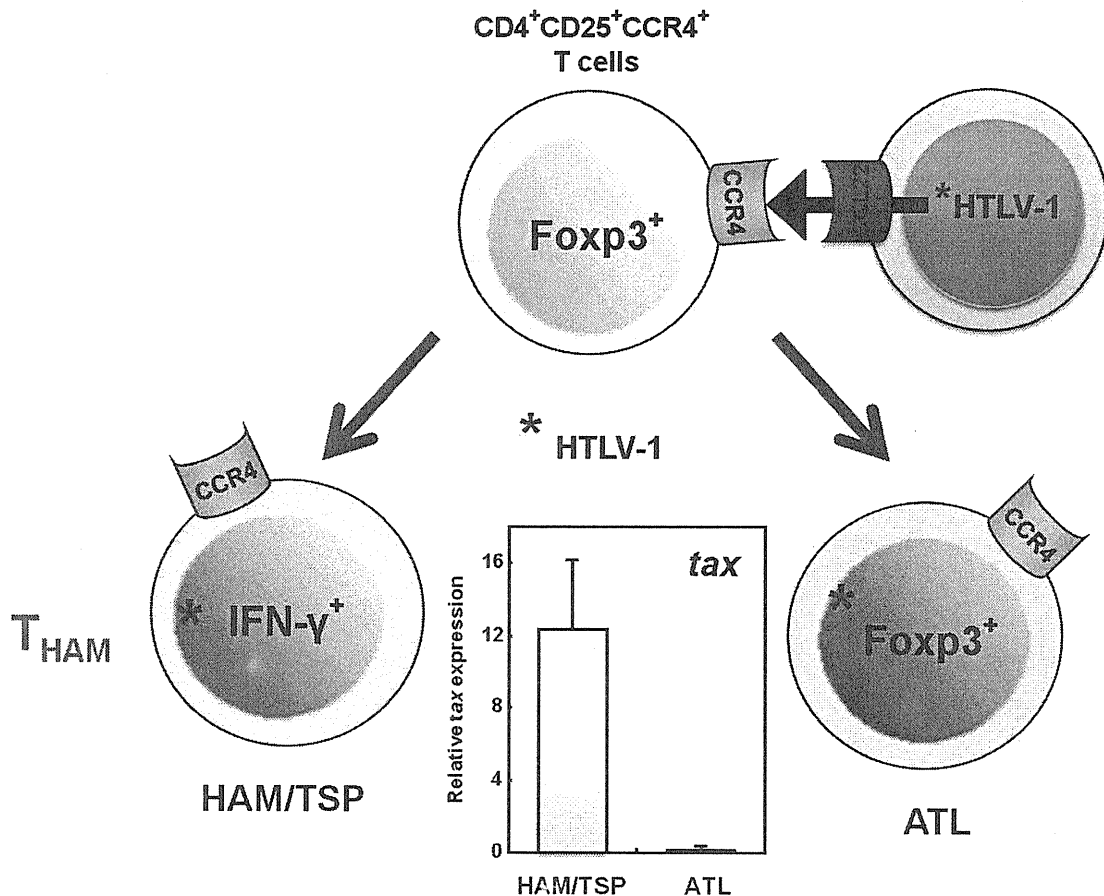**Figure 5.** Differential fate of HTLV-1-infected $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP and ATL patients. After HTLV-1 infection, $CD4^+CD25^+CCR4^+$ T cells in HAM/TSP patients, which are primarily Th2 and Treg cells before infection, become $IFN-\gamma^+Foxp3^-$ T cells (T_{HAM} cells) with high 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 $IFN-\gamma^+Foxp3^-$ 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 ($exFoxp3^+$ 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.
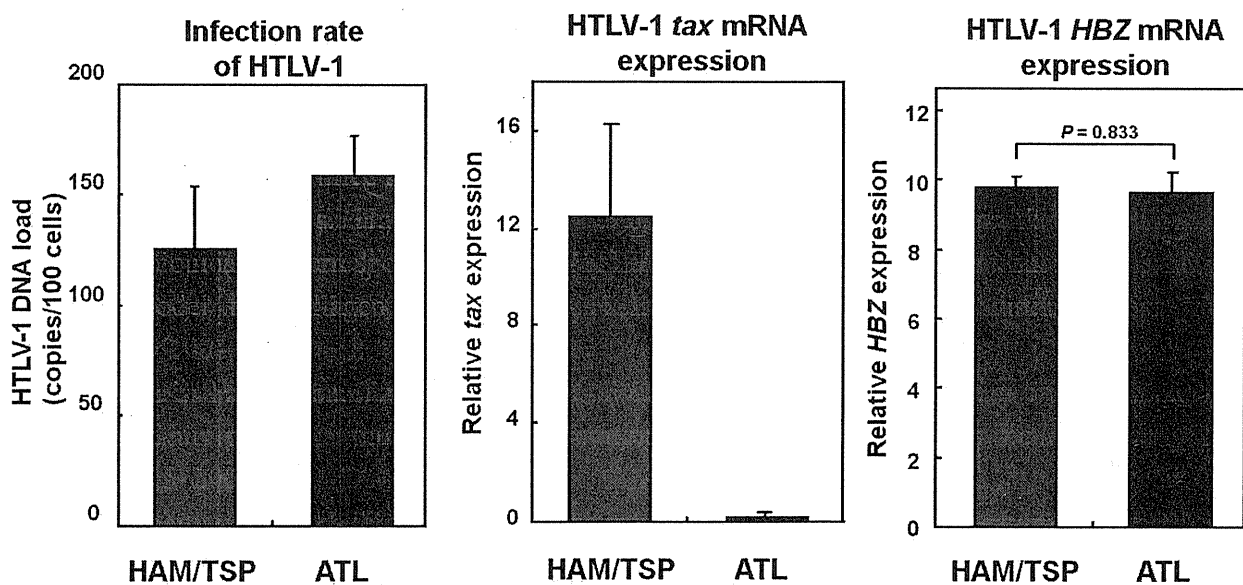
7. Mechanisms Underlying Increased HTLV-1 Tax Expression in HAM/TSP Patients

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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Mini Review

Human T-lymphotropic virus type 1 (HTLV-1) and innate immunity

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Human T-cell lymphotropic virus type 1 (HTLV-1) is a T lymphotropic human retrovirus that causes adult T-cell leukemia/lymphoma (ATL) and is associated with immunological disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A higher viral load in HTLV-1-infected individuals increases the risk of HAM/TSP and ATL; furthermore, it affects the disease severity of HAM/TSP. Therefore, the precise immune mechanisms controlling HTLV-1-infected cells must be further characterized. In this regard, the role of HTLV-1-specific CD8+ cytotoxic T lymphocytes (CTLs) has been studied intensively. However, there are few reports describing the role of innate immunity in controlling the proliferation of HTLV-1-infected cells.

Natural killer (NK) and invariant natural killer T (iNKT) cells are the cellular components of innate immunity that regulate the immune response to general viral infection and cancers. Dendritic cells (DCs) play important roles in the activation of these NK and iNKT cells as well as CTLs. In this review, we summarize the characteristics of DCs, NK cells, and iNKT cells in individuals infected with HTLV-1.

In the peripheral blood of HAM/TSP and ATL patients, the decreased number and impaired functionality of DCs, NK cells, and iNKT cells have been reported. Even in asymptomatic carriers, the functions of these cell populations are perturbed by HTLV-1 infection, while their frequencies are comparable to those of healthy individuals. These observations suggest that abnormalities of DCs, NK cells, and iNKT cells are implicated in the pathogenesis of HTLV-1-associated diseases via insufficient viral control.

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is a human retrovirus that causes persistent infection in the host. While most infected persons remain asymptomatic carriers (ACs), 3–5% develop a T-cell malignancy termed adult T-cell leukemia (ATL), and another 0.25–3% develop a chronic progressive inflammatory neurologic disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)[1]. One of the most important pathogenic factors in HAM/TSP is the increased HTLV-1 provirus load in the peripheral blood and cerebrospinal fluid[2,3], suggesting that the immune control of the virus is inadequate in affected persons. A higher HTLV-1 provirus load increases the risk of HAM/TSP and ATL[2,4]; therefore, the precise immune mechanisms controlling HTLV-1-infected cells must be characterized in more detail. With regard to the host defense mechanisms involved in HTLV-1 infection, the role of HTLV-1specific CD8^{+} cytotoxic T lymphocytes (CTLs) has been studied[5]. The HTLV-1specific CTL response is critical for the maintenance of a low viral load[5]. Despite the high frequency of HTLV-1specific CTLs, the number of HTLV-1infected T cells is surprisingly high in HAM/TSP patients[3]. We and other researchers have reported that the maturation and function of HTLV-1specific CTLs are impaired in HAM/TSP patients, although in vitro studies have shown that these CTLs exert cytolytic activity against HTLV-1-expressing target cells[6,7]. Therefore, we hypothesize that there may be another non-CTL cell population that contributes to the control of HTLV-1infected T cells.

Besides CTLs, there are several cell populations that have cytolytic activity against virus-infected cells in the human immune system (Fig. 1), e.g., natural killer (NK) cells, natural killer T (NKT) cells, and γδ T cells, which are cellular components of innate immunity. Dendritic cells (DCs) play an important role in the activation of these cell populations and CTLs. Because there is little evidence suggesting a role for γδ T cells in the pathogenesis of HTLV-1-associated disorders, this review focuses on the role of DCs, NK cells, and NKT cells in HTLV-1-associated diseases by comparing with the role of these cells in HIV-1 infection.

Dendritic cells and HTLV-1

Immature DCs are located in peripheral tissues and can effectively capture antigens, leading to their maturation via the expression of major histocompatibility complex (MHC) class I/II and co-stimulatory molecules such as CD80, CD86, and CD40. Mature DCs are professional antigen-presenting cells that are uniquely able to prime naïve T cells. DCs consist of two main subsets: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). These cells play important roles in the regulation of innate and adaptive immunity (Fig. 1). mDCs can induce the activation of invariant NKT (iNKT) cells by the surface expression of the CD1d / glycolipid complex. pDCs secrete type 1 interferon (IFN) after antigen capture. Type 1 IFN induces the activation of NK cells and promotes the activation of iNKT cells by mDCs.

An in vitro study indicated that cell-free HTLV-1 effectively infects DCs, leading to the transmission and transformation of CD4^{+} T cells[8]. This study suggested the mechanism of HTLV-1 transmission and that the HTLV-1 infection of DCs plays a role in the pathogenesis of HTLV-1associated disorders. In fact, HTLV-1infected DCs are observed in the peripheral blood of HTLV-1infected individuals[9,10], and infected pDCs have an impaired ability to produce type I IFN[10]. In addition, we recently reported that the frequency of mDCs and pDCs is significantly decreased in patients with both HAM/TSP and ATL[11]. These studies imply that the decreased number and impaired functionality of DCs are implicated in pathogenesis by interfering with innate immunity.
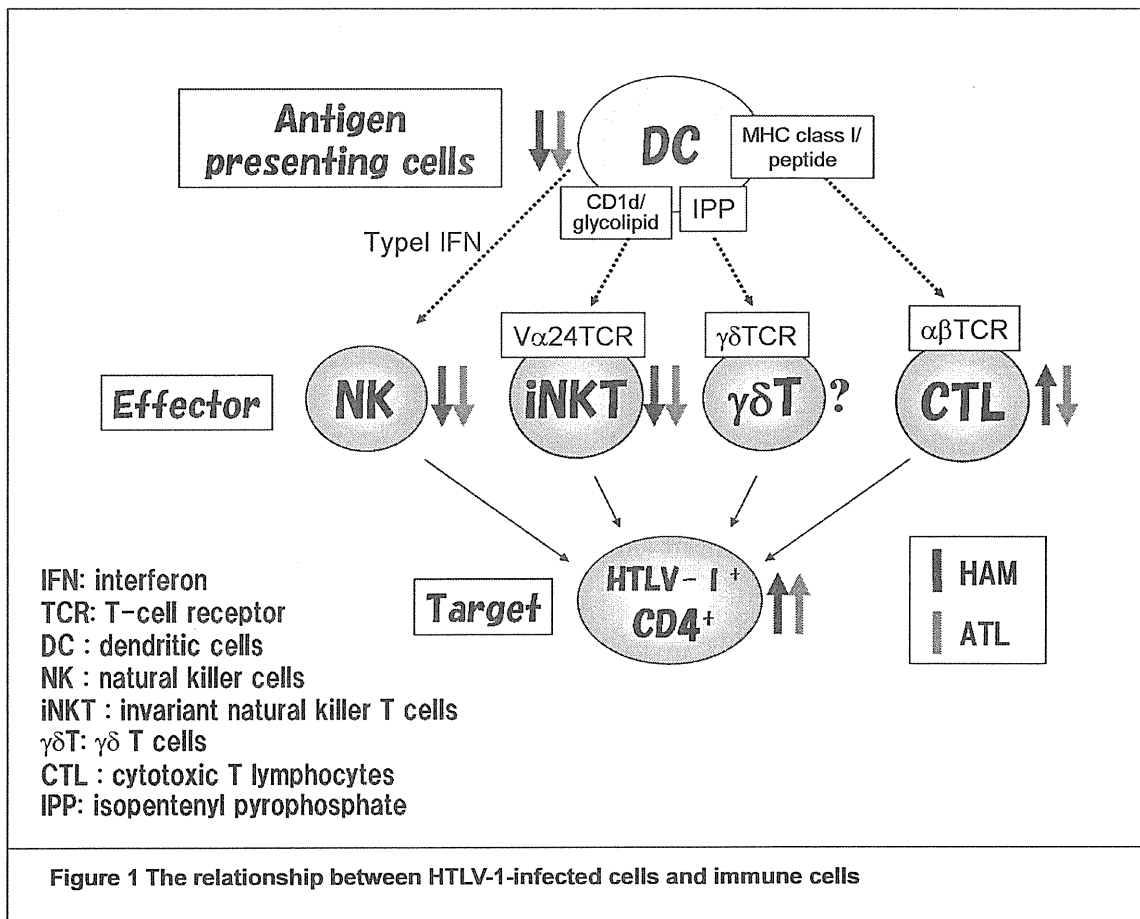
Natural killer cells and HTLV-1

NK cells are major components of the innate immune system and comprise 10–15% of peripheral blood mononuclear cells (PBMCs) in normal individuals. They have direct and indirect cytolytic activity against tumor cells and virus-infected cells by producing perforins, granzymes, and IFN-γ. Human NK cells can be divided into two subsets on the basis of their cell-surface markers: CD56^{+}CD16^{+} and CD56^{bright}CD16- NK cells. CD56^{+}CD16^{+} NK cells are the major population of NK cells and have natural cytotoxic activity.

CD56^{bright}CD16- NK cells are not cytotoxic but have the capacity to produce large amounts of IFN-γ upon activation. The activity of NK cells is regulated by a balance between positive and negative signals from different activating and inhibitory NK receptors. CD94/NKG2 receptor family is expressed on CD8+ T cells and γδT cells as well as NK cells, and is involved in the absence or presence of HAM/TSP by modulating the activities of those cell populations[12,13].

In HIV-1-infected individuals, the number and function of the NK cell subsets are impaired [14], as observed in HTLV-1-infected individuals. We and other investigators have

reported that the number of CD56+CD16+ NK cells in HAM/TSP and ATL patients are significantly reduced as compared to that in healthy controls[11,15]. The activity of NK cells in HAM/TSP patients was significantly decreased as compared to that in healthy controls[15]. In addition, the HTLV-1 infection of primary CD4+ T cells leads to their escape from NK cell-mediated cytotoxicity; HTLV-1 p12^I downregulates the expression of intercellular adhesion molecule-1 (ICAM-1) and -2 on the cell surface of infected CD4+ T cells that result in the reduced adherence of NK cells to these HTLV-1infected CD4+ T cells[16].

Figure 1 The relationship between HTLV-1-infected cells and immune cells

Natural killer T cells and HTLV-1

Natural killer T (NKT) cells, a unique T-cell subpopulation, constitute a subset of lymphocytes that share the features of innate and adaptive immune cells. Unlike conventional T cells, NKT cells express a T-cell receptor (TCR) that recognizes glycolipids instead of protein antigens. Moreover, these

cells share properties and receptors with NK cells. They rapidly produce granzymes and perforins upon stimulation. Among the CD3+ T cells in human blood, 10–25% express NK cell surface molecules such as CD161, and these cells are classified as NKT cells. A small population of T cells within this NKT cell subset expresses a highly conserved Vα24Jα18 TCR chain that preferentially as-

sociates with Vβ11. These T cells are referred to as invariant NKT (iNKT) cells. Activation of human iNKT cells requires the presentation of glycolipids such as α-galactosylceramide (α-GalCer) on the MHC class I-like molecule CD1d (Fig. 1). α-GalCer induces the rapid production of cytokines and potent antitumor and antipathogen responses by iNKT cells. CD4– iNKT cells preferentially induce the Th1 response and are more important than CD4+ iNKT cells in controlling viral infection and cancer[17].

	HTLV-1 infection	ATL		HAM		AC	
		Freq.	Function	Freq.	Function	Freq.	Function
mDCs	+[9]	↓↓[10,11]	↓↓	↓[11]	↓	→[10,11]	↓
pDCs	+[10]	↓[10,11]	↓↓	↓[10,11]	↓	→[10,11]	↓[10]: IFNα production
NK cells	–[21]	↓↓[11]	↓↓	↓[11,15]	↓[15]: cytolytic activity	→[11]	↓[15]: cytolytic activity
iNKT cells	+[11]	↓↓[11]	↓↓	↓↓[11,22]	↓[11]: intracellular perforin	→[11]	↓[11]: intracellular perforin

Table 1. The immunological conditions of DCs, NK cells, and NKT cells in HTLV-1-infected individuals

DCs, dendritic cells; mDCs, myeloid DCs; pDCs, plasmacytoid DCs; NK, natural killer cells; NKT, natural killer T-cells; iNKT, invariant NKT cells; IFN, interferon; Freq., frequency; ATL, adult T-cell leukemia; HAM, HTLV-1-associated myelopathy; ACs, asymptomatic carriers

HIV-1-infected subjects had a reduced number of iNKT cells in the peripheral blood as compared to healthy donors[18,19]. The proliferative potential and INF-γ production of residual iNKT cells were impaired in HIV-1-infected individuals[20]; likewise, patients with HTLV-1-associated disorders had a decreased frequency of iNKT cells in the peripheral blood[11]. Interestingly, unlike HIV-1 infection, CD4– iNKT cells were preferentially decreased by HTLV-1 infection[11]. The production of perforin in iNKT cells was impaired in ACs and HAM/TSP patients[11]. In addition, there was an inverse correlation between the frequency of iNKT cells and the HTLV-1 proviral load in the peripheral blood of HTLV-1-infected individuals[11]. Notably, in vitro stimulation of peripheral blood cells with α-GalCer led to an increase in the number of iNKT cells and a subsequent decrease in the number of HTLV-1-infected T-cells in samples from ACs[11]. These results suggest that iNKT cells contribute to the immune defense against HTLV-1, and iNKT cell depletion plays an important role in the pathogenesis of HAM/TSP and ATL.

Conclusion

In Figure 1 and Table 1, we summarize the immunological conditions of DCs, NK cells, and iNKT cells in HTLV-1-infected individuals. In ACs, the functions of these cell populations are perturbed by HTLV-1 infection, whereas their frequencies are comparable to those of healthy individuals. These conditions may suggest the latent immunosuppressive state of HTLV-1 carriers. In patients with ATL and HAM/TSP, not only the functional impairment of DCs, NK cells, and iNKT cells but also the decreased number of these cell populations has been observed. These conditions may contribute to inadequate viral control and have an important role in the pathogenesis of HTLV-1associated disorders.

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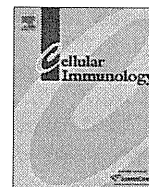
Advantage of higher-avidity CTL specific for Tax against human T-lymphotropic virus-1 infected cells and tumors

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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.

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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 $\alpha 1$ and $\alpha 2$ domains from human HLA-A2.1 and $\alpha 3$ 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 $\alpha 3$ 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 $190 \times g$ 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
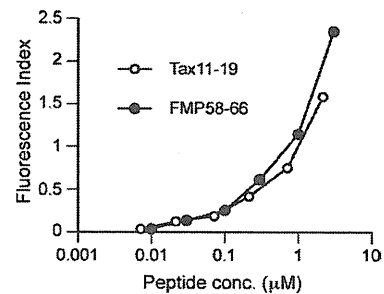

Fig. 1. Comparison of HLA-A2 binding curves between Tax11-19 and FMP58-66 peptide in T2-binding assay. The binding affinity of Tax11-19 for HLA-A2 molecule is almost as strong as that of FMP58-66 in influenza A virus.

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).
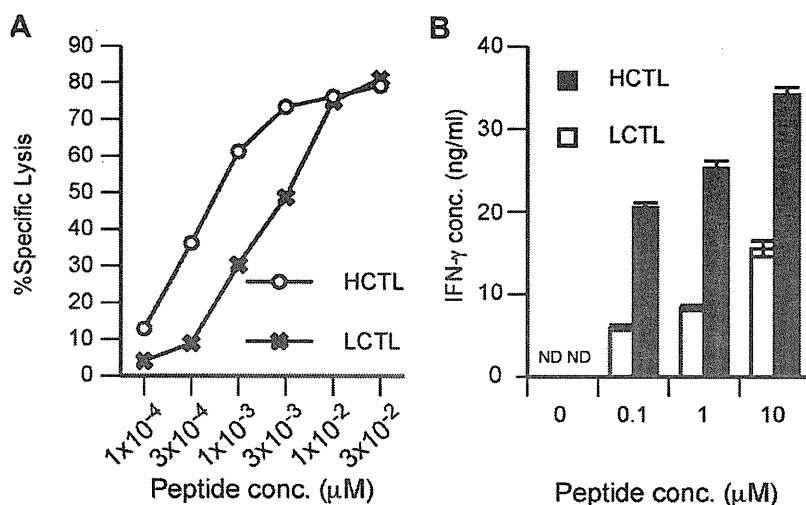
Fig. 2. Difference in functional avidity between HCTLs and LCTLs. (A) Recognition by the Tax11-19 peptide specific CTLs, HCTL and LCTL, of Tax11-19 antigenic peptide from 10^{-4} to 10 μM when presented on C1R.AAD target cells. The effector to target-cell (E/T) ratio was 20:1. Error bars were omitted 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.
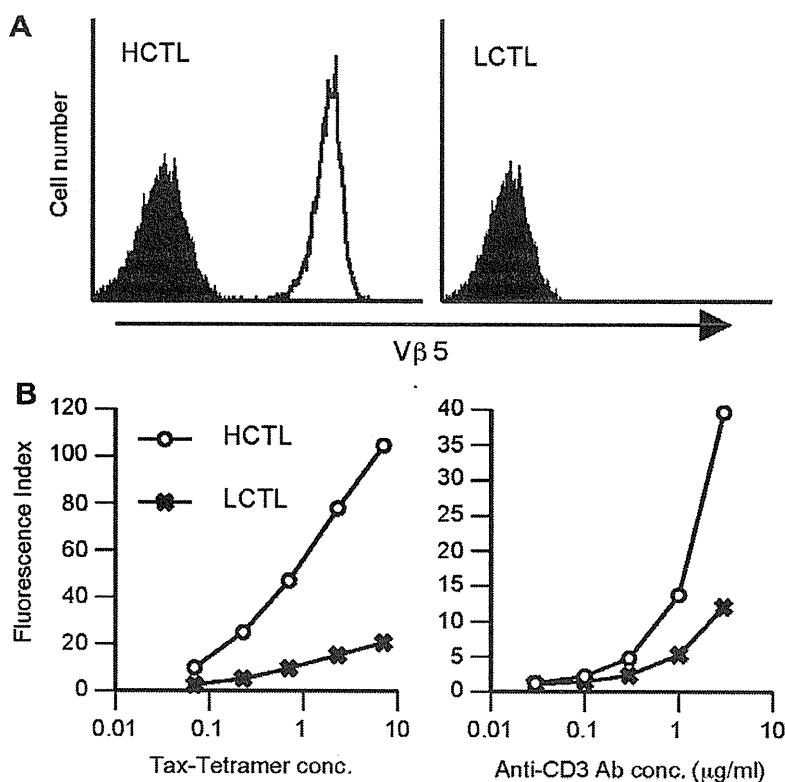

Fig. 3. TCR Vβ usage and expression level of TCR complex on Tax-specific CTLs with different functional avidity. (A) Comparison of Vβ usage pattern between HCTLs and LCTLs cytometry analysis (FCM). No Vβs among available anti-Vβ antibodies were detected in LCTL but only Vβ5 was detected in HCTL. (B) 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
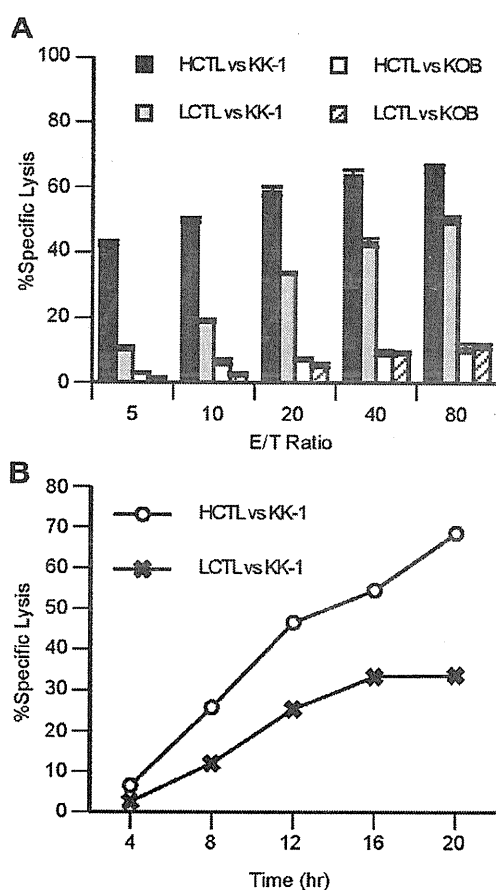
A

B

A

B

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
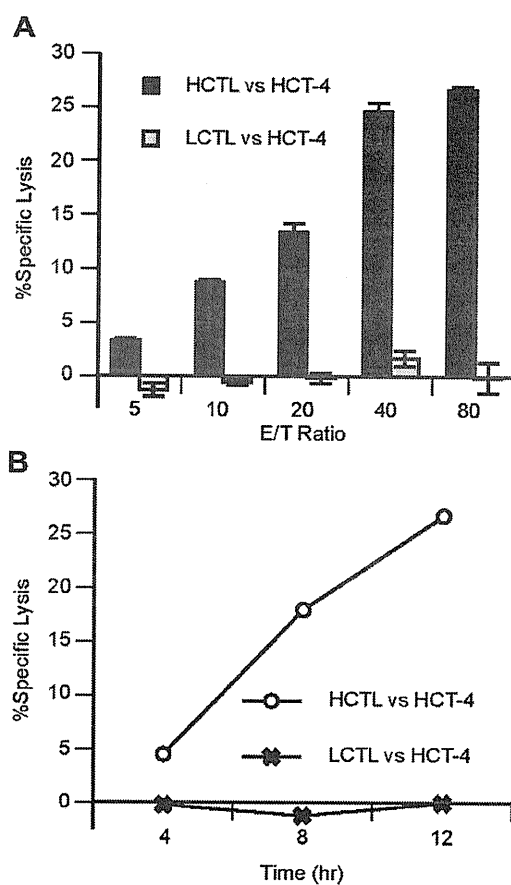
of anti-tumor immunity, the in vivo relevance of differences in functional avidity has been established by demonstrating that high-avidity CTLs clear tumor antigens more efficiently than low-avidity CTL [34–38]. In HTLV-1 infection, however, while there is increasing body of evidence that CTL quality 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