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.

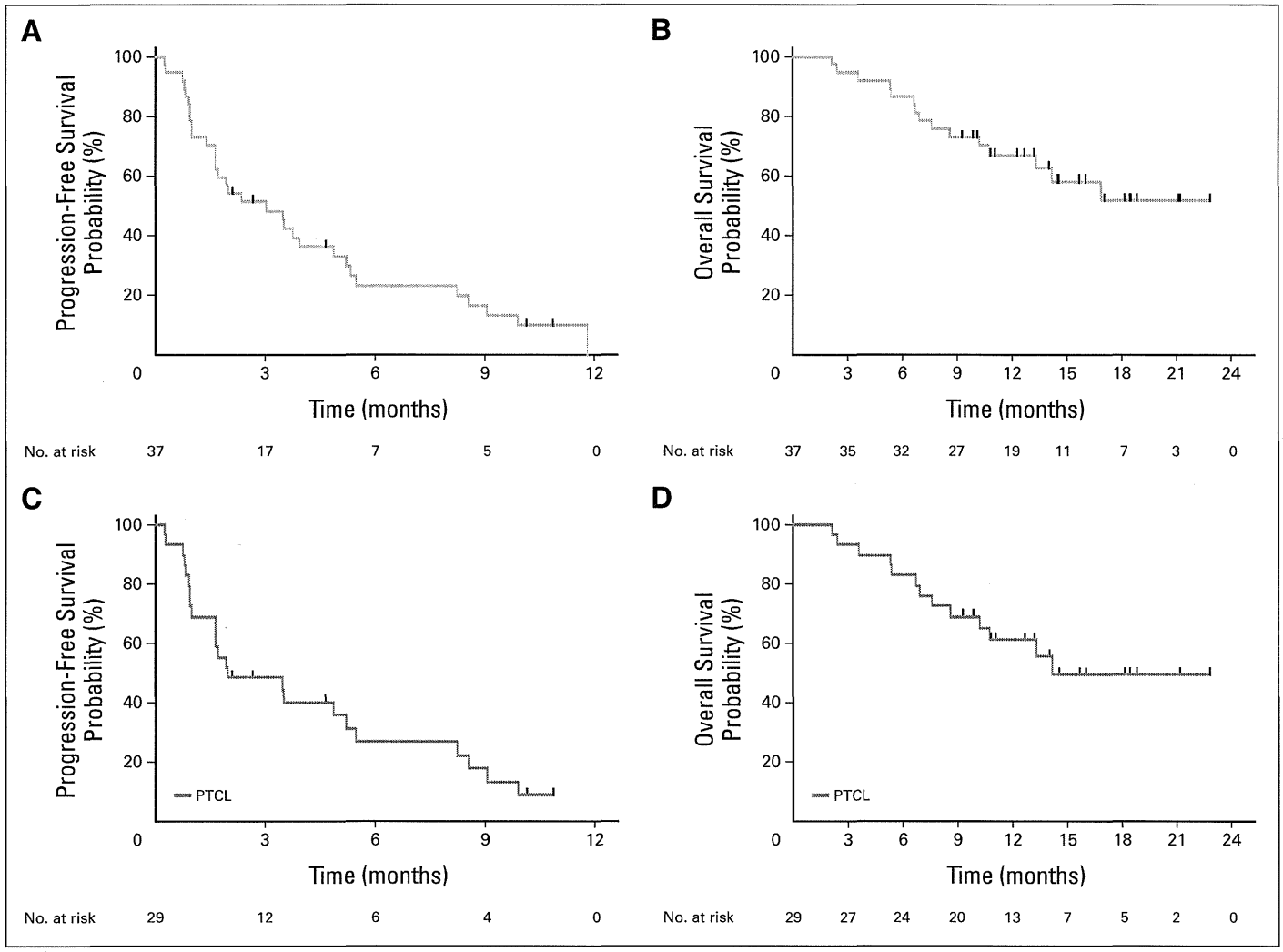

Fig 1. Kaplan-Meier curves of (A) estimated progression-free survival (median, 3.0 months), (B) overall survival (median not reached), (C) progression-free survival in patients with peripheral T-cell lymphoma (PTCL; median, 2.0 months), and (D) overall survival in patients with PTCL (median, 14.2 months).

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 ($\pm$ standard deviation) in plasma after the eighth infusion were 45.9 $\pm$ 9.3 and 29.0 $\pm$ 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

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

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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AUTHOR CONTRIBUTIONS

Conception and design: Michinori Ogura, Takashi Ishida, Kiyohiko Hatake, Kensei Tobinai, Kunihiro Tsukasaki, Hiroshi Inagaki, Shiro Akinaga, Ryuzo Ueda
Financial support: Shiro Akinaga
Provision of study materials or patients: Michinori Ogura, Takashi Ishida, Kiyohiko Hatake, Masafumi Taniwaki, Kiyoshi Ando, Kensei Tobinai, Katsuya Fujimoto, Kazuhito Yamamoto, Toshihiro Miyamoto, Naokuni Uike, Mitsune Tanimoto, Kunihiro Tsukasaki, Kenichi Ishizawa
Collection and assembly of data: Michinori Ogura, Takashi Ishida, Kiyohiko Hatake, Masafumi Taniwaki, Kiyoshi Ando, Kensei Tobinai, Katsuya Fujimoto, Kazuhito Yamamoto, Toshihiro Miyamoto, Naokuni Uike, Mitsune Tanimoto, Kunihiro Tsukasaki, Kenichi Ishizawa
Data analysis and interpretation: Michinori Ogura, Takashi Ishida, Kensei Tobinai, Junji Suzumiya, Hiroshi Inagaki, Kazuo Tamura, Masao Tomonaga, Ryuzo Ueda
Manuscript writing: All authors
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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.

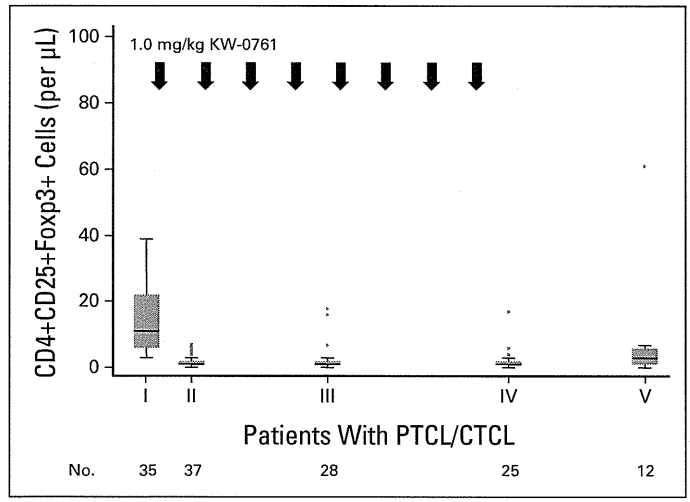

Fig A1. T-cell subset analysis. Numbers of CD4+CD25+Foxp3+ (regulatory T) cells are presented. Blood samples collected at times indicated in the protocol were analyzed. Blood samples were taken (I) just before the first mogamulizumab infusion, (II) just before the second infusion, (III) just before the 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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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.

*Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan; †Department of Tumor Immunology, Aichi Medical University School of Medicine, Aichi 480-1195, Japan; ‡Department of Hematology, Oita Prefectural Hospital, Oita 870-8511, Japan; and §Department of Hematology, Imamura Bun-in Hospital, Kagoshima 890-0064, Japan

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Address correspondence and reprint requests to Dr. Takashi Ishida, Department of Medical Oncology and Immunology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-chou, Mizuho-ku, Nagoya, Aichi 467-8601, Japan. E-mail address: itakashi@med.nagoya-cu.ac.jp

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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Materials and Methods

Primary human cells

Blood samples were obtained from healthy volunteers, HTLV-1 asymptomatic carriers (ACs), and ATL patients. Mononuclear cells were isolated with Ficoll-Paque (Pharmacia, Peapack, NJ). Genotyping of HLA-DR, HLA-DQ, and HLA-DP was performed using an WAKFlow HLA-typing kit (WAKUNAGA Pharmacy, Hiroshima, Japan). Diagnosis and classification of clinical subtypes of ATL were according to the criteria proposed by the Japan Lymphoma Study Group (32). All donors provided informed written consent before sampling, according to the Declaration of Helsinki, and the current study was approved by the institutional ethics committees of Nagoya City University Graduate School of Medical Sciences.

Cell lines

ATN-1, MT-1, TL-Om1, and ATL102 are ATL cell lines; MT-2, MT-4, and TL-Su are HTLV-1–immortalized lines; and K562 is a chronic myelogenous leukemia blast crisis cell line (8, 33). Genotyping of HLA-DR, HLA-DQ, and HAL-DP was performed using a WAKFlow HLA-typing kit.

Expansion of HBZ-specific T cells

PBMCs from ATL patients or HTLV-1 ACs were suspended in RPMI 1640 (Cell Science and Technology Institute, Sendai, Japan) supplemented with 10% human serum and 10 μM synthetic HBZ-derived peptides at a cell concentration of 2×10^6/ml. The peptides were purchased from Invitrogen (Carlsbad, CA). The cell suspension (2×10^6 cells) was cultured at 37°C in 5% CO_2 for 2 d, and an equal volume of RPMI 1640 supplemented with 100 IU/ml IL-2 was added. After subsequent culture for 5 d, an equal volume of ALyS505N (Cell Science and Technology Institute) supplemented with 100 IU/ml IL-2 was added, and the cells were cultured with appropriate medium (ALyS505N with 100 IU/ml IL-2) for an additional 7 d.

Abs and flow cytometry

PerCP-conjugated anti-CD8 mAb (SK1; eBioscience, San Diego, CA) and PE-conjugated anti-CD4 mAb [SFCI12T4D11 (T4); Beckman Coulter, Fullerton, CA] were used. For assessing HLA class II expression, PE-conjugated anti–HLA-DR (G46-6; BD Biosciences, San Jose, CA), anti–HLA-DQ (HLA-DQ1; BioLegend, San Diego, CA), or appropriate isotype-control mAbs were used. For intracellular IFN-γ and TNF-α staining, the expanded cells were cocultured with or without target cells or synthetic peptides at 37°C in 5% CO_2 for 3 h, after which brefeldin A (BD Biosciences) was added at 2 μg/ml. The cells were then incubated for an additional 2 h. Subsequently, they were fixed in 10% formaldehyde and stained with FITC-conjugated anti–IFN-γ (45.15; Beckman Coulter) or allophycocyanin-conjugated anti–TNF-α (MAb11; eBioscience) mAbs with 0.25% saponin for 60 min at room temperature. To determine HLA restriction, HLA-blocking experiments were conducted. The expanded cells were preincubated with 20 μg/ml anti–HLA-DR (L243; BioLegend), 20 μg/ml anti–HLA-DQ (1SPVL3; Beckman Coulter), or appropriate isotype control mAbs (20 μg/ml) at 37°C in 5% CO_2 for 1 h, after which they were stimulated with the peptide or the cell lines (ATN-1 and K562). Cells were analyzed on a FACSCalibur (BD Biosciences) with the aid of FlowJo software (Tree Star, Ashland, OR).

Quantitative RT-PCR

Total RNA was isolated with RNeasy Mini Kits (QIAGEN, Tokyo, Japan). Reverse transcription from the RNA to first-strand cDNA was carried out using High Capacity RNA-to-cDNA Kits (Applied Biosystems, Foster City, CA). *HBZ* and *β-actin* mRNA were amplified using TaqMan Gene Expression Assays with the aid of an Applied Biosystems StepOnePlus. The primer set for *HBZ* was as follows: sense, 5′-TCGACCTGAGCTTTAAACTTACCTAGA-3′ and antisense, 5′-GACACAGGCAAGCATCGAAA-3′. All values given are means of triplicate determinations.

Results

T cell responses against synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein

Because it was reported that HTLV-1 Tax-specific T cells were induced in some ATL patients after allogeneic HCT (10, 11), we initially tried to expand HBZ-specific T cells using PBMCs from an ATL patient who received allogeneic HCT with reduced-intensity conditioning and has been in complete remission (CR) for >3 y (patient #1 after HCT). PBMCs were stimulated with a mixture of 1 16-mer and 19 20-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein (peptides number 1–20, Fig. 1), at a concentration of 10 μM each. The expanded cells were analyzed by forward scatter height and side scatter height levels, and the lymphocyte population was determined and plotted to show CD4 and CD8 positivity (Fig. 2A, *left panels*). The expanded CD8 T cells responded weakly to stimulation with these 20 overlapping peptides relative to controls without peptide stimulation, as assessed by IFN-γ production (Fig. 2A, *upper middle panels*) but not TNF-α (Fig. 2A, *lower middle panels*). In contrast, the expanded CD4 T cells responded to stimulation by the 20 overlapping peptides by producing both IFN-γ (Fig. 2A, *upper right panels*) and TNF-α (Fig. 2A, *lower right panels*). Because the response of the stimulated and expanded CD4 T cells was stronger than the CD8 response, we focused on the CD4 T cell response against HBZ in patient #1 after HCT.

PBMCs from this patient (#1 after HCT) were stimulated with a mixture of five overlapping peptides consisting of peptides 1–4, 5–8, 9–12, 13–16, and 17–20 (Fig. 1). The expanded CD4 T cells responded to the peptide mixture 9–12 better than to control (no peptides). They produced both IFN-γ (Fig. 2B, *upper panels*) and TNF-α (Fig. 2B, *lower panels*). The expanded CD4 T cells responded very weakly to the peptide mixtures 13–16 and 17–20 by producing TNF-α but not IFN-γ. No responses were observed against the peptide mixtures 1–4 or 5–8 (Fig. 2B). These data indicate that the epitope of HBZ recognized by CD4 T cells from the patient was present in peptides 9–12, within HBZ aa residues 81–130 (Fig. 1).

Next, PBMCs from the same patient were stimulated with four synthetic peptides: 9, 10, 11, and 12. The expanded CD4 T cells responded to peptide 12 by producing both IFN-γ (Fig. 2C, *upper panels*) and TNF-α (Fig. 2C, *lower panels*). The cells did not respond significantly to the other peptides (9, 10, or 11). These results narrow down the specific epitope of HBZ recognized by the CD4 T cells from the patient to a sequence within peptide 12: HBZ aa 111–130 (Fig. 1).

Determination of the minimum epitope sequence of HBZ recognized by CD4 T cells

Seven synthetic peptides (12-1, 12-2, 12-3, 12-4, 12-5, 12-6, 12-7) representing parts of peptide 12 were prepared (Fig. 3A). Responses of the CD4 T cells, which had been stimulated by peptide 12, to these different peptides were tested. The expanded CD4 T cells responded better to peptides 12, 12-1, 12-2, 12-3, and 12-4

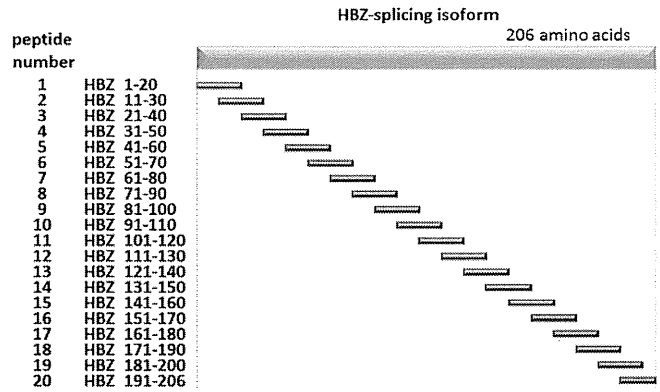

FIGURE 1. Synthetic peptides derived from spliced HBZ. Schematic of 19 20-mer and 1 16-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein.

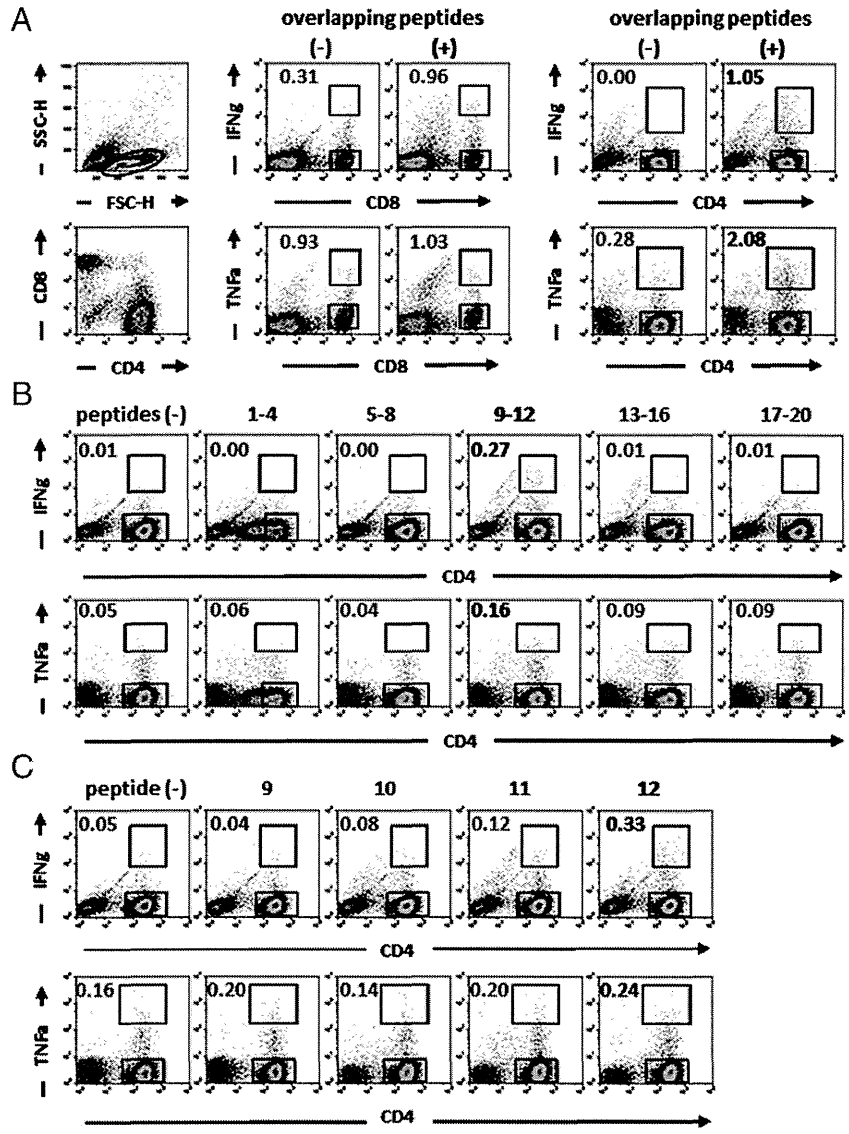

FIGURE 2. T cell responses against synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein. (**A**) PBMCs from patient #1 after HCT were expanded by stimulating with a mixture of 19 20-mer and 1 16-mer synthetic peptides overlapping by 10 aa and covering the entire sequence of the spliced HBZ protein. The responses of expanded CD8 and CD4 T cells to each of the overlapping peptides were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate ($CD8^+$ or $CD4^+$ and IFN-γ^+ or TNF-α^+ cells) relative to the cells in the lower gate ($CD8^+$ or $CD4^+$ and IFN-γ^- or TNF-α^- cells) is indicated in each flow cytometry panel. (**B**) PBMCs from patient #1 after HCT were expanded by stimulating with five overlapping peptide mixtures consisting of peptides 1–4, 5–8, 9–12, 13–16, and 17–20. (**C**) PBMCs from patient #1 after HCT were expanded by stimulating with four synthetic peptides: 9, 10, 11, and 12. The responses of expanded CD4 T cells to each synthetic peptide were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments.

by producing both IFN-γ and TNF-α. These cells did not respond to peptides 12-5, 12-6, or 12-7 (Fig. 3B). These data indicate that the N terminus of the minimum epitope sequence of HBZ recognized by the CD4 T cells from the patient is arginine, located at HBZ114 (Fig. 3A). Because the expanded CD4 T cells responded to peptide 12-4, the C terminus of the minimum epitope sequence of HBZ must be inside of alanine, located at HBZ125.

Next, three synthetic peptides (12-4-1, 12-4-2, 12-4-3; sequences were HBZ114–124, HBZ114–123, and HBZ114–122, respectively) were prepared to determine the C terminus of the minimum epitope sequence of HBZ (Fig. 3C). The expanded CD4 T cells responded to peptides 12-1 and 12-4 (positive controls) but not to 12-4-1, 12-4-2, 12-4-3, or a negative control peptide 12-7 (Fig. 3D). These data demonstrate that the minimum epitope sequence of HBZ recognized by the CD4 T cells from the patient was RRRAEKKAADVA (HBZ114–125).

Determination of the HLA allele on which the identified HBZ-derived peptides are presented to CD4 T cells

We investigated whether HBZ-specific CD4 T cells also recognized naturally processed and presented peptides. Thus, we initially determined *HBZ* expression by ATL or HTLV-1–immortalized cell lines and found that it was expressed by all of the lines tested (ATN-1, MT-1, MT-2, MT-4, TL-Su, TL-Om1, ATL102), regardless of their *Tax* mRNA expression (Fig. 4A, below the graph). *HBZ* expression levels of these established lines were almost as high as those of PBMCs containing >50% ATL cells obtained from 12 patients with the acute or chronic type of disease. K562 did not express *HBZ*, as might be expected, and all primary ATL cells tested were HBZ^+, consistent with an earlier study (Fig. 4A) (25). Next, we assessed the expression of HLA class II by the cell lines. The ATL or HTLV-1–immortalized cell lines tested were all positive for both HLA-DR and HLA-DQ (Fig. 4B). These observations indicate that ATN-1, MT-1, MT-2, MT-4, TL-Su, TL-Om1, and ATL102 had the potential to present the HBZ-derived peptides on their HLA-DR or HLA-DQ molecules.

Next, we examined the responses of HBZ-specific CD4 T cells from patient #1 after HCT against K562 or HBZ-expressing lines of different HLA types. The responses of HBZ-specific CD4 T cells to the lines were evaluated without the addition of peptide. The CD4 T cells that had been expanded from patient #1 after HCT using peptide 12 responded to peptide 12-1 (positive control) but not to K562, which expressed no *HBZ* (negative control) (Fig. 4C, *upper six panels*). When tested against ATL or HTLV-1–immortalized cell lines, the CD4 T cells responded strongly to ATN-1 and TL-Su (Fig. 4C, *lower panels*). Comparing the HLA class II types of the donor of the effector CD4 T cells (patient #1 after HCT) with ATN-1 and TL-Su showed that HLA-DRB1*15:01 and

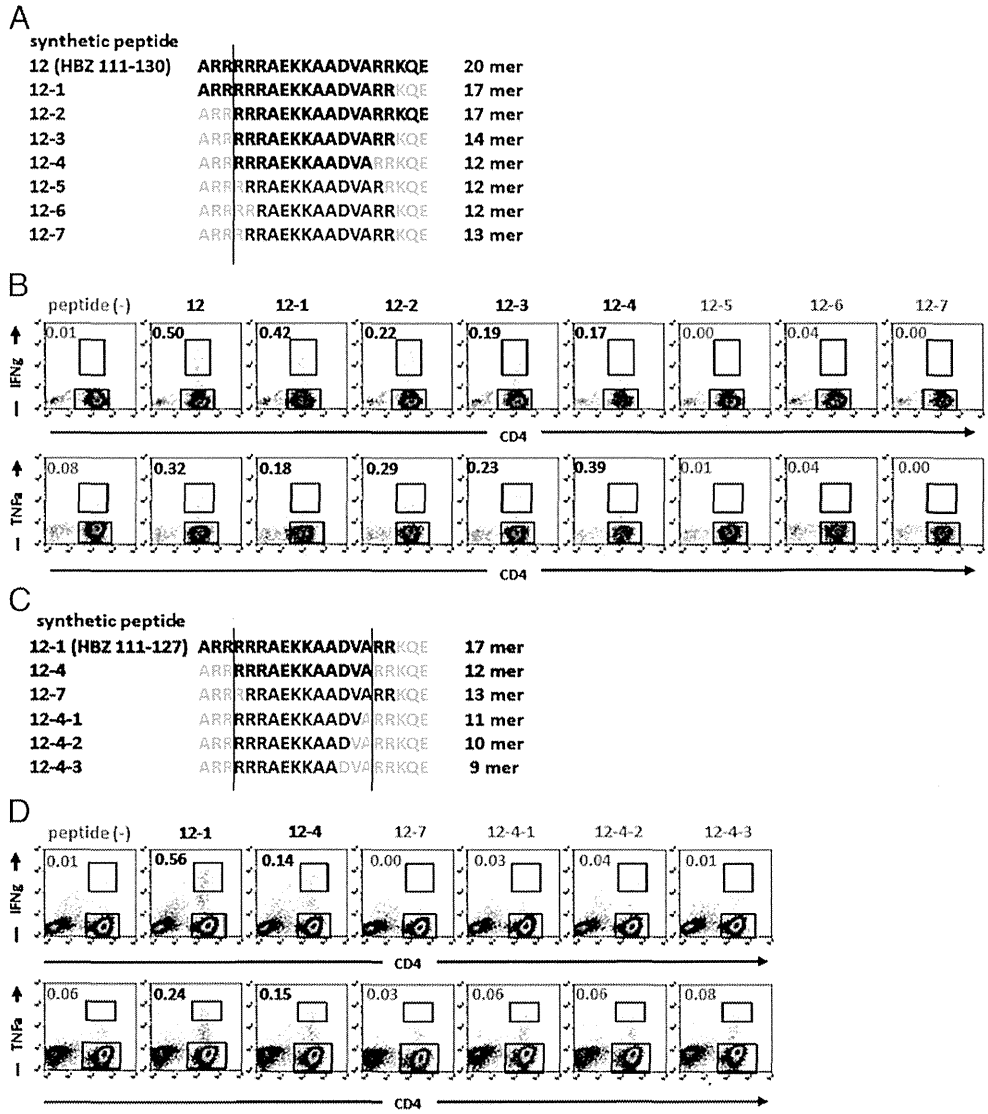

FIGURE 3. Determination of the minimum epitope sequence of HBZ recognized by CD4 T cells. (**A**) Schematic diagram of seven synthetic peptides (12-1, 12-2, 12-3, 12-4, 12-5, 12-6, 12-7) from peptide 12. They were prepared to determine the N terminus of the sequence representing the minimum epitope of HBZ recognized by the CD4 T cells. (**B**) PBMCs from patient #1 after HCT were expanded by peptide 12. The responses of expanded CD4 T cells to each synthetic peptide (12, 12-1, 12-2, 12-3, 12-4, 12-5, 12-6, 12-7) were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments. (**C**) Schematic diagram of three synthetic peptides (12-4-1, 12-4-2, 12-4-3) prepared to determine the C terminus of the sequence representing the minimum epitope of HBZ recognized by the CD4 T cells. (**D**) The responses of expanded CD4 T cells to each synthetic peptide (12-1, 12-4, 12-7, 12-4-1, 12-4-2, 12-4-3) were evaluated by the production of IFN-γ or TNF-α. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments.

HLA-DQB1*06:02 were shared by all three (Table I). In addition, the CD4 T cells responded to MT-2, TL-Om1, and ATL102 to a lesser degree (Fig. 4C, *lower panels*); these three lines were found to share HLA-DRB1*15:02 and HLA-DQB1*06:01 (Table I). Together, these results indicate that the HBZ-specific CD4 T cell responses from patient #1 after HCT were restricted by HLA-DRB1*15:01 or HLA-DQB1*06:02, as well as by HLA-DRB1*15:02 or HLA-DQB1*06:01. In contrast, the peptide-sensitized CD4 T cells did not respond to MT-1 or MT-4 (Fig. 4C, *lower panels*), consistent with the present observations that the epitope of HBZ recognized by such CD4 T cells was restricted by HLA-DRB1*15:01/HLA-DQB1*06:02 and HLA-DRB1*15:02/HLA-DQB1*06:01.

Next, we tested whether HLA-DR or HLA-DQ restricted the presentation of the HBZ-derived peptide. CD4 T cells expanded by peptide 12 no longer responded to specific stimulation by peptide 12 in the presence of anti–HLA-DR–blocking mAb by producing IFN-γ (Fig. 5A, *upper left panels*), but it did respond in the presence of the isotype-control mAb (Fig. 5A, *upper right panels*). These CD4 T cells also still responded to peptide 12 in the presence of anti–HLA-DQ–blocking mAb (Fig. 5A, *lower left panels*) and its isotype control (Fig. 5A, *lower right panels*). In addition, in the presence of anti–HLA-DR–blocking mAb, CD4 T cells expanded by peptide 12 no longer responded to ATN-1 (Fig. 5B, *left panels*), which carried HLA-DRB1*15:01/HLA-DQB1*06:02 (Table I) and expressed *HBZ* mRNA (Fig. 4A). However, they did respond by producing IFN-γ and TNF-α in the presence of the isotype control (Fig. 5B, *left panels*). These CD4 T cells also still responded to ATN-1 in the presence of anti–HLA-DQ–blocking mAb and its isotype control (Fig. 5B, *right panels*). Furthermore, HBZ-specific CD4 T cell responses to K562 (negative control) were not affected by anti–HLA-DR, anti–HLA-DQ, or their isotype mAbs (Fig. 5C). These observations from Ab-blocking experiments, together with the results shown in Fig. 4, indicate that the epitope sequence of HBZ recognized by the CD4 T cells from patient #1 after HCT were restricted by HLA-DR, specifically HLA-DRB1*15:01 and HLA-DRB1*15:02.

Clinical significance of the specific CD4 T cell response against HBZ

The data presented thus far pertained to CD4 T cells obtained from only one patient (patient #1 after HCT). Therefore, we used HBZ peptide 12 to stimulate and expand 28 PBMC samples obtained from 27 other HTLV-1–infected individuals who carried HLA-DRB1*15:01 or HLA-DRB1*15:02. PBMCs were obtained from 10 HTLV-1 ACs, 10 ATL patients who had not undergone allogeneic HCT, and 8 ATL patients after allogeneic HCT. Among them, PBMCs from one individual (patient #2) were tested at different disease stages (i.e., CRs before and after allogeneic HCT). HBZ-specific CD4 T cell responses were absent in all 10

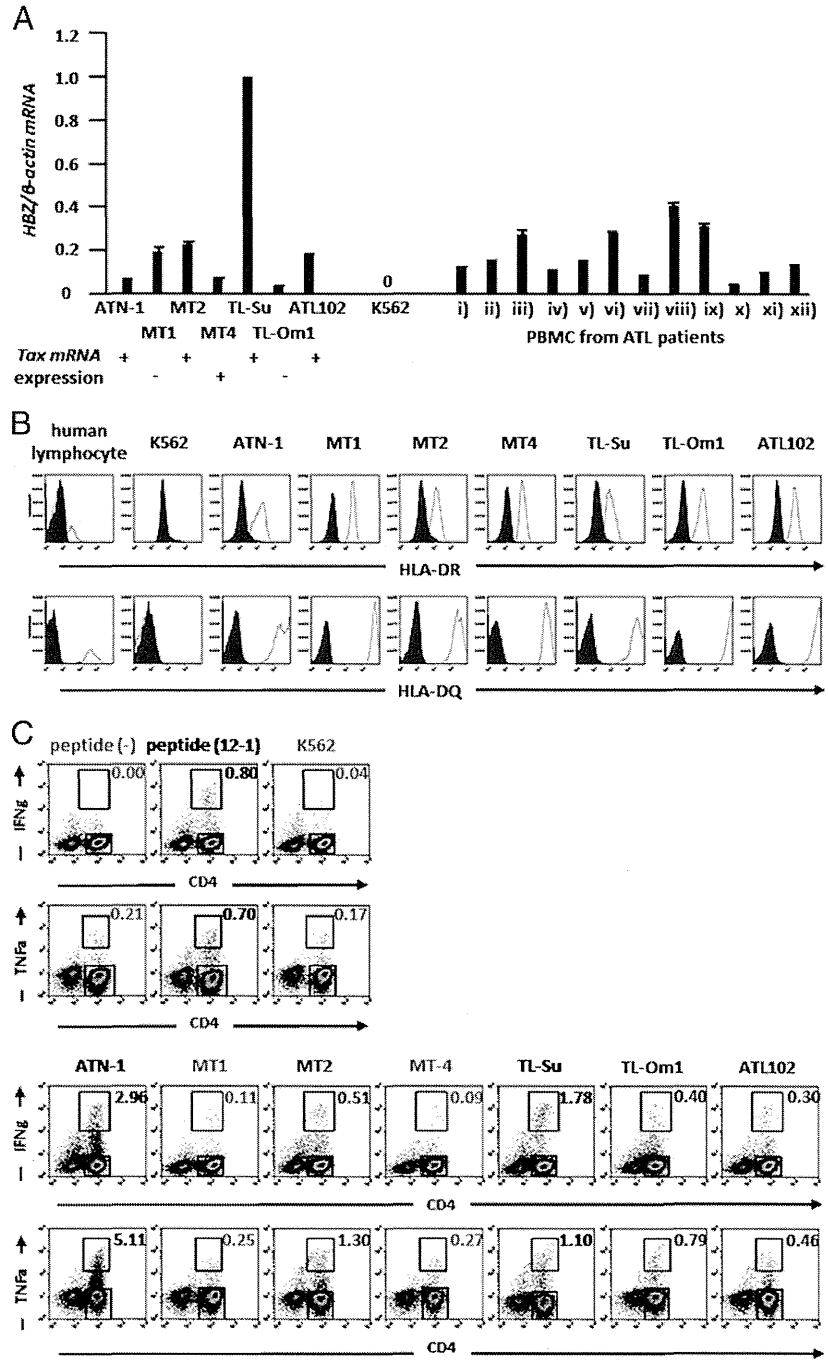

FIGURE 4. Responses of HBZ-specific CD4 T cells from patient #1 after HCT to ATL or HTLV-1–immortalized cell lines. (**A**) *HBZ* expression in ATL and HTLV-1–immortalized cell lines, K562, or PBMCs from ATL patients was analyzed by qRT-PCR by dividing the *HBZ* expression level by the *β-actin* expression level, resulting in an *HBZ/β-actin* mRNA ratio with the expression level in TL-Su set at unity. Data shown are means of triplicate experiments; error bars represent SD. *Tax* mRNA expression of each ATL and HTLV-1–immortalized cell line is indicated, as determined in our previous study (8). (**B**) HLA-DR and HLA-DQ expression in ATL cell lines, HTLV-1–immortalized lines, or K562, as analyzed by flow cytometry. The cell lines were stained with anti–HLA-DR mAb (*upper panels*, open graphs), anti–HLA-DQ mAb (*lower panels*, open graphs), or the corresponding isotype-control mAbs (filled graphs). (**C**) The expanded CD4 T cells were cocultured or not with the synthetic peptide 12-1. Negative controls without peptide stimulation (*upper left panels*) and positive controls with peptide stimulation (*upper middle panels*) are shown. The expanded CD4 T cells were cocultured with target cell lines in the absence of peptide stimulation. CD4 T cells did not respond to K562, which expressed no HBZ and acted as the negative control (*upper right panels*). The CD4 T cell responses to ATL or HTLV-1–immortalized cell lines, which expressed *HBZ*, with different HLA types were evaluated (*lower panels*). The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments.

HTLV-1 ACs, as well as in all 10 nontransplanted ATL patients (of whom 9 were in CR after systemic chemotherapy and the other was of smoldering type under observation only). In contrast, specific CD4 T cell responses to HBZ were observed in three of the eight additional ATL patients who were in CR after allogeneic HCT (patients #2, #3, and #4). The CD4 T cells from patient #2 and #4 after HCT responded to HBZ peptide 12 by producing both IFN-γ and TNF-α (Fig. 6, *right panels*). In patient #3, no TNF-α response was observed, but there was a clear IFN-γ response to HBZ peptide 12 (Fig. 6, *lower left panels*). Thus, specific CD4 T cell responses against HBZ were observed in four of nine recipients after allogeneic HCT (44%) but in no other ATL patients. Among the patients examined in this study, one patient with acute-type ATL received systemic chemotherapy and achieved CR. Subsequently, she received allogeneic HCT from an HLA-A, B, DR-matched HTLV-1 noninfected sibling donor and maintained CR (patient #2 after HCT). Although HBZ-specific CD4 T cell responses were not present at CR before allogeneic HCT in this patient (Fig. 6, *upper left panels*), they developed after transplantation (Fig. 6, *upper right panels*).

Table I. HLA information

	HLA-DRB1		HLA-DQB1		HLA-DPB1	
ATN-1	*04:05	*15:01	*04:01	*06:02	*05:01	*05:01
MT-1	*04:01	*09:01	*03:01	*03:03	*04:02	*05:01
MT-2	*04:04	*15:02	*03:02	*06:01	*05:01	*09:01
MT-4	*01:01	*16:02	*05:01	*05:02	*05:01	*05:01
TL-Su	*09:01	*15:01	*03:03	*06:02	*02:01	*17:01
TL-Om1	*15:02	*15:02	*06:01	*06:01	*09:01	*09:01
ATL102	*04:04	*15:02	*03:02	*06:01	*05:01	*09:01
Patient #1 after HCT	*04:05	*15:01	*04:01	*06:02	*02:01	*06:01

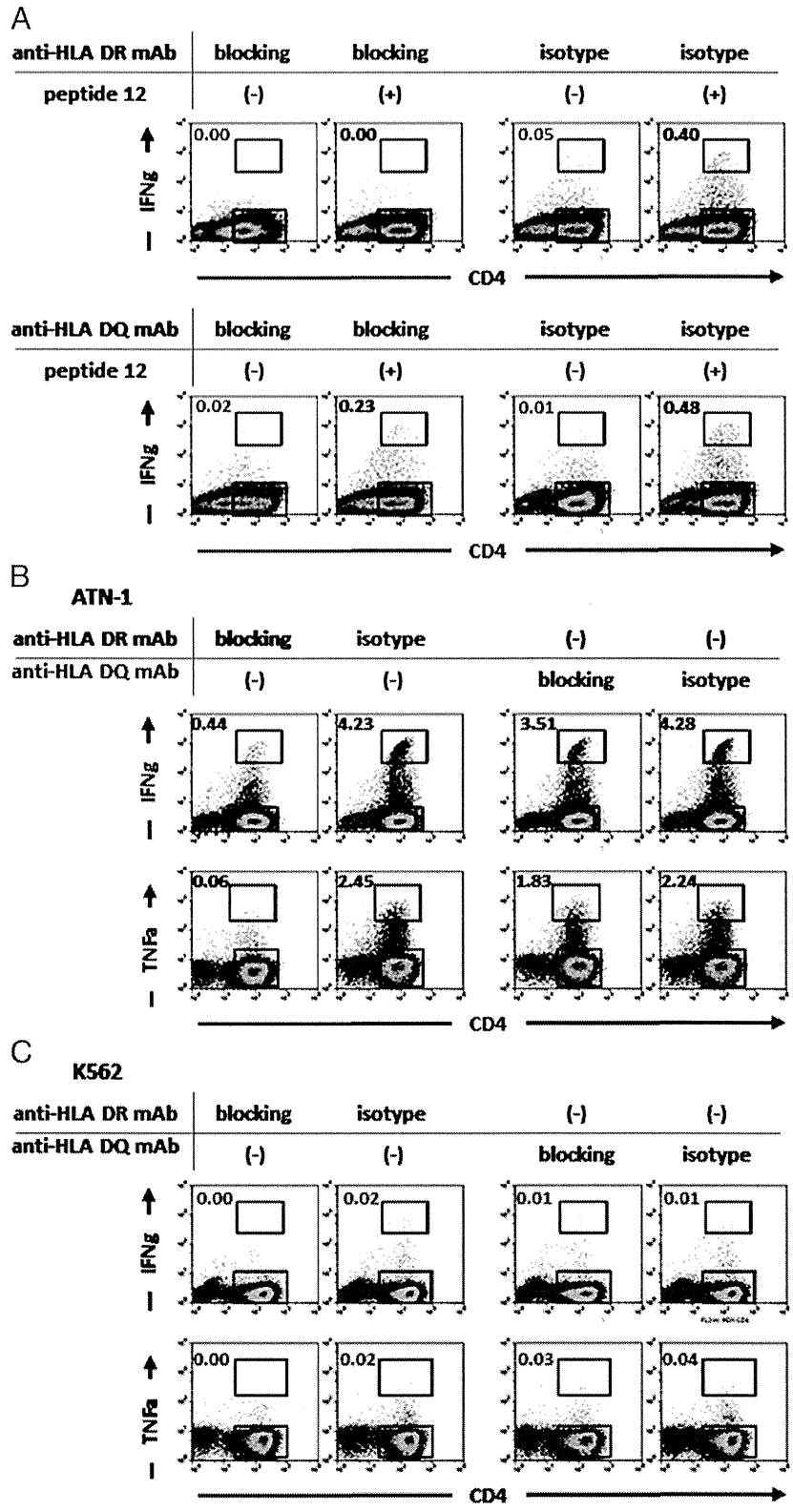

FIGURE 5. Determination of the HLA alleles restricting the presentation of HBZ-derived peptides to HBZ-specific CD4 T cells. (**A**) Responses of HBZ-specific CD4 T cells were evaluated, with or without HBZ peptide 12, in the presence of anti–HLA-DR–blocking mAb (*upper left panels*), anti–HLA-DQ–blocking mAb (*lower left panels*), or the corresponding isotype-control mAb (anti-HLA-DR isotype mAb, *upper right panels*; anti-HLA-DQ isotype mAb, *lower right panels*). Responses of HBZ-specific CD4 T cells to ATN-1, which carries HLA-DRB1*15:01/HLA-DQB1*06:02 and expresses *HBZ* mRNA (**B**), and to K562 (negative control) (**C**) were also evaluated in the presence of HLA-blocking mAbs or their isotype controls, without peptide stimulation. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. Each result is representative of three independent experiments.

Discussion

In the current study, we demonstrated the presence of HBZ-specific CD4 T cells in an ATL patient after allogeneic HCT and determined the minimum sequence of a novel HLA-DRB1*15:01–restricted HBZ-derived epitope to be RRRAEKKAADVA (HBZ114–125). HBZ peptides including the sequence HBZ114–125 were also presented on HLA-DRB1*15:02 and recognized by CD4 T cells. To the best of our knowledge, this is the first report to identify naturally processed and presented HLA-DR–restricted epitopes derived from HBZ on the surface of ATL cells. In an earlier study, an HBZ peptide–specific CTL line was established from an HLA-A*02:01^{+} individual, using peptides derived from the HBZ sequence. The peptides were selected by computer algorithms available at the BioInformatics and Molecular Analysis Section Web site (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and the SYFPEITHI Web site (http://www.syfpeithi.de/) for strong binding affinity to the HLA-A*02:01 molecule. However, the established CTL line recognized the corresponding peptide-pulsed

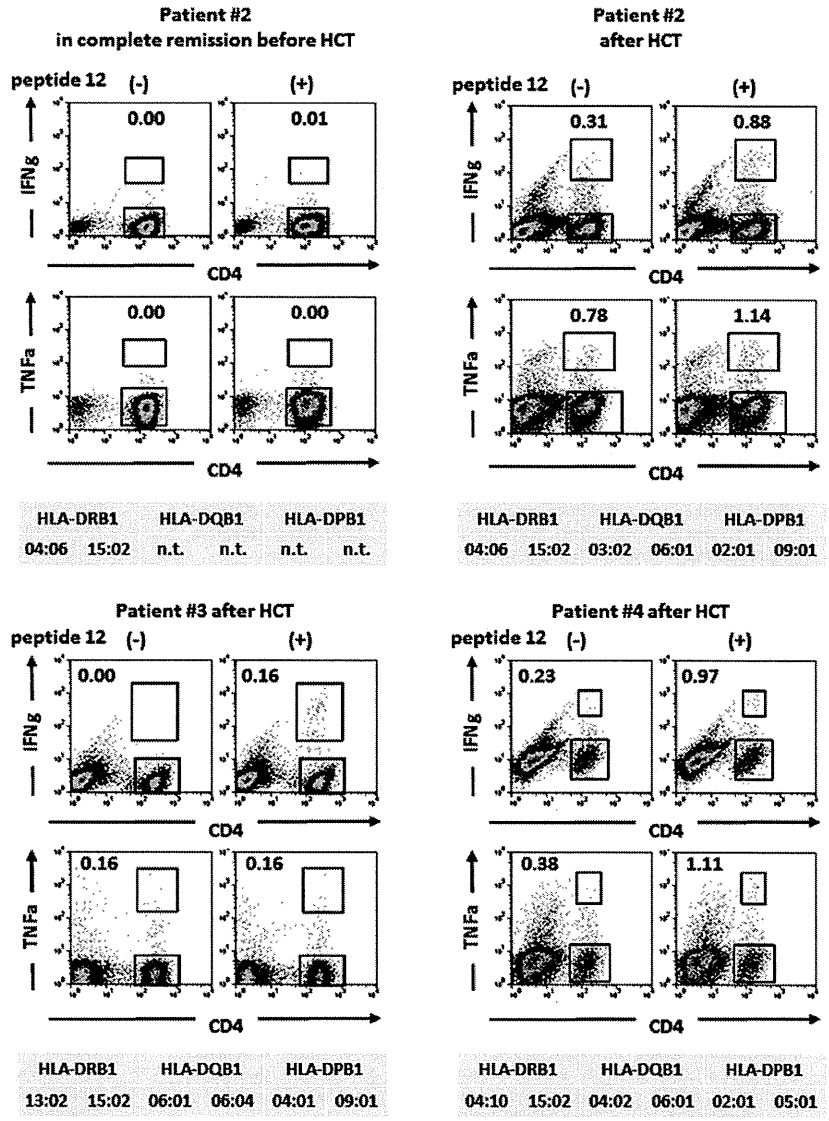

FIGURE 6. HBZ-specific CD4 T cell responses in additional ATL patients. PBMCs from additional ATL patients (#2, #3, and #4) carrying HLA-DRB1*15:02 were expanded by stimulation with HBZ peptide 12. The responses of expanded CD4 T cells to peptide 12 were evaluated by the production of IFN-γ or TNF-α. Although no HBZ-specific CD4 T cell response was observed in patient #2 in CR before allogeneic HCT (*upper left panels*), they developed after transplantation (*upper right panels*). HBZ-specific CD4 T cell responses were also observed in patient #3 (*lower left panels*) and patient #4 (*lower right panels*) in CR after allogeneic HCT. The percentage of responding cells in the upper gate relative to the cells in the lower gate is indicated in each flow cytometry panel. The HLA type of each patient is indicated below the flow cytometry panels. Each result is representative of three independent experiments. n.t., Not tested.

HLA-A*02:01$^+$ cells but not ATL cells (29). Therefore, it was not determined whether HBZ-derived peptides could be naturally presented on cells from HTLV-1–infected people. Another earlier study (30) demonstrated that HBZ expression was a critical determinant of viral persistence in the chronic phase of HTLV-1 infection. That novel study was performed using experimentally validated epitope-prediction software (34), but it did not determine the HBZ-derived epitope sequence or the corresponding HLA allele presenting it.

In the current study, no HLA-DRB1*15:01–restricted or HLA-DRB1*15:02–restricted HBZ-specific CD4 T cell response was observed in any ATL patients who had not undergone allogeneic HCT or in any HTLV-1 ACs. We surmise that HTLV-1 transmission from mothers to infants through breast milk in early life induces tolerance to HBZ, but not to Tax, by unknown mechanisms, resulting in insufficient HBZ-specific T cell responses in HTLV-1–infected individuals. This would be consistent with the persistent expression of HBZ in HTLV-1–infected cells (2, 25). In addition, insufficient HBZ-specific T cell responses may be due, in part, to the fact that the majority of the *HBZ* mRNA is retained in the nucleus, which may inhibit its translation (35), and probably leads to a low level of HBZ protein expression in HTLV-1–infected cells (29). In contrast, the finding that HLA-DRB1*15:01–restricted or HLA-DRB1*15:02–restricted HBZ-specific CD4 T cell responses were detected in ATL patients after allogeneic HCT requires explanation. Our hypothesis is that, after allogeneic HCT, the reconstituted immune system from donor-derived hematopoietic stem cells can recognize virus protein HBZ as foreign, although its expression is low, and HBZ-specific immune responses are provoked because of the lack of tolerance induction under these circumstances. In one patient with acute-type disease, HBZ-specific CD4 T cell responses were not observed in PBMCs at the time of CR before HCT, but they became detectable after allogeneic HCT. This observation supports our hypothesis. An earlier study (36) reported that HBZ-specific T cell responses were detected in some patients with HTLV-1–associated myelopathy (HAM). Unlike ATL, HAM can occur in individuals infected with HTLV-1 by any route of transmission, such as sexual intercourse (37). Therefore, some patients with HAM, infected with HTLV-1 after reaching adulthood (i.e., who became infected after their immune system had fully matured), may recognize virus protein HBZ as foreign, and HBZ-specific immune responses may be provoked. From this point of view, detection of HBZ-specific T cell responses might be expected in some HTLV-1 ACs, infected after becoming adults, but we did not see this in the present study.

In conclusion, we report the presence of HBZ-specific CD4 T cell responses in ATL patients who were in CR but only after allogeneic HCT. These responses potentially contribute to the

graft-versus-HTLV-1 effect. Novel strategies that enhance the posttransplantation allogeneic anti–HTLV-1 effect targeting HBZ, which never provokes graft-versus-host disease, could lead to improved outcomes of allogeneic HCT for ATL.

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TLR4 and NLRP3 inflammasome activation in monocytes by *N*-propionyl cysteaminylphenol-maleimide-dextran (NPCMD)

Yu Mizote [a,b], Kazumasa Wakamatsu [c], Shosuke Ito [c], Akiko Uenaka [d], Yoshihiro Ohue [b], Koji Kurose [b], Midori Isobe [b], Akira Ito [e], Yasuaki Tamura [f], Hiroyuki Honda [g], Toshiharu Yamashita [h], Satoshi Nohara [i], Mikio Oka [b], Kowichi Jimbow [h], Eiichi Nakayama [d,*]

[a] *Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan*
[b] *Department of Respiratory Medicine, Kawasaki Medical School, Kurashiki, Japan*
[c] *Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake, Japan*
[d] *Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Kurashiki, Japan*
[e] *Department of Chemical Engineering, Faculty of Engineering, Kyusyu University, Fukuoka, Japan*
[f] *Department of Pathology, Sapporo Medical Univeristy School of Medicine, Sapporo, Japan*
[g] *Department of Biotechnology, School of Engineering, Nagoya University, Nagoya, Japan*
[h] *Department of Dermatology, Sapporo Medical Univeristy School of Medicine, Sapporo, Japan*
[i] *Meito Sangyo Co., Nagoya, Japan*

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ABSTRACT

Background: *N*-propionyl cysteaminylphenol-maleimide-dextran (NPCMD) is a toxic tyrosinase substrate developed to treat melanoma.
Objective: We investigated the effect of NPCMD on innate immune responses in monocytes.
Methods: $CD14^+$ monocytes and a monocytic cell line, THP-1, were stimulated with NPCMD in vitro. Cytokines in the culture supernatants were determined by ELISA and flow cytometry.
Results: NPCMD stimulated $CD14^+$ monocytes and THP-1 cells to secrete TNFα, IL-6 and IL-8, but not IL-10 or IL-12. TNFα secretion from THP-1 cells stimulated with NPCMD was inhibited by addition of an anti-TLR4 mAb in culture. Moreover, NPCMD stimulated production of pro-IL-1β in $CD14^+$ monocytes and monocytic cell line THP-1 cells and activated the NLRP3-inflammasome, resulting in production of mature IL-1β. Use of ASC and NLRP3-deficient THP-1 cell lines established involvement of the NLRP3 inflammasome in an IL-1β secretion in treatment with NPCMD. Inhibition of IL-1β secretion by an endocytosis inhibitor, cytochalasin B, and a lysosomal enzyme cathepsin B inhibitor, CA-074 Me, suggested the involvement of lysosomal rupture and leakage of cathepsin B into the cytosol in NLRP3 activation by NPCMD.
Conclusion: The immunopotentiating effect of NPCMD mediated by TLR4 and NLRP3 inflammasome activation could be useful for eliciting effective adaptive immune responses against melanoma and other tumors.

1. Introduction

Melanogenesis is a biosynthetic pathway in the cytosolic organelle melanosome in a melanocyte and a melanoma cell. The enzyme tyrosinase catalyzes oxidative conversion of L-tyrosine via dopaquinone to a melanin pigment [1,2]. Therapeutic agents specific to melanoma have been studied in terms of utilizing this unique biosynthetic pathway. Monobenzone (hydroquinone monobenzyl ether) is a strong inducer of skin depigmentation (vitiligo) and also causes application-related dermatitis [3–5]. Its depigmenting action depends on its conversion by tyrosinase and the subsequent formation of benzoquinone, which binds to cysteine residues in

Abbreviations: 4-*S*-CAP, 4-*S*-cysteaminylphenol; alum, aluminum hydroxide; APDC, (2R,4R)-4-aminopyrrolidine-2, 4-dicarboxylic acid; ATP, adenosine triphosphate; CBA, cytometric bead array; CMD, carboxymethyl dextran; DAMP, danger-associated molecular pattern; defASC, ASC-deficient THP-1; defNLRP3, NLRP3-deficient THP-1; FBS, fetal bovine serum; LAL, limulus amebocyte lysate; LPS, lipopolysaccharide; MIL, maleimide linker; MIL-CMD, maleimide linker conjugated carboxymethyl dextran; MTT, methylthiazole tetrazolium; NPCMD, *N*-propionyl-4-*S*-cysteaminylphenol-maleimide-dextran; NPrCAP, *N*-propionyl-4-*S*-cysteaminylphenol; PAMP, pathogen-associated molecular pattern; PBMCs, peripheral blood mononuclear cells; RQ, relative quantification.

* *Corresponding author at*: Faculty of Health and Welfare, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701-0193, Japan. Tel.: +81 86 462 1111x54954; fax: +81 86 464 1109.
E-mail address: nakayama@mw.kawasaki-m.ac.jp (E. Nakayama).

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melanosome and other proteins. Subsequently, the haptenated proteins sensitize skin [6]. It is strongly cytotoxic to melanoma cells, which probably uptake monobenzone specifically compared to other cell types [7]. Its cytotoxicity is independent of the presence of the tyrosinase enzyme [6,7] and therefore it is not in use systemically. Jimbow and his colleagues have developed less toxic and feasible therapeutic chemicals for melanoma using tyrosine analogs [8]. A sulfur-amine analog of tyrosine, 4-*S*-cysteaminylphenol (4-*S*-CAP), was produced and an *N*-protected analog of 4-*S*-CAP, *N*-propionyl-4-*S*-cysteaminylphenol (NPrCAP), was stable for degradation [9]. Furthermore, recently, to increase the solubility of NPrCAP, carboxymethyl dextran (CMD) was conjugated to it using a maleimide linker (MIL). Thus, NPrCAP-CMD (NPCMD) was expected to diffuse efficiently in tumor tissue. These phenols have been shown to be good substrates for tyrosinase [9], are selectively incorporated into melanoma cells and showed cytotoxicity in vitro and in vivo [9–11]. Moreover, adaptive immunity elicited against melanoma was shown to be involved in an NPrCAP-mediated anti-melanoma effect [12].

Inflammasomes are cytosolic sensors that rapidly activate the caspase-1 protease in response to various pathogen-associated molecular patterns (PAMPs) or host-derived signals of cellular stress (danger-associated molecular patterns, DAMPs) [13]. Caspase-1 cleaves and activates two pro-inflammatory cytokines, IL-1β and IL-18. Memory T-cell responses play an important role in adaptive immunity. There is some evidence of innate activation of memory T-cell responses without involving T-cell antigen receptor signaling [14,15]. Recently, it was shown that memory CD8 T-cells in some bacterial infections were activated by IL-18 released following NLRC4 inflammasome activation in the absence of T-cell antigen receptor activation [16]. In this study, we investigated the activation of innate immune responses by NPCMD instead of its direct effect on melanoma. We show that NPCMD stimulated $CD14^+$ monocytes and monocytic cell line THP-1 cells to secrete TNFα via TLR4, and IL-1β by NLRP3 activation. Efficient activation of innate immunity by NPCMD could facilitate adaptive immunity against melanoma and other tumors.

2. Materials and methods

2.1. Reagents

All chemicals were of the highest purity available. 3-Mercaptopropionic acid, *N,N'*-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, *N,N*-dimethylformamide, and *N*-hydroxysuccinimide were purchased from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Dojindo Laboratories (Kumamoto, Japan). Carboxymethyl dextran (M.W. 10,000) and *N*-(2-aminoethyl) maleimide hydrochloride were synthesized at Meito Sangyo Co. (Nagoya, Japan). 4-*S*-CAP and NPrCAP were prepared by the method of Padgette et al. [17] and Tandon et al. [9], respectively. Adenosine triphosphate (ATP), cytochalasin B, dextranase, lipopolysaccharide (LPS), methylthiazole tetrazolium (MTT) and polymyxin B were purchased from Sigma–Aldrich (St. Louis, MO). The caspase-1 inhibitor z-YVAD-fmk and (2R, 4R)-4-aminopyrrolidine-2, 4-dicarboxylic acid (APDC) were from Enzo Life Sciences (Farmingdale, NY). CA-074 Me was from Merck Millipore (Billerica, MA). A double strand DNA analog poly (dA:dT) was from InvivoGen (San Diego, CA). Aluminum hydroxide (alum) was from Katayama Chemical (Osaka, Japan).

2.2. Synthesis of N-propionyl cysteaminylphenol-maleimide-dextran (NPCMD)

1.4 g (12.2 mmol) *N*-hydroxysuccinimide and 2.3 g (12.0 mmol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were added to 5.4 g (0.54 mmol) carboxymethyldextran in 52 mL water, and were stirred for 1 h at room temperature. 1.56 g (8.83 mmol) *N*-(2-aminoethyl) maleimide hydrochloride in 52 mL of 0.2 mol/L boric buffer (pH 8.5) was stirred in a reactor adding the above reaction mixture. After 18 h at room temperature, the reaction mixture was washed by flowing water through the dialysis membrane (M.W. 1000) overnight. The solution was dried to a powder under freeze-drying conditions to give 5.33 g of maleimide linker conjugated carboxymethyl dextran (MIL-CMD) (99%). 2.6 g MIL-CMD in 200 mL water was added slowly to 0.33 g NPrCAP-SH in 35 mL THF and stirred for 1 h at room temperature. The reaction mixture was evaporated to remove THF and filtrated. The filtrate was washed by flowing water through the dialysis membrane (M.W. 1000) overnight. The resulting solution was dried to a powder under freeze-drying conditions to give 2.37 g NPCMD (91%).

2.3. Blood samples

Peripheral blood was drawn from healthy donors after obtaining written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich). $CD3^+$, $CD14^+$, $CD19^+$ and $CD56^+$ cells were purified from PBMCs using CD3, CD14, CD19 and CD56 microbeads, respectively, using an autoMACS (Miltenyi Biotec, Auburn, CA). The residual cells were used as $Lineage^-$ cells.

2.4. Cell lines

THP-1, an acute monocytic leukemia cell line was obtained from ATCC. NLRP3-deficient THP-1 (defNLRP3) and ASC-deficient THP-1 (defASC) were obtained from InvivoGen. The medium used to maintain these cell lines was RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (JRM Bioscience, Lenexa, KA).

2.5. Quantitative real-time RT-PCR

Total RNA was obtained from cells using an RNeasy Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. 500 nanograms of each sample were subjected to cDNA synthesis using a PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Two-step real-time RT-PCR was run on an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). The primers were: *ASC* 5′-TGGTCAGCTTCTACCTGGAGACCTA-3′ (forward), 5′-CTTGGCTGCCGACTGAGGAG-3′ (reverse), *IL-1β* 5′-ACAGATGAAGTGCTCCTTCCA-3′ (forward), 5′-GTCGGAGATTCGTAGCTGGAT-3′ (reverse), *NLRP3* 5′-CTGCGATCAACAGGAGAGACCTTT-3′ (forward), 5′-ACCCATCCACTCCTCTTCAATGCT-3′ (reverse), *GAPDH* 5′-GCTCTCTGCTCCTCCTGTTC-3′ (forward), 5′-ACGACCAAATCCGTTGACTC-3′ (reverse). The TaqMan probes were: *ASC* 5′-FAM-TCACCGCTAACGTGCTGCGCGACAT-TAMRA-3′, *IL-1β* 5′-FAM-CTCT GCCCTCTGGATGGCGG-TAMRA-3′, *NLRP3* 5′-FAM-TGCACGTGTTTCGAATCCCACTGTGA-TAMRA-3′, *GAPDH* 5′-HEX-AGCCACATCGCTCAGACACCATGGG-BHQ1-3′. PCR was performed with FastStart Universal Probe Master (ROX) (Roche Applied Science, Upper Bavaria, Germany), the primer pair, the TaqMan probe, and cDNA solution. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The mRNA expression level of each target gene was normalized to the expression level of GAPDH.

2.6. MTT assay

$CD3^+$, $CD14^+$, $CD19^+$, $CD56^+$, $Lineage^-$ and THP-1 cells (1×10^5) were cultured in a 96-well round culture plate in 10% FBS-RPMI 1640 medium with NPCMD for 1 day at 37 °C. After incubation, the medium was removed and serum-free RPMI1640 medium

containing MTT (0.5 mg/mL) was added. After an additional incubation for 3 h at 37 °C, the medium was removed and DMSO was added to each well. The absorbance was read at 535 nm.

2.7. Endotoxin detection

Endotoxin was estimated using Limulus Amebocyte Lysate (LAL) Kinetic-QCL (Lonza, Allendale, NJ) according to the manufacturer's instructions.

2.8. ELISA to detect dextran

NPCMD (1 μg/mL) with various amounts of polymyxin B or dextranase in a coating buffer were adsorbed onto a 96-well ELISA plate (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. After washing and blocking, mouse anti-dextran mAb (STEMCELL Technologies, Vancouver, Canada) was added and incubation was done for 2 h at 37 °C. After washing, a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (MBL, Nagoya, Japan) was added and incubation was done for 1 h at 37 °C. After washing and development, absorbance was read at 490 nm.

2.9. Cytokine detection

Supernatants from cultures of $CD3^+$, $CD14^+$, $CD19^+$, $CD56^+$, $Lineage^-$ and THP-1 cells (1×10^5) treated with NPCMD were collected and the amounts of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNFα were estimated using a Cytometric Bead Array (CBA) kit (BD Biosciences, San Jose, CA) by FACS Canto II.

2.10. IL-1β and TNFα ELISA

THP-1 cells (1×10^5) were treated with the indicated amounts of NPCMD, NPrCAP, MIL-CMD, alum, ATP or poly (dA:dT) in the presence or absence of LPS. The cytokines in the culture supernatants or cell lysates were estimated by DuoSet Sandwich ELISAs (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. For the inhibition assay, the indicated amounts of anti-TLR4 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), polymyxin B, z-YVAD-fmk, cytochalasin B, CA-074 Me and APDC were added to the assay culture.

2.11. Statistical analysis

The values are expressed as the mean ± S.D. of individual samples. The significance of the results was determined using the Student's *t* test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Endotoxin-like activity of NPCMD

As shown in Fig. 1A, to increase the solubility of NPrCAP and make it diffuse efficiently in tissue, CMD was conjugated using the MIL. First, we examined the endotoxin-like activity of NPrCAP and its CMD conjugate (NPCMD) with an LAL test. As shown in Fig. 1B, moderate endotoxin-like activity was detected in NPCMD, but not its components, NPrCAP or MIL-CMD alone at an equivalent amount included in NPCMD. To examine the possibility that the endotoxin-like activity observed in the NPCMD preparation was indeed due to LPS contaminating the preparation, we examined the effect of polymyxin B treatment of NPCMD in an endotoxin assay. As shown in Fig. 1C, while the endotoxin activity of LPS was diminished completely, the endotoxin-like activity of NPCMD was not diminished by the treatment. On the other hand, while no reduction in endotoxin activity of LPS was observed by dextranase treatment, the endotoxin-like activity of NPCMD was reduced by

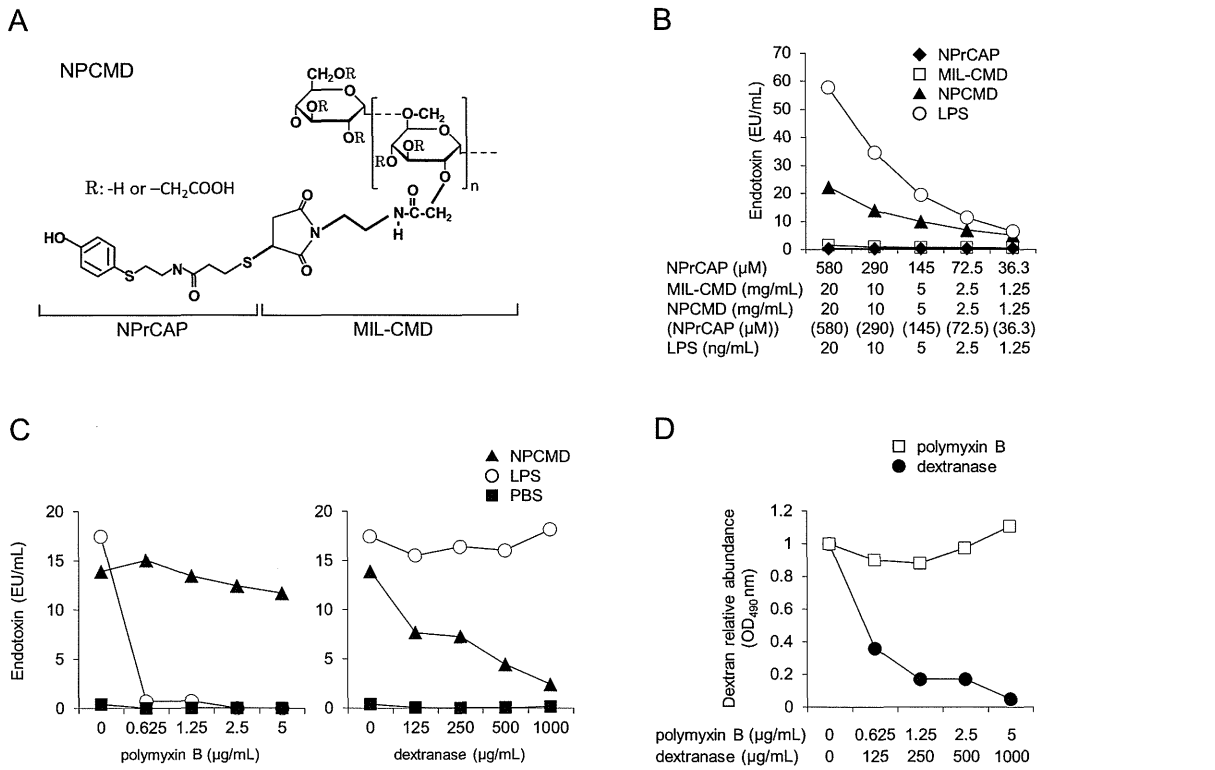

Fig. 1. Endotoxin assay of NPCMD. (A) Structural formula of NPCMD. (B) and (C) LAL test for endotoxin. (D) ELISA for dextran in NPCMD (1 μg/mL) using an anti-dextran mAb. In (C) and (D), NPCMD (10 mg/mL), LPS (5 ng/mL) and PBS (control) were pre-treated with polymyxin B or dextranase. These results are representative from three independent experiments.

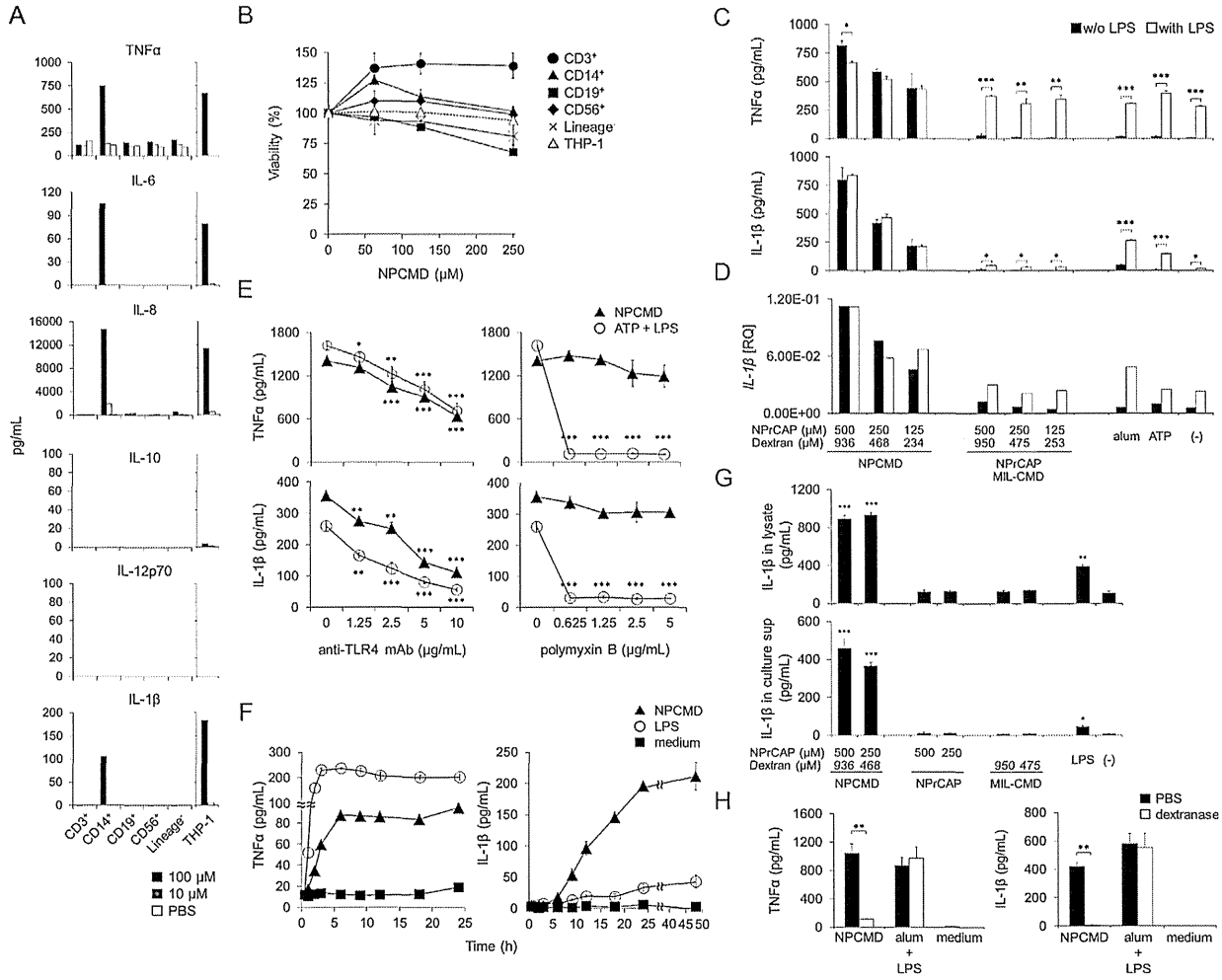

Fig. 2. Cytokine release from CD14$^+$ monocytes and THP-1 cells after treatment with NPCMD. (A) CD3$^+$, CD14$^+$, CD19$^+$, CD56$^+$, Lineage$^-$ and THP-1 cells (1×10^5) were treated with NPCMD (containing 100 or 10 μM NPrCAP) for 48 h. Cytokines in the culture supernatants were determined with a CBA kit. The assays were done in duplicate and the values represent the mean. (B) CD3$^+$, CD14$^+$, CD19$^+$, CD56$^+$, Lineage$^-$ or THP-1 cells (1×10^5) were treated with NPCMD at the indicated concentrations. Viability of cells was measured after 24 h by MTT assay. The viability (percentage) was calculated relative to untreated (control) cells. The assays were done in triplicate and the values represent the mean ± S.D. (C) and (D) THP-1 cells (1×10^5) were treated with NPCMD, NPrCAP plus MIL-CMD, alum (25 μg/mL) or ATP (1 μM) with or without (w/o) LPS (100 pg/mL) for 18 h. In (D), the mRNA level of IL-1β was quantified by real-time RT-PCR. RQ, relative quantification. In (C), the assays were done in triplicate and the values represent the mean ± S.D. In (D), the assays were done in duplicate and the values represent the mean. (E) THP-1 cells (1×10^5) were treated with NPCMD (containing 200 μM NPrCAP) or ATP (1 μM) plus LPS (50 pg/mL) in the presence of the indicated amounts of anti-TLR4 mAb or polymyxin B for 18 h. The assays were done in triplicate and the values represent the mean ± S.D. (F) THP-1 cells (5×10^4) were treated with NPCMD (containing 200 μM NPrCAP) or LPS (1 ng/mL) for the indicated times. The assays were done in triplicate and the values represent the mean ± S.D. (G) THP-1 cells (1×10^5) were treated with NPCMD, NPrCAP, MIL-CMD or LPS (1 ng/mL) for 12 h. After treatment, the cells were lysed by cycles of freeze-thawing. The assays were done in triplicate and the values represent the mean ± S.D. (H) THP-1 cells (1×10^5) were treated with NPCMD (containing 200 μM NPrCAP) or alum (25 μg/mL) plus LPS (50 pg/mL) for 18 h after no treatment or pre-treatment with dextranase (0.5 mg/mL). TNFα and IL-1β in the supernatant in (C), (E), (F), (G) and (H), and pro-IL-1β and mature IL-1β in the cell lysate in (G) were determined by ELISA. Statistical analyses were performed with the Student's *t* test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the same treatment. Dextran degradation by dextranase treatment determined by ELISA using an anti-dextran mAb (Fig. 1D) was consistent with the reduction in endotoxin-like activity in NPCMD in Fig. 1C.

Collectively, the findings show that the conjugated product NPCMD had moderate endotoxin-like activity in the LAL test, while its components NPrCAP and MIL-CMD showed no such activity. The endotoxin-like activity of NPCMD was dependent on dextran in the molecule.

3.2. Cytokine release from CD14$^+$ monocytes and the monocytic cell line THP-1 following treatment with NPCMD

CD3$^+$, CD14$^+$, CD19$^+$ and CD56$^+$ cells were purified using magnetic beads coated with the respective antibody and treated with NPCMD. As shown in Fig. 2A, only CD14$^+$ cells were reactive to NPCMD and secreted TNFα, IL-6, IL-8 and IL-1β, but not IL-10 or IL-12p70. Essentially similar results were obtained with the monocytic cell line THP-1. While NPCMD was toxic to melanoma as NPrCAP [11,12,18,19], no cytotoxicity was observed with PBMCs and THP-1 cells following treatment with NPCMD in an MTT assay (Fig. 2B). We investigated secretion of TNFα and IL-1β from THP-1 cells treated with NPCMD, and its components NPrCAP or MIL-CMD at an equivalent amount included in NPCMD. As shown in Fig. 2C, those cytokine secretions were observed only by treatment with NPCMD, but not its component NPrCAP or MIL-CMD alone or their mixture. The effect of LPS on TNFα or IL-1β secretion from THP-1 cells treated with NPCMD was then investigated. No augmentation of cytokine secretion by addition of LPS was observed (Fig. 2C). Quantitative real-time RT-PCR analysis showed a dose-dependent elevation of *IL-1β* mRNA levels in THP-1 cells treated with NPCMD (Fig. 2D). We then examined whether the secretion of TNFα and IL-1β was due to activation of the TLR4 pathway by antibody blocking using an anti-TLR4 mAb. As shown in Fig. 2E, the secretion of TNFα and IL-1β was blocked by addition of an anti-TLR4 mAb to the culture. These findings suggested that

NPCMD stimulated monocytes and THP-1 cells through the TLR4 pathway and secreted TNFα and IL-1β. Moreover, TNFα or IL-1β production from THP-1 cells by LPS plus ATP, but not by NPCMD, was diminished by treatment with polymyxin B, suggesting no involvement of LPS in those cytokine secretions in NPCMD stimulation. As shown in Fig. 2F, secretion of TNFα occurred as early as 2 h after NPCMD treatment, being comparable to LPS, while IL-1β secretion was only observed 6 h after treatment, indicating secretion of IL-1β following TNFα. IL-1β secretion was shown to occur by caspase-1 mediated degradation of pro-IL-1β to its mature form. Activation of caspase-1 occurs by activation of a multi-protein complex known as inflammasome. Pro-caspase-1 formed a multiplex by binding to the effector site of inflammasome, autocatalizing the molecule to active caspase-1 and cleaving pro-IL-1β to produce its active form. Therefore, production of pro-IL-1β was necessary before inflammasome activation to produce IL-1β. The results in Fig. 2G suggested that pro-IL-1β was produced in the cytosol of THP-1 in the treatment with NPCMD and IL-1β was secreted in the culture supernatant. Furthermore, as shown in Fig. 2H, TNFα and IL-1β secretion from THP-1 cells by NPCMD was diminished by dextranase treatment, indicating its dextran-dependent activation consistent with the results shown in Fig. 1. Endotoxin-like activity of NPCMD through the TLR4 pathway likely primed monocytes and THP-1 cells to produce pro-IL-1β in the cytosol, and thereafter secrete IL-1β efficiently with the same NPCMD stimulation.

3.3. *Involvement of NLRP3 inflammasome activation for IL-1β secretion from THP-1 cells treated with NPCMD*

The effect of z-YVAD-fmk, a peptide inhibitor of active caspase-1 on IL-1β secretion from THP-1 cells treated with NPCMD was examined. As shown in Fig. 3A, dose-dependent inhibition of IL-1β secretion, but not TNFα secretion, was observed. A similar effect was observed in IL-1β secretion from THP-1 cells treated with alum plus LPS or ATP plus LPS, both of which were shown to stimulate NLRP3 inflammasome activation to produce IL-1β in THP-1 cells [20,21].

To investigate whether NLRP3 inflammasome activation was involved in IL-1β secretion from THP-1 cells treated with NPCMD, we utilized ASC- and NLRP3-deficient THP-1 cell lines. As shown in Fig. 3B, no *ASC* or *NLRP3* mRNA expression was observed in ASC-deficient or NLRP3-deficient THP-1 cells, respectively. As shown in Fig. 3C, while TNFα secretion from either ASC or NLRP3 deficient THP-1 cells was observed by treatment with NPCMD, as well as alum plus LPS or ATP plus LPS, no IL-1β secretion was observed from either cell type. However, IL-1β secretion was observed in NLRP3-deficient, but not ASC-deficient THP-1 cells, treated with a double stranded DNA analog poly (dA:dT), which is a ligand of the AIM2 inflammasome plus LPS [22]. These findings suggested that NPCMD stimulated TNFα as well as IL-6 and IL-8 secretion from THP-1 cells through TLR4 signaling and IL-1β secretion through NLRP3 inflammasome activation. To secrete IL-1β, accumulation of pro-IL-1β is necessary [23]. The results suggested that pro-IL-1β was preformed through TLR4 signaling by NF-κB activation and cleaved it to mature IL-1β by active caspase-1 produced by autocleavage of pro-caspase-1 following NLRP3 inflammasome activation by NPCMD.

NLRP3 inflammasome activation was shown to occur following endocytosis of bacterial PAMPs and endogenous danger signals like DAMPs, resulting in lysosomal rupture. Therefore, we examined the effect of the endocytosis inhibitor cytochalasin B and the lysosomal enzyme cathepsin B inhibitor CA-074 Me as shown in Figs. 3D and E, respectively. Cytochalasin B inhibited IL-1β secretion from THP-1 cells treated with NPCMD or alum plus LPS, but not ATP plus LPS. CA-074 Me inhibited IL-1β secretion from THP-1 cells treated with NPCMD, as well as alum plus LPS or ATP plus LPS. Furthermore, ROS have been shown to cause NLRP3 inflammasome activation [13,24]. The ROS inhibitor APDC, however, showed only marginal inhibition of IL-1β secretion from THP-1 cells treated with NPCMD or ATP plus LPS. No inhibition was observed with alum plus LPS treatment.

4. Discussion

In this study, we showed that NPCMD, but not its components NPrCAP or MIL-CMD alone or their mixture, gives rise to a positive reaction in a standard LAL test used to detect endotoxin in a dextran-dependent manner that was included in the molecule. These findings suggested that the positive reaction of NPCMD in the LAL test was likely due to the conformation derived from combining NPrCAP and MIL-CMD. In this regard, dextran contains α-1, 6 glycoside glucose predominantly. In the LAL test, β-glucan (β1-3 glucose) from fungus is known to give a positive reaction [25]. Although the positivity of NPCMD in the LAL test may not be directly linked to its endototoxin-like activity, NPCMD indeed showed endotoxin-like activity. NPCMD stimulated $CD14^+$ monocytes and monocytic cell line THP-1 cells to secrete TNFα, IL-6 and IL-8, but not IL-10 or IL-12 by itself. Moreover, TNFα secretion from THP-1 cells was inhibited by the addition of an anti-TLR4 mAb in culture. Production of IL-1β and IL-18 was mediated by inflammasome activation, which resulted in autocleavage of pro-caspase-1 bound to inflammasome by its proximity in multiplex formation to active caspase-1. Pro-IL-1β and pro-IL-18 preformed in the cytosol were cleaved to proinflammatory cytokine IL-1β and IL-18, respectively, by caspase-1. NPCMD stimulated production of pro-IL-1β and pro-IL-18 (data not shown) via NF-κB activation (data not shown) in monocytic cell line THP-1 cells, and activated the NLRP3 inflammasome resulting in production of mature IL-1β and IL-18. Use of ASC and NLRP3-deficient THP-1 cell lines established the involvement of NLRP3 inflammasome in IL-1β and IL-18 secretion in stimulation with NPCMD. While TNFα was secreted from either ASC-deficient or NLRP3-deficient THP-1 cells in stimulation with NPCMD, no IL-1β secretion was observed. A double stranded DNA analog, poly (dA:dT), which is the ligand to AIM2 [22] containing the ASC domain activated NLRP3-deficient, but not ASC-deficient, THP-1 cells to secrete IL-1β, established target specificity of NPCMD. The precise mechanisms of NLRP3 activation by NPCMD in this study remain unknown. However, inhibition of IL-1β secretion by the endocytosis inhibitor cytochalasin B and a lysosomal enzyme cathepsin B inhibitor, CA-074 Me, suggested involvement of lysosomal rupture and leakage of cathepsin B into the cytosol.

In a recent study, we suggested that NPrCAP can be activated in melanoma cells by tyrosinase leading to the quinone-hapten NPrCAQ, which binds to melanosome or other proteins through their cysteine residues to form possible neo-antigens, thus triggering an immunological response [26]. NPCMD was also oxidized to a quinone form, similar to NPrCAQ (Supplementary Fig. 1). However, the effect of NPCMD to induce cytokine production in monocytes or the monocytic cell line THP-1 is independent of melanosomal oxidation because another analog, NPr (2-*S*) CAP-MIL-CMD, that was not a tyrosinase substrate could induce cytokine production similar to NPr (4-*S*) CAP-MIL-CMD (NPCMD).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.11.006.

Several mechanisms of NLRP3 inflammasome activation have been studied extensively. First, extracellular ATP stimulates the purinergic $P2X_7$ receptors, triggering potassium efflux and inducing recruitment of the pannexin-1 membrane pore [27]. Pore formation allows extracellular NLRP3 agonists to enter into