We then assessed CT antigen-specific cellular immune responses in ATLL patients. As the majority of ATLL patients are in a severe anemic state as a characteristic of the disease and also due to intensive chemotherapy, we collected PBMCs from patients in partial or complete remission except Pt. #8 with an indolent ATLL type. Considering this limitation of available sample sizes and predominant humoral immune responses against NY-ESO-1, we focused on NY-ESO-1 as a parameter of cellular immune responses. With sufficient amounts of PBMCs from nine ATLL patients (Pt #1, 2, 4, 8, 13, 14, 19, 27, and 43), NY-ESO-1 expression by ATLL cells was confirmed by RT-PCR in each patient, except for Pt. #13 (no detection) and #43 (unavailability of sample) (Supplemental Table 2). $CD8^+$ T cells were pre-sensitized with autologous $CD4^-CD8^-$ PBMCs pulsed with a pool of NY-ESO-1 peptides, and antigen-specific $CD8^+$ T cells were analyzed with NY-ESO-1/HLA tetramers corresponding to the HLA allele of each patient. NY-ESO-1-specific $CD8^+$ T cells were detected in ATLL Pt. #2, 4, 14, and 43 (Figure 3A). The results indicate that NY-ESO-1-specific $CD8^+$ T cell responses spontaneously develop in a subset of patients harboring NY-ESO-1 expressing ATLL.

NY-ESO-1-specific $CD8^+$ T cells produce cytokines and recognize an autologous ATLL cell line.

With the presence of NY-ESO-1-specific $CD8^+$ T cells in ATLL patients, we further analyzed their cytokine production in response to a pool of NY-ESO-1 peptides or autologous tumor cells. Sufficient amounts of PBMCs for the cytokine analysis were available from three ATLL patients (Pt. #4, #14 and 19), in which NY-ESO-1 expression was confirmed by RT-RCR (Supplemental Table 2). NY-ESO-1-specific $CD8^+$ T cells were detected with NY-ESO-1/MHC tetramers in two of them (Pt. #4 and

14), but relevant tetramers were not available for another patient (Pt. #19). NY-ESO-1-specific $CD8^+$ T cells prepared from these three patients by pre-sensitization with NY-ESO-1 peptides-pulsed $CD4^-CD8^-$ PBMCs produced IFN-γ and/or TNF-α by intracellular cytokine staining (Figure 3B). In Pt #14, the frequency of NY-ESO-1-specific $CD8^+$ T cells producing IFN-γ was much higher than NY-ESO-1-specific $CD8^+$ T cells detected by NY-ESO-1/HLA-Cw*0304 tetramer, suggesting that this patient may have $CD8^+$ T cells recognizing other epitopes of NY-ESO-1 (Figure 3A and B). Taken together, NY-ESO-1-specific $CD8^+$ T cells were detected in five out of nine (55.6%) ATLL patients.

We also examined whether NY-ESO-1-specific $CD8^+$ T cells from Pt. #4 recognized autologous ATLL cells. They produced IFN-γ and TNF-α against autologous ATLL cells expressing NY-ESO-1, but not against a control HLA-matched ATLL cell line (ATL-102) without NY-ESO-1 expression (Figure 3C). Collectively, these data indicate that NY-ESO-1-specific $CD8^+$ T cells are present in ATLL patients and are able to recognized and kill autologous leukemic cells.

$CD4^+$ T cell responses against NY-ESO-1 in ATLL patients

To determine whether NY-ESO-1-specific $CD4^+$ T cells were present in these ATLL patients, $CD4^+$ T cells derived from PBMCs obtained from ATLL patients (Pt #1, 2, 4, 8, 13, 14, 19, 27, and 43) were pre-sensitized by $CD4^-CD8^-$ PBMCs pulsed with a pool of NY-ESO-1 peptides, and assessed for cytokine production by intracellular cytokine staining. Out of nine patients, NY-ESO-1-specific $CD4^+$ T cells were detected only in one patient (Pt #19), who was in complete remission after receiving allogeneic hematopoietic stem cell transplantation (Figure 4). Thus, NY-ESO-1-specific $CD4^+$ T cells are present in a subset of ATLL patients, but a much lower frequency than $CD8^+$

T cell responses, partly because the presence of ATLL cells, which are CD4 positive, may make the detection difficult and possibly because $CD25^{+}CD4^{+}$ Tregs are present in the $CD4^{+}$ T cell fraction.

Discussion

Since the initial description of ATLL as a unique type of T cell leukemia/lymphoma,[1] various therapeutic attempts have been made. Yet, the prognosis of ATLL is still poor despite advances in our knowledge regarding the oncogenic process of the disease.[10,23] Here, we have examined CT antigen expression and its immunogenicity in ATLL patients to explore the potential for immunotherapy of ATLL by targeting CT antigens. We found that CT antigens such as NY-ESO-1, MAGE-A3, and MAGE-A4 were highly expressed in ATLL. In particular, the frequencies of NY-ESO-1 and MAGE-A4 expression (61.4% and 61.4%, respectively) at the mRNA level were higher than or comparable to those in other malignancies. For example, the frequency of NY-ESO-1 and MAGE-A4 expression was 32-45% and 28%, respectively, in malignant melanoma, 24-33% and 63-90.2% in esophageal cancer, 30-43% and 57% in ovarian cancer, and 18-35% and 33% in bladder cancer.[19,32,33] In addition, we have revealed that NY-ESO-1 was immunogenic in ATLL patients and elicited specific humoral and cellular immune responses in a subset of patients. These data strongly support CT antigens as novel targets for ATLL immunotherapy.

The frequency of humoral immune responses in ATLL patients was much higher against NY-ESO-1 compared with other CT antigens such as MAGE-A3 and MAGE-A4. Although NY-ESO-1 expression by ATLL cells is presumably required for antibody induction, the level of NY-ESO-1 mRNA expression did not reflect the induction of humoral immune responses (Supplemental Table 2). This lack of correlation appears to be in part due to immunological properties of NY-ESO-1 antigen itself.[19,26] It has been shown that polymeric structures of NY-ESO-1 through disulfide bonds and their interaction with Calreticulin-TLR4 on immature DC surface are

required to induce phagocytosis of NY-ESO-1 protein and deliver danger signals for making the protein immunogenic.[34] In addition, some non-MAGE-A members of the MAGE family are ubiquitously expressed, and therefore possibly in more stable tolerance compared with NY-ESO-1. These properties of NY-ESO-1 and MAGE proteins might make the former more immunogenic than the latter.

Despite high immunogenicity of NY-ESO-1, the frequency (13.6%, 3/22) of primary ATLL patients who spontaneously developed NY-ESO-1 antibody responses against NY-ESO-1 expressing leukemic cells was lower when compared with the frequencies in patients with malignant melanoma or non-small-cell lung cancers (NSCLC) expressing NY-ESO-1 (approximately 50%).[19,26] This may reflect the feature of ATLL that tumor cells from the majority of ATLL patients express Foxp3, a key transcription factor for $CD25^+CD4^+$ Tregs.[6,9,35-37] ATLL cells from a subset of patients indeed appear to function as Tregs and contribute to profound immunosuppression that hampers the host's immune responses.[6,36] Alternatively or additionally, the chemokine CCL22 produced by ATLL cells might enhance the migration of CCR4-expressing $CD25^+CD4^+$ Tregs to tumor sites.[38] It remains to be determined whether Tregs in ATLL patients or ATLL cells themselves suppress NY-ESO-1-specific immune responses. Yet, it has been shown that helper T cell responses against NY-ESO-1 are subject to active suppression by Tregs in patients with solid tumors and healthy individuals.[30,37] Further studies are required to understand immunosuppressive property of ATLL in order to enhance immune responses against CT antigens in ATLL patients.

NY-ESO-1-specific cellular immune responses were detected in a significant number (five out of nine, 55.6%) of ATLL patients in partial or complete remission. This indicates that reducing the number of ATLL cells before CT antigen-targeted

immunotherapy may be a crucial component to successfully induce/augment antigen-specific $CD8^+$ T cells. We have recently reported that humanized anti-CCR4 mAb, KW-0761, showed clinically significant anti-tumor activity as a salvage therapy for patients with relapsed ATLL.[8,39] Pt. #2, #4, and #14 from whom we detected NY-ESO-1-specific $CD8^+$ T cells, were in complete or partial remission after anti-CCR4 mAb treatment.[8,39] As CCR4 is expressed on ATLL cells as well as $CD25^+CD4^+FOXP3^+$ Tregs, in addition to T-helper type 2 cells,[8,40,41] anti-CCR4 mAb treatment can reduce not only ATLL cells but also endogenous $CD25^+CD4^+FOXP3^+$ cells[42], thereby contributing to evoking NY-ESO-1-specific $CD8^+$ T cell responses. Indeed, the high frequency of NY-ESO-1-specific $CD8^+$ T cells detectable in vitro in ATLL patients could be in part due to the absence of Tregs. Thus, combining CT antigen-targeted immunotherapy following reduction of endogenous Tregs as well as ATLL cells by anti-CCR4 mAb treatment would be an ideal strategy for ATLL immunotherapy.

NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cell responses observed in Pt. #19 who was in a complete remission after allogeneic hematopoietic stem cell transplantation suggest an association of immune responses against CT antigens with a graft-versus-ATLL effect. This indicates that CT antigen-targeted immunotherapy combined with allogeneic stem cell transplantation may augment the efficacy of the current allogeneic hematopoietic stem cell transplantation for treating ATLL. In addition, as HTLV-1 Tax-specific $CD8^+$ T cells reportedly contribute to graft-versus-ATLL effects,[43] a combination immunotherapy with CT antigen and Tax after stem cell transplantation might also be therapeutically effective. Further, considering the reported high efficacy of NY-ESO-1-targeted adoptive T cell therapy against malignant melanoma and synovial cell sarcoma,[44,45] similar adoptive T cell therapy for ATLL could also be effective.

ATLL is, to our knowledge, the first example of high expression of CT antigens in leukemia or lymphomas as detailed analyses of CT antigen expression in other hematologic disorders have been limited.[21,22] It contrasted with a previous report showing that T-cell lymphoma lacks expression of CT antigens, except SCP-1. As a likely mechanism for transcriptional activation of the CT antigen genes in malignant cells, it has been suggested that the expression of CT antigen is induced by CpG island hypomethylation at the promoter regions.[18] An inflammatory environment created by infection may also trigger NY-ESO-1 expression. It cannot be excluded that viral components may promote this hypomethylation at the promoter regions or elicit inflammatory environment, thereby inducing CT antigen expression. Yet, we failed to observe any correlation between viral gene expression such as Tax and HBZ and CT antigen expression (Supplemental Table 2). In addition, high titers of HTLV-1 Gag/Env antibody responses were not associated with the induction of humoral immune responses against CT antigens (Supplemental Table 2). Peripheral T-cell lymphomas, not otherwise specified (PTCL-NOS) are particularly heterogeneous; yet, the CCR4 positive subset of PTCL-NOS might be a distinct disease entity whose clinicopathological features and genomic profiles were reportedly very similar to the lymphoma type of ATLL.[24,46,47] PTCL-NOS, especially the CCR4 positive subset, have a very poor prognosis like ATLL, and no standard treatment strategies are available.[48] Notably, we have found that CCR4-positive PTCL-NOS also expressed NY-ESO-1 at a high frequency (unpublished data). These data, when taken together, indicate that the frequent expression of CT antigens in ATLL may not be induced by HTLV-1 infection itself, but rather it may constitute a novel subtype of T cell leukemia/lymphoma that expresses CCR4 and CT antigens regardless of HTLV-1 infection.

A small population (approximately 5%) of HTLV-1-infected individuals progresses to ATLL after a long latency period of about 50-70 years.[2] Although the detailed mechanisms of this leukemogenesis from HTLV-1 infection to ATLL have not yet been well elucidated, host immune responses against HTLV-1-infected cells have been suspected to play an important role.[8,16,49] Interestingly, some of HTLV-1-infected asymptomatic HTLV-1 carriers harbored significant titers of antibody against NY-ESO-1, MAGE-A3 and MAGE-A4, despite that CT antigens including NY-ESO-1, MAGE-A3 and MAGE-A4 were not detected at the mRNA level in their PBMCs (Supplemental Figure 1). This discrepancy raises several possibilities; for example, the number or the frequency of HTLV-1-infected cells expressing CT antigens in circulating PBMCs may be too low to detect the antigen. Alternatively, it is possible that HTLV-1-infected cells expressing CT antigens may be eliminated in HTLV-1-infected asymptomatic carriers by anti-CT antigen-specific immune responses as tumor immunosurveillance,[50] while antibody responses may linger on. Future studies with a large cohort of HTLV-1-infected asymptomatic carriers need to address the kinetics of their CT antigen expression during a long latency period and their immune responses against the antigen in the course of ATLL development. The studies will not only provide a rationale for including CT antigens as possible targets of ATLL immunotherapy but also contribute to our understanding of the multi-step oncogenesis of ATLL and devising preventive strategies for ATLL by targeting CT antigens.

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Authorship contribution

H.N., T.I., S.G., L.J.O., R.U., and S.S. designed the research; H.N., Y.M., T.I., S.G., E.S., F.M., D.S., A.I. and Y.F. performed experiments; T.I., F.M., A.I, A.U., H.I. and R.U. collected samples and obtained clinical data; H.N., Y.M., T.I., S.G., E.S., Y.F., R.U., and S.S. analyzed data; H.N., Y.M., T.I., S.G., E.S., R.U., and S.S. wrote the paper.

Disclosure of Conflicts of Interest

The authors have no potential conflicts of interest.

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Table 1. Summary of CT antigen expression in ATLL cells from primary ATLL patients*

	Number of Pts	NY-ESO-1	MAGE	
			A3	A4
Age (mean 60.6)				
≥ 60	29	20	12	20
< 60	28	15	6	15
Sex				
Male	30	17	8	17
Female	27	18	10	18
Disease type				
Aggressive	40	25 (62.5%)	14 (35.0%)	26 (65.0%)
Acute	37	22	13	24
Lymphoma	3	3	1	2
Indolent	17	9 (52.9%)	4 (23.5%)	9 (52.9%)
Chronic	11	7	2	8
Smoldering	6	3	2	1

* mRNA expression of NY-ESO-1, MAGE-A3, and MAGE-A4 in ATLL cells from primary ATLL patients was analyzed with RT-PCR. CT antigen expression was summarized based on several clinical parameters.

Figure legends

Figure 1. NY-ESO-1, MAGE-A3, and MAGE-A4 are widely expressed by primary ATLL cells.

(A) Representative result of RT-PCR analysis for mRNA expression of NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, SSX-4 and SCP-1. (B) ATLL samples were subjected to immunohistochemical staining for NY-ESO-1 (E978) mAb and pan-MAGE (57B) mAb (bar = 50 μm). These experiments were performed independently at least twice with similar results.

Figure 2. Humoral immune responses against NY-ESO-1 are detected in a subset of patients with ATLL.

Sera were collected from 43 primary ATLL patients and antibody responses against 10 CT antigens (NY-ESO-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, CT-10, SSX-1, SSX-2, and SSX-4) were analyzed by ELISA as described in Methods. This experiment was performed at least twice with similar results.

Figure 3. NY-ESO-1-specific $CD8^+$ T cells are detected in ATLL patients.

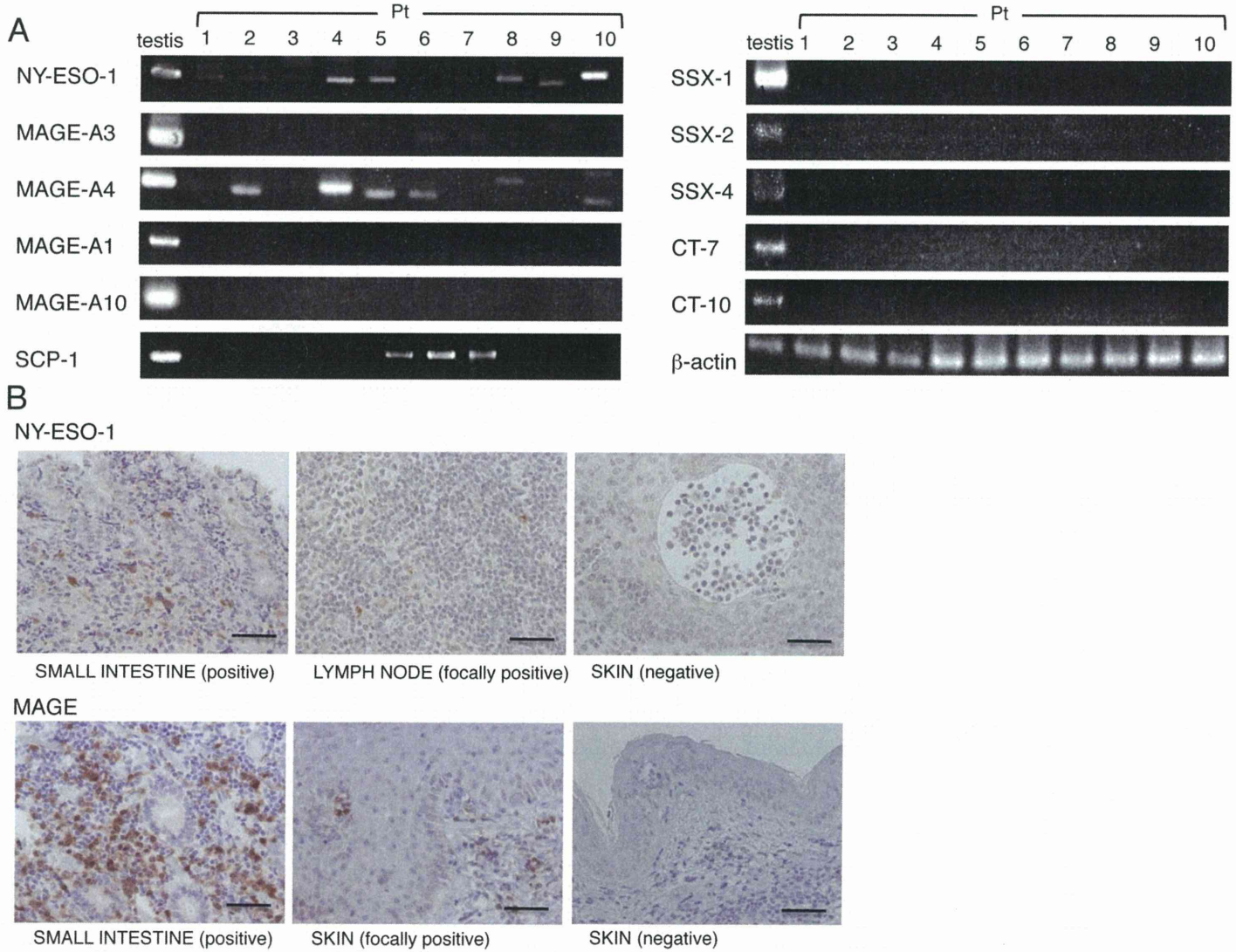
$CD8^+$ T cells derived from PBMCs of Pt #1, 2, 4, 8, 13, 14, 19, 27, and 43 were pre-sensitized by $CD4^-$ $CD8^-$ PBMCs pulsed with NY-ESO-1 peptides covering the entire sequence of NY-ESO-1 as described in Methods. (A) Induction of specific $CD8^+$ T cells was analyzed by staining with NY-ESO-1/HLA tetramers indicated. Cytokine (IFN-γ and TNF-α) secreting capacity of NY-ESO-1-specific $CD8^+$ T cells was analyzed by intracellular cytokine staining for recognition of (B) autologous activated-T-cell APCs

pulsed with NY-ESO-1 peptides or (C) autologous ATLL cells. These experiments were performed independently at least twice with similar results.

Figure 4. NY-ESO-1-specific $CD4^+$ T cells are present in an ATLL patient (Pt. #19) receiving an allogeneic hematopoietic stem cell transplantation.

$CD4^+$ T cells were pre-sensitized by $CD4^-$ $CD8^-$ PBMCs pulsed with NY-ESO-1 peptide covering the entire sequence of NY-ESO-1 as described in Methods. Induction of NY-ESO-1-specific $CD4^+$ T cells was analyzed by intracellular cytokine staining using autologous activated-T-cell APCs pulsed with NY-ESO-1 peptides. These experiments were performed independently twice with similar results.

Figure 1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testis 1 2 3 4 5 6 7 8 9 10
SSX-1
SSX-2
SSX-4
CT-7
CT-10
β-actin
B
NY-ESO-1
SMALL INTESTINE (positive)
LYMPH NODE (focally positive)
SKIN (negative)
MAGE
SMALL INTESTINE (positive)
SKIN (focally positive)
SKIN (negative)