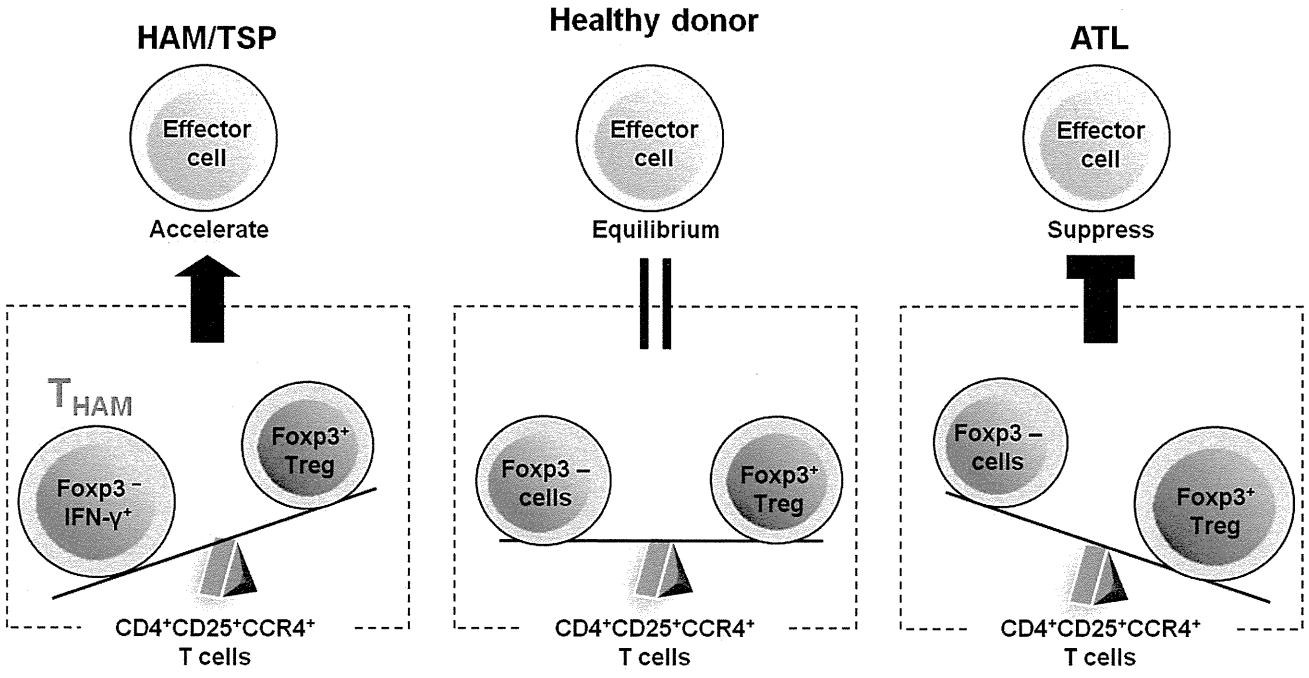
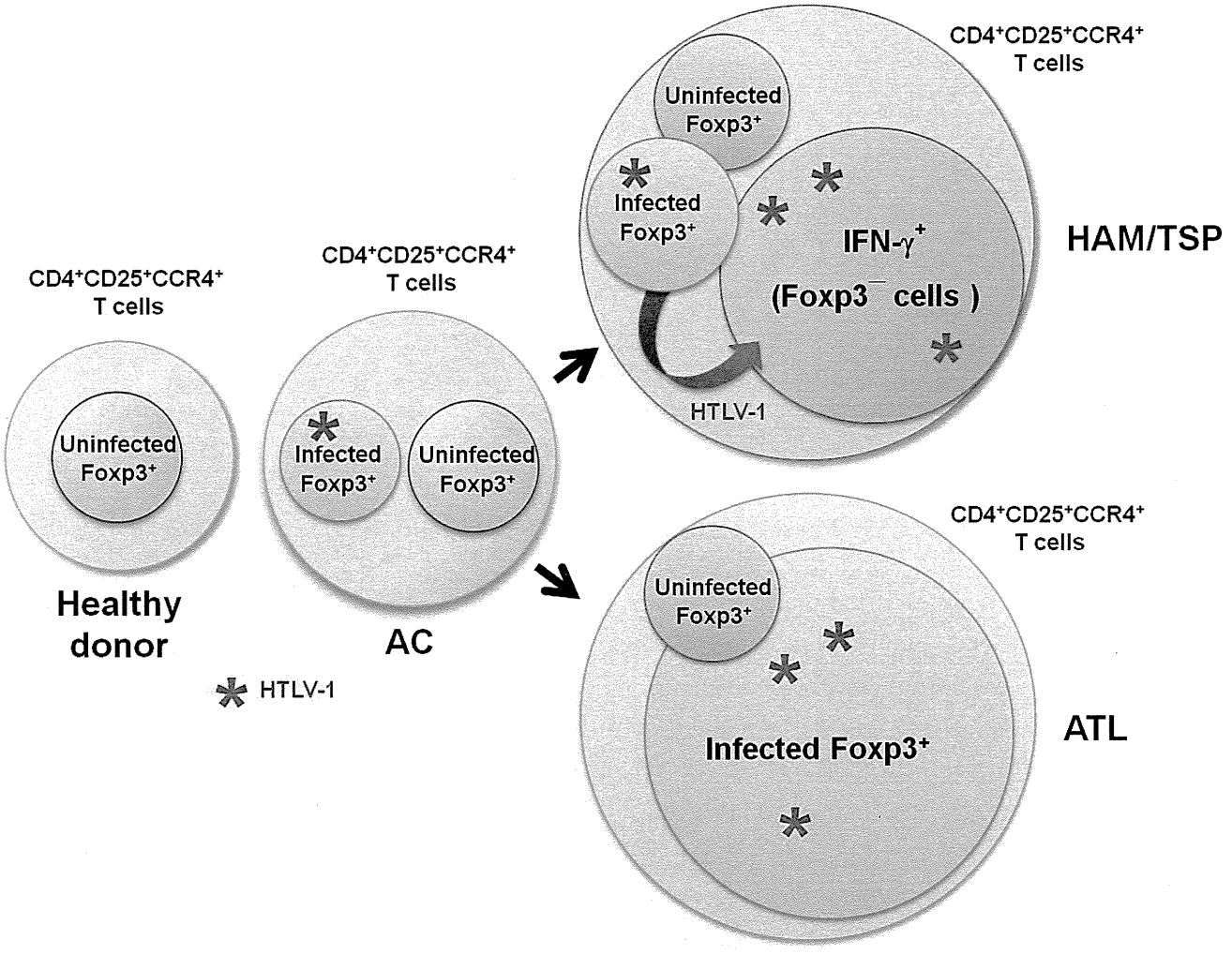
Figure 3. Differential immune responses and T_{HAM}/Treg ratios in $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP and adult T cell leukemia/lymphoma (ATL) patients.



5. Increased Numbers of $CD4^+Foxp3^+$ Cells in HAM/TSP Patients

Recently, it has been reported that the number of $CD4^+Foxp3^+$ cells increases in HTLV-1-infected asymptomatic carriers, and is even higher in patients with HAM/TSP [61]. Although this report initially appears to conflict with the observations described above, it may not. In contrast to the decreased number of $CD4^+$ T cells in patients with human immunodeficiency virus (HIV) infection, the number of HTLV-1 infected $CD4^+$ T cells—most of which are CD4+CD25+CCR4+ T cells—in HAM/TSP patients is greatly increased. Therefore, although the percentage of $Foxp3^+$ cells among the $CD4^+CD25^+CCR4^+$ T cells is lower, the overall number of $CD4^+Foxp3^+$ cells in HAM/TSP patients may be higher than that in healthy donors (Figure 4). Indeed, when we analyzed the number of $Foxp3^+$ cells in healthy donors and HAM/TSP patients, we found it to be nearly equivalent between the two groups or slightly higher in HAM/TSP patients [54]. This difference (from slightly high to higher) would depend on the number of HTLV-1-infected $CD4^+$ T cells in the samples tested. Importantly, Toulza *et al.* demonstrated that the rate of CTL-mediated lysis was negatively correlated with the number of HTLV-1-Tax^- $CD4^+Foxp3^+$ cells, but not with the number of $Tax^+CD4^+Foxp3^+$ cells [61], again suggesting that HTLV-1-infected Treg cells lose their regulatory function, while HTLV-1-uninfected Treg cells contribute substantially to immune control of HTLV-1 infection.

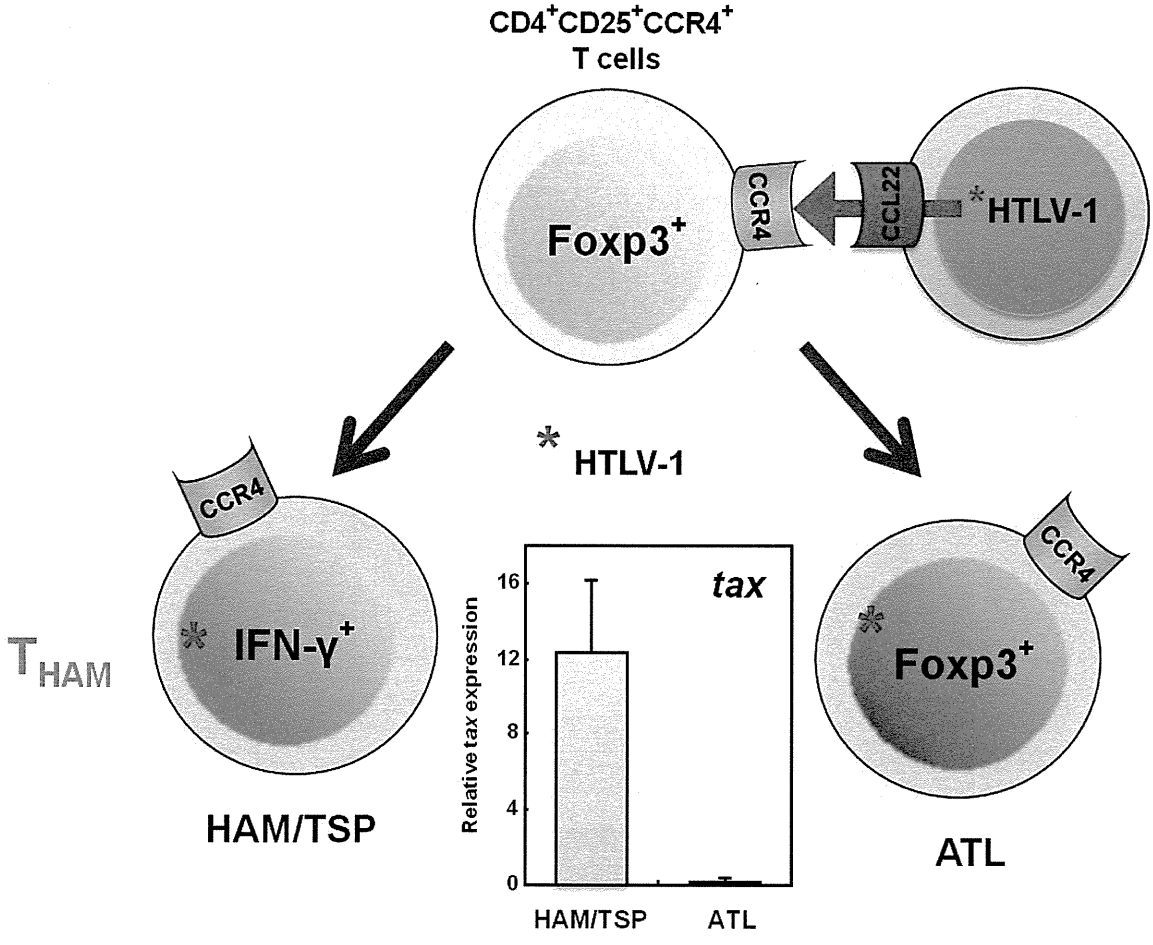
Figure 4. Scheme of proportion of each cellular component in $CD4^+CD25^+CCR4^+$ T cells of healthy donors, asymptomatic carriers (AC), and patients with HAM/TSP or ATL. Although the proportion of $Foxp3^+$ cells among the $CD4^+CD25^+CCR4^+$ T cells is lower in HAM/TSP patients, the overall number of $CD4^+Foxp3^+$ cells in HAM/TSP patients is higher than that in healthy donors. In ATL patients, the majority of $CD4^+CD25^+CCR4^+$ T cells are $Foxp3^+$ cells.



6. Does the T_{HAM} Cell Population Include exFoxp3$^+$ Cells?

According to Hieshima *et al.*'s recent delineation of the molecular mechanism underlying HTLV-1 tropism to $CCR4^+CD4^+$ T cells [60], HTLV-1 Tax does not induce expression of CCR4, but Tax does induce expression of CCL22, which is the ligand for CCR4. Therefore, HTLV-1-infected T cells produce CCL22 through Tax and selectively interact with $CCR4^+CD4^+$ T cells, resulting in preferential transmission of HTLV-1 to $CCR4^+CD4^+$ T cells (Figure 5). In HTLV-1-seronegative healthy individuals, $CD4^+CD25^+CCR4^+$ T cell populations primarily consist of suppressive T cell subsets, such as Treg and Th2 cells [61]. However, as described above, cells of this T cell subset become Th1-like cells that overproduce IFN-γ in patients with HAM/TSP, while leukemogenesis develops and maintains the $Foxp3^+$ Treg phenotype in ATL patients (Figure 5).

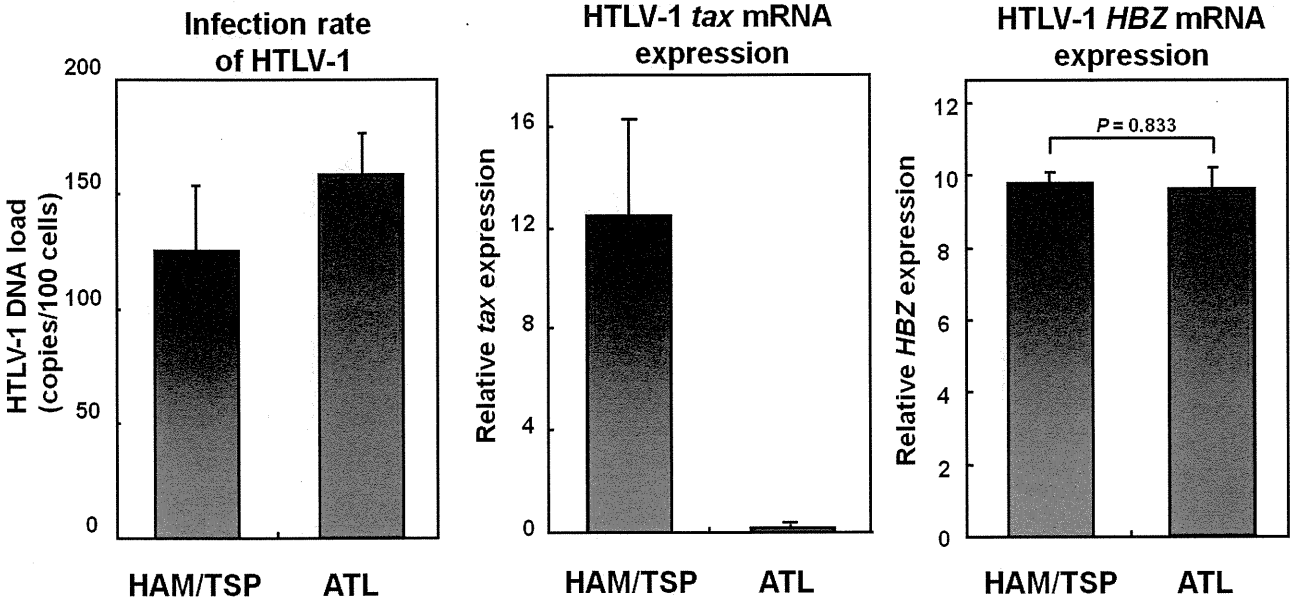
Figure 5. Differential fate of HTLV-1-infected $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP and ATL patients. After HTLV-1 infection, $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients, which are primarily Th2 and Treg cells before infection, become IFN-γ^+Foxp3$^-$ T cells (T_{HAM} cells) with high levels of intracellular HTLV-1 *tax* expression. In ATL patients, leukemogenesis develops and the $Foxp3^+$ Treg phenotype is maintained.



To determine whether HTLV-1 expression contributes to the differential fate of HTLV-1-infected $CD4^+CD25^+CCR4^+$ T cells between HAM/TSP and ATL patients, differences in the HTLV-1 proviral load and the HTLV-1 *tax* mRNA and HTLV-1 *HBZ* mRNA expression of these populations were analyzed (Figure 6). Although HTLV-1 *tax* mRNA expression in $CD4^+CD25^+CCR4^+$ T cells was found to be significantly higher in HAM/TSP patients than in ATL patients, HTLV-1 proviral DNA loads and *HBZ* mRNA expression levels were found to be equivalent in the two groups [54] (Figure 6). This high HTLV-1 Tax expression in HAM/TSP $CD4^+CD25^+CCR4^+$ T cells ($Foxp3^-$) and low HTLV-1 Tax expression in ATL $CD4^+CD25^+CCR4^+$ T cells ($Foxp3^+$) suggests that intracellular HTLV-1 expression may act as a "switch" that directs T cell plasticity from $Foxp3^+$ Treg cells to IFN-γ^+Foxp3$^-$ T cells. Indeed, a recent report highlighted that loss of Foxp3 in Treg cells and acquisition of IFN-γ may result in conversion of suppressor T cells into highly autoaggressive lymphocytes (ex$Foxp3^+$ cells), which can contribute to the development of autoimmune conditions [62,63]. These findings support the hypothesis that HTLV-1 *tax* may be one of the exogenous retrovirus genes responsible for immune dysregulation through its interference in the equilibrium between inflammation and tolerance.

This hypothesis is currently being tested as a means of elucidating the precise molecular mechanisms by which HTLV-1 influences the fate and function of $CD4^+CD25^+CCR4^+$ T cells, especially $Foxp3^+$ Treg cells. Further research investigating this hypothesis using animal models is required, as is further work to pathologically identify the $exFoxp3^+$ cells in the spinal cord lesions of HAM/TSP patients.

Figure 6. Increased HTLV-1 *tax* mRNA expression in $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients. The HTLV-1 proviral load in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP and ATL patients was quantified by real-time PCR (left panel, n = 3). Expression levels of HTLV-1 *tax* mRNA (center panel, HAM/TSP: n = 4, ATL: n = 3) and *HBZ* mRNA (right panel, n = 5) in $CD4^+CD25^+CCR4^+$ T cells from HAM/TSP and ATL patients were quantified by real-time RT-PCR. Data are presented as mean ± standard error.



7. Mechanisms Underlying Increased HTLV-1 Tax Expression in HAM/TSP Patients

As described above, higher levels of HTLV-1 Tax expression have been observed in HAM/TSP patients [11], and a correlation between Tax expression and disease risk [64] has been identified. Both findings, together with experimental evidence [65] and theoretical justification [66] for selective proliferation of HTLV-1 expressing T cells *in vivo*, indicate that increased HTLV-1 provirus expression may play an important role in the pathogenesis of HAM/TSP. However, the molecular mechanisms underlying the increased levels of HTLV-1 provirus expression in HAM/TSP patients are not understood. Evidence continues to accumulate that the genomic integration site of HTLV-1 provirus affects the level of provirus expression. Continued accumulation of evidence is aided by the availability of the human genome sequence, which has enabled large-scale research into HTLV-1 integration sites. This research has demonstrated that the provirus integration sites of HTLV-1 *in vivo* are not randomly distributed within the human genome but rather associated with transcriptionally active regions [67,68]; that the frequent integration into these transcription units is associated with increased levels of provirus expression; and, importantly, that the increased number of integration sites in

transcription units is associated with HAM/TSP [68]. Future research should endeavor to elucidate the mechanisms underlying the immune dysregulation observed in HAM/TSP patients.

8. Conclusion

HTLV-1 initiates persistent infection of $CD4^+$ T cells and results in the development of HAM/TSP, a chronic neuroinflammatory disorder characterized by very high strong cellular and humoral immune responses. Because a higher viral load in HTLV-1-infected individuals increases the risk of HAM/TSP and is associated with high cellular and humoral immune responses, HTLV-1 infection-induced immune dysregulation may play an important role in the development and pathogenesis of this disease. The recent discovery of Treg cells has provided new opportunities for and generated increased interest in elucidating the mechanisms underlying the induction of immune activation by HTLV-1-infected T cells. Among the $CD4^+$ T helper cell populations that play a central role in adaptive immune responses, the $CD4^+CD25^+CCR4^+$ T cell population, which primarily consists of suppressive T cell subsets, such as the Treg and Th2 subsets, in healthy individuals, is the predominant viral reservoir of HTLV-1 in both ATL and HAM/TSP patients. Interestingly, cells of this T cell subset become Th1-like cells, overproducing IFN-γ in HAM/TSP patients, while leukemogenesis develops and maintains the $Foxp3^+$ Treg phenotype in ATL patients. These results indicate that HTLV-1 may intracellularly induce T cell plasticity from Treg to IFN-γ^+ T cells, which may contribute to the development of HAM/TSP. As such, these results support the hypothesis that HTLV-1 is one of the exogenous retrovirus genes responsible for immune dysregulation through its interference in the equilibrium maintained among host immune responses. Because the majority of immune disorders are of unknown etiology, the discovery of HTLV-1 and its association with inflammatory conditions has greatly enhanced our understanding of the pathogenic mechanisms underlying organ-specific immune disorders. Further investigation of the mechanism underlying HTLV-1 action in the immune system may result in identification of new molecular pathways that will further elucidate the basic mechanisms underlying immune-mediated disorders.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Advantage of higher-avidity CTL specific for Tax against human T-lymphotropic virus-1 infected cells and tumors

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ABSTRACT

Strong CTL response can be observed and associated with the control of proviral load in human T-lymphotropic virus type 1 (HTLV-1) infection. However, there are few details with regard to how HTLV-1 specific CTLs work against HTLV-1 infected cells and adult T-cell leukemia cells (ATLs). In this study, using Tax-specific CTL lines with high- and low-functional avidity developed from HLA-A2-transgenic mice, we showed that higher avidity CTLs specific for Tax expressing larger numbers of TCRs and better binding strength to the antigen-HLA-A2 complex are much more efficient at eliminating HTLV-1 infected cells and, in particular, ATL tumor cells with the ability of recognizing a latent level of Tax product detected only with a real-time PCR. These findings suggest that such higher avidity CTLs specific for Tax in HTLV-1 could be responsible for preventing the development of HTLV-1 infection by detecting trace amount of antigens.

1. Introduction

The human T-lymphotropic virus type 1 (HTLV-1) causes two distinct types of disease: a $CD4^+$ T cell malignancy known as adult T cell leukemia (ATL) [1,2] and a range of inflammatory disease, of which HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the best recognized and most widely studied [3,4]. In patients with HTLV-1 infection, the proviral load of HTLV-1 is usually stable over time [5]. However, the factors determining the set point of proviral load in each person remain to be elucidated. In particular, CTLs are active in individuals with low proviral load, in whom immunosurveillance could be more effective [6,7]. Several studies have reported that high-levels of HTLV-1-specific CTL activity can be observed in HAM/TSP patients and some asymptomatic HTLV-1 carriers, while ATL patients apparently lack HTLV-1-specific CTL activity, although it can be sporadically induced during the remission stages or after mitogenic stimulation with multiple in vitro antigenic stimulations of peripheral blood mononuclear cells [8,9]. One of the major target antigens by HTLV-1-specific CTLs in human is Tax protein [10,11], which is a molecule responsible for T-cell immortalization [12,13]. CTLs induced in ATL patients in remission are able to lyse autologous tumor cells in vitro [14]. These observations suggest that HTLV-1-specific CTLs could play a critical role in host immunosurveillance against ATLs.

While the number of HTLV-1-specific CTLs elicited is unquestionably important [7], recent studies have identified an additional parameter, functional avidity, as critical in determining the efficiency of viral clearance [15–18]. T-cell avidity is a measure of the sensitivity of T cells recognizing a cognate antigen. High-avidity CTLs are those that can recognize antigen-presenting cells (APCs) bearing very low levels of peptide-major histocompatibility complex (MHC) antigen, whereas low-avidity CTLs require much more peptide-MHC antigen to be activated or to exert effector function [15,19–21].

In this study, in order to clarify whether Tax-specific CTLs with higher avidity are critical as a deterrent to control the proliferation of ATL and the expansion of HTLV-1 infection, we developed two CTL lines specific for Tax11-19 antigen having high- and low-avidity from HLA-A2 transgenic mice in vitro. Using these CTLs, we demonstrate not only that Tax product is a critical antigen but also in particular that the specific CTLs with higher avidity for Tax11-19 have a selective advantage on recognition of human ATLs and HTLV-1 infected cells compared with those with low avidity in vitro.

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2. Materials and methods

2.1. Synthetic peptides

The Tax11-19 peptide, LLFGYPVYV, was purchased from Asahi Technoglass (Chiba, Japan) and used as an HLA-A2-restricted CTL antigen [11].

2.2. Cells

C1R.AAD cell line (HMYC1R transfected with HLA chimeric molecule containing α1 and α2 domains from human HLA-A2.1 and α3 from mouse H-2D^d) was described previously [22]. Cell lines were maintained in culture medium (CTM; 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA)) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

HTLV-1-infected human ATL cell lines, KK-1 and KOB, were derived from the peripheral blood and ascites of ATL patients, respectively [23,24]. Human IL-2 dependent T cell line (HCT-4) was derived from the cerebrospinal fluid of a HAM/TSP patient [25]. KK-1, KOB, and HCT-4 were used as a target. Cells were maintained in CTM with 100 units/ml of recombinant human IL-2 (Imunace®35, Shionogi, Osaka, Japan).

2.3. Mice

Transgenic HHD-2 mice (gift from Dr. François Lemonnier, Institute Pasteur, Paris, France) were bred in our colony at the Institute of the Experimental Animals at St. Marianna University. HHD-2 mice are characterized by knock-out of the murine β_2-microglobulin gene, as well as murine H-2D^b, transgenic expression of human HLA-A2.1 with a covalently-linked human β_2-microglobulin and a murine D^b-derived α3 domain to allow interaction with mouse CD8 [26]. All animal studies were approved by the Institute of Experimental animals at St. Marianna University.

2.4. Binding assay

Peptide binding to HLA-A2 molecules was measured using T2 mutant cell lines as described previously [27,28]. T2 cells (3×10^5/well) were incubated overnight in 96-well plates with culture medium (a 1:1 mixture of RPMI 1640 and Eagle-Hank's amino acid (EHAA) containing 2% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin) with 10 μg/ml human β_2-microglobulin (Sigma–Aldrich, St. Louis, MO) and different peptide concentration. On the following day, cells were washed at 190 × *g* for 5 min twice with cold PBS containing 2% FBS and incubated for 30 min at 4 °C with anti-HLA-A2.1 BB7.2 mAb (1/100 dilution of hybridoma supernatant) and 5 μg/ml FITC-labeled goat anti-mouse Ig (BD PharMingen, San Diego, CA). Cells were washed twice after each incubation; subsequently, HLA-A2.1 expression was measured by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). HLA-A2.1 expression was quantified as fluorescence index (FI) according to the formula: FI = ((geometric mean fluorescence with peptide − geometric mean fluorescence without peptide)/geometric mean fluorescence without peptide). $FI_{0.5}$ is the concentration required to give an FI of 0.5, meaning a 50% increase in HLA-A2 on the cell surface. Background fluorescence without BB7.2 was subtracted for each individual value. To compare the different peptides, $FI_{0.5}$ was calculated from the titration curve for each peptide. Each sample was tested in triplicate. Values were expressed as mean in triplicate.

2.5. CTL generation in HHD-2 transgenic mice

The method for generating antigenic peptide-specific CTL lines form HHD mice was described previously [28,29]. Mice aged more than 8 weeks were immunized subcutaneously in the base of the tail with 100 μl of an emulsion containing 1:1 incomplete Freund's adjuvant (IFA), antigenic CTL peptide and cytokines (50 nmol Tax (11-19) peptide, 25 nmol HBV core 128–140 helper epitope, 3 μg of rmIL-12 and 3 μg of rmGM-CSF). Mice were boosted 2 weeks later, with the spleens removed 10–14 days after the boost. Immune spleen cells (2.5×10^6/well) were stimulated in 24-well plates with autologous spleen cells (5×10^6/well) pulsed for 30 min with 10 μM Tax11-19 peptide for the development of low-avidity CTL lines (LCTL) or with 10 nM for high-avidity CTL lines (HCTL) in CTM supplemented with 10% T-stim® (Collaborative Biochemical Products, Bedford, MA). Following a minimum of four in vitro stimulations with the peptide-pulsed syngeneic spleen cells, two CTL lines were maintained by weekly restimulation with 1×10^6 cells/well with 4×10^6 peptide-pulsed mitomycin C-treated syngeneic spleen cells as feeders.

2.6. Cytotoxic assay

CTL activity was measured with ^{51}Cr-labeled target cells. Target cells (1×10^6) were pulsed in 100 μl of 150 μCi ^{51}Cr for 1 h and were washed three times, with 5000 cells/well then added to 96-well round-bottom plates containing different peptide concentrations. Effector cells were introduced followed by additional incubation. Supernatants were then harvested and analyzed. The percentage of specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was determined from target cells that had been incubated in the absence of effector cells, while maximum release was determined in the presence of 2% TRITON® X-100 Detergent (CALBIOCHEM, La Jolla, CA). Each sample was tested in triplicate. Values were expressed as means ± SEM of triplicates.

2.7. IFN-γ ELISA assay

IFN-γ in the culture supernatant harvested at 24 h was determined using an ELISA kit (R&D, Minneapolis, MN) according to the manufacture's instructions. All samples were analyzed in triplicate. Values were expressed as means ± SEM of triplicates.

2.8. TCR Vβ screenings of CTLs

We assessed a Vβ usage pattern between HCTL and LCTL using Vβ TCR screening kit by a flow cytometry analysis (BD Bioscience Phamingen, San Diego, CA).

2.9. Flow cytometry

We used a PE-Tax11-19/HLA-A*0201 tetramer-LLFGYPVYV (Medical & Biological Laboratories, Nagoya, Japan) and PE-hamster anti-mouse CD3ε Ab (145-2C11, BD Bioscience Phamingen, San Diego, CA). Cells were centrifuged and washed twice with PBS containing 0.5% BSA, and then resuspended in 1% BSA/PBS. Cells were incubated 40 min at 4 °C with the antibody and then washed three times. The tetramer and anti CD3ε Ab were titrated for staining simultaneously.

In order to compare the affinity of T cell receptor between HCTLs and LCTLs, indexes were calculated using the following two equations: ratio of geometric mean (RGM) = (geometric mean using tetramer or anti-CD3ε Ab)/(geometric mean using control Ab). Each sample was tested in triplicate.

2.10. Western blotting

KK-1, KOB, and HCT-4 were lysed using standard lysis buffer (20 mM Tris–HCl, 250 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail (Roche, Mannheim, Germany)). Lysates were stored at −80 °C until use. Protein concentration was determined using the Bradford method (Bio-Rad protein assay reagent; Bio-Rad laboratories, Hercules, CA). Equal amounts (30 μg) of protein were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Following the transfer, membranes were blocked with Difco Skim milk (BD Bioscience, San Diego, CA) overnight at 4 °C. The working concentrations of the first Abs were 1 μg/ml for anti-Tax Ab (Lt-4) [30] and anti murine β-actin Ab (SIGMA, St. Louis, MO), and 1:10,000 for HRP-conjugated anti-mouse IgG Ab (SIGMA, St. Louis, MO). The membrane was washed, and was reacted with the appropriate second antibody. Finally, signals were visualized using the extended cavity laser (ECL) system (GE Healthcare Bio-sciences KK, Tokyo, Japan).

2.11. Real-time reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen, Carisbad, CA). First-stand cDNA was synthesized with random hexamers and reverse transcriptase (ReverTraAce; Toyobo, Japan) using 1 μg of total RNA in a reaction volume of 20 μl. Real-time PCR reactions were carried out using TaqMan® Universal Master Mix (Applied Biosystems, Carisbad, CA). ABI Prism 7500 SDS was programmed to an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers and probe for detecting the HTLV-1 Tax or GAPDH mRNA were used as described previously [31]. Relative quantification of mRNA was performed using the comparative threshold cycle method with GAPDH as an endogenous control. For each sample, target gene expression was normalized against the expression of GAPDH. To determine relative expression levels, the following formula was used: target gene expression = $2^{-(Ct[target]-Ct[GAPDH])}$. Each sample was tested in triplicate. Values were expressed as means ± SEM of triplicates.

3. Results

3.1. Binding affinity of Tax11-19 for HLA-A2 molecule

Before attempting to develop Tax-specific CTL lines from HLA-A2 transgenic HHD mice, we evaluated the binding affinity of Tax11-19 peptide by T2 binding assay, which measures the cell surface stabilization of HLA-A2 molecules. Tax11-19 peptide displayed a binding capacity for the HLA-A2 molecule that was nearly equal to that of the positive control, the highly antigenic influenza virus matrix peptide (FMP58-66) [32] ($FI_{0.5}$ = 0.329 for Tax11-19, 0.284 μM for FMP58-66) (Fig. 1). These data suggest that Tax11-19 would be a very strong antigenic peptide restricted to the HLA-A2 molecule.

3.2. Recognition of Tax11-19 peptide by CTL lines of different avidity

Based on the observation that Tax11-19 showed strong antigenicity inducing specific CTLs, we next attempted to develop low-avidity CTLs (LCTL) and high-avidity CTLs (HCTL) from HLA-A2 transgenic mice. HCTL were generated by weekly stimulation using low concentrations (10 nM) of the Tax peptide pulsed onto APCs, while LCTLs were also generated using 10 μM of the Tax peptide pulsed onto APCs. Using these different CTL lines, we examined Tax-specific CTLs-mediated cytotoxicity with Tax peptide titrated over a range of concentrations. The titration curve showed a

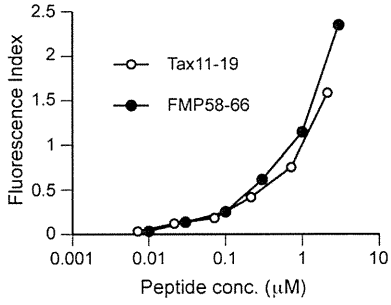

Fig. 1. Comparison of HLA-A2 binding curves between Tax11-19 and FMP58-66 peptide in T2-binding assay. The binding affinity of Tax11-19 for HLA-A2 molecule is almost as strong as that of FMP58-66 in influenza A virus.

0.5–1 $\log_{10}$ difference in functional avidity measured as the peptide concentration necessary to produce 50% lysis (Fig. 2A). Similarly, we examined their properties in antigen-specific IFN-γ production from these CTL lines (Fig. 2B). With a 24 h assay, HCTLs showed more IFN-γ production than LCTLs even at lower concentration of Tax antigen. These data suggest that the two different CTL lines specific for Tax have different functional avidity.

3.3. Different Vβ usage and binding ability to Tax-tetramer between high- and low-avidity CTLs

In order to confirm whether these CTLs with different avidity possessed different TCR structures, we assessed the difference in Vβ usage pattern between HCTLs and LCTLs using flow cytometric analysis (FCM). On FCM, antibodies available for screening were those for Vβ 2, 3, 4, 5, 6, 7, 8.1, 8.2, 8.3, 9, 10, 11, 12, 13, 14, and 17. On FCM, no Vβ were detected in LCTLs, while only Vβ5 was detected in HCTLs (Fig. 3A). The data suggested that the major TCR repertoire of HCTL is Vβ5, indicating that these two Tax-specific CTL lines have different TCR structures.

We next compared the binding affinity of TCR between HCTL and LCTL using Tax11-19/HLA-A2 tetramer-LLFGYPVYV and anti-CD3 Ab (Fig. 3B). On FCM with both Tax11-19-tetramer and anti-CD3 Ab titration, HCTLs showed a stronger fluorescence than LCTLs (Fig. 3B). On Tax11-19-tetramer assay, the ratio of fluorescence index (HCTL/LCTL) was ~5-fold at any titrated concentration, and it took 1.5 logs more tetramer to achieve the same level of staining. In the titration of anti-CD3 Ab, the ratio was ~3-fold and also it required about 3-fold more antibody to reach the same level of staining. These findings suggested that HCTLs not only have higher TCR affinity but also express greater numbers of TCR molecules on their surface when compared with LCTLs.

3.4. Recognition of human ATL targets by Tax-specific CTLs from HHD mice

We further examined whether these murine CTL lines with different functional avidity could induce cytotoxic activity against human ATL targets. We used the HTLV-1-infected human ATL cell lines, KK-1 (HLA-A2) and KOB (HLA-A30) as target cells derived from peripheral blood and ascitis of ATL patients, respectively [23,24]. These murine CTL lines did not show strong cytotoxicity against human ATL lines as against murine targets with a 4 h assay, as it was previously reported that species specificity between murine CD8 and the α3 domain of human HLA-A2 may reduce the recognition ability by CTLs [33]. However, on a 12 h assay, cytotoxicity against human ATL was observed in an HLA-A2 restricted manner (Fig. 4A). HCTLs were especially more efficient at killing at low E/T ratios. Furthermore, on kinetics assay, HCTLs showed more efficient cytotoxicity against the human ATL target (KK-1) than LCTLs (Fig. 4B).

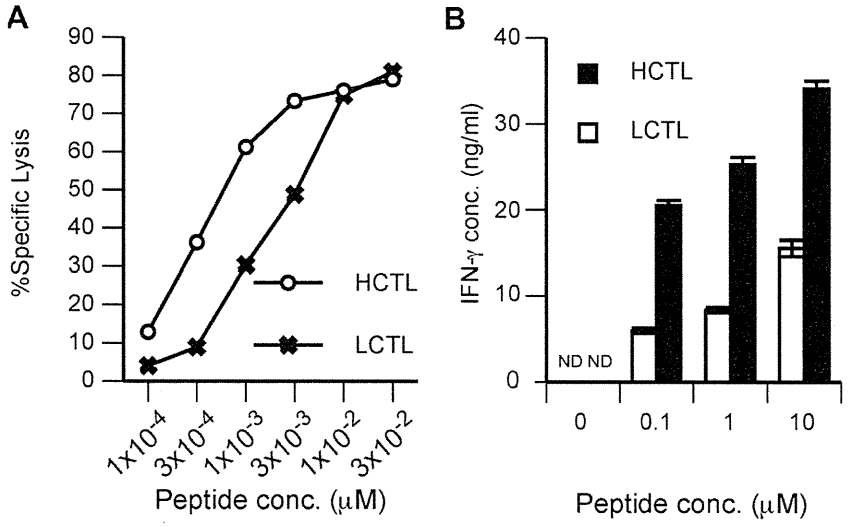

Fig. 2. Difference in functional avidity between HCTLs and LCTLs. (A) Recognition by the Tax11-19 peptide specific CTLs, HCTL and LCTL, of Tax11-19 antigenic peptide from 10^{-4} to 10 μM when presented on C1R.AAD target cells. The effector to target-cell (E/T) ratio was 20:1. Error bars were omitted because all SEMs were <3.5%. (B) Comparison of Tax11-19-specific IFN-γ production between HCTLs and LCTLs. A total of 200,000 CTL cells were cultured with 100,000 mytomicin-c treated C1R.AAD cell with 0.1–10 μM Tax11-19 peptide. Culture supernatants at 24 h were assayed using IFN-γ ELISA kit according to the manufacturer's instructions. ND, not detected.

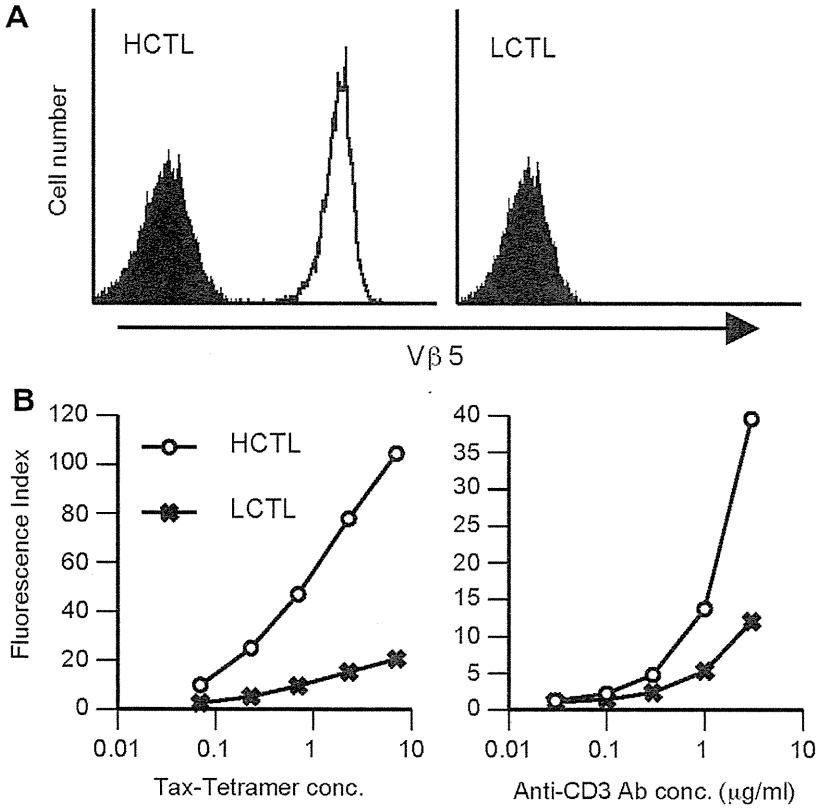

Fig. 3. TCR Vβ usage and expression level of TCR complex on Tax-specific CTLs with different functional avidity. (A) Comparison of Vβ usage pattern between HCTLs and LCTLs cytometry analysis (FCM). No Vβs among available anti-Vβ antibodies were detected in LCTL but only Vβ5 was detected in HCTL. (B) Comparison of binding curves for human Tax11-19-tetramer and anti-CD3ε Ab between HCTLs and LCTLs. HCTLs consistently showed a stronger fluorescence index than LCTLs; for Tax11-19-tetramer, the ratio of fluorescence index (LCTL/HCTL) was ~5-fold, and for anti-CD3ε Ab, it was ~3-fold.

3.5. Recognition of HTLV-1 infected human T cells by Tax-specific CTL from HHD mice

Next, in order to examine a comparison of the cytotoxicity against HTLV-1 infected non-tumor cells, we used HTLV-1 infected human T cells (HCT-4) derived from a patient with HAM/TSP [25]. On a 12 h lytic assay, HCTLs showed more efficient cytotoxicity against the HTLV-1 infected human T cells while LCTLs were not able to kill the targets under the these experimental conditions (Fig. 5A). At no time point was there detectable killing by LCTLs (Fig. 5B). These findings suggested that the superior recognition ability by the CTLs with higher functional avidity may have a more

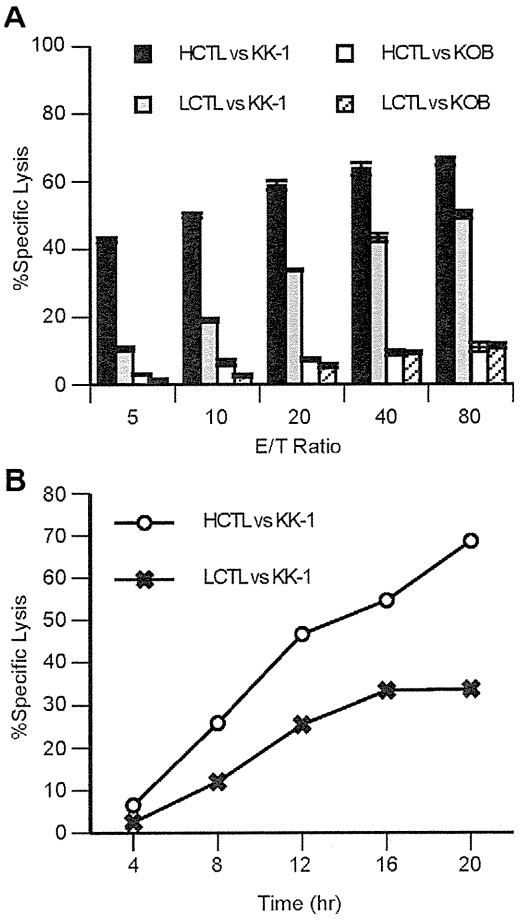

Fig. 4. Recognition pattern of human ATL targets by Tax-specific CTLs. (A) Comparison of cytotoxicity for human ATL targets (KK1, HLA-A2; KOB, HLA-A30) between HCTLs and LCTLs. (12 h ^{51}Cr release assay) (B) Comparison of kinetics of Tax-specific CTL-mediated cytotoxicity (E:T ratio = 40:1) between HCTLs and LCTLs. Similar results were obtained in three different experiments.

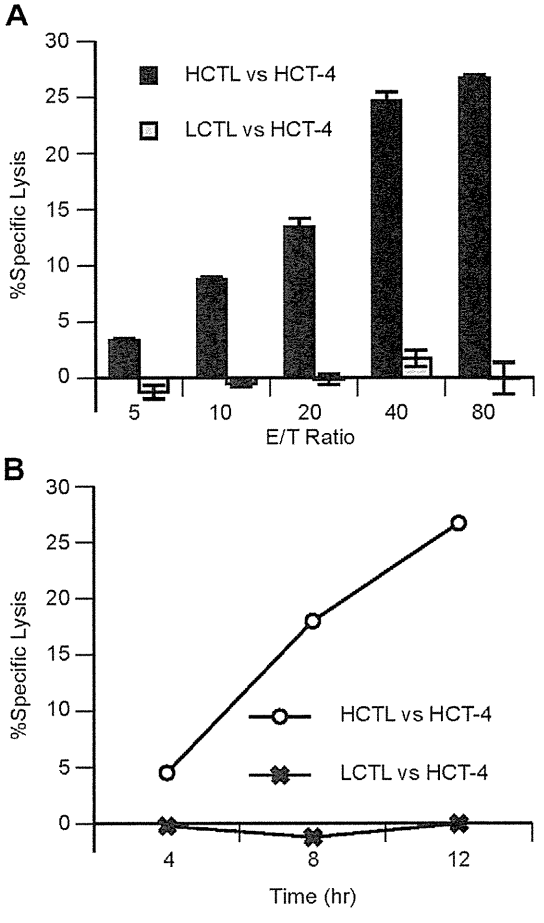

Fig. 5. Recognition pattern of HTLV-1 infected human T cell line by Tax-specific CTLs. (A) Comparison of cytotoxicity for human IL-2 dependent HTLV-1 infected cell, HCT-4 (HLA-A2), between HCTLs and LCTLs. (12 h ^{51}Cr release assay). (B) Comparison of kinetics of Tax-specific CTL-mediated cytotoxicity (E:T = 40:1) between HCTLs and LCTLs. Similar results were obtained in four different experiments.

striking effect in the case of recognizing normal cells infected with the virus.

3.6. Expression of Tax product in human ATL tumors and HTLV-1 infected T cell target

The cytotoxicity data against human targets indicated that higher functional avidity in CTLs is critical for efficient cytotoxicity against tumor or infected normal cell targets in humans. However, the amount of Tax antigen expressed in target cells that could be recognized by higher avidity CTLs was unclear. Therefore, we investigated how much Tax products could be yielded in these human ATL and HTLV-1 infected target cells. Using western blotting (Fig. 6A), Tax protein was detected in KOB and HCT-4 target cells, but not in KK-1. Since KK-1 cells were recognized by HCTLs more strongly than by LCTLs, we further evaluated the level of Tax mRNA produced in KK-1 using real-time PCR. The expression levels of Tax mRNA in KK-1 were around one thousand-fold lower than that in KOB (Fig. 6B). These results demonstrated that Tax11-19-specific higher avidity CTLs showed more efficient cytotoxicity against ATL by recognizing very small amount of Tax product detected only with real-time PCR.

4. Discussion

HTLV-1 infection elicits a strong CTL response, with Tax protein being the major target of HTLV-1-specific CTLs [10,11]. In the field of anti-tumor immunity, the in vivo relevance of differences in functional avidity has been established by demonstrating that high-avidity CTLs clear tumor antigens more efficiently than low-avidity CTL [34–38]. In HTLV-1 infection, however, while there is increasing body of evidence that CTL quality from the aspect of functional avidity of CTL might be crucial for the efficient control of HTLV-1 infection [17,39], little is known about how the functional avidity of HTLV-1 virus-specific CTLs is related to the control of HTLV-1-infected cells and tumors. Furthermore, the virus is latent in the tumor cells and it is difficult to detect expression of viral proteins [40–42]. This is the reason why there has not been direct evidence on whether Tax11-19 works as a definitive CTL antigen in HLA-A2-restricted patients with HTLV-1 infection and ATLs. The present study provides clear evidence regarding the notion that high avidity CTLs specific for Tax protein play a greater role in the specific destruction of ATL and HTLV-1-infected cells using Tax-specific CTLs with different functional avidity generated from HLA-A2 transgenic HHD mice, with human ATL lines and HTLV-1 infected cells acting as targets. As Tax11-19 peptide antigen binds HLA-A2 with almost as high affinity as FMP58-66 in influenza A virus (Fig. 1), which has one of the highest affinity peptides among HLA-A2 restricted peptide antigens [27,28], we developed CTL lines specific for Tax11-19, HCTL and LCTL, for which we found the optimum antigen-presenting conditions for the induction and maintenance of the CTL lines were 10 nM- and

10 μM-peptide pulsing APCs, respectively. The 1000-fold difference of such antigenic concentration resulted in the CTL lines with differences of functional avidity in antigen-specific cytotoxicity and IFN-γ production (Fig. 2). These different avidity CTLs also had different repertoires of TCRVβ, suggesting the structure of TCR in the major repertoire of two lines were distinct (Fig. 3A). In order to compare TCR affinity for the human Tax-tetramer, the mismatch of which to murine CD8 could permit assessment of the strength of TCR ligation to peptide/MHC complex more closely without the influence of CD8 binding [43], we titrated the tetramer and evaluated the effect of the number of TCR molecules expressed at the same time. Higher avidity Tax-specific CTLs showed higher fluorescene on both Tax-tetramer (~5-fold) and anti-CD3Ab (~3-fold) staining (Fig. 3B), thus suggesting that CTL might acquire higher avidity state by possessing the different structure of the TCR as well as by increasing the number of TCR molecules expressed although other factors could also play a role for determining the avidity of CTLs [15].

HTLV-1 Tax, a critical viral protein for HTLV-1 leukemogenesis, is the most likely target for HTLV-1 specific CTL in HTLV-1-infected individuals [10,11]. In HTLV-1-infected patients with HLA-A2, the Tax11-19-specific CTL response is predominantly detected in culture [44]. However, few details are known about the recognition mechanism by Tax-specific CTLs because of the difficulty of developing CTL lines specific for Tax11-19 antigen [9]. Although both HCTLs and LCTLs developed from HLA-A2 transgenic mice were not able to induce cytotoxicity against the human HLA-A2-restricted ATL line, KK-1, on 4 h assay because of the mismatch between the murine CD8 and human α3 domain [22], HCTLs clearly showed more efficient cytotoxicity than LCTLs with longer-term assay of more than 4 h (Fig. 4). Furthermore, the use of the human IL-2-dependent HTLV-1-infected non-tumor cell, HCT-4, clearly brought out the difference in cytotoxic efficacy between HCTL and LCTL (Fig. 5). These findings could be direct evidence not only that Tax11-19 might be naturally processed for presentation as a CTL antigen in both ATL tumor cells and virus-infected cells but also that the higher avidity CTL for Tax11-19 could be more critical in clearing HTLV-1-infected cells as well as ATL tumors in HLA-A2-restricted patients. In addition, HCTLs could more strongly recognize a latent level of Tax product detected only with a real-time PCR, not detectable with western blotting in the ATL target (Fig. 6). Furthermore, HCTLs also possessed higher elimination potential against HTLV-1 infected non-tumor targets when compared with LCTLs (Figs. 4 and 5).

The present findings are consistent with previous reports showing that the lytic efficiency of $CD8^+$ T cell response was inversely correlated with the proviral load and the rate of proviral expression in patients with HTLV-1 infection [17]. These data also strongly support the notion that induction of high avidity CTLs is critical for development of more effective vaccines against cancer and chronic viral infection such as HTLV-1and HIV. In addition, based on the observation that the high-avidity CTLs expressed a greater number of TCR molecules when compared with the low-avidity CTLs (Fig. 3B), such more multivalent TCR display might be one of the critical factors in establishing functional high avidity, leading to more efficient TCR cell therapy in the future [45].

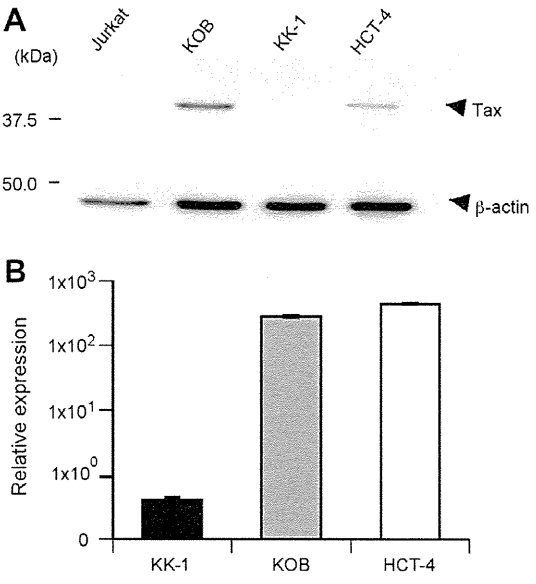

Fig. 6. Expression of Tax product in human ATL tumors and HTLV-1 infected T cell targets. (A) Tax protein is detected in KOB and HCT-4 by western blotting, but not in KK-1. Jurkat cells were used as a negative control. (B) Comparison of mRNA production of Tax by real-time PCR among KOB, KK-1 and HCT-4. Tax production in KK-1 was detected by real-time PCR, but not in a western blotting. Jurkat cells as a negative control gave no detectable signal with the Tax-primer.

Conflict of interest

The authors declare no conflict of interest.

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Overexpression of *SPACIA1/SAAL1*, a Newly Identified Gene That Is Involved in Synoviocyte Proliferation, Accelerates the Progression of Synovitis in Mice and Humans

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Objective. **To identify novel genes associated with dysregulated proliferation of activated synovial fibroblasts, which are involved in arthritic joint destruction.**

Methods. **We performed transcriptome analysis to identify genes that were up-regulated in the foot joints of mice with collagen-induced arthritis (CIA). The effect of candidate genes on proliferation of synovial fibroblasts was screened using antisense oligodeoxynucleotides and small interfering RNAs (siRNAs). We characterized the expression and function of a novel gene, synoviocyte proliferation–associated in collagen-induced arthritis 1 (*SPACIA1*)/serum amyloid A–like 1 (*SAAL1*) using antibodies and siRNA and established transgenic mice to examine the effect of SPACIA1/SAAL1 overexpression in CIA.**

Results. **Human and mouse *SPACIA1/SAAL1* encoded 474 amino acid proteins that shared 80% homology. SPACIA1/SAAL1 was primarily expressed in the nucleus of rheumatoid arthritis (RA) synovial fibroblasts and was highly expressed in the hyperplastic lining of inflamed synovium. In addition, its expression level in RA- or osteoarthritis (OA)–affected synovial tissue was positively correlated with the thickness of the synovial lining. Furthermore, *SPACIA1/SAAL1* siRNA inhibited the proliferation of synovial fibroblasts, especially tumor necrosis factor α–induced synovial fibroblasts, by blocking entry into the S phase without inducing apoptosis. Finally, transgenic mice overexpressing *SPACIA1/SAAL1* exhibited early onset and rapid progression of CIA.**

Conclusion. **These results suggest that SPACIA1/SAAL1 is necessary for abnormal proliferation of synovial fibroblasts and its overexpression is associated with the progression of synovitis in mice and humans. Thus, therapy targeting SPACIA1/SAAL1 might have potential as an inhibitor of synovial proliferation in RA and/or OA.**

Synovitis is a common characteristic of rheumatoid arthritis (RA) and knee osteoarthritis (OA). The major pathologic features of synovitis are hyperplasia of the synovial lining, inflammatory cell infiltration, and

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high stromal cell density (1). Activated synovial fibroblasts, which are a major component of synovial lining hyperplasia, are important in the pathogenesis of synovitis because they secrete cytokines and chemokines, leading to the exacerbation of inflammation. They also produce matrix metalloproteinases and cathepsins, which destroy bone and cartilage (2). As a result, the activated synovial fibroblasts form pannus, a type of granulation tissue that erodes the joint (3). Therefore, reducing the number of activated synovial fibroblasts is a promising therapeutic strategy for arthritis. For example, adenoviral gene transfer of cyclin-dependent kinase inhibitors, such as p16 or p21, inhibits synovial cell proliferation and has demonstrated high therapeutic efficacy in animal models of RA (4,5). Similarly, intraarticular injection of anti-Fas IgM monoclonal antibodies induces apoptosis in synoviocytes and infiltrating lymphocytes, which leads to improvement of RA (6).

Although these findings suggest that synoviocyte proliferation is important in the pathogenesis of RA, the exact molecular mechanism of the disease is not known. To clarify the mechanism of the abnormal proliferation of synovial fibroblasts in RA, we used gene expression and functional analyses to seek novel genes that are involved in this process. Herein we describe a novel gene, synoviocyte proliferation–associated in collagen-induced arthritis 1 (*SPACIA1*)/serum amyloid A–like 1 (*SAAL1*), which was found to be up-regulated in the foot joints of mice with collagen-induced arthritis (CIA). Knockdown of this gene inhibited the proliferation of human RA synovial fibroblasts (RASFs) in vitro. Finally, we demonstrated that overexpression of *SPACIA1*/*SAAL1* is associated with the progression of synovitis in mice and humans.

MATERIALS AND METHODS

Ethical considerations. All human and animal experimental protocols in this study (nos. 443 and 31M0912T2, respectively) were approved by the Ethics Review Committee of St. Marianna University School of Medicine. Written informed consent was obtained from all patients prior to collection of joint tissue samples.

Animals. Male DBA/1J mice were purchased from Japan SLC. DBA/1J mice and transgenic mice were raised under conventional conditions at our facilities. Tap water and food were provided ad libitum.

Induction and assessment of CIA. For the microarray experiments, 10 DBA/1J mice (7–8 weeks old) were immunized twice with 100 μg bovine type II collagen (CII), as described previously (7). Seven days after the second injection of CII, collagen-injected mice and control mice were killed and foot joints were removed from their forepaws.

To induce CIA in *SPACIA1*-overexpressing mice, CII was administered as described above, except that the amount of CII administered in the second injection was reduced to 50 μg. Subsequently, the mice were assessed 3 times per week, and an arthritis score was assigned based on the grading system described by Hughes et al (8). In addition, blood samples were collected from the tail vein once per week to measure anti-CII antibody titers. Forty-two days after the first injection, the mice were weighed and killed, and samples of their blood, spleen, and knee joints were collected.

DNA microarray. Total RNA was extracted and pooled from the foot joint samples from the mice with CIA and from the control mice. Messenger RNA (mRNA) was isolated using the PolyATtract mRNA Isolation System (Promega). Double-stranded complementary DNA (cDNA) was synthesized from 1 μg mRNA, followed by preparation of biotinylated complementary RNA (cRNA) with the BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). Biotin-labeled cRNA, which was fragmented according to the Affymetrix procedure, was hybridized to Murine Genome U74v2 microarrays. Subsequently, these microarrays were stained with streptavidin–phycoerythrin and scanned with a GeneArray scanner. The intensity data from each array were normalized using Microarray Suite version 5.0 software. To determine the $\log_2$ ratio of signal intensities, we compared the normalized data from mice with CIA to the data from the control group, using Data Mining Tool version 3.0 software. The microarrays, scanner, and software sets were all obtained from Affymetrix, and all of the annotation data for the identified probe sets were obtained from the NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx).

Design and use of antisense oligodeoxynucleotides (ODNs) and small interfering RNAs (siRNAs) in cell proliferation assays. To screen candidate genes, we designed antisense phosphorothioated ODNs near the start codon, using Oligo software (Molecular Biology Insights). We also designed siRNAs, using the design program on the Takara Bio web site (http://www.takara-bio.co.jp/rnai/intro.htm). The ODNs and siRNAs were chemically synthesized at Hokkaido System Science. Their sequences are shown in Supplementary Tables 1 and 2 (available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

RASFs were isolated and cultured as described previously (9). Twenty-four hours before transfection, the cells were trypsinized, seeded on a 24-well plate, and cultured overnight. To determine the effect of antisense ODNs and siRNAs on the proliferation of RASFs, we transfected 100 n*M* antisense ODN or 100 n*M* siRNA into the cells with Oligofectamine (Invitrogen) and then cultured the cells for 66 hours or 96 hours, respectively. Subsequently, we used the Cell Counting Kit-8 assay (Dojindo) to determine the relative number of viable cells.

Similarly, to determine the effect of *SPACIA1* siRNA on RASF proliferation induced by serum or tumor necrosis factor α (TNFα), we used *SAAL1* (also known as *SPACIA1*) siRNA, c-*fos* siRNA, and negative control siRNA (On-Target plus SMARTpool; Dharmacon). RASFs were then trypsinized and seeded in 48-well plates. After 18 hours, 33 n*M* siRNA was transfected into the cells with Lipofectamine 2000 (Invitrogen) in Opti-MEM medium. Four hours later, the culture medium