even if they harbor high frequencies of total FOXP3⁺ TILs. Further studies are

required in various types of cancers to assess not only the number of FOXP3⁺ TILs but also the composition of FOXP3⁺ subpopulations among TILs, and to evaluate such parameters in terms of their relationship with the extent of anti-tumor immune responses and the prognosis of patients.

How do Treg cells infiltrate into tumor tissues?

As a likely mechanism by which Treg cells become abundant in tumor tissues, it has been proposed that tumor cells and/or tumor infiltrating macrophages produce the chemokine (C–C motif) ligand 22 (CCL22), which chemo-attracts and recruits to tumor tissues FOXP3⁺CD4⁺ Treg cells expressing C–C chemokine receptor type 4 (CCR4) (Figure 2 and [9,15[°],20^{°°},21]). Other combinations of chemokines and chemokine receptors, such as CCR10–CCL28 and CXCR3–CXCR3 ligands (such as CXCL9, 10, and 11), also reportedly contribute to Treg-cell infiltration [22,23]. It remains obscure whether conventional T cells can differentiate into suppression-competent FOXP3⁺ Treg cells in tumor microenvironments in humans. After the promoted migration to tumor tissues from the circulation, FOXP3⁺CD4⁺ Treg cells are activated and expand presumably via recognizing tumor-associated antigens or

Figure 2



FOXP3⁺CD4⁺ Treg cells infiltrate into tumor tissues through chemo-attraction mainly via CCR4–CCL22 and recognize self-antigens including tumor antigens present in tumor tissues. Proliferated/differentiated FOXP3⁺CD4⁺ Treg cells (mainly eTreg cells) efficiently suppress the activation of tumor antigen-specific effector T cells. To evoke and augment anti-tumor immune responses, Treg cells can be targeted at (1) Treg-cell migration, (2) Treg-cell proliferation/differentiation and (3) Treg-cell-mediated suppression.

self-antigens released from dying tumor cells (Figure 2). Indeed, Treg cells in cancer patients recognize a broad range of tumor antigens including NY-ESO-1 and Survivin, and suppress tumor-antigen-specific effector T cells [24–26,27[•],28], as previously shown with mice [29]. Compared with tumor-reactive effector or memory CD4⁺ T cells, natural FOXP3⁺ Treg cells may be better at recognizing tumor-associated self-antigens because of their TCR repertoires being more self-reactive than those of conventional T cells and their higher level expression of T cell accessory molecules including adhesion molecules (such as LFA-1) indicative of their ‘antigen-primed’ states [30,31]. This Treg-cell dominant immune-suppressive tumor microenvironment implies that cancer vaccines composed of proteins or long peptides (>15 amino acids) of tumor antigens may preferentially activate tumor-antigen-specific Treg cells, rather than antigen-specific effector T cells [32], augmenting suppression by the former on the latter unless proper strategies to block Treg-cell activation or suppressive function are taken.

Treg cells suppress the activation of tumor-antigen-specific T cells

Are natural Treg cells indeed suppressing the activation and expansion of tumor-antigen-specific effector T cells in healthy individuals and cancer patients? Direct evidence for the case was provided by immune responses to NY-ESO-1, one of the most immunogenic cancer/testis antigens [6,33]. For example, *in vitro* NY-ESO-1 peptide stimulation of peripheral blood lymphocytes after

depletion of CD25⁺CD4⁺ Treg cells was able to activate NY-ESO-1-specific naive CD4⁺ T-cell precursors in healthy individuals and in melanoma patients who possessed NY-ESO-1-expressing tumors but failed to develop anti-NY-ESO-1 Ab [34[•],35[•]]. In contrast, most NY-ESO-1-specific CD4⁺ T cells in melanoma patients who had spontaneously developed anti-NY-ESO-1 Ab were derived from a memory population and could be activated even in the presence of CD25⁺CD4⁺ Treg cells [35[•]]. In addition, following vaccination of ovarian cancer patients with an HLA-DP-restricted NY-ESO-1 peptide, the development of NY-ESO-1-specific high-avidity effector T cells from naive T cells was hampered by the presence of CD25⁺CD4⁺ Treg cells, although the vaccination could expand low-avidity NY-ESO-1-specific CD4⁺ T cells present in an effector/memory fraction before the vaccination [36]. These results collectively indicate that healthy individuals and cancer patients harbor potentially tumor-reactive T cells, whose activation and expansion are suppressed by natural Treg cells, and that Treg-cell depletion is able to activate and expand NY-ESO-1-specific high-avidity T cells from naive T-cell precursors, allowing their differentiation into high-avidity effector T cells capable of mediating potent anti-tumor immune responses.

Immunotherapy targeting Treg cells

Depletion of Treg cells or their functional alteration

As Treg cells constitutively express the high-affinity IL-2 receptor, CD25 (IL-2 receptor α -chain) can be suitable for Treg-cell depletion [37]. In animal models,

administration of cell-depleting anti-CD25 monoclonal Ab (mAb) before tumor inoculation resulted in tumor eradication [12,38]; in humans, removal of CD25⁺CD4⁺ Treg cells from PBLs induced tumor antigen-specific T cells *in vitro* as discussed earlier [34[°],35[°]]. In clinical trials with anti-CD25 mAb or denileukin diftitox (DAB₃₈₉IL-2), which is an immunotoxin-conjugated IL-2, some studies have shown the potential of CD25⁺ T cell depletion to augment anti-tumor immune responses; yet, other similar studies failed to obtain clinically evident augmentation [39–42]. Since activated effector T cells also express CD25 and their production of IL-2 is required for the expansion of CD8⁺ CTLs [43], CD25-based cell depletion may reduce activated effector T cells as well, canceling the effect of Treg-cell depletion to augment anti-tumor immunity.

Chemokine receptors are another candidate for Treg-cell depletion. CCR4 was shown to be specifically expressed by FOXP3^{hi}CD45RA⁻CD25^{hi} eTreg cells, but not by CD45RA⁺FOXP3^{lo}CD4⁺ naive Treg cells or most effector T cells in peripheral blood (Figure 1 and [20^{°°}]). With depletion of CCR4⁺ T cells and subsequent *in vitro* cancer/testis antigen NY-ESO-1 peptide stimulation, NY-ESO-1-specific CD4⁺ T cells were efficiently activated in a similar manner as observed following CD25⁺ T-cell depletion. CCR4⁺ T-cell depletion also augmented *in vitro* induction of NY-ESO-1-specific CD8⁺ T cells in melanoma patients. In addition, CCR4⁺ eTreg cells were predominant among melanoma-infiltrating FOXP3⁺ T cells and much higher in frequency compared with those in peripheral blood [20^{°°}]. Anti-CCR4 mAb is therefore instrumental for evoking and augmenting anti-tumor immunity in cancer patients by selectively depleting eTreg cells and is now being tested in the clinic.

Other molecules predominantly expressed by Treg cells can also be targeted for cell depletion as well as functional manipulation. GITR (glucocorticoid-induced TNF-receptor family related protein) is a co-stimulatory molecule expressed at low levels on resting CD4⁺ and CD8⁺ T cells and constitutively on FOXP3⁺CD4⁺ Treg cells at high levels [44]. Activation of GITR signaling with agonistic anti-GITR mAb or GITR ligands can inhibit the suppressive activity of FOXP3⁺CD4⁺ Treg cells and make effector T cells resistant to FOXP3⁺CD4⁺ Treg-cell-mediated suppression [44–46]. Another candidate molecule is OX40, a co-stimulatory molecule of the TNF receptor family. It is transiently expressed on activated T cells and constitutively expressed on FOXP3⁺CD4⁺ Treg cells. Previous studies using agonistic anti-OX40 mAb have shown that the mAb mediates anti-tumor effects by attenuating FOXP3⁺CD4⁺ Treg-mediated suppression and activating effector T-cell function [47,48]. Therapies targeting GITR and OX40 are currently in clinical trials.

Immune-checkpoint blockade with possible effects on Treg cells

Immune-checkpoint blockade by mAb such as anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) mAb and anti-programmed cell death protein 1 (PD-1) mAb is currently being tested extensively with various types of solid tumors and has provided promising clinical results [10]. In fact, Ipilimumab, a humanized anti-CTLA-4 mAb, was approved by the FDA for the treatment of malignant melanoma based on the clinical data from phase III clinical trials [49^{°°},50]. CTLA-4 is constitutively expressed by FOXP3⁺CD4⁺ Treg cells and is up regulated by CD4⁺ and CD8⁺ effector T cells after activation. It was originally thought that anti-CTLA-4 mAb would block an inhibitory signal on activated CD4⁺ and CD8⁺ effector T cells and recover their anti-tumor activity [51,52]. Recent animal studies using anti-CTLA-4 mAb lacking the ADCC (antibody-dependent cellular cytotoxicity) activity by modulating Fc portion or Fc receptor knockout mice have shown that anti-tumor activity of anti-CTLA-4 mAb was attributed to depletion of FOXP3⁺CD4⁺ Treg cells from tumor tissues, rather than direct activation of effector T cells [53[°],54[°],55[°]]. Indeed, decreased numbers of FOXP3⁺ Treg cells in tumor tissues following anti-CTLA-4 mAb (Ipilimumab, IgG1 subclass) treatment were strongly correlated with clinical benefit [56,57]. In addition, the crucial roles of CTLA-4 for FOXP3⁺CD4⁺ Treg-cell function was revealed in animal studies, which showed that specific deficiency of CTLA-4 in FOXP3⁺CD4⁺ Treg cells impaired their suppressive function and thereby augmented anti-tumor immunity [58,59].

While most anti-PD-1 mAbs and anti-PD-L1 mAbs investigated in the clinic are of the IgG4 subclass [60–62], which is not cell-depleting, it remains to evaluate Treg-cell function in tumor tissues because PD-1 is also highly expressed by tumor-infiltrating Treg cells ([63] and H.N. and S.S., unpublished data). As the PD-1 mAb CT-011 is of the IgG1 subclass, it is of interest to compare clinical benefits among the anti-PD-1 mAbs with different isotypes [64]. Immune-checkpoint blockade targeting other molecules such as LAG3 is also under clinical studies (<http://clinicaltrials.gov/>). Considering the expression of these molecules on FOXP3⁺CD4⁺ Treg cells as well, it is worth exploring whether mAbs specific for the molecules may have some effects on FOXP3⁺CD4⁺ Treg cells, in particular, those in tumor tissues.

Chemicals or drugs preferentially affecting Treg cells

In addition to biologicals for Treg-cell depletion or functional alteration, anti-cancer drugs such as cyclophosphamide and fludarabine can selectively affect Treg cells [65,66], presumably because natural Treg cells are physiologically in a more proliferative state than other

T cells via recognizing self-antigens or commensal microbes. Supporting this effect, multiple peptide vaccine combined with a single dose of cyclophosphamide reduced the number of Treg cells and induced strong immune responses against multiple tumor antigen peptides, with longer survival of renal cell carcinoma patients [66]. In addition to such radiomimetic drugs affecting proliferative T cells, there may be other chemicals that differentially affect Treg cells and effector T cells via exploiting, for example, different cytokine or metabolic sensitivities, thereby tipping their balance toward the dominance of effector T cells to augment anti-tumor immunity.

Conclusions and perspectives

The vital roles of Treg cells in tumor immunity are now widely accepted and Treg-cell targeting therapy is under active investigation. For clinical application of these therapies, there are some issues to be considered. One is how deleterious autoimmunity possibly accompanying Treg-cell depletion can be circumvented. In addition to optimizing the degree and duration of the depletion, it is critical to target a Treg-cell subpopulation, rather than whole FOXP3⁺ cells, to evoke effective anti-tumor immunity while avoiding autoimmunity. One possible way is to specifically control eTreg cells, which are predominant in tumor tissues and most suppressive. For example, cell-depleting anti-CCR4 mAb and anti-CTLA-4 mAb deplete eTreg cells but not naive Treg cells because the latter expresses CCR4 and CTLA-4 at much lower levels than the former. The preserved naive Treg cells are sufficient to suppress serious autoimmunity [14^{**},20^{**}]. It also needs to consider that Treg cells and activated effector T cells share common phenotypes; for example, both express CD25, CTLA-4, PD-1 and GITR, although at different levels. This implies that cell-depleting mAbs specific for these molecules could deplete tumor-reactive activated effector T cells as well, reducing the anti-tumor effects by Treg-cell depletion [45]. The timing and dose of mAb administration can be essential factors for differential control of Treg cells and effector T cells involved in tumor immunity. It is also critically important to monitor tumor-infiltrating Treg cells, in particular Treg-cell subpopulations, rather than circulating Treg cells, before and after immunological intervention to predict the efficacy of Treg-cell depletion or functional modulation for evoking or augmenting anti-tumor immunity [7]. It is envisaged that combinations of Treg-cell targeting (e.g., by reducing Treg cells or attenuating their suppressive activity in tumor tissues) with the activation of tumor-specific effector T cells (e.g., by cancer vaccine) will make the current cancer immunotherapy more effective in the clinic.

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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-2R γ^{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