TH1/TH2 cytokine quantification

Amounts of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, TNF-α, and TNF-β in patients' sera were quantified by a cytofluorometry-based ELISA system (Flowcytomix, Bender Medsystems GmbH, Austria). Standard curves for each cytokine were generated using the reference cytokine concentrations supplied by the manufacturer. Cytokines in sera from patients at different time points were estimated according to the manufacturer's instructions. Raw data of the FC bead assay were analyzed by FlowCytomixPro2.3 software.

Statistical analysis

The statistical analyses of immunological parameters and prognostic factors (PFS or OS) were performed using Wilcoxon signed-rank test and Kaplan-Meier method, respectively, with JMP software, version 9.0.3 (SAS Institute Inc., Cary, NC, USA).

Additional files

Additional file 1: The mean fluorescent intensity (MFI) of the surface expression of immunological molecules. Tabular data.

Additional file 2: Schedule for DC vaccination combined with sunitinib in this clinical trial. Supplementary figure.

Additional file 3: Data from an individual patient. Supplementary figure.

Additional file 4: Computed tomography (CT) images. Supplementary figure.

Abbreviations

RCC: Renal cell carcinoma; mRCC: Metastatic RCC; TKI: Tyrosine kinase inhibitor; MDSCs: Myeloid-derived suppressor cells; Tregs: Regulatory T cells; PBMCs: Peripheral blood mononuclear cells; GM-CSF: Granulocyte macrophage colony-stimulating factor; DCs: Dendritic cells; TNF-α: Tumor necrosis factor α; PGE2: Prostaglandin E2; OS: Overall survival; PFS: Progression-free survival; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; RECIST: Response Evaluation Criteria in Solid Tumors; PBS: Phosphate-buffered saline.

Competing interests

Department of Immunotherapeutics is an endowed department supported by financial contributions from Medinet Co. Ltd. (Yokohama, Japan). Dr. Kazuhiro Kakimi received research support from Medinet Co. Ltd. The costs of the entire DC culture production and part of the immunological assays were covered by Medinet Co. Ltd. The study sponsors had no involvement in study design; collection, analysis, and interpretation of data; writing the report; and the decision to submit the report for publication. No potential conflicts of interest were disclosed by the other authors.

Authors' contributions

Conceived and designed the study: HM, YE, YH and KK. Performed the clinical study: HM, YE, HK, TN, HF, MS, TH, and KK. Analyzed the data: HM and KK. Wrote the paper: HM, YE, YH and KK. All authors read and approved the final manuscript.

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Denatured Mammalian Protein Mixtures Exhibit Unusually High Solubility in Nucleic Acid-Free Pure Water

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Abstract

Preventing protein aggregation is a major goal of biotechnology. Since protein aggregates are mainly comprised of unfolded proteins, protecting against denaturation is likely to assist solubility in an aqueous medium. Contrary to this concept, we found denatured total cellular protein mixture from mammalian cell kept high solubility in pure water when the mixture was nucleic acids free. The lysates were prepared from total cellular protein pellet extracted by using guanidinium thiocyanate-phenol-chloroform mixture of TRIzol, denatured and reduced total protein mixtures remained soluble after extensive dialysis against pure water. The total cell protein lysates contained fully disordered proteins that readily formed large aggregates upon contact with nucleic acids or salts. These findings suggested that the highly flexible mixtures of disordered proteins, which have fully ionized side chains, are protected against aggregation. Interestingly, this unusual solubility is characteristic of protein mixtures from higher eukaryotes, whereas most prokaryotic protein mixtures were aggregated under identical conditions. This unusual solubility of unfolded protein mixtures could have implications for the study of intrinsically disordered proteins in a variety of cells.

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Introduction

Proteins perform an extraordinary array of functions in cells [1]. To understand the behavior of proteins in living cells, we must consider the extremely high intracellular concentrations of macromolecules. The cytoplasmic protein concentration has been estimated to be 100 mg/mL [2], and the total macromolecular concentration (including proteins, lipids, nucleic acids, and sugars) could be as high as 400 mg/mL [3]. Proteins have therefore evolved to exert their biological functions under highly crowded conditions, which raises the question of how they maintain solubility in such a dense milieu. Intrinsically unstructured proteins display unusually high solubility, and studying these molecules may elucidate the mechanisms underlying this phenomenon.

Proteins must fold into unique three-dimensional structures and interact specifically with particular molecules to function correctly. However, some proteins exist in an intrinsically unstructured form, lacking stable secondary and tertiary structural elements, but retaining full functionality. These intrinsically disordered proteins (IDPs) are unfolded *in vitro*, but may adopt functional conformations *in vivo*, although several lines of indirect evidence indicate that IDPs remain disordered in the cell [4,5]. The capacity for folding or remaining intrinsically unstructured mainly depends on the interplay between water molecules and the characteristic amino acid composition that dictate the hydrophobicity, charge, and flexibility [6,7]. Generally, IDPs lack bulky hydrophobic residues such as Ile, Leu, and Val, as well as aromatic residues such as Trp, Tyr, and Phe but are enriched in polar residues such as Arg, Gly, Gln, Ser, Pro, Glu and Lys, and the secondary structure-breaking amino acids Gly and Pro [6,7]. This composition results in high solubility in water despite being highly unstructured. Much work has been done on prediction of IDPs from protein sequences, and this class of proteins are much more abundant in eukaryotes than in prokaryotes [8,9]. Although the predicted disorder depends on the program used, intrinsically disordered regions (IDRs) account for 8–10% of protein sequences in prokaryotes and 30–41% in eukaryotes [10,11]. The majority of cellular proteins are predicted to adopt fully folded biologically active conformations, but IDRs are abundant. Unlike globular proteins, IDPs show unusually high solubility following heat treatment. Kim *et al* (2000) demonstrated that 20% of total proteins in Jurkat T-cell lysates are heat-resistant and remain soluble after boiling [12]. The resultant soluble protein fractions

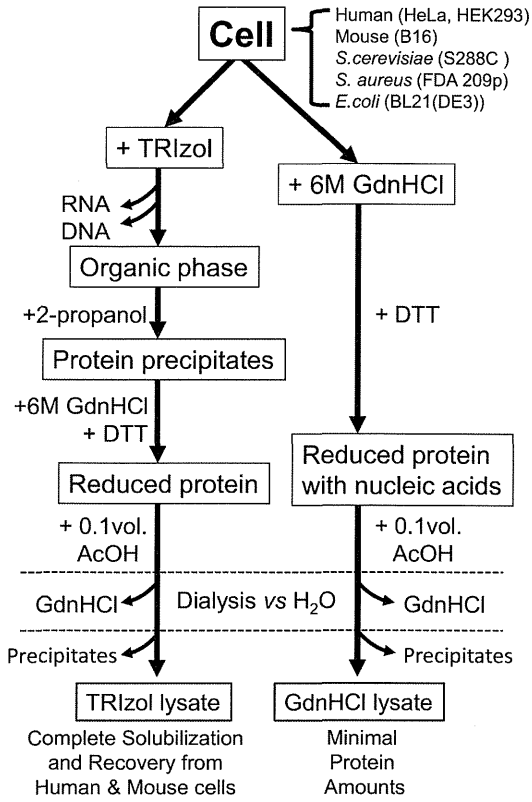

Figure 1. Schematic representation of the preparation of total cell protein lysates.
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are enriched in IDPs and are a valuable resource for proteomic research [13,14].

It is widely accepted that denaturing proteins exposes hydrophobic residues that are normally buried in the native conformation, and aggregation is mainly mediated by the resulting hydrophobic or electrostatic interactions between individual molecules. Hydrophobic interactions mainly occur between neighboring denatured protein molecules, whereas electrostatic interactions mainly occur between denatured proteins and anionic nucleic acid polymers. Removal of nucleic acids is therefore critical for efficient oxidative refolding of globular proteins from bacterial inclusion bodies [15]. The refolding efficiency can be improved by altering the ionic strength, pH, and using additives [16], but the final yield of refolded protein is often decreased substantially due to the presence of misfolded protein molecules that seed aggregation during purification steps. Poor protein solubility is a commonly encountered problem, and maintaining proteins in soluble conditions is the conventional approach for ensuring biological activity is maintained. The opposite approach of intentional denaturation is unusual, but may work well for maintaining the solubility of IDPs.

The unusual high solubility of mammalian IDPs appeared to be characteristic of proteins from higher eukaryotes, since most prokaryotic protein mixtures aggregated under similar denaturing conditions. Although the detailed mechanism is unclear, this unusual solubility presumably reflects the amino acid composition of eukaryotic IDPs, and likely reflects key evolutionary differences.

Materials and Methods

Cell culture

Human cell lines HeLa S3 and HEK293 PEAKrapid, and the mouse cell line B16 melanoma-F10 were purchased from ATCC. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, PAA laboratories, Austria) and penicillin/streptomycin (Wako, Osaka, Japan). *S. cerevisiae*, S288C (National Bio-Resource Project of the MEXT, Japan) was grown in YPD media at 30°C for 24 h. *S. aureus* (FDA 209P) in brain Bacto heart infusion medium (BD Biosciences), and *E. coli* BL21 (DE3) (Novagen) in LB medium were grown at 37°C for 24 h. *E. coli* BL21 (DE3) containing pET23a-human β-actin plasmid DNA were used to express human β-actin. Transformed cells were cultured in LB at 37°C, expression was induced with 0.4 mM IPTG, and growth continued for 3 h.

Isolation of nucleic acid-free total cell proteins

Total cell proteins were isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Briefly, sub-confluent mammalian cells cultured on a 100 mm dish were washed twice with PBS, lysed in 5 mL TRIzol, scraped off and transferred to a centrifuge tube. Proteins were recovered in the organic phase following addition of chloroform, and precipitated by addition of 2-propanol. Protein precipitates were extensively washed with 0.3 M GdnHCl in 95% ethanol at least five times, to give a white protein pellet that was washed three times with ethanol. Ethanol-wet pellets were used directly as they were poorly soluble in 6 M GdnHCl after drying. Cell pellets of *S. cerevisiae*, *S. aureus*, *and E. coli* (0.2 g wet weight were dissolved in 1 mL TRIzol and treated as described above.

Preparation of protein lysates in salt-free water

Total cell proteins were dissolved in 6 M GdnHCl containing 0.1 M Tris-HCl pH 8.5, and the protein concentration was adjusted for each experiment using values determined from the absorbance at 280 nm, assuming 1 absorbance unit at 280 = 1 mg/mL. Disulfide bonds were then reduced with 0.1 M DTT at 37°C for 1 h, and a 0.1 volume of acetic acid was added. The resultant protein solutions were dialyzed extensively against Milli-Q water using a Slide-A-Lyzer (3.5K MWCO, Thermo Fisher Scientific, Waltham, MA) at 4°C for 48 h. The Milli-Q water was changed every few hours initially then every 12–16 h. Residual nucleic acids in TRIzol lysate were determined with Quant-iT PicoGreen dsDNA and RiboGreen RNA Assay Kit (Life Technologies, Carlsbad, CA). To determine the solubility in TRIzol lysates, initial protein concentrations were adjusted to 1 mg/mL before starting dialysis. Aggregated proteins and the remaining soluble proteins were separated by centrifugation at 14,000×*g* for 15 min at 4°C, and each was solubilized in 8 M urea prior to protein concentration measurement using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Determination of protein solubility in lysates containing additives

Nucleic acid-free total cell protein lysates (TRIzol lysates) from HeLa cells in Milli-Q water and with a protein concentration of 2–3 mg/mL and a pH of 5 and an electrical conductivity <20 μS/cm were used for assays. dNTPs (Thermo) 16S-rRNA and 23S-rRNA from *E. coli* MRE600 (Boehringer Mannheim, Germany), tRNA from baker's yeast (Type X-SA, Sigma, St. Louis, MO), or DNA from calf thymus (phenol-chloroform extracted, <2000 bp,

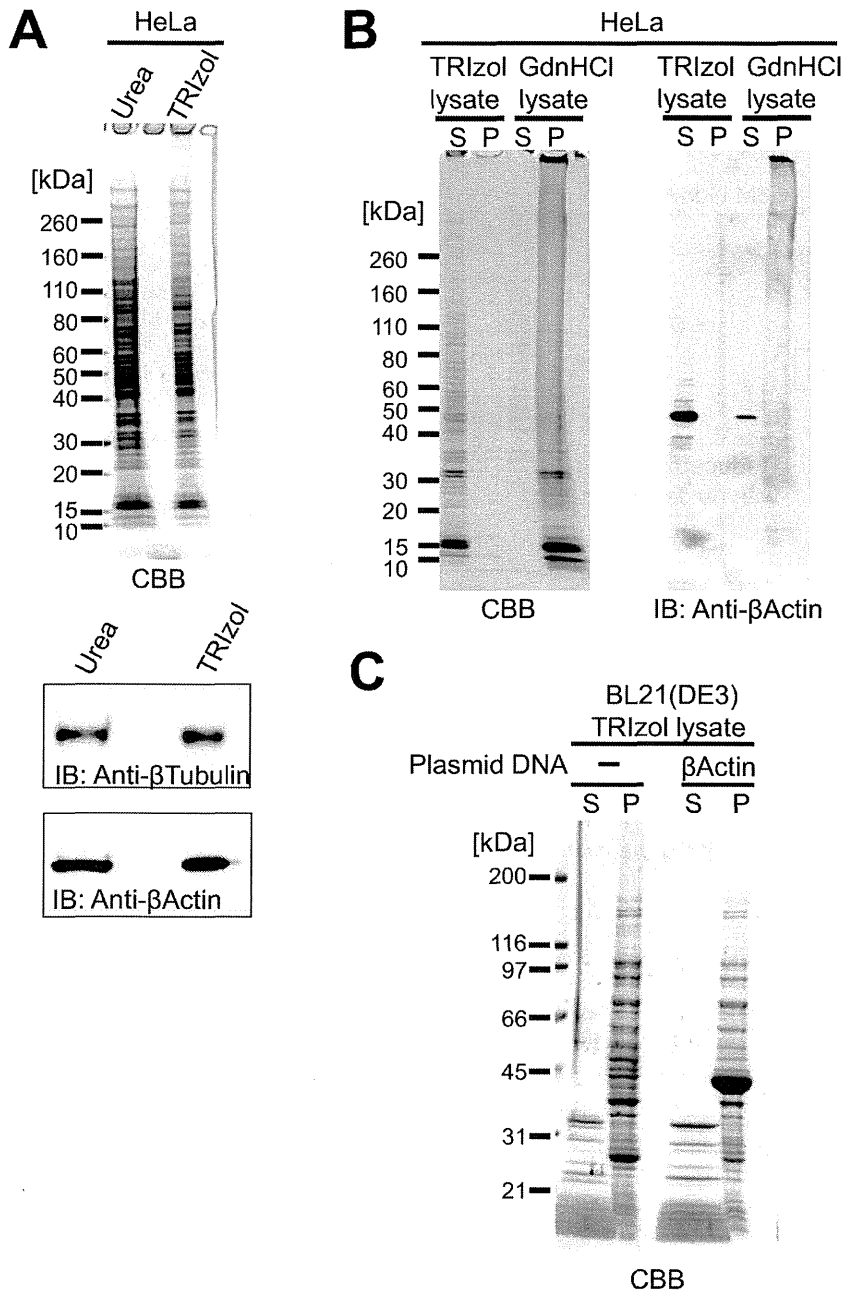

Figure 2. Analysis of total cell protein lysates. A. SDS-PAGE of total cell proteins lysed with 8 M urea or precipitated from the organic phase of TRIzol homogenates (an equivalent number of HeLa cells were used in each case). Endogenous proteins were also analyzed using western blotting. **B.** SDS-PAGE of total cell protein TRIzol and GdnHCl lysates. Equivalent amounts of protein in soluble fractions (S) and precipitates (P) following centrifugation were loaded. **C.** SDS-PAGE of TRIzol lysates prepared from *E. coli* BL21(DE3) expressing or not expressing human β-actin.
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WAKO) were mixed with TRIzol lysates to give a protein concentration of 0.1 mg/mL, and incubated for 60 min at 4°C. After centrifugation at 14,000×*g* for 15 min at 4°C, the concentration of soluble proteins was determined by Bradford protein assay (Bio-Rad).

Plasmid transfection and functional assays

Plasmids for expression of the enhanced GFP (pEGFP-N1; Clontech, Mountainview, CA) and firefly luciferase (Luc; pGL3-basic; Promega, Madison, WI) were used to transfect HEK293 PEAKrapid cells using 293 fectin (Invitrogen) which were subsequently cultured for 24 h. To prepare native protein lysates, cells were lysed with Glo Reporter Lysis Buffer (GLB, Promega). The fluorescence intensity of EGFP-containing lysates was analyzed using a Multi Microplate Reader MTP-800 (Hitachi, Japan) at Ex/Em: 480/530 nm. Luminescence of Luc-containing lysates was measured using a steady Glo assay kit (Promega) and Luminometer Junior LB9509 (Berthold Technologies, Dak Ridge, TN).

Table 1. Solubility of denatured and nucleic acid-free total cell proteins in pure water.

Source	Solubility in TRIzol lysate (%)*
H. Sapien, M. musculus (HeLa, Hek293, B16–F10)	95.9±1.7
S.cerevisiae (S288C)	59.1±8.4
S. aureus (FDA209p)	47.3±0.9
E.coli (BL21(DE3))	32.9±3.0

*Initial protein concentrations were adjusted to 1 mg/mL before dialysis.
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Western blotting

Endogenous and transiently expressed reporter protein levels were verified by Western blotting using conventional procedures using the following primary antibodies; anti-β-actin (13E5, Cell Signaling Technologies, Beverly, MA), β-tubulin (Cell Signaling Technologies), GFP (mFX75, Wako), Luciferase (MBL, Nagoya, Japan). Membranes were treated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology), and positive signals were measured using a chemiluminescence system.

NMR

NMR spectra were recorded at 37°C on a Varian Unity INOVA 600 spectrometer (Varian, CA). 3Hmutwil and *S*-carboxymethylated mouse lysozyme were prepared as described previously [17–19], and 0.1 mM samples of ^{15}N-labeled proteins were dissolved in TRIzol lysates or distilled water containing 10% D_2O. The pH was adjusted to 2 using HCl, and NH signal assignments from ^{1}H-^{15}N-labeled HSQC spectra were assigned as described [19].

Results

Preparation of nucleic acid-free total cell protein lysates

In order to prepare nucleic acid-free total protein lysates from cultured mammalian cells under native conditions, we removed nucleic acids using three different approaches; selective precipitation with polyethylenimine [20], extensive digestion with nuclease, and chromatographically using an anion-exchange column. Unfortunately, neither method produced a satisfactory yield or purity. In contrast, phenol-chloroform extraction was efficient at ensuring total separation of nucleic acids and denatured total cell proteins. The guanidinium thiocyanate-phenol-chloroform mixture that constitutes TRIzol reagent, that is regularly used for RNA preparation [21], was used to homogenize cells, and proteins extracted using this reagent have been successfully recovered for proteomic research [22–24]. In this extraction procedure, proteins are fractionated into the organic phase and precipitated by addition of 2-propanol (Fig. 1), and 90% of total cellular proteins can be recovered, which is considerably higher than was achieved by homogenizing cells directly in 8 M Urea (Fig. 2A). After extensive washes with 0.3 M guanidine hydrochloride (GdnHCl)-95% ethanol, or in ethanol, total cellular proteins were recovered as a tightly packed white pellet following centrifugation, which was used directly or stored at −20°C as a wet pellet to avoid the difficulties associated with resuspending dried pellets in denaturant solutions. When dissolved in 6 M GdnHCl, proteins formed a slightly cloudy solution that clarified following reduction with dithiothreitol (DTT). Proteins were successfully solubilized following dialysis against pure water at acidic pH (Fig. 2 and Table 1) to give a yield of approximately 6 mg/mL from HeLa cells. The pH of the TRIzol lysate was between 5 and 5.8, which was the same as the dialysis solution, confirming dialysis had gone to completion. The electrical conductivity of the TRIzol lysate was less than 20 μS/cm, which was estimated to be less than 1 mM of electrolytes and is probably mostly residual GdnHCl. All Trizol lysates confirmed to show UV absorption spectrum has a peak maximum at approximately 280 nm. The residual nucleic acids in HeLa TRIzol lysate were less than 1 ng/mL in 1 mg/mL of protein by fluorescent nucleic acids detection methods, thus the lysates were virtually nucleic acids free. As shown in Figure 2A, denatured mammalian proteins in nucleic acid-free water showed unexpectedly high solubility compared with the extensive insoluble aggregation observed in denaturant containing nucleic acids (Fig. 1, 2B). Total cellular proteins from another eukaryote (*Saccharomyces cerevisiae*) and two prokaryotes (*Staphylococcus*

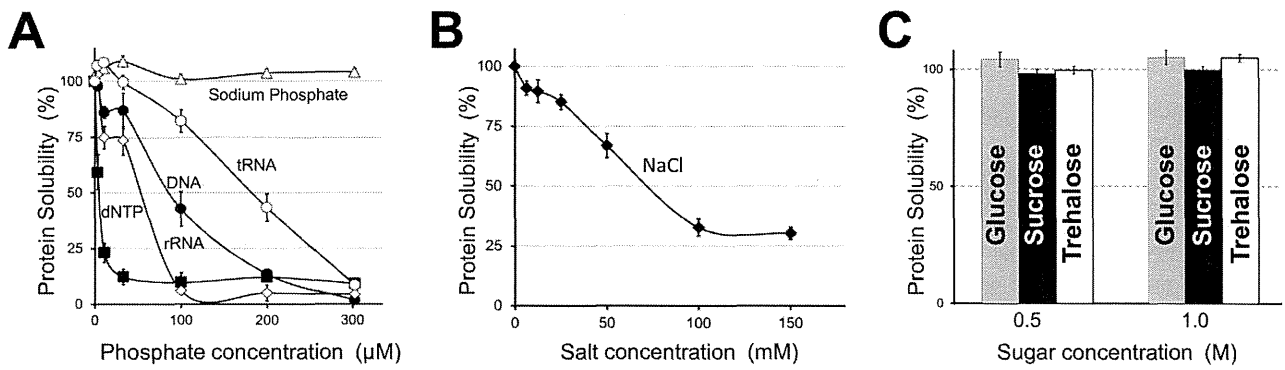

Figure 3. Effect of additives on protein solubility in HeLa cell TRIzol lysates. The solubility of proteins remaining after addition of nucleic acids (**A**), sodium chloride (**B**) or carbohydrates (**C**) was determined.
doi:10.1371/journal.pone.0113295.g003

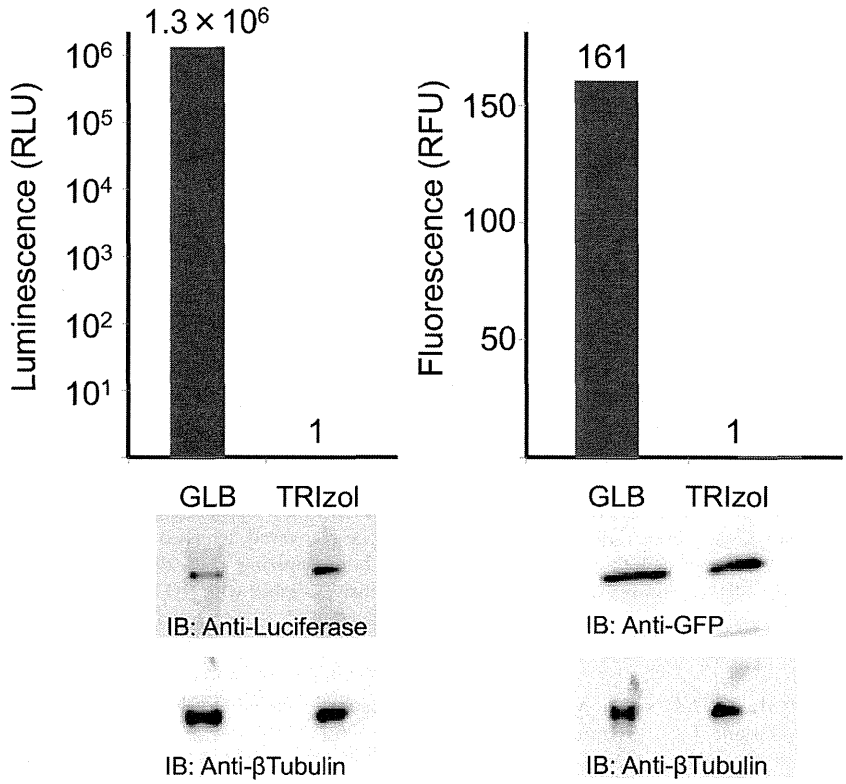

Figure 4. Biological activity of reporter proteins in physiological buffer or TRIzol lysates. Hek293 cells expressing either GFP or Luc were directly lysed in physiological Glo Lysis buffer (GLB) or TRIzol, and GFP or Luc activity were measured. Reporter proteins were verified by western blotting.
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aureus and *Escherichia coli*) showed reduced solubility (Table 1). However, low molecular weight (<20 kDa) denatured proteins from *E. coli* were soluble in these conditions, whereas most higher molecular weight (>30 kDa) proteins were insoluble (Figure 2C). Recombinant human β -actin is expressed in inclusion bodies in *E. coli*, and is known to be highly insoluble in the denatured form [25]. This protein remained insoluble in the *E. coli* total protein lysate even in nucleic acid-free conditions (Fig. 2C). In contrast, denatured β-actin from HeLa cells showed high solubility in nucleic acid-free conditions (Fig. 2B). The high solubility and resistance to aggregation of mammalian total cellular proteins in nucleic acid-free pure water compared to prokaryotic proteins (Table 1) presumably reflects an evolutionary divergence, which is consistent with the high abundance of IDPs in eukaryotes but not in prokaryotes.

Effect of additives on the solubility of TRIzol-solubilized proteins from HeLa cells

Nucleic acids at a concentration of 100−300 μM of phosphate group (30–100 μg/mL) induced precipitation of TRIzol-solubilized proteins from HeLa cells. Interestingly, the triphosphate group of dNTPs appeared to be a strong inducer of precipitation of denatured proteins, whereas monophosphate anions showed no such effect in the concentration range studied (Fig. 3A). The ionic strength was also important for solubility; the solubility of proteins in physiological saline decreased to 30% (Fig. 3B). However, non-ionic solutes such as sugars did not affect protein solubility (Fig. 3C), indicating that coulomb interactions between denatured proteins and additives contributed to protein solubility in TRIzol lysates. Although the pH can be an important factor affecting the net charge of protein molecules, this proved difficult to determine to analyze here, because addition of ionic buffers rapidly induced protein aggregation at all pH values tested.

Protein conformation in TRIzol lysates

To confirm that proteins were completely denatured in TRIzol lysates, HEK293 cells expressing Luciferase or GFP were examined. As shown in Figure 4, both cell types were successfully lysed in Glo Reporter Lysis Buffer (GLB, Promega) under native conditions in which the reporter protein function was maintained. While ensuring the same number of each cell type was used, reporter proteins were successfully recovered in soluble but denatured (non-functional) form in TRIzol lysates. In a previous study, immunoglobulin light chain derived amyloidogenic 3Hmut at pH 2 [19] and *S*-carboxylmethylated mouse lysozyme at pH 5 [17] were confirmed to be fully disordered using heteronuclear NMR spectroscopy [18,26]. Using these disordered proteins as probes, ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra were compared for HeLa TRIzol lysates. As shown in Figure 5, the overall spectra for both proteins exhibited similar crowded resonances indicative of fully denatured proteins. These results confirmed that the proteins in the HeLa cell lysates were fully unfolded and highly soluble.

Discussion

In this study, we observed that fully denatured mammalian total cell protein mixtures showed unusually high solubility in nucleic acid-free pure water. This unusual solubility appeared to be

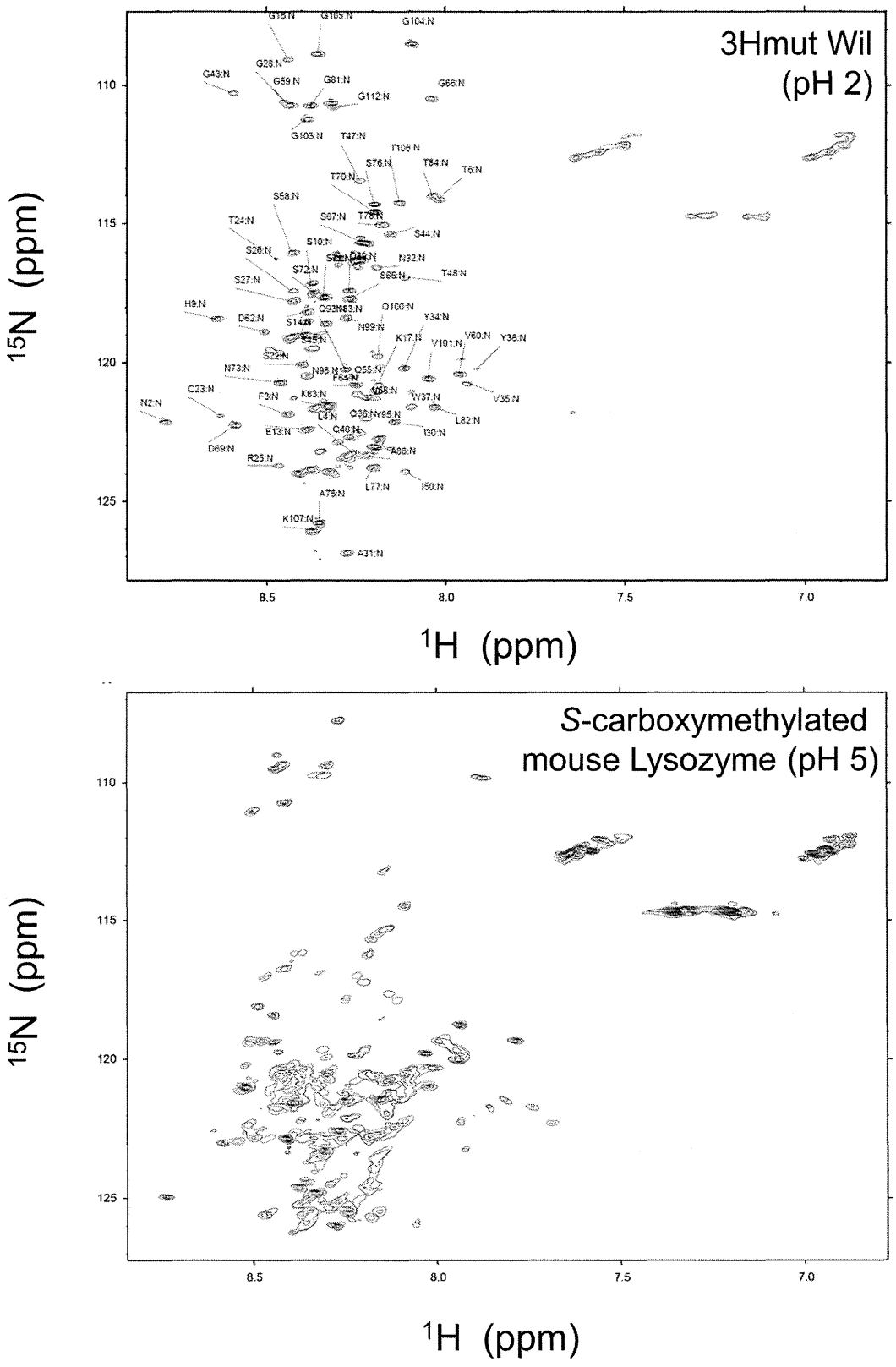

Figure 5. Effect of water soluble denatured protein mixtures on the solution structure of intrinsically disordered proteins. ^{1}H-^{15}N HSQC spectra of the intrinsically disordered 3HmutWil and *S*-carboxymethylated mouse lysozyme in the absence (black) and presence (red) of HeLa cell TRIzol lysates. Assignments of 3HmutWil in the absence of lysate are indicated.
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characteristic of higher eukaryotes, since proteins from a lower eukaryote (yeast) and two prokaryotes were largely aggregated under comparable conditions. This observation likely reflects a key evolutionary divide since IUPs are known to be highly abundant in mammalian cells [10,11]. Within the single-celled organisms studied, denatured proteins from the eukaryote *Saccharomyces cerevisiae* showed higher solubility than did those from two prokaryotes (Table 1). This trend was consistent with previous studies on IDPs from *Saccharomyces cerevisiae* and *E. coli* [27]. Therefore, in a salt-free and nucleic acid-free environment, the solubility of denatured proteins is highly correlated with the flexibility of the polypeptide and the proportion of hydrophilic residues in the protein chain.

Reconstitution of nucleic acids into TRIzol lysates from the HeLa cells indicated that these polyanionic macromolecules strongly promote the aggregation of denatured proteins. As shown in Figure 3A, tRNA, which is a tightly folded molecule, induced protein aggregation to a lesser degree than did dNTPs that have exposed phosphate groups. Thus, the electrostatic interactions between nucleic acids and denatured protein molecules could be a strong trigger for protein aggregation. This may explain why mammalian recombinant proteins frequently aggregate in bacterial cells. In our previous study, recombinant proteins isolated from bacterial inclusion bodies were found to be tightly associated with nucleic acid [15].

The ionic strength of TRIzol lysates also affected protein solubility significantly (Fig. 3B). Unfolded purified proteins, including integral membrane proteins, have been successfully solubilized in pure water previously [6,7,28–30]. Furthermore, the structural and dynamic properties of proteins in 8 M urea and pure water were shown to be similar using NMR [31]. Soluble proteins in pure water are predicted to be highly flexible due to strong intramolecular and intermolecular electrostatic repulsion [30]. In this study, pure water had a pH of 5.6, presumably due to the atmospheric carbon dioxide concentration. At this pH, ionizable groups on Lys, Arg, His, Asp, and Glu residues are potentially fully charged in the absence of counter ions, which maximizes the hydration of unfolded proteins. The high entropy of the denatured proteins in the HeLa cell TRIzol lysates is presumably the reason for the high solubility. Since aggregation requires productive collisions between protein molecules, enthalpy-entropy compensation theory can explain the unusually high solubility of denatured mammalian proteins in pure water [32,33].

Although the detailed mechanism is unclear, flexible polypeptide chains classified as IDPs may competitively suppress intermolecular interactions between otherwise insoluble hydrophobic polypeptides. Mammalian proteins in TRIzol lysates retained solubility for more than 6 months at 4°C. Importantly, approximately 30% of fully disordered proteins in mixtures from human cells maintained solubility in physiological saline (Fig. 3B). Competitive suppression of protein aggregation may partially explain the extraordinarily high solubility of mammalian proteins in living cells.

Upon screening of aggregation-prone protein domains, highly charged intrinsically disordered flexible sequences termed entropic bristles served as effective solubilizers in fusion partner proteins [33–35]. This suggests that disordered regions in IDPs enhance protein solubility via entropic effects, and pure water may enhance this effect. Chemical protein cationization of Cys residues is a powerful approach for solubilization of denatured proteins [17,36,37], which also enhances protein flexibility via electrostatic effects. Enhancing protein flexibility therefore appears to be a productive strategy for increasing the solubility of disordered proteins.

Solubilization of proteins is essential for their use in biotechnological and medical applications. Maintaining the biologically active 'native' conformation is the preferred approach for soluble proteins. In the case of denatured proteins, especially those of mammalian origin, nucleic acid-free pure water may be a useful solvent for the alternative approach of solubilizing disordered proteins. This alternative approach could be applied in numerous ways. For example, surgically removed cancer tissues contain immunologically important antigens that induce cancer immunity [38–40], and the insoluble fraction of tumor cell homogenates in PBS lysed by sonication contain tumor antigens eliciting cytotoxic T-lymphocytes [41]. The method of extracting denatured proteins in high yield established in this study may therefore be useful for preparation of cancer vaccines.

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Author Contributions

Conceived and designed the experiments: JF YA KK. Performed the experiments: JF HF RK TH HT YA. Analyzed the data: RK HN HM. Contributed to the writing of the manuscript: JF RK HN.

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ORIGINAL REPORT

Multicenter Phase II Study of Mogamulizumab (KW-0761), a Defucosylated Anti-CC Chemokine Receptor 4 Antibody, in Patients With Relapsed Peripheral T-Cell Lymphoma and Cutaneous T-Cell Lymphoma

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ABSTRACT

Purpose
CC chemokine receptor 4 (CCR4) is expressed by peripheral T-cell lymphomas (PTCLs) and is associated with poor outcomes. Mogamulizumab (KW-0761) is a defucosylated humanized anti-CCR4 antibody engineered to exert potent antibody-dependent cellular cytotoxicity. This multicenter phase II study evaluated the efficacy and safety of mogamulizumab in patients with relapsed PTCL and cutaneous T-cell lymphoma (CTCL).

Patients and Methods
Mogamulizumab (1.0 mg/kg) was administered intravenously once per week for 8 weeks to patients with relapsed CCR4-positive PTCL or CTCL. The primary end point was the overall response rate, and the secondary end points included safety, progression-free survival (PFS), and overall survival (OS).

Results
A total of 38 patients were enrolled, and 37 patients received mogamulizumab. Objective responses were noted for 13 of 37 patients (35%; 95% CI, 20% to 53%), including five patients (14%) with complete response. The median PFS was 3.0 months (95% CI, 1.6 to 4.9 months), and the median OS was not calculated. The mean maximum and trough mogamulizumab concentrations (± standard deviation) after the eighth infusion were 45.9 ± 9.3 and 29.0 ± 13.3 μg/mL, respectively. The most common adverse events were hematologic events, pyrexia, and skin disorders, all of which were reversible and manageable.

Conclusion
Mogamulizumab exhibited clinically meaningful antitumor activity in patients with relapsed PTCL and CTCL, with an acceptable toxicity profile. Further investigation of mogamulizumab for treatment of T-cell lymphoma is warranted.

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INTRODUCTION

Mature T/natural killer (NK)-cell neoplasms comprise approximately 20 subclassified heterogeneous groups of non-Hodgkin lymphomas (NHLs) that account for approximately 10% of NHLs in Western countries[1-3] and approximately 25% of NHLs in Japan.[4,5] Mature T/NK-cell neoplasms are largely subdivided into peripheral T-cell lymphoma (PTCL) and cutaneous T-cell lymphoma (CTCL), and different treatment strategies are used for each of these entities.[1,6]

According to the WHO classification, PTCL includes peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), angioimmunoblastic T-cell lymphoma (AITL), and anaplastic large-cell lymphoma (ALCL).[1-3] Cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) and CHOP-like regimens have been widely used as the standard first-line treatment for patients with PTCL.[7,8] With the exception of those patients with anaplastic lymphoma kinase-positive ALCL, the efficacy of these combination therapies is unsatisfactory because those who achieve remission eventually experience relapse and poor outcomes.[3,9] Several agents have been approved by the US Food and Drug Administration for the treatment of relapsed or refractory (Rel/Ref) PTCL: pralatrexate, romidepsin for Rel/Ref PTCL, and brentuximab vedotin for Rel/Ref ALCL. The overall response rates

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(ORRs) were reported to be 29% and 25% for PTCL and 86% for ALCL, respectively.[10-12]

CTCL can be classified as mycosis fungoides (MF), Sézary syndrome, or cutaneous ALCL. The majority of cases of CTCL in Japan consist of MF.[13] The therapeutic approaches and outcomes for these conditions are primarily dependent on disease stage.[6,7,14] Patients with advanced stage CTCL who relapse after systemic chemotherapies and those with transformed MF have particularly poor outcomes.[15,16] Recently, the US Food and Drug Administration approved agents for Rel/Ref CTCL treatment, including vorinostat, denileukin diftitox, and romidepsin, with ORRs of 30%, 30%, and 34%, respectively.[17-19] However, there are few treatment options or approved agents for CTCL in Japan, partly because of its low prevalence here.[5,12,13]

CC chemokine receptor 4 (CCR4) is a marker for type 2 helper T cells or regulatory T (Treg) cells and is expressed on tumor cells in approximately 30% to 65% of patients with PTCL.[20,21] CCR4-positive patients (eg, in the PTCL-NOS subgroup) have a shorter survival time when compared with CCR4-negative patients.[21-23] Further, CCR4 expression increases with advancing disease stage in patients with MF/Sézary syndrome.[24]

Mogamulizumab (KW-0761) is a humanized anti-CCR4 monoclonal antibody with a defucosylated Fc region that enhances antibody-dependent cellular cytotoxicity.[25,26] In vitro antibody-dependent cellular cytotoxicity assay and in vivo studies in a humanized mouse model revealed that mogamulizumab exhibited potent antitumor activity against T-cell lymphoma cell lines and against primary CTCL cells from patients.[26-28]

In a phase I study of patients with relapsed adult T-cell leukemia-lymphoma (ATL) and PTCL/CTCL, mogamulizumab was well tolerated up to a dose of 1.0 mg/kg. An ORR of 31% (five of 16) was obtained, including one partial response (PR) among three patients with PTCL/CTCL.[29] Mogamulizumab yielded an ORR of 50% (13 of 26) for relapsed CCR4-positive ATL in a subsequent phase II study.[30] In the United States, a phase I/II study for patients with Rel/Ref CTCL revealed that mogamulizumab was well tolerated with an ORR of 37% (14 of 38, 8% complete response [CR], 29% PR) and a median PFS of 341 days.[31]

The present report describes the results of a multicenter phase II study in Japan that was designed to assess the efficacy and safety of mogamulizumab in patients with relapsed CCR4-positive PTCL or CTCL.

PATIENTS AND METHODS

Study Design and Treatment

This was a multicenter, single-arm phase II study conducted at 15 Japanese centers. At least 35 patients were required to detect a lower limit of the 95% CI that exceeded the 5% threshold, and the expected ORR for mogamulizumab was 25% with a statistical power of 90%.[10,29]

All patients gave written informed consent before enrollment. Patients received intravenous infusions of 1.0 mg/kg mogamulizumab once per week for 8 weeks. Dose modification of mogamulizumab was not allowed. Oral antihistamine and acetaminophen were given before each dose of mogamulizumab as premedication.[29,30] A systemic corticosteroid (hydrocortisone 100 mg intravenously) was also administered before the first dose of mogamulizumab to prevent an infusion reaction. The same dose of hydrocortisone was administered before the second and subsequent administrations at the investigators' discretion. The plasma concentrations of mogamulizumab and antimogamulizumab antibodies in plasma were determined by using enzyme-linked immunosorbent assays.[29,30] Blood samples were collected from all patients who received at least one dose of mogamulizumab at times determined by the protocol for pharmacokinetic analyses. Maximum plasma mogamulizumab concentration and trough concentration parameters were calculated from 0 to 7 days after the eight doses. T-cell subsets and NK cell distribution were also investigated by flow cytometry during and after mogamulizumab treatment. This study was conducted in accordance with the Declaration of Helsinki and in compliance with Good Clinical Practices. The protocol was approved by the institutional review board at each participating institution.

Patients

Patients who were ≥ 20 years of age and who had CCR4-positive PTCL or CTCL with relapse after their last systemic chemotherapy were eligible for participation. Patients who were refractory to their most recent therapy were not eligible for this study. Histopathological subtypes were assessed and reclassified by the Independent Pathology Review Committee according to the 2008 WHO classification.[1] CCR4 expression was determined by immunohistochemistry by using an anti-CCR4 monoclonal antibody (KM2160) and was confirmed by central review, as described previously.[29] In brief, CCR4 expression was classified according to the proportion of stained tumor cells (negative, < 10%; 1+, 10% to <25%; 2+, 25% to < 50%; 3+, ≥ 50%). Staging of nodal/extranodal and/or cutaneous lesions was performed if the lesions met the following requirements: nodal and extranodal lesions were > 1.5 cm in measurable length on cross-sectional computed tomography images, cutaneous lesions were identifiable on visual inspection, and peripheral blood abnormal lymphocyte count was ≥ 1,000/μL and comprised ≥ 5% of total leukocytes. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Other notable eligibility criteria regarding laboratory values were as follows: neutrophil count ≥ 1,500/μL, platelet count ≥ 50,000/μL, hemoglobin level ≥ 8.0 g/dL, AST level ≤ 2.5× the upper limit of normal (ULN), ALT level ≤ 2.5× the ULN, total bilirubin level ≤ 1.5× the ULN, and serum creatinine level ≤ 1.5× the ULN. Patients were excluded if they had any severe complications, such as CNS involvement or a bulky lymphoma mass requiring emergent radiotherapy, a history of allogeneic stem-cell transplantation, active concurrent cancers, an active infection, or positivity for hepatitis B virus DNA, hepatitis B surface antigen, hepatitis C virus antibody, or human immunodeficiency virus antibody.

Efficacy and Safety Assessment

The primary objective was to assess the best overall response, and the secondary objectives included assessments of the best response according to disease site, progression-free survival (PFS), and overall survival (OS). Efficacy was evaluated by the Independent Efficacy Assessment Committee according to modified response criteria based on the International Working Group Criteria.[32,33] Cutaneous lesions were evaluated by using the modified Severity Weighted Assessment Tool.[34] In addition, treatment efficacy in patients with CTCL was evaluated by using a Global Response Score.[35] Responses were assessed after the fourth and eighth mogamulizumab infusions and at 2 and 4 months after the end of treatment. Treatment was discontinued if progressive disease (PD) was evident. PD and survival were monitored until at least 4 months after the completion of dosing. For safety evaluations, adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for AEs, version 4.0.

Statistical Analysis

PFS and OS were analyzed by using the Kaplan-Meier method. PFS was defined as the time from the first dose of mogamulizumab to progression, relapse, or death by any cause (whichever occurred first). OS was measured from the day of the first dose to death by any cause.

RESULTS

Patient Characteristics

Sixty-five patients were screened, and 64 biopsy specimens were histologically confirmed as PTCL or CTCL by the Independent Pathology Review Committee. In total, 50 (78%) of the 64 screened

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patients were CCR4-positive. Of these, 38 eligible patients were enrolled in the study and 37 received at least one infusion of mogamulizumab. One patient withdrew because of an infectious complication before dosing. Patient characteristics, histopathology subtypes, and previous systemic therapies are shown in Table 1.

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic*	Patients (N = 37) No.	%	Patients With PTCL (n = 29) No.	%	Patients With CTCL (n = 8) No.	%
Age, years						
Median	64		67		50	
Range	33-80		33-80		36-70	
≥ 65	18	49	17	59	1	13
Sex						
Male	23	62	20	69	3	38
Female	14	38	9	31	5	63
ECOG performance status						
0	24	65	19	66	5	63
1	12	32	10	34	2	25
2	1	3	0	0	1	13
Elevated LDH level†	21	57	18	62	3	38
Bone marrow involvement	7	19	7	24	0	0
No. of previous systemic regimens						
Median	2		2		3	
Range	1-6		1-5		1-6	
1	14	38	13	45	1	13
2	15	41	12	41	3	38
≥ 3	8	22	4	14	4	50
Types of systemic therapy						
Chemotherapy	37	100	29	100	8	100
CHOP/CHOP-like regimen	36	97	29	100	7	88
DeVIC	6	16	4	14	2	25
CHASE	5	14	5	17	0	0
Single-agent therapy	5	14	0	0	5	63
Other	10	27	10	34	0	0
Auto-PBSCT	3	8	3	10	0	0
Radiotherapy	9	24	5	17	4	50
Intensity of CCR4 expression‡						
1+	6	16	4	14	2	25
2+	6	16	4	14	2	25
3+	25	68	21	72	4	50
Histopathology by central review						
PTCL-NOS	16	43	16	55		
AITL	12	32	12	41		
ALCL, ALK negative	1	3	1	4		
MF	7	19			7	88
c-ALCL	1	3			1	13

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; ALK, anaplastic lymphoma kinase; c-ALCL, cutaneous anaplastic large-cell lymphoma; CHASE, cyclophosphamide, cytosine arabinoside, etoposide, and dexamethasone; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CTCL, cutaneous T-cell lymphoma; DeVIC, dexamethasone, etoposide, ifosfamide, and carboplatin; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; MF, mycosis fungoides; NOS, not otherwise specified; PBSCT, peripheral-blood stem-cell transplantation; PTCL, peripheral T-cell lymphoma.

*Of the 38 patients enrolled, 37 received at least one infusion of mogamulizumab.

†Elevated LDH level: higher LDH level than upper limit of the normal range.

‡The denominator used for the intensity of CC chemokine receptor 4 (CCR4) expression is based on subjects who were positive for CCR4 by immunohistochemistry.

Of the 37 patients who received mogamulizumab, 25 (68%) completed the planned course of eight infusions. Nine patients (24%) discontinued treatment because of PD, and three patients (8%) due to serious AEs.

Efficacy

The ORR for the 37 treated patients was 35% (13 of 37; 95% CI, 20% to 53%), and 14% of patients (five of 37) achieved a CR, of which one was unconfirmed (Table 2). Reponses (CR/PR) were observed in at least one patient with each subtype of disease, but the ORR differed between subtypes. The ORR was 34% (10 of 29; 95% CI, 18% to 54%) in patients with PTCL (three of 16 for PTCL-NOS, six of 12 for AITL, and one of one for ALCL, anaplastic lymphoma kinase-negative) and 38% (three of eight; 95% CI, 9% to 76%) in those with CTCL (two of seven for MF and one of one for cutaneous ALCL). In addition, ORR in patients with CTCL was 50% (four of eight; 95% CI, 16% to 84%) according to the Global Response Score.

Total ORR did not significantly correlate with CCR4 expression level, patient age, or the number of previous chemotherapy regimens. The response rates for lymph node and cutaneous lesions were 33% (11 of 33) and 58% (seven of 12), respectively.

The median PFS was 3.0 months (95% CI, 1.6 to 4.9 months) for the entire population and 2.0 months for patients with PTCL. Although the median OS was not reached for the entire population at the

Table 2. Best Response (N = 37)

Parameter	No. of Patients	No. of Patients With Best Response CR/CRu	PR	SD	PD	Response Rate (%)*
Overall response	37	5	8	13	11	35
Histopathology by central review						
PTCL	29	5†	5	9	10	34
PTCL-NOS	16	1	2	6	7	19
AITL	12	3	3	3	3	50
ALCL, ALK negative	1	1†	0	0	0	100
CTCL	8	0	3	4	1	38
MF	7	0	2	4	1	29
c-ALCL	1	0	1	0	0	100
Age, years						
< 65	19	1†	6	7	5	37
≥ 65	18	4	2	6	6	33
Intensity of CCR4 expression						
1+	6	1	1	3	1	33
2+	6	1	2	2	1	50
3+	25	3†	5	8	9	32
No. of previous systemic regimens						
1	14	3	3	6	2	43
2	15	1	1	6	7	13
≥ 3	8	1†	4	1	2	63

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; ALK, anaplastic lymphoma kinase; c-ALCL, cutaneous anaplastic large-cell lymphoma; CCR4, CC chemokine receptor 4; CR, complete response/complete remission; CRu, uncertain complete response/uncertain complete remission; CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; NOS, not otherwise specified; PD, progressive disease; PR, partial response/partial remission; PTCL, peripheral T-cell lymphoma; SD, stable disease.

*Response rate (%): 100 × number of responders/number of subjects in each category included in the efficacy analysis set.

†Among the patients who showed CR/CRu, one showed CRu.

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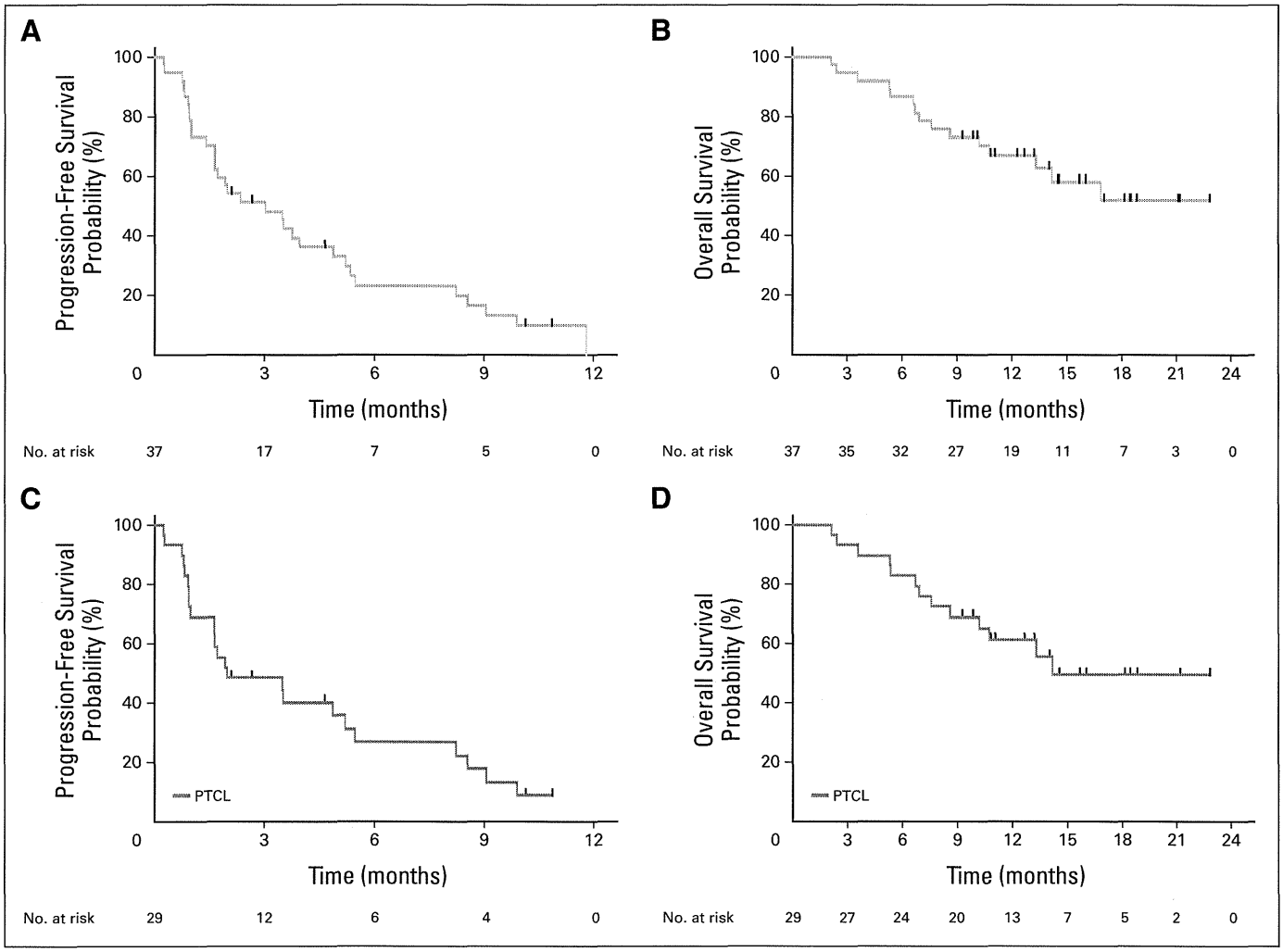

Fig 1. Kaplan-Meier curves of (A) estimated progression-free survival (median, 3.0 months), (B) overall survival (median not reached), (C) progression-free survival in patients with peripheral T-cell lymphoma (PTCL; median, 2.0 months), and (D) overall survival in patients with PTCL (median, 14.2 months).

time of this report, it was 14.2 months for patients with PTCL (Fig 1). Moreover, the median PFS of all 13 responders was 5.5 months, and for PTCL responders (n = 10), it was 8.2 months.

Safety

The most common treatment-related AEs of all grades and treatment-related AEs of grade 3/4 were lymphocytopenia (81%, 73%), neutropenia (38%, 19%), and leukocytopenia (43%, 14%), whereas the most common nonhematologic AE was pyrexia (30%; grade 2 or lower) (Table 3). Lymphocytopenia occurred in 30 patients (81%) and was noted after the first dose in 26 of these patients. For 19 of the patients, lymphocyte counts were $< 800/\mu L$ (grades 2 to 4) before the first dosing. The lymphocyte count ultimately recovered to normal or baseline levels in all patients.

Infusion reaction (24%; grade 2 or lower) occurred primarily at the first infusion, after which it became less frequent, and all patients recovered. No infusion prolongation/interruption was caused by the infusion reaction.

In addition, treatment-related skin disorders were commonly reported (all grades, 51%; grade 3/4, 11%) when grouped according to system organ class. Of the 19 patients who suffered from skin disorder complications, 15 patients experienced improvement, whereas the remaining patients discontinued treatment because of PD or switched to other post treatments. One patient who had a history of psoriasis before the study treatment developed two serious skin disorders (toxicoderma and psoriasis vulgaris) during the study period.

Fifteen serious treatment-related AEs were observed among eight patients (22%); these AEs included grade 3 polymyositis in one patient, grade 2 cytomegalovirus retinitis in two patients, and grade 4 second primary malignancy in one patient with AITL. All patients improved over time, and there were no deaths related to AEs.

Pharmacokinetics and Pharmacodynamics

The mean maximum mogamulizumab concentration and trough mogamulizumab concentration (± standard deviation) in plasma after the eighth infusion were 45.9 ± 9.3 and 29.0 ± 13.3 μg/mL, respectively. Antimogamulizumab antibodies were not detected after dosing in any patients. These results were consistent with the findings of a previous study of patients with ATL.[30] As an exploratory study, we assessed the effect of mogamulizumab on the number of CD4+/CD25+/Foxp3+ cells (the Treg cell subset) and CD45+/CD16+/CD56+ cells (the NK cell subset). Patients given

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Table 3. Treatment-Related Adverse Events (N = 37)

Adverse Event*	All Grades		Grade ≥ 3	
	No.	%	No.	%
Hematologic				
Lymphocytopenia	30	81	27	73
Leukocytopenia	16	43	5	14
Thrombocytopenia	14	38	1	3
Neutropenia	14	38	7	19
Anemia	5	14	2	5
Febrile neutropenia	1	3	1	3
Nonhematologic				
Pyrexia	11	30	0	0
Infusion reaction	9	24	0	0
ALT increased	8	22	1	3
ALP increased	8	22	1	3
Hypophosphatemia	6	16	1	3
Hypokalemia	2	5	1	3
Infection	1	3	1	3
Oral candidiasis	1	3	1	3
Pneumonia	1	3	1	3
Herpes esophagitis	1	3	1	3
Polymyositis	1	3	1	3
Second primary malignancy†	1	3	1	3
Skin and subcutaneous tissue disorders (SOC)	19	51	4	11
Rash papular	6	16	1	3
Rash erythematous	5	14	1	3
Psoriasis	2	5	1	3
Rash maculopapular	2	5	1	3
Toxic skin eruption	2	5	1	3

Abbreviations: ALP, alkaline phosphatase; SOC, System Organ Class (according to the Medical Dictionary for Regulatory Activities).
*Treatment-related adverse events that were reported in at least 15% of patients or that were of grade 3-4 severity.
†Diffuse large B-cell lymphoma was reported in one patient with angioimmunoblastic T-cell lymphoma.

mogamulizumab exhibited a profound depletion of the Treg cell subset during treatment, and cell levels had not returned to baseline 4 months after the last dose (Fig A1). Mogamulizumab also caused a modest decrease in the NK cell subset during treatment (data not shown).

DISCUSSION

This report described results from a single-arm, open-label multicenter phase II study of mogamulizumab in patients with relapsed CCR4-positive PTCL and CTCL.

Mogamulizumab showed promising antitumor activity, with an ORR of 35% (95% CI, 20% to 53%) and a CR/unconfirmed CR of 14%. These data were consistent with those reported with relapsed ATL.[30] It is notable that all three patients who relapsed after autoperipheral blood stem-cell transplantation responded to mogamulizumab. The total ORR is comparable to that of other US Food and Drug Administration-approved drugs, such as pralatrexate and romidepsin.[10,11] However, the present study differed from previous studies in several important respects. Firstly, the patient population was smaller than in the pralatrexate or romidepsin studies. Secondly, since it has been reported that CCR4 expression correlated with advanced disease,[24] it is important to note that although these two studies enrolled relapsed and refractory patients irrespective of their CCR4 expression status, the present study only recruited relapsed patients who were CCR4-positive. However, almost all patients in the present study had good PS compared with those patients in the previous studies. Thirdly, all patients with MF (n = 7) in the present study had relapsed after systemic chemotherapies and were presumed to have advanced stage disease, because all of these patients exhibited clinical skin tumors. Further, four of these seven patients exhibited clinically abnormal lymph node swelling, which does not usually occur at stages lower than IIB.[14,15]

In future study, PFS may also be improved by a longer continuous dosing schedule, such as a phase I/II study for CTCL.[31]

Although the number of patients was relatively small in the present study, the ORR for the AITL group (50%; six of 12) seemed noteworthy, while appearing relatively low in patients with PTCL-NOS (19%; three of 16). However, the three patients with PTCL-NOS who responded to mogamulizumab achieved durable PFS (9.0, 10.1+, and 10.8+ months; +, censored). Further studies are needed to identify which CCR4-positive T-cell lymphoma patients are most likely to benefit from mogamulizumab therapy.

There was no definite correlation between ORR and patient characteristics, such as age, CCR4 expression level, or number of previous systemic regimens. Although our study only included CCR4-positive patients with PTCL and CTCL, a recent US phase I/II study of mogamulizumab included both CCR4-positive and CCR4-negative patients with CTCL.[31] In that study, mogamulizumab exhibited efficacy irrespective of CCR4 expression (positive or negative) or CCR4 expression level, with a continuous dosing schedule.[31] Further studies are needed to define if CCR4 positivity represents a useful predictive biomarker in either PTCL or CTCL.

CCR4-positivity was confirmed in 78% of the 64 screened patients, a higher rate than previously reported.[20,21] However, it is possible that this variation in CCR4 positivity was due to differences in immunohistochemistry assay sensitivity. In our ongoing CTCL phase III study, our protocol permitted recruitment of both CCR4 positive and negative CTCL patients (NCT01728805). This is because the detection limit of CCR4 positivity may not be yet fully established, and mogamulizumab might have antitumor activity against CCR4-negative tumors through the depletion of CCR4-positive regulatory T cells,[36] thus enhancing pre-existing CD8+ cytolytic T-lymphocytes. Based on the latter new concept, an investigator-initiated trial of mogamulizumab against CCR4-negative solid tumors has been initiated (UMIN000010050).

Most of the AEs associated with mogamulizumab were mild and reversible. One patient suffered from polymyositis, an immune-related serious AE, after seven doses of mogamulizumab. The patient improved after steroid pulse therapy, treatment with tacrolimus hydrate, and continuous rehabilitation. Although drug-induced myositis was a possible cause, the relationship between mogamulizumab and myositis was not determined, even after detailed investigation. In our study, skin rash could also represent an immune-related AE, as other immunotherapies, including ipilimumab and zanolimumab, cause similar skin toxicity.[18,36-38] In addition, this may relate to the antitumor mechanism of mogamulizumab, because CCR4 contributes to skin-specific lymphocyte homing.[39] Indeed, a previous study revealed that patients who developed skin disorders ultimately had better therapeutic responses to treatment.[30] In the present study, of the

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13 patients who developed grade 2 to 3 skin disorders, five patients achieved CR/PR. Of the 24 patients who developed grade 1 or no skin disorders, eight patients achieved CR/PR. Hence, no clear correlation between skin disorders and response rate was observed in the present study.

As shown in Figure A1, mogamulizumab caused a significant and persistent reduction in the number of Treg cells. This may be responsible for the increased incidence of skin disorders seen in patients with ATL.[30,40] Skin disorders were observed in 19 patients (51%), with grade 3/4 in four cases (11%). This was lower than the proportion of patients who developed skin disorders (67%, 22% in grade 3/4) in a previous study.[30] One patient (4%) with ATL developed Stevens-Johnson syndrome (SJS)[30] and four patients with ATL developed SJS/toxic epidermal necrolysis in postmarketing surveillance of mogamulizumab[40]; however, no cases of SJS/toxic epidermal necrolysis were observed in the present study. Similarly, four of 21 patients with ATL (19%) developed symptoms consistent with SJS[41] after treatment with pralatrexate, whereas no SJS was observed in patients with PTCL[10] after pralatrexate treatment. The risk of severe skin disorders may therefore be lower in patients with PTCL, compared with patients with ATL.

In conclusion, this phase II study revealed that mogamulizumab had promising efficacy and tolerability in patients with relapsed CCR4-positive PTCL and CTCL. Given its novel mechanism of action and favorable toxicity profile compared with multiagent cytotoxic chemotherapy, we might expect the use of mogamulizumab in combination with other agents. Further preclinical and clinical studies of combination therapy will be needed.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The following review committees and medical experts participated in this trial. Takashi Terauchi, Research Center for Cancer Prevention and Screening National Cancer Center; Ukihide Tateishi, Yokohama City University Graduate School of Medicine; Junichi Tsukada, University of Occupational and Environmental Health; Koichi Nakata, University of Occupational and Environmental Health; Shigeo Nakamura, Nagoya University Graduate School of Medicine; Koichi Ohshima, Kurume University School of Medicine; Tetsuo Nagatani, Hachioji Medical Center of Tokyo Medical University; Akimichi Morita, Nagoya City University Graduate School of Medical Sciences; Kuniaki Ito, National Cancer Center Hospital East; Noriko Usui, Jikei University School of Medicine; Hirokazu Nagai, Clinical Research Center National Hospital Organization Nagoya Medical Center.

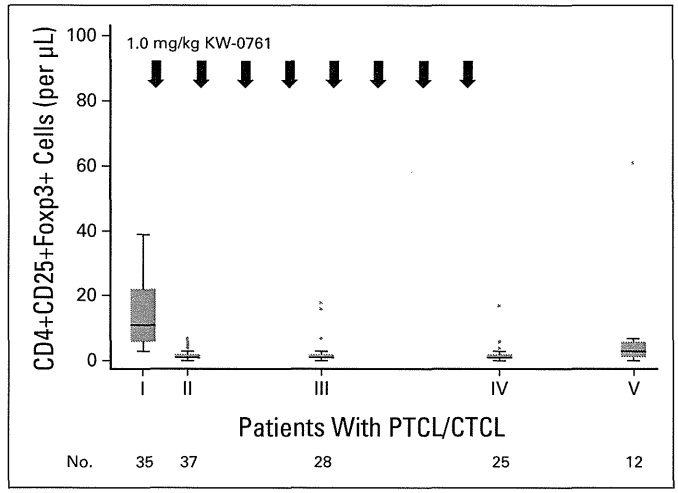

Fig A1. T-cell subset analysis. Numbers of CD4+CD25+Foxp3+ (regulatory T) cells are presented. Blood samples collected at times indicated in the protocol were analyzed. Blood samples were taken (I) just before the first mogamulizumab infusion, (II) just before the second infusion, (III) just before the fifth infusion, (IV) 1 week after the eighth infusion, and (V) 4 months after the eighth infusion. The number of samples used for analysis at each point is indicated below the graph. CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

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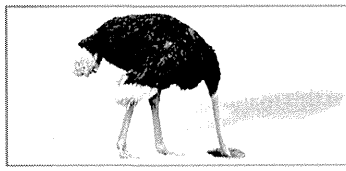
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HTLV-1 bZIP Factor–Specific CD4 T Cell Responses in Adult T Cell Leukemia/Lymphoma Patients after Allogeneic Hematopoietic Stem Cell Transplantation

Tomoko Narita,* Takashi Ishida,* Ayako Masaki,* Susumu Suzuki,*,† Asahi Ito,* Fumiko Mori,* Tomiko Yamada,* Masaki Ri,* Shigeru Kusumoto,* Hirokazu Komatsu,* Yasuhiko Miyazaki,‡ Yoshifusa Takatsuka,§ Atae Utsunomiya,§ Akio Niimi,* Shinsuke Iida,* and Ryuzo Ueda†

We document human T lymphotropic virus type 1 (HTLV-1) bZIP factor (HBZ)-specific CD4 T cell responses in an adult T cell leukemia/lymphoma (ATL) patient after allogeneic hematopoietic stem cell transplantation (HCT) and identified a novel HLA-DRB1*15:01–restricted HBZ-derived naturally presented minimum epitope sequence, RRRAEKKAADVA (HBZ114–125). This peptide was also presented on HLA-DRB1*15:02, recognized by CD4 T cells. Notably, HBZ-specific CD4 T cell responses were only observed in ATL patients after allogeneic HCT (4 of 9 patients) and not in nontransplanted ATL patients (0 of 10 patients) or in asymptomatic HTLV-1 carriers (0 of 10 carriers). In addition, in one acute-type patient, HBZ-specific CD4 T cell responses were absent in complete remission before HCT, but they became detectable after allogeneic HCT. We surmise that HTLV-1 transmission from mothers to infants through breast milk in early life induces tolerance to HBZ and results in insufficient HBZ-specific T cell responses in HTLV-1 asymptomatic carriers or ATL patients. In contrast, after allogeneic HCT, the reconstituted immune system from donor-derived cells can recognize virus protein HBZ as foreign, and HBZ-specific immune responses are provoked that contribute to the graft-versus-HTLV-1 effect. *The Journal of Immunology*, **2014, 192: 940–947.**

Adult T cell leukemia/lymphoma (ATL) is a distinct hematologic malignancy caused by human T lymphotropic virus type 1 (HTLV-1) (1, 2). ATL is resistant to conventional chemotherapeutic agents, and only limited treatment options are available (3). Although early efforts using myeloablative chemoradiotherapy together with autologous hematopoietic stem cell rescue for ATL were associated with a high incidence of relapse and fatal toxicities (4), allogeneic hematopoietic stem cell transplantation (HCT) has been explored as a promising alternative treatment, achieving long-term remission in a proportion of patients with ATL (5, 6). The potential benefit of allogeneic HCT for ATL patients is considered to be due to the high immunogenicity of HTLV-1–infected cells (7–12), which was associated with the existence of posttransplant graft-versus-HTLV-1 and/or graft-versus-ATL effects (13, 14).

HTLV-1 was the first retrovirus to be directly associated with a human malignancy (15, 16), and ~20 million people worldwide are estimated to be infected with this virus (17). Among the HTLV-1 regulatory and accessory genes, *Tax* transforms rodent cells and immortalizes human primary T cells (18–20). In addition, Tax-transgenic mice develop spontaneous tumors (21–24). Another HTLV-1 component gene, *HBZ*, promotes the proliferation of ATL cells (25). Transgenic mice expressing HTLV-1 bZIP factor (HBZ) in their CD4 T cells share many symptoms and immunological features with HTLV-1–infected humans (26). Thus, both *Tax* and *HBZ* are thought to play critical roles in ATL oncogenesis, but there is a marked contrast between them in their expression profiles in primary ATL cells: HBZ expression is constitutive whereas Tax expression is frequently suppressed or minimal in ATL cells (25, 27, 28). Because immune responses against Tax were reported to be strong (7, 8), impaired Tax expression is thought to lead to a survival advantage for HTLV-1–infected cells in the host (2). These observations raise a simple question as to why the expression of Tax, but not HBZ, is impaired, despite both being HTLV-1–derived Ags seen by the human immune system as foreign. In other words, why is it that only HBZ, but not Tax, is constitutively expressed in ATL cells, although it was reported that HBZ is an immunogenic protein recognized by HBZ-specific CTL clones (29, 30). Although several studies (29–31) have been performed to determine the immunogenicity of HBZ, the precise immunological significance of HBZ in HTLV-1–infected individuals has not been fully established. Therefore, the aim of the current study was to clarify the clinical role of HBZ-specific immune responses in HTLV-1–infected individuals.

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Abbreviations used in this article: AC, asymptomatic carrier; ATL, adult T cell leukemia/lymphoma; CR, complete remission; HAM, human T lymphotropic virus type 1–associated myelopathy; HBZ, human T lymphotropic virus type 1 bZIP factor; HCT, hematopoietic stem cell transplantation; HTLV-1, human T lymphotropic virus type 1.

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