Autologous Tax-Specific CTL Therapy in a Primary Adult T Cell Leukemia/Lymphoma Cell–Bearing NOD/Shi-scid, IL-2Rγnull Mouse Model

Ayako Masaki, Takashi Ishida, Susumu Suzuki, Asahi Ito, Fumiko Mori, Fumihiko Sato, Tomoko Narita, Tomiko Yamada, Masaki Ri, Shigeru Kusumoto, Hirokazu Komatsu, Yuetsu Tanaka, Akio Niimi, Hiroshi Inagaki, Shinsuke Iida and Ryuzo Ueda

*J Immunol* 2013; 191:135-144; Prepublished online 3 June 2013;
doi: 10.4049/jimmunol.1202692
http://www.jimmunol.org/content/191/1/135

**References**

This article *cites 44 articles*, 18 of which you can access for free at: http://www.jimmunol.org/content/191/1/135.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Autologous Tax-Specific CTL Therapy in a Primary Adult T Cell Leukemia/Lymphoma Cell–Bearing NOD/Shi-scid, IL-2Rγnull Mouse Model

Ayako Masaki,* Takashi Ishida,* Susumu Suzuki,*,† Asahi Ito,* Fumiko Mori,* Fumihiko Sato,*‡ Tomoko Narita,* Tomiko Yamada,* Masaki Ri,* Shigeru Kusumoto,* Hirokazu Komatsu,* Yuetsu Tanaka,† Akio Niimi,* Hiroshi Inagaki,* Shinsuke Iida,* and Ryuzo Ueda†

We expanded human T-lymphotropic virus type 1 Tax-specific CTL in vitro from PBMC of three individual adult T cell leukemia/lymphoma (ATL) patients and assessed their therapeutic potential in an in vivo model using NOG mice bearing primary ATL cells from the respective three patients (ATL/NOG). In these mice established with cells from a chronic-type patient, treatment by i.p. injection of autologous Tax-CTL resulted in greater infiltration of CD8-positive T cells into each ATL lesion. This was associated with a significant decrease of ATL cell infiltration into blood, spleen, and liver. Tax-CTL treatment also significantly decreased human soluble IL-2R concentrations in the sera. In another group of ATL/NOG mice, Tax-CTL treatment led to a significant prolongation of survival time. These findings show that Tax-CTL can infiltrate the tumor site, recognize, and kill autologous ATL cells in mice in vivo. In ATL/NOG mice with cells from an acute-type patient, whose postchemotherapeutic remission continued for >18 mo, antitumor efficacy of adoptive Tax-CTL therapy was also observed. However, in ATL/NOG mice from a different acute-type patient, whose ATL relapsed after 6 mo of remission, no efficacy was observed. Thus, although the therapeutic effects were different for different ATL patients, to the best of our knowledge, this is the first report that adoptive therapy with Ag-specific CTL expanded from a cancer patient confers antitumor effects, leading to significant survival benefit for autologous primary cancer cell–bearing mice in vivo. The present study contributes to research on adoptive CTL therapy, which should be applicable to several types of cancer. The Journal of Immunology, 2013, 191: 135–144.

Adult T cell leukemia/lymphoma (ATL) is a distinct hematologic malignancy caused by human T-lymphotropic virus type 1 (HTLV-1) (1–4). ATL patients have a very poor prognosis for which no standard treatment strategy is available (5, 6). Over the last decade, allogegeneic hematopoietic stem cell transplantation has evolved into a potential approach to treat ATL patients. However, only a small fraction of patients can benefit from transplantation, such as those who are younger, have achieved sufficient disease control, and have an appropriate stem cell source (7, 8). Therefore, the development of alternative treatment strategies for ATL patients is an urgent issue.

HTLV-1 Tax, a virus-encoded regulatory gene product, is required for the virus to transform cells (9) and is thought to be indispensable for oncogenesis. Therefore, Tax has been considered as a molecular target for immunotherapy against ATL (10–14). However, it was reported that the level of Tax expression in HTLV-1–infected cells decreases during disease progression, and Tax transcripts are detected only in ~40% of established ATL cases (15). Moreover, weak or absent responses to Tax were observed in ATL patients (16), leading to controversy as to whether Tax is an appropriate target for immunotherapy of ATL. In this context, we have recently reported the potential relevance of Tax as a target for ATL immunotherapy. Tax-specific CTL recognized HLA/Tax–peptide complexes on autologous ATL cells and killed them, even when their Tax expression was so low that it could only be detected by RT-PCR but not at the protein level in vitro (17). However, in general, tumors develop in a complex and dynamic microenvironment in humans (18–20). Therefore, antitumor activities of cancer-specific CTL should be evaluated under conditions including the cancer microenvironment. In addition, susceptibility to CTL is different in established cell lines and primary tumor cells isolated directly ex vivo from patients, especially autologous tumor cells, with the latter certainly being more relevant for evaluating antitumor effects of CTL. Based on these considerations, we expanded Tax-specific CTL in vitro from PBMC of ATL patients and tested in this study the potential significance of Tax as a target for ATL immunotherapy in an in vivo model consisting of NOD/Shi-scid, IL-2Rγnull (NOG) mice (21) bearing the autologous primary ATL cells (ATL/NOG).
Materials and Methods

Primary human cells

Primary ATL cells were obtained from three individual patients of which patient 1 had chronic-type and patients 2 and 3 were acute. Diagnosis and classification of clinical subtypes of ATL was according to the criteria proposed by the Japan Lymphoma Study Group (22). Mononuclear cells were isolated from blood or lymph node cells with Ficoll-Paque (Phar- macia, NJ). Primary ATL cells were separated using anti-human CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by means of an autoMACS Pro (Miltenyi Biotec). Genotyping of HLA-A, -B, and -C was performed using an HLA-typing Kit (WAKFLOW HLA-typing kit; Wakunaga Pharmacy, Hiroshima, Japan). The disease activity of patient 1 was stable; this patient had been carefully observed under a wait-and-see policy for ∼4 y prior to sampling. Both patients 2 and 3 received systemic chemotherapies and achieved complete remissions. Patient 2 remained in remission for ∼18 mo, but in patient 3, ATL relapsed after only 6 mo in remission. Thus, patient 3 subsequently again received systemic chemotherapy for his relapsed ATL. In patients 2 and 3, primary ATL cells were obtained at first diagnosis, and PBMC for CTL expansion were obtained in remission. They were cryopreserved until use. 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, and TL-Oml are ATL cell lines, TL-Su, TCM-Kan, and MT-4 are HTLV-1-immortalized lines, and K562 is a chronic myelogenous leukemia blast crisis cell line, as previously described (17).

Expansion of HTLV-1 Tax-specific CTL

PBMC from the ATL patients were suspended in RPMI 1640 (Cell Science and Technology Institute, Sendai, Japan) supplemented with 10% human serum and 0.1 μM Tax epitope peptide (LLFGYPVV or SFHSLHLL; Invitrogen, Carlsbad, CA) at a cell concentration of 2.0×10⁶/ml. The cells were cultured at 37°C in 5% CO₂ for 2 h after which brefeldin A (BD Biosciences) was added at 10 μg/ml. Intracellular IFN-γ staining, the expansion of ATL cells from all three patients were positive and HLA-A*24:02/Tax 301–309 tetramer-positive cells, 7.89×10⁶/ml (7.53×10⁵/ml; 7.53×10⁵/ml), and 29 d (18.8×10⁵/ml; 16.1×10⁵/ml) after tumor inoculations. Control RPMI 1640 was i.p. injected in the same manner. The infiltration of ATL cells into the organs, and the levels of human sIL-2R in the sera 31 d after tumor inoculation were determined.

Immunopathological analysis

H&E staining and immunostaining by anti-CD4 (4B12; Novocastra, Wetzlar, Germany), CD25 (4C9; Novocastra), and CD8 (C8/144B; DakoCytomation, Glostrup, Denmark) was performed on formalin-fixed, paraffin-embedded sections, using a Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) with the Bond polymer refine detection kit (Leica Microsystems).

Statistical analysis

The differences between groups regarding the percentage of ATL cells in mouse whole blood cells, liver, and spleen cell suspensions and human sIL-2R concentrations in the serum were examined with the Mann-Whitney U test. Survival analysis was done by the Kaplan-Meier method, and survival curves were compared using the log-rank test. All analyses were performed with SPSS Statistics 17.0 (SPSS, Chicago, IL). In this study, p = 0.05 was considered significant.

Results

Expression of HTLV-1 Tax-specific CTL

PBMC from the ATL patients were suspended in RPMI 1640 (Cell Science and Technology Institute, Sendai, Japan) supplemented with 10% human serum and 0.1 μM Tax epitope peptide (LLFGYPVV or SFHSLHLL; Invitrogen, Carlsbad, CA) at a cell concentration of 2.0×10⁶/ml. The cells were cultured at 37°C in 5% CO₂ for 2 h after which brefeldin A (BD Biosciences) was added at 10 μg/ml. Intracellular IFN-γ staining, the expansion of ATL cells from all three patients were positive and HLA-A*24:02/Tax 301–309 tetramer-positive cells, 7.89×10⁶/ml (7.53×10⁵/ml; 7.53×10⁵/ml), and 29 d (18.8×10⁵/ml; 16.1×10⁵/ml) after tumor inoculations. Control RPMI 1640 was i.p. injected in the same manner. The infiltration of ATL cells into the organs, and the levels of human sIL-2R in the sera 33 d after tumor inoculation were determined.

Immunopathological analysis

H&E staining and immunostaining by anti-CD4 (4B12; Novocastra, Wetzlar, Germany), CD25 (4C9; Novocastra), and CD8 (C8/144B; DakoCytomation, Glostrup, Denmark) was performed on formalin-fixed, paraffin-embedded sections, using a Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany) with the Bond polymer refine detection kit (Leica Microsystems).

Statistical analysis

The differences between groups regarding the percentage of ATL cells in mouse whole blood cells, liver, and spleen cell suspensions and human sIL-2R concentrations in the serum were examined with the Mann-Whitney U test. Survival analysis was done by the Kaplan-Meier method, and survival curves were compared using the log-rank test. All analyses were performed with SPSS Statistics 17.0 (SPSS, Chicago, IL). In this study, p = 0.05 was considered significant.

Results

Tax expression in ATL cells from patients

The inoculated primary ATL cells from all three patients were positive for CD4 and CD25 (Fig. 1A, left panel, Fig. 1C, 1D, top left panels). Tax proteins were weakly detected in a subpopulation of ATL cells from all patients by flow cytometry (Fig. 1A, right two panels, and Fig. 1C, 1D, top right two panels). The Tax/human β-actin mRNA levels of the ATL cells from patients 1, 2, and 3, were 0.192±0.005 (SD), 0.492±0.054, and 0.080±0.009, respectively, when the value of TL-Su was set at unity as previously described (17) (Fig. 2D). Although the short time of in vitro culture changes the expression levels of Tax in primary ATL cells (17, 24), the result presented in this study was obtained at the same time as performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia, Second Edition, and were approved by the Ethics Committee of the Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences.

CD4-positive primary ATL cells were separated from PBMC of patient 1 and suspended at 1×10⁶ cells per 0.2 ml RPMI 1640, which were i.p. inoculated into each of 20 NOG mice. The inoculated ATL cells consisted of several blood samplings. The primary ATL-bearing mice were divided into two groups of 10 each. One group was used for evaluation of ATL cell organ infiltration and to measure levels of human soluble IL-2R (sIL-2R) in sera using the human sIL-2R immunoassay kit (R&D Systems, Minneapolis, MN) 27 d after tumor inoculation. The other group was used for evaluation of survival. Each group was further divided into two groups of five each for autologous Tax-CTL or control (0.2 ml RPMI 1640) injections. Autologous Tax-CTL suspended in 0.2 ml RPMI 1640 were i.p. injected 2 (mononuclear cells, 4.59×10⁶/mouse; CD8-positive and HLA-A*24:02/Tax 301–309 tetramer-positive cells, 7.89×10⁶/ml), 7 (3.57×10⁶; 9.71×10⁶), 12 (3.26×10⁶; 5.49×10⁶), 20 (3.12×10⁶; 5.48×10⁶), and 23 (2.51×10⁶; 4.22×10⁶) after ATL cell inoculations. Control RPMI 1640 was i.p. injected in the same manner.

PBMC of patient 2, consisting of ∼80% of CD4⁺/CD25⁺ ATL cells, were suspended in 0.2 ml of RPMI 1640 and i.p. inoculated into each of six NOG mice. The primary ATL-bearing mice were divided into two groups of three for each autologous Tax-CTL or control injections. Autologous Tax-CTL suspended in 0.2 ml RPMI 1640 were i.p. injected 2 (mononuclear cells, 7.50×10⁶/mouse; CD8-positive and HLA-A*24:02/Tax 301–309 tetramer-positive cells, 18.1×10⁶/ml), 7 (6.75×10⁶; 22.3×10⁶), 14 (5.95×10⁶; 20.7×10⁶), 21 (5.70×10⁶; 22.3×10⁶), and 23 d (6.04×10⁶; 21.3×10⁶) after ATL cell inoculations. Control RPMI 1640 was i.p. injected in the same manner. The infiltration of ATL cells into the organs, and the levels of human sIL-2R in the sera 33 d after tumor inoculation were determined.

The inoculated primary ATL cells from all patients were positive for CD4 and CD25 (Fig. 1A, left panel, Fig. 1C, 1D, top left panels). Tax proteins were weakly detected in a subpopulation of ATL cells from all patients by flow cytometry (Fig. 1A, right two panels, and Fig. 1C, 1D, top right two panels). The Tax/human β-actin mRNA levels of the ATL cells from patients 1, 2, and 3, were 0.192±0.005 (SD), 0.492±0.054, and 0.080±0.009, respectively, when the value of TL-Su was set at unity as previously described (17) (Fig. 2D). Although the short time of in vitro culture changes the expression levels of Tax in primary ATL cells (17, 24), the result presented in this study was obtained at the same time as performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia, Second Edition, and were approved by the Ethics Committee of the Center for Experimental Animal Science, Nagoya City University Graduate School of Medical Sciences.
the in vitro experiments were performed, showing Tax-specific CTL responses against autologous ATL cells (Fig. 2A–C).

**Adoptively transferred autologous Tax-specific CTL**

Flow cytometric analyses of the expanded and adoptively transferred Tax-CTL of patient 1 at days 2, 7, 12, 20, and 23 are presented. The lymphocyte population was identified by forward light scatter-height (FSC-H) and side scatter-height (SSC-H) values (Fig. 1B, left panels) and is plotted to show CD8 and HLA-A*02:01/Tax tetramer positivity (right panel). The inoculated primary ATL cells from patient 2 were positive for CD4 and CD25 (top left panel). Tax protein was weakly detected in a subpopulation of ATL cells (top right two panels). Autologous adoptively transferred Tax-CTL from patient 2 are presented. Lymphocyte population is determined by FSC-H and SSC-H levels (bottom left panel) and plotted to show CD8 and HLA-A*02:01/Tax tetramer positivity (bottom right panel). The inoculated primary ATL cells from patient 3 were positive for CD4 and CD25 (top left panel). Tax protein was weakly detected in a subpopulation of ATL cells (top right two panels). Autologous adoptively transferred Tax-CTL from patient 3 are also shown in Fig. 1C and 1D, bottom panels, respectively.

**Tax-specific CTL responses against autologous ATL cells in vitro**

The adoptively transferred Tax-CTL from patient 1 were cocultured with autologous ATL cells, ATL cell lines, HTLV-1-immortalized lines, or K562, and their responses were evaluated by IFN-γ production in vitro (Fig. 2A, 2D). HLA-A*24:02/Tax301–309 tetramer-positive fractions of these expanded CD8-positive cells produced IFN-γ when cocultured with autologous ATL cells, TL-Su, or ATN-1. These tetramer-positive cells did not respond to MT-1, MT-4, or TCL-Kan. These results indicate that only target cells having both HLA-A*24:02 and Tax were recognized. The tetramer-negative fractions of these expanded CD8-positive cells also produced IFN-γ when stimulated with autologous ATL cells. This suggests that they recognize unidentified Tax-derived epitopes, Ags derived from HTLV-1 components other than Tax, or ATL-related tumor Ags not of viral origin such as cancer-testis Ags (25). The tetramer-negative fractions of these expanded CD8-positive cells also produced IFN-γ when stimulated with TCL-Kan. Because both patient 1 and TCL-Kan share HLA-A*02:07, -B*46:01, and -C*01:02, the tetramer-negative cells might be recognizing unidentified Tax-derived epitopes, other HTLV-1 Ags or ATL tumor Ag-derived epitopes presented on a different shared MHC allele. These effector cells did not respond to K562 by IFN-γ production, showing that they had no NK activity.

The adoptively transferred Tax-CTL from patient 2 were tested next. HLA-A*02:01/Tax11–19 tetramer-positive fractions of these expanded CD8-positive cells specifically produced IFN-γ when stimulated with 0.1 μM of the corresponding peptide. These cells also respond to target cells including autologous ATL cells in a manner restricted by Tax expression and the appropriate HLA type as did patient 1 (Fig. 2B, 2D).

The adoptively transferred Tax-CTL from patient 3 were also tested. Although HLA-A*24:02/Tax301–309 tetramer-positive fractions of these expanded CD8-positive cells responded to TL-Su and the corresponding peptide by producing IFN-γ, they did not respond to autologous ATL cells or ATN-1, the Tax expression of which was relatively low (Fig. 2C, 2D).

**Macroscopic findings in ATL/NOG mice with cells from patient 1 treated or not treated with adoptive autologous Tax-CTL**

Ten primary ATL cell-bearing mice were evaluated for the efficacy of treatment by adoptive transfer of autologous Tax-CTL. The appearance of the mice treated with Tax-CTL and of the controls is shown in Fig. 3, top and bottom panels, respectively. In general, spleens were much more enlarged in the control mice than in the CTL-treated mice.

**Flow cytometric analyses of infiltrating ATL cells in organs of ATL/NOG mice with cells from patient 1**

The percentage of CD4-positive ATL cells in whole blood of control NOG mouse 1 was 0.57% (i.e., 0.57% [human CD45-positive population] × 100.0% [human CD4-positive CD8-negative cells] = 0.57%). In control NOG mice 2, 3, 4, and 5 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5, the percentages of ATL cells in whole blood, calculated in the same manner, were 1.57, 2.53, 0.18, and 0.94% and 0.22, 0.17, 0.01, 0.59, and 0.02%, respectively (Fig. 4A). Thus, Tax-CTL treatment significantly reduced the percentage of ATL cells present in the blood of these mice (p = 0.047; Fig. 5A, left panel).
The percentages of CD8-positive CD4-negative T cells in the whole blood of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.10, 0.55, 0.00, 0.55, and 0.03%, respectively (Fig. 4A, bottom panels).

The percentage of CD4-positive ATL cells in spleen cell suspensions of control NOG mouse 1 was 0.43% (i.e., 0.46% [human CD45-positive population] \( \times \) 94.35% [human CD4-positive CD8-negative cells] = 0.43%). In control NOG mice 2, 3, 4, and 5 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5, the percentages of ATL cells in the spleen cell suspensions, calculated in the same manner, were 3.24, 1.83, 1.97, and 5.32% and 0.24, 0.09, 0.02, 0.11, and 2.98%, respectively (Fig. 4B). Thus, Tax-CTL treatment significantly reduced the percentage of ATL cells present in the spleens of these mice as well as in the blood (\( p = 0.047 \); Fig. 5A, middle panel). Again, the percentages of CD8-positive CD4-negative T cells in the spleen cell suspensions of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.10, 0.29, 0.02, 0.07, and 2.26%, respectively (Fig. 4B, bottom panels).

The percentages of CD4-positive ATL cells in liver cell suspensions were also quantified. In control NOG mouse 1, this value was 0.25% (i.e., 0.26% [human CD45-positive population] \( \times \) 94.62% [human CD4-positive CD8-negative cells] = 0.25%). In control NOG mice 2, 3, 4, and 5 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5, the percentages of ATL cells in the liver cell suspensions, calculated in the same manner, were 0.50, 0.64, 0.42, and 2.00% and 0.10, 0.05, 0.02, 0.02, and 0.18%, respectively (Fig. 4C). Thus, Tax-CTL treatment also significantly reduced the percentage of ATL cells present in the livers of these mice (\( p = 0.009 \); Fig. 5A, right panel). The percentages of CD8-positive CD4-negative T cells in the liver cell suspensions of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.01, 0.16, 0.02, 0.01, and 0.12%, respectively (Fig. 4C, bottom panels).

Microscopy findings in spleens of ATL/NOG mice receiving cells from patient 1 with or without adoptive autologous Tax-CTL therapy

In the control NOG mice, large atypical cells with irregular and pleomorphic nuclei proliferated with a multifocal pattern and...
replaced normal splenic architecture. Immunopathological analyses of control mouse 4 are shown in Fig. 4D (three left panels). The atypical cells were positive for CD4 and CD25 (data not shown), but negative for CD8, consistent with their identity as infiltrating ATL cells. In the Tax-CTL–treated NOG mice, atypical cells proliferated with a patchy pattern. Immunopathological analyses of Tax-CTL-treated NOG mouse 5 are shown in Fig. 4D (right three panels). The atypical cells were positive for CD4 and CD25 (data not shown), but negative for CD8, again consistent with ATL cell infiltration. ATL tumor-infiltrating CD8-positive cells were also present, consistent with the flow cytometric analyses showing the presence of CTL (Fig. 4B).

**Tax-CTL treatment significantly decreases human sIL-2R concentrations in serum of NOG mice bearing primary ATL cells from patient 1**

We measured human sIL2R concentrations in serum as a reliable surrogate marker reflecting ATL tumor burden (26) in the mice. The serum sIL-2R concentrations in control NOG mice 1, 2, 3, 4 and 5 and Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 28,087, 36,924, 34,611, 36,906, and 42,955 and 0, 0, 0, 0, and 1.061 pg/ml, respectively. Thus, Tax-CTL treatment significantly decreased the ATL tumor burden present in these mice ($p = 0.007$; Fig. 5B).

**Tax-CTL treatment results in a significant prolongation of survival of primary patient 1 ATL cell–bearing NOG mice**

Tax-CTL recipients had a significant benefit in terms of prolongation of survival compared with controls (Fig. 6A; $p = 0.002$). In order to estimate the ATL cell tumor burden during CTL treatment in both groups, flow cytometry analyses of whole blood cells were performed. Thirty-one days after ATL cell inoculation, the percentage of CD4-positive CD8-negative ATL cells in the blood of control NOG mouse 1 was 2.48% (i.e., 2.49% [human CD45-positive population] × 99.60% [human CD4-positive and CD8-negative cells] = 2.48%). In control NOG mice 2, 3, 4, and 5 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5, the percentages of ATL cells in whole blood, calculated in the same manner, were 0.62, 0.56, 0.77, and 1.22% and 0.20, 1.59, 0.11, 0.04, and 0.05%, respectively. At this time, the percentages of CD8-positive CD4-negative T cells in the whole blood of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.32, 1.09, 0.15, 0.19, and 0.07%, respectively (Fig. 6B, top panels).

In the same animals, 38 d after ATL cell inoculation, the percentages of CD4-positive ATL cells in the whole blood of control NOG mice 1, 2, 3, 4 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 1.59, 5.83, 1.79, and 0.88% and 0.08, 2.88, 0.06, 0.00 and 0.04%, respectively. Control NOG mouse 5 sickened and died on day 34 due to ATL progression. At this time, the percentages of CD8-positive CD4-negative T cells in the blood of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.21, 3.72, 0.08, 0.08, and 0.01%, respectively (Fig. 6B, top panel).

Forty-five days after ATL cell inoculation, the percentage of CD4- and CD25-positive ATL cells in the whole blood of control NOG mouse 1 was 4.60% (i.e., 4.70% [human CD45-positive population] × 97.77% [human CD4-positive and CD25-positive cells] = 4.60%). In control NOG mice 2, 3, and 4 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5, the percentages of ATL cells in whole blood, calculated in the same manner, were 7.07, 1.26, and 1.11 and 0.05, 6.96, 0.04, 0.01, and 0.02%, respectively (Fig. 6B, bottom, second panel).

Seventy-nine days after ATL cell inoculation, the percentages of CD4-positive ATL cells in the whole blood of control NOG mouse 3 and in Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.45, and 0.00, 1.27, 0.01, 0.00, and 0.00%, respectively. Control NOG mouse 1, 2, and 4 sickened and died on days 47, 47, and 78, respectively (Fig. 6B, bottom panels). At this time, the percentages of CD8-positive CD4-negative T cells in the whole blood of Tax-CTL–treated NOG mice 1, 2, 3, 4, and 5 were 0.00, 1.11, 0.01, 0.00, and 0.00%, respectively (Fig. 6B, bottom panels).

Throughout the study, no toxicity attributable to CTL injections was observed in any of the mice that had received cells from patient 1.

**FIGURE 3.** Macroscopic findings in ATL/NOG mice with cells from patient 1 with or without adoptive autologous Tax-CTL therapy. The appearance of mice treated with Tax-CTL and of the controls is shown in the bottom and top panels, respectively. Spleens were much more enlarged in the control mice compared with CTL-treated mice.
In the blood of CTL-treated mouse 2, not only the CD4-positive ATL cells, but also relatively high levels of CD8-positive cells persisted more than in the other CTL-treated mice. We surmise that the residual ATL cells might persistently stimulate adoptively transferred CD8-positive cells, leading to the expansion of these T cells in the mouse.

**FIGURE 4.** Analyses of ATL cell infiltration of cells from patient 1 into the organs. Human CD45-positive cells in ATL/NOG mice plotted to show CD4 and CD8 expression in blood (A), spleen (B), and liver (C). The CD4-positive, CD8-negative cells are ATL cells, and the CD8-positive, CD4-negative cells are the adoptively transferred cells. CD8Low populations observed in the spleen (B) and liver (C) cells from CTL-treated mice are nonspecific signals. The percentage of each cell type is indicated in each panel. (D) Microscopy findings in spleens of mice with or without adoptive autologous Tax-CTL therapy. Immunopathological analyses of control mouse 4 are shown. The atypical cells were positive for CD4, but negative for CD8, consistent with ATL cell infiltration (left three panels). Immunopathological analyses of Tax-CTL–treated NOG mouse 5 indicate atypical cells positive for CD4, but negative for CD8, consistent with ATL cell infiltration. ATL tumor-infiltrating CD8-positive cells were also observed (right three panels). No toxicity attributable to CTL injections was observed in any of the mice. Original magnification ×200.

**FIGURE 5.** Therapeutic efficacy of adoptively transferred autologous Tax-CTL in a NOG mouse bearing primary ATL cells from patient 1. (A) The percentages of ATL cells in whole blood, spleen, or liver cell suspensions of each autologous primary ATL-bearing NOG mouse. Tax-CTL treatment led to a significant decrease of ATL cell infiltration into blood, spleen, and liver. (B) Human sIL-2R concentration in the serum of each autologous primary ATL-bearing NOG mouse. Tax-CTL treatment significantly decreased human sIL-2R concentrations in serum in the primary ATL cell–bearing NOG mice.
Therapeutic efficacy of adoptive autologous Tax-CTL in ATL/NOG mice receiving cells from patient 2

ATL cell infiltrations into the organs were evaluated by flow cytometry. The percentage of CD4-positive CD25-positive ATL cells in the whole blood of control NOG mouse 1 was 15.3\% (i.e., \(16.7\% \times 91.5\%\) [human CD45-positive CD8-negative cells] = 15.3\%). In control NOG mice 2 and 3 and in Tax-CTL–treated NOG mice 1, 2, and 3, the percentages of ATL cells in the bone marrow, calculated in the same manner, were 4.4 and 15.3\% and 3.3, 5.8, and 5.4\%, respectively (Figs. 7A, 8A, left panel).

The percentage of CD4-positive CD25-positive ATL cells in the bone marrow of control NOG mouse 1 was 0.71\% (i.e., 0.88\% [human CD45-positive population] \times 80.54\% [human CD4-positive CD8-negative cells] = 0.71\%). In control NOG mice 2 and 3 and in Tax-CTL–treated NOG mice 1, 2, and 3, the percentages of ATL cells in the bone marrow, calculated in the same manner, were 0.52 and 1.81\% and 0.23, 0.11, and 0.13\%, respectively (Figs. 7B, 8A, right panel).

Immunopathological analyses of liver demonstrated that in the control NOG mice, large atypical cells with irregular and pleomorphic nuclei proliferated with a patchy or focal pattern. The atypical cells were positive for CD4 (Fig. 7C, top panels) and CD25 (data not shown), consistent with their being infiltrating ATL cells.

In the Tax-CTL–treated NOG mice, there were few areas infiltrated...
by atypical cells. Images of CTL-treated mice are shown in Fig. 7C, bottom panels. The CD4- and CD25-positive cells are ATL cells. The percentages of each cell type are indicated in each panel. (C) Microscopy findings in livers of mice with or without adoptive autologous Tax-CTL therapy. No toxicity attributable to CTL injections was observed in any of the mice. Original magnification ×100.

FIGURE 7. Analyses of patient 2 ATL cell infiltration into the organs. Human CD45-positive cells in ATL/NOG mice plotted to show CD4 and CD25 expression in blood (A) and bone marrow (B). The CD4- and CD25-positive cells are ATL cells. The percentages of each cell type are indicated in each panel. (C) Microscopy findings in livers of mice with or without adoptive autologous Tax-CTL therapy. No toxicity attributable to CTL injections was observed in any of the mice. Original magnification ×100.

FIGURE 8. Therapeutic efficacy of adoptively transferred autologous Tax-CTL in a patient 2 primary ATL cell–bearing NOG mice. (A) The percentages of CD4- and CD25-positive ATL cells in whole blood and bone marrow of each autologous primary ATL-bearing NOG mouse. (B) Human sIL-2R concentration in serum of each autologous primary ATL-bearing NOG mouse. Tax-CTL treatment significantly decreased human sIL-2R concentrations in serum in the primary ATL cell–bearing NOG mice.
Therapeutic efficacy of adoptive autologous Tax-CTL in the ATL/NOG mice with cells from patient 3

In this case, Tax-CTL treatment did not show any therapeutic efficacy in controlling CD4-positive CD25-positive ATL cell infiltration into blood, spleen, liver, or bone marrow, as determined by flow cytometric analyses. There were also no significant differences between CTL-treated and control NOG mice in their serum human sIL-2R concentrations. Again, no toxicity attributable to CTL injections was observed in any of the mice. Collectively, the conclusion in this study must be that autologous Tax-CTL treatment did not decrease the ATL tumor burden present in these mice.

Discussion

In the current study, therapeutic efficacy of adoptive patient-autologous Tax-CTL against two out of three patients’ ATL cells was documented in vivo in ATL/NOG mice. In the mouse model with cells from patient 1, infiltration of substantial amounts of CD8-positive T cells into each ATL lesion was observed in the Tax-CTL–treated mice, associated with a significant decrease of ATL cell infiltration into blood, spleen and liver, relative to controls. Tax-CTL treatment significantly decreased human sIL-2R concentrations in the serum (reflecting reduced ATL tumor burden). The efficacy of CTL treatment was also assessed by survival analysis using other ATL/NOG mice. Tax-CTL treatment led to a significant prolongation of survival time compared with control ATL/NOG mice. Adverse events such as organ disorders caused by CTL treatment were not observed in any of the mice. These findings show that Tax-specific CTL infiltrated the tumor site, recognized, and killed autologous ATL cells in mice in vivo. Although Tax expression of the inoculated primary ATL cells from patient 1 (which were cultured in vitro) was low as assessed by flow cytometry (Fig. 1A), potent autologous CTL activity was observed in ATL/NOG mice in vivo. This was partially due to the fact that ATL cells present at the site of active cell proliferation, such as spleen or liver in ATL/NOG mice, expressed substantial amounts of Tax, but it was minimally expressed by the tumor cells in a quiescent state such as in the blood (17). In mice with ATL cells from patient 2, the therapeutic efficacy of adoptive patient-autologous Tax-CTL was also confirmed by decreased ATL cell infiltration into the organs and the levels of human sIL-2R concentrations in the serum. In contrast to these two cases, in mice with cells from patient 3, no therapeutic efficacy was seen in vivo. This is consistent with the finding that the adoptively transferred Tax-CTL did not respond to autologous ATL cells in vitro (Fig. 2C). Although the precise reason for this decreased susceptibility of patient 3 primary ATL cells to autologous Tax-CTL in vitro and in vivo is unclear, it is possible that it may reflect the clinical features of the individual ATL patient. Thus, the clinical manifestation in patient 1, the most susceptible in mice in vivo, was stable disease, with the patient under observation in a watch-and-wait approach. Clinical manifestations of patient 2, moderately susceptible in the mouse model, were aggressive, but the patient did achieve long-term remission. The disease course in patient 3, in contrast, was aggressive, and no long-term remission could be achieved. Thus, although the therapeutic efficacy of Tax-CTL in ATL/NOG mice was different in the three different patients, to the best of our knowledge, this is the first demonstration, to our knowledge, that adoptive therapy with Ag-specific CTL expanded from a cancer patient mediates a potent antitumor effect, leading to significant survival benefit for autologous primary cancer cell–bearing mice in vivo (patient 1). The present study not only provides a strong rationale for exploiting Tax as a possible target for ATL immunotherapy, but also contributes to research supporting the efficacy of adoptive CTL therapy for other types of cancer.

NOG mice have severe, multiple immune dysfunctions, such that human healthy immune cells engrafted into them retain essentially the same functions as in humans (27, 28). In addition, primary human cancer cells also engraft and survive in NOG mice by interacting with murine cells in the microenvironment; thus, NOG mice have contributed to analyzing the pathogenesis of several human cancers, especially hematopoietic malignancies, and evaluating the effects of therapeutic agents (17, 29–32). The primary ATL cells tested in this study could be maintained by serial transplantation in NOG mice, but could not be maintained long-term (>1 mo) in vitro in IL-2–containing media (data not shown). These findings indicate that the ATL cells survived and proliferated in a murine microenvironment-dependent manner. That is to say, the present ATL model should more truly reproduce human ATL in vivo including the tumor microenvironment, compared with any other current models, especially those that use established tumor cell lines.

It is generally accepted that increased regulatory T (Treg) cells in the tumor microenvironment play an important role in tumor escape from host immunity in several different types of cancer (33, 34). Therefore, depletion of Treg cells in the vicinity of tumors is a potentially promising strategy for boosting tumor-associated Ag-specific immunity (35–38). We have shown that a therapeutic anti-CCR4 mAb does deplete Treg cells in vitro (39, 40) and in vivo in humanized mice (27). Furthermore, we confirmed the CD25+CD4+FOXP3+ Treg depletion activity mediated by the humanized anti-CCR4 mAb mogamulizumab (KW-0761) in humans (41–44). Therefore, a combination of Tax-CTL adoptive immunotherapy with mogamulizumab to act not only as an anti-ATL agent but also to deplete Treg cells would be promising.

Acknowledgments

We thank Chiiori Fukuyama for excellent technical assistance and Naomi Ochiai for excellent secretarial assistance.

Disclosures

Nagoya City University Graduate School of Medical Sciences has received research grant support from Kyowa Hakko Kirin for works provided by T.I. T.I. received honoraria from Kyowa Hakko Kirin. The other authors have no financial conflicts of interest.

References
