pathogenesis of HAM/TSP. MPs are infected with HTLV-I *in vitro* and *in vivo* [13-18], and dendritic cells have been shown to effectively transfer cell-free virus to $CD4^+$ T cells [18]. HTLV-I-infected dendritic cells can stimulate both $CD4^+$ and $CD8^+$ T cells [17]. Moreover, HTLV-I infection of $CD14^+$ cells and the concomitant expression of IL-15 mediate spontaneous degranulation and IFN-γ expression in $CD8^+$ T cells [19]. Pathological studies have confirmed the presence of inflammatory monocyte/macrophages as well as $CD4^+$ T cells and $CD8^+$ T cells in the central nervous system (CNS) of HAM/TAP patients [20,21]. These findings suggest that virus-infected or activated MPs may play a role in immune regulation and disease progression in patients with HTLV-I-associated neurological diseases.

MPs are widely distributed immune cells that maintain tissue homeostasis and provide a first line of defense against invading pathogens. MPs have been shown to present antigens bound by major histocompatibility complex (MHC) molecules and to activate $CD4^+$ T helper cells or cytotoxic $CD8^+$ T cells [22]. The abilities to combat microbial infection and clear debris are intimately tied to MP activation and follow degenerative, inflammatory, infectious, and ischemic insults. However, under inflammatory conditions, differential MP population and activation of MPs are related to immunopathogenesis and disease progression. Human peripheral monocytes contain two major subsets, the $CD14^+CD16^-$ and $CD14^{low}CD16^+$ monocytes [23]. The $CD14^{low}CD16^+$ monocytes express higher levels of proinflammatory cytokines than $CD14^+CD16^-$ monocytes, with a higher capacity for antigen presentation, and are increased in inflammatory and infectious diseases in humans [24]. Macrophage/microglial inflammatory activities have been shown to influence a number of neurodegenerative diseases including human immunodeficiency virus (HIV)-associated dementia, Alzheimer's disease, Parkinson's disease, stroke, brain and spinal cord trauma [25]. In HAM/TSP, the expression of proinflammatory cytokines such as IL-1β, TNF-α and IFN-γ is detected in peripheral blood mononuclear cells (PBMCs) as well as in perivascular infiltrating macrophages and microglia in the spinal cords of patients with HAM/TSP [26,27]. Moreover, HTLV-I Tax has been reported to induce the human proIL-1β gene promoter in monocytic cells [28]. Thus, MPs of patients with HAM/TSP might be activated under inflammatory conditions and play a role in immunopathogenesis of this disorder.

In this study, we demonstrate that $CD14^+$ cells of patients with HAM/TSP showed an inflammatory phenotype as evidenced by high expression of HLA-DR and CX_3CR1, proinflammatory cytokines (TNF-α and IL-1β) and acceleration of HTLV-I Tax expression in $CD4^+$ T cells. Minocycline, which is tetracycline derivative and a known inhibitor of activated macrophage/microglia [29], significantly inhibited TNF-α and IL-1β expressions in cultured $CD14^+$ cells of patients with HAM/TSP. Moreover, treatment with minocycline demonstrated inhibition of IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP, resulting from inhibition of MP activation by minocycline. These results demonstrate that $CD8^+$ T cell activation of patients with HAM/TSP can be suppressed through down-regulation of MP activation, and suggest a novel treatment strategy in patients with HTLV-I associated neurological disease.

Results

High CX_3CR1 and HLA-DR expression in monocytes of patients with HAM/TSP

To characterize $CD14^+$ cell subsets in PBMCs of HAM/TSP patients, the expression of monocyte markers CD14 and CD16 was examined by flow cytometry in NDs, ACs and patients with HAM/TSP. Figure 1A demonstrates a representative dot plot of MP populations of a ND and a patient with HAM/TSP. Group analysis did not show significant differences between $CD14^+CD16^-$ and $CD14^{low}CD16^+$ frequencies in MP population among NDs, ACs, and patients with HAM/TSP (data not shown). Previous reports demonstrated that $CD14^{low}CD16^+$ monocytes expressed higher levels of CX_3CR1 (a fractalkine receptor) and HLA-DR, proinflammatory cytokines and higher potency in antigen presentation in human inflammatory and infectious diseases [23,24]. We therefore compared CX_3CR1 and HLA-DR expression on $CD14^{low}CD16^+$ monocytes among the groups. A representative dot plot shows that both CX_3CR1 and HLA-DR expression was higher in $CD14^{low}CD16^+$ subset of a patient with HAM/TSP than that of a ND (Figure 1A). In NDs, the $CD14^{low}CD16^+$ subset expressed both CX_3CR1 and HLA-DR (mean+/-standard deviation (SD) = 7.572+/-6.748, n = 10; Figure 1B). In contrast, the $CD14^{low}CD16^+$ subset of patients with HAM/TSP had significantly higher levels of both CX_3CR1 and HLA-DR expression (mean+/-SD = 51.88+/-24.42, n = 12; Figure 1B). CX_3CR1 and HLA-DR expression in $CD14^{low}CD16^+$ subset of ACs was significantly lower than those in patients with HAM/TSP, and at comparable levels with those in NDs (mean+/-SD = 15.04+/-13.31, n = 6; Figure 1B). These results demonstrated that the $CD14^{low}CD16^+$ subset in patients with HAM/TSP showed significantly high expression of CX_3CR1 and HLA-DR, compared to NDs and ACs.

Given the high expression of CX_3CR1 and HLA-DR on the $CD14^{low}CD16^+$ subset in patients with HAM/TSP, we asked whether these changes in MP subsets were related to biomarkers of disease activity in HAM/TSP. We previously reported that $CD14^+$ cells induced degranulation and IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP *in vitro* [19]. We therefore analyzed the

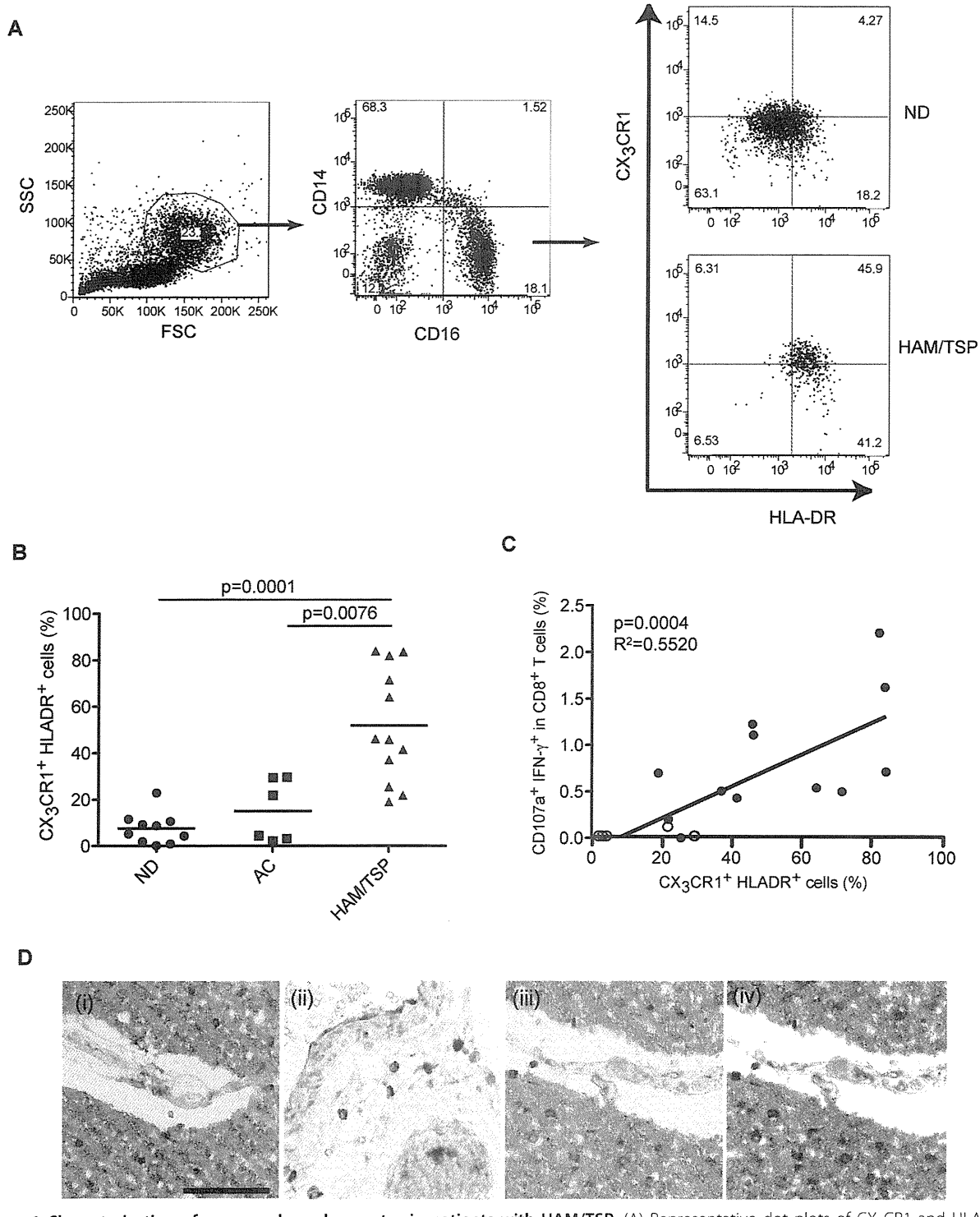

Figure 1 Characterization of mononuclear phagocytes in patients with HAM/TSP. (A) Representative dot plots of CX_3CR1 and HLA-DR expression in $CD14^{low}$ $CD16^+$ cells of a ND and a HAM/TSP patient. (B) Comparison of frequencies of CX_3CR1^+ HLA-DR^+ cells in $CD14^{low}$ $CD16^+$ mononuclear phagocytes of NDs, ACs and HAM/TSP patients. The data were obtained from ten NDs, six ACs and twelve HAM/TSP patients. The $CD14^{low}CD16^+$ subset of HAM/TSP patients had significantly higher levels of both CX_3CR1 and HLA-DR expression, compared to NDs ($p = 0.0001$) and ACs ($p = 0.0076$) by Mann-Whitney test. The horizontal line represents the mean. (C) The frequency of CX_3CR1^+HLA-DR^+ cells was shown to be significantly correlated with spontaneous degranulation/IFN-γ expressions in $CD8^+$ T cells of HTLV-I-infected patients, including ACs ($n = 6$, opened circle) and patients with HAM/TSP ($n = 12$, closed circle) by simple linear regression analysis ($P = 0.0004$, $R^2 = 0.5520$). (D) Localization of CX_3CR1^+ cells in the spinal cord of a HAM/TSP patient. Parenchyma (i) and meninges (ii) were stained with antibodies for CX_3CR1 (brown). Parenchyma was stained with antibody for CX_3CR1 (brown in iii), and double-stained with CX_3CR1 and CD68 (red in iv). CX_3CR1^+ cells were positive for CD68. Magnifications, ×20. Black bar = 40 μm.

relationship between CX_3CR1/HLA-DR expression on $CD14^{low}CD16^+$ subset and degranulation/IFN-γ expression in $CD8^+$ T cells of HTLV-I-infected patients. CX_3CR1/HLA-DR expression on $CD14^{low}CD16^+$ subset was significantly correlated with degranulation/IFN-γ expression in $CD8^+$ T cells of HTLV-I-infected patients (Figure 1C; P = 0.0004, R^2 = 0.552). These results suggested that activation of MP *in vivo* could be related to $CD8^+$ T cell activation of patients with neurologic inflammatory disease.

Immunohistochemical analysis further demonstrated that CX_3CR1^+ cells were detected in the spinal cord of a patient with HAM/TSP (Figure 1D). CX_3CR1^+ cells were detected around the blood vessels and in the parenchyma and the meninges in the HAM/TSP spinal cord (Figure 1Di and 1Dii, respectively), suggesting a recruitment of CX_3CR1^+ cells from the periphery to the spinal cord parenchyma and meninges. Moreover, CX_3CR1^+ cells in the parenchyma were morphologically bigger (Figure 1Diii) and positive for CD68 (Figure 1Div), probably corresponding to MPs. These results further support the idea that CX_3CR1^+ cells might be recruited from peripheral blood to the spinal cord in patients with HAM/TSP.

$CD14^+$ cells express TNF-α and IL-1β and increase HTLV-I Tax expression in $CD4^+$ T cells of patients with HAM/TSP

To further investigate MP activation in HAM/TSP patients, we examined TNF-α and IL-1β expression in cultured PBMCs of ND and HAM/TSP patients. After culture of total PBMCs for 24 hours, the frequency of $CD14^+$ cells that expressed TNF-α was first examined by flow cytometry. $CD14^+$ cells expressing TNF-α was significantly elevated in HAM/TSP patients, compared to NDs (Figure 2A). IL-1β was detected in PBMC culture supernatants of HAM/TSP patients but not of NDs (Figure 2B). Since relative expression of IL-1β mRNA dramatically increased in $CD14^+$ cells after culture (data not shown), IL-1β detected in the culture supernatants would be released from the MPs of HAM/TSP patients.

In addition to the production of various proinflammatory cytokines, activated or virus-infected MPs, such as infection with HIV, effectively transfer or promote productive virus upon interaction with T cells [30,31]. Although $CD14^+$ cells of patients with HAM/TSP are activated and also infected with HTLV-I at low levels, we wished to determine if there would be an increase in HTLV-I production in $CD4^+$ T cells of patients with HAM/TSP after interaction with autologous $CD14^+$ cells. To address whether $CD14^+$ cells promote HTLV-I production in $CD4^+$ T cells of patients with HAM/TSP, we examined HTLV-I Tax expression of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells cocultured with or without autologous $CD14^+$ cells of patients with HAM/TSP, compared to those of ACs. As shown in Figure 2Ci, in patients with HAM/TSP, 5.8-9.5% of $CD4^+CD25^+$ T cells expressed HTLV-I Tax proteins at baseline. After coculture with autologous $CD14^+$ cells, HTLV-I Tax expression was dramatically increased in $CD4^+CD25^+$ T cells (14.1-15.9%, p = 0.0226; Figure 2Ci). While HTLV-I Tax expression was also detected in 0.8-4.4% of $CD4^+CD25^-$ T cells, an increase after coculture with $CD14^+$ cells was lower than in $CD4^+CD25^+$ T cells (Figure 2Cii). Since the increase of Tax expression was not detected in $CD4^+$ T cells without cell-cell contact with $CD14^+$ cells (data not shown), the increased expression of HTLV-I Tax in $CD4^+$ T cells by the addition of $CD14^+$ cells was cell-dependent. By contrast, both $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells of ACs showed lower expression of Tax proteins (< 1%), which did not change after coculture with autologous $CD14^+$ cells (Figure 2C). Thus, $CD14^+$ cells could accelerate Tax expression in HTLV-I-infected $CD4^+$ T cells of patients with HAM/TSP.

Minocycline inhibited MP activation and spontaneous lymphocyte proliferation of patients with HAM/TSP

Since various therapeutic agents have been developed for neuroinflammatory diseases specifically aimed at the inhibition of activated MPs, we attempted to examine the inhibition of MP function in patients with HAM/TSP using minocycline, which is known as an inhibitor of monocyte/macrophage activation. To evaluate inhibitory effect of minocycline on activated MP of patients with HAM/TSP, we examined TNF-α expression in cultured PBMCs of patients with HAM/TSP by treatment with minocycline. As shown in Figure 3A, the frequency of $CD14^+$ cells expressing TNF-α was significantly inhibited at 10 μM of minocycline treatment in HAM/TSP patients (Figure 3A; closed bar, p = 0.0313). The cultured $CD4^+$ T cells also expressed TNF-α, but minocycline did not inhibit TNF-α expression in $CD4^+$ T cells (Figure 3A; opened bar). As demonstrated previously (Figure 2B), IL-1β was detected in the supernatants of cultured PBMCs of patients with HAM/TSP; the release of IL-1β from these cultured HAM/TSP PBMCs was also inhibited by 10 μM of minocycline treatment (p = 0.0078; Figure 3B). These results demonstrated that minocycline inhibited the expression of proinflammatory cytokines from MPs, but not from $CD4^+$ T cells, of patients with HAM/TSP.

An additional established measure of HAM/TSP T cell activation *ex vivo* is the well-described observations of increased spontaneous lymphoproliferation [5]. In addition to the expression of HTLV-I Tax and a variety of cytokines in PBMCs of HTLV-I-infected patients that are associated with spontaneous lymphoproliferation [32-34], the activation of MP is also involved in spontaneous

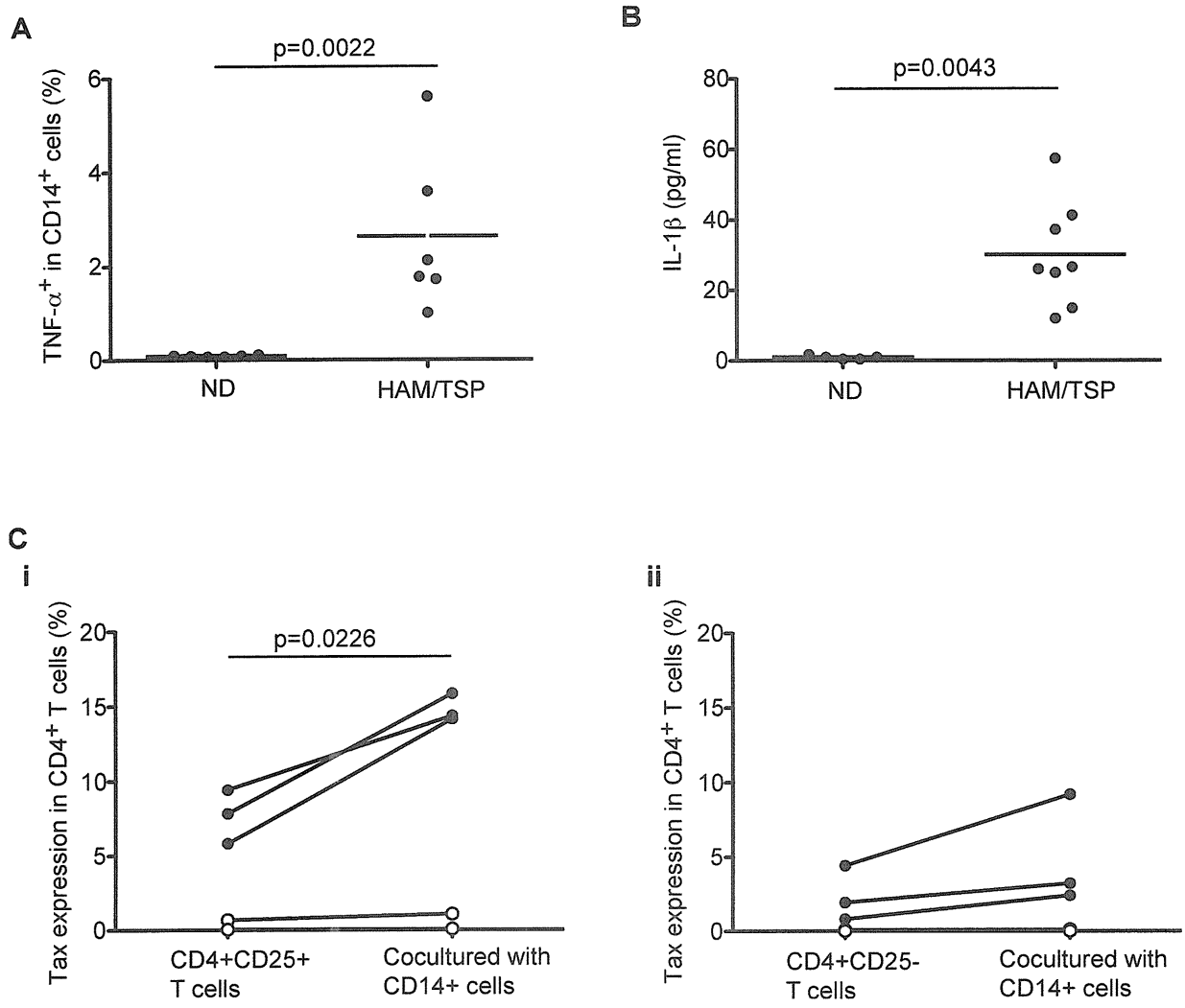

Figure 2 Activated CD14$^+$ cells in patients with HAM/TSP. (A) TNF-α expression in CD14$^+$ cells of NDs and HAM/TSP patients after culture for 24 hours. The data were obtained from six NDs and six HAM/TSP patients. CD14$^+$ cells expressing TNF-α was significantly elevated in HAM/TSP patients, compared to NDs by Mann-Whitney test ($p = 0.0022$). The horizontal line represents the mean. (B) Detection of IL-1β in PBMC culture supernatants of NDs and HAM/TSP patients after culture for 24 hours. The data were obtained from five NDs and eight HAM/TSP patients. IL-1β expression in HAM/TSP patients was significantly higher in those cells of NDs by Mann-Whitney test ($p = 0.0043$). The horizontal line represents the mean. (C) Tax expressions in CD4$^+$CD25$^+$ T cells (i) and CD4$^+$CD25$^-$ T cells (ii) cocultured with or without autologous CD14$^+$ cells of ACs ($n = 2$, opened circle) and patients with HAM/TSP ($n = 3$, closed circle) for 18 hours.

lymphoproliferation of patients with HAM/TSP [5]. To address the inhibitory effects of minocycline on spontaneous lymphoproliferation, uptake of [^{3}H] thymidine as a marker of proliferation was examined in PBMCs of two patients with HAM/TSP after treatment with minocycline. In minocycline-treated HAM/TSP PBMCs, the spontaneous lymphoproliferation was inhibited in a dose-dependent manner (Figure 3C). Since the treatment with minocycline did not inhibit HTLV-I Tax expression in both T cells and CD14$^+$ cells (data not shown), these results showed that minocycline can downregulate MP activation, such as proinflammatory cytokine expression.

Minocycline inhibits spontaneous degranulation and IFN-γ expression in CD8$^+$ T cell of patients with HAM/TSP

MPs play an indispensable role in the induction of antigen-specific CTL responses by capturing viral antigen and presenting peptide through MHC class I to CD8$^+$ T cells. In patients with HAM/TSP, HTLV-I-infected or activated MPs collaborate with CD8$^+$ T cell to induce spontaneous degranulation and high IFN-γ expression [19]. Since we have demonstrated that minocycline has inhibitory effects on activated MPs (Figure 3), minocycline might also inhibit MP function such as triggering adaptive immune responses. To determine if inhibition of MPs affects CD8$^+$

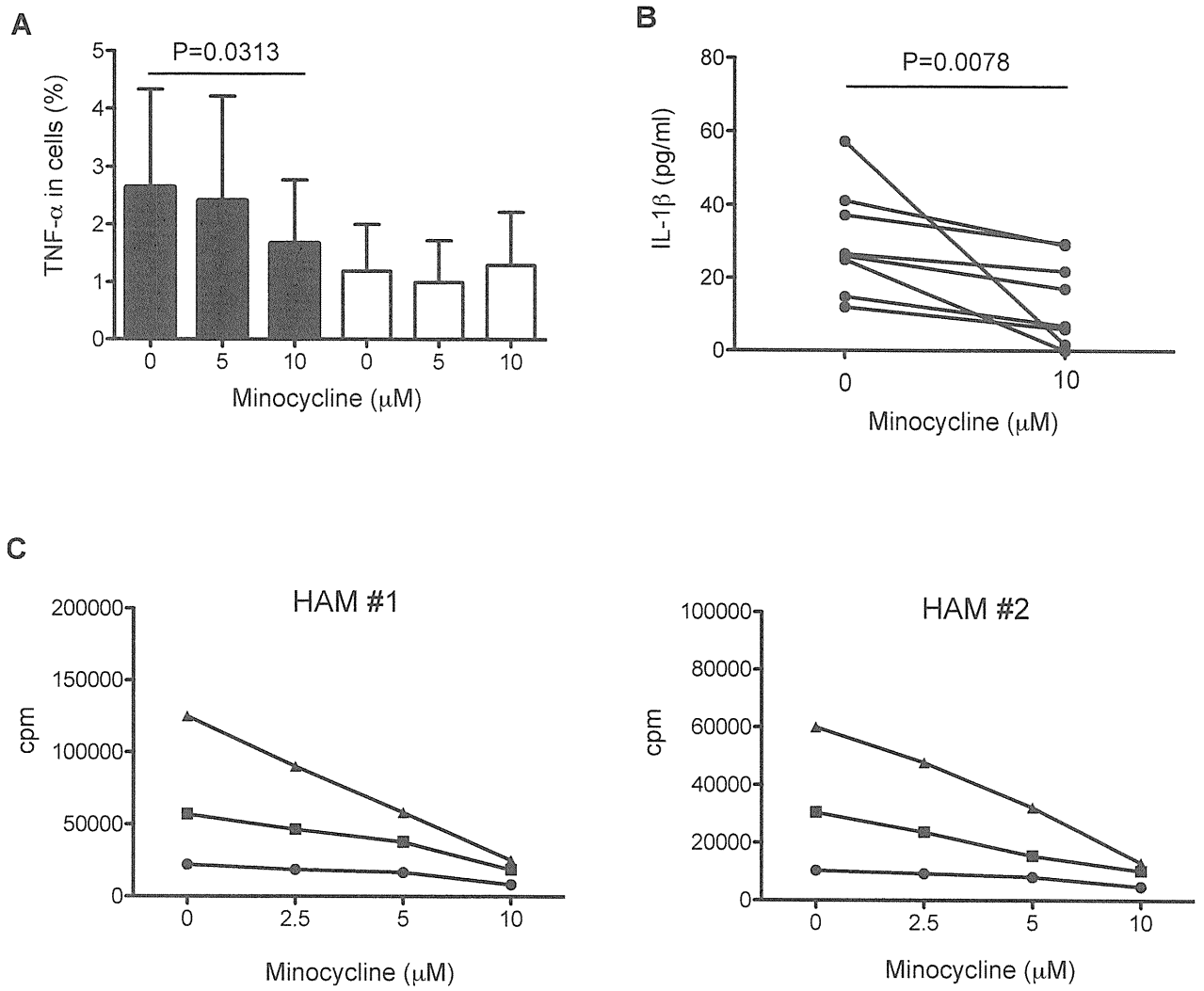

Figure 3 Minocycline inhibited TNF-α expression and IL-1β release in patients with HAM/TSP. (A) Dose-dependent inhibitory effects of minocycline on TNF-α expressions in $CD14^+$ cells (closed bar) and $CD4^+$ T cells (opened bar) of HAM/TSP patients (n = 6). The PBMCs were cultured with 0, 5 and 10 μM of minocycline for 24 hours. The frequency of $CD14^+$ cells expressing TNF-α was significantly inhibited at 10 μM of minocycline treatment in HAM/TSP patients (p = 0.0313; Wilcoxon matched-pairs signed rank test). Error bars represent SD. (B) Inhibition of IL-1β release in PBMC culture supernatants of HAM/TSP patients by 10 μM of minocycline after culture for 24 hours (n = 8). The release of IL-1β from these cultured HAM/TSP PBMCs was significantly inhibited by minocycline treatment (p = 0.0078; Wilcoxon matched-pairs signed rank test). (C) Inhibitory effects of minocycline on spontaneous lymphoproliferation in HAM/TSP patients. The PBMCs from two HAM/TSP patients (HAM#1 and #2) were cultured with 0, 2.5, 5 and 10 μM of minocycline, and pulsed with 1 μCi $[^3H]$ thymidine for 4 hours at 3 days (closed circle), 4 days (closed square) and 5 days (closed triangle). The average cpm from each well in triplicate was plotted.

T cell responses in HAM/TSP, we examined the effect of minocycline on expression of CD107a, a marker of degranulation, and IFN-γ in $CD8^+$ T cells of patients with HAM/TSP. As previously reported [19], CD107a and IFN-γ were spontaneously expressed in $CD8^+$ T cells of a patient with HAM/TSP after PBMC culture for 24 hours without any exogenous stimuli, but not in those cells of a ND. In Figure 4A, representative dot plots show that treatment with minocycline inhibited CD107a and IFN-γ expression in $CD8^+$ T cells of a patient with HAM/TSP. Group analysis showed significant, dose-dependent, inhibitory effects of minocycline on spontaneous degranulation and IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP (Figure 4B). These results demonstrated that spontaneous degranulation and IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP were inhibited by treatment with minocycline.

Minocycline inhibits antigen-specific $CD8^+$ T cells responses in patients with HAM/TSP

To confirm whether treatment with minocycline could inhibit antigen-specific $CD8^+$ T cell responses of

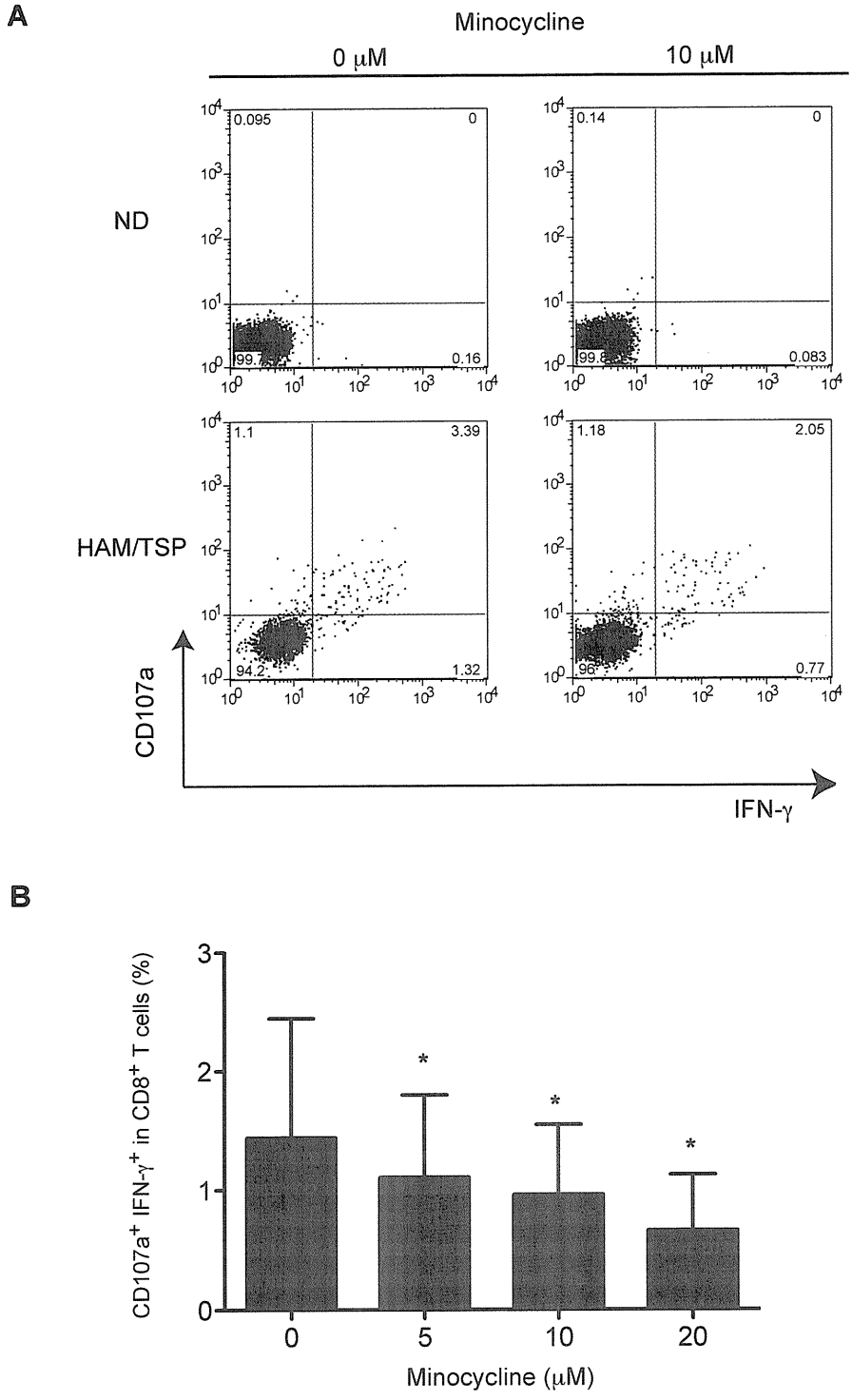

Figure 4 Minocycline inhibited spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107a and IFN-γ expression in $CD8^+$ T cells of a ND and a HAM/TSP patient after culture for 24 hours with or without 10 μM of minocycline. (B) Inhibitory effects of minocycline on degranulation/IFN-γ expression in $CD8^+$ T cells of eight HAM/TSP patients after culture for 24 hours. Spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients was significantly inhibited by minocycline treatment (*p = 0.0078; all by Wilcoxon matched-pairs signed rank test). Error bars represent SD.

patients with HAM/TSP, we examined CD107a and IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP, who were subtyped as HLA-A*201, by stimulation with a known immunodominant HLA-A2-binding HTLV-I Tax11-19 peptide [35]. As previously reported [36], cytotoxicity (CD107 expression) can be triggered at peptide concentrations 10- to 100-fold less than those required for inflammatory cytokine (IFN-γ) production in primary virus-specific human $CD8^+$ T cells. In $CD8^+$ T cells of a patient with HAM/TSP, after stimulation with the low peptide concentration (0.1 ng/ml) for 5 hours, the majority of responding cells degranulated, but produced little or no detectable IFN-γ (Figure 5A). As the peptide concentration was increased, more cells exhibited dual effector functions of degranulation and IFN-γ production (Figure 5A). Thus, $CD8^+$ T cells exhibited inflammatory changes following cytotoxic responses depending on the quantity of antigen stimulation. Figure 5B shows representative dot plots of CD107a and IFN-γ expressions in $CD8^+$ T cells of a HLA-A*201$^+$ patient with HAM/TSP after the Tax11-19 stimulation with or without minocycline treatment. As the peptide concentration increased, more cells exhibited both degranulation and IFN-γ production in $CD8^+$ T cells of a HAM/TSP patient (Figure 5B, upper dot plots). Interestingly, as the cells were treated with minocycline, both degranulation and IFN-γ production were detected in Tax11-19-specific $CD8^+$ T cells, but the frequency of $CD107a^+IFN\text{-}\gamma^+$ cell population did not increase in $CD8^+$ T cells stimulated with increased Tax11-19 peptides (Figure 5B, lower dot plots). These results suggested that minocycline inhibited the activation of Tax-specific $CD8^+$ T cells (Figure 5B, lower dot plots). In addition, IFN-γ expression was reduced, but total CD107a expression did not change in Tax11-19-specific $CD8^+$ T cells after minocycline treatment (Figure 5B, lower dot plots). Three HLA-A*201$^+$ HAM/TSP patients showed that minocycline treatment inhibited 40% of $CD107a^+IFN\text{-}\gamma^+$ expressions, but not total CD107a expressions, in $CD8^+$ T cells after stimulation with Tax11-19 (Figure 5C). These results demonstrated that treatment with minocycline reduced the inflammatory responses (IFN-γ expression), but retained anti-viral cytotoxic response (total CD107a expression) in Tax11-19-specific $CD8^+$ T cells of HAM/TSP patients.

Minocycline down-regulated MHC class I expression on MPs of patients with HAM/TSP

As $CD8^+$ T cells are stimulated by antigenic peptides that are presented by MHC class I molecules expressed on the surface of antigen-presenting cells, we asked whether the effect of minocycline that modulates the inflammatory response in Tax-specific $CD8^+$ T cells of patients with HAM/TSP might be associated with decreased capacity of antigen-presentation in MPs. To clarify the capacity of antigen-presentation in MPs, we examined MHC class I expression on MPs of patients with HAM/TSP after culture with or without minocycline treatment. Figure 6A shows representative histograms of MHC class I expression on $CD14^+$ cells in a patient with HAM/TSP before and after culture for 5 and 18 hours. MHC class I expression on $CD14^+$ cells of a patient with HAM/TSP gradually increased after culture (Figure 6A). After treatment with minocycline, MHC class I expression on $CD14^+$ cells gradually decreased, compared to those on $CD14^+$ cells without minocycline (Figure 6A). Group analysis including three patients with HAM/TSP showed that mean fluorescent intensities of MHC class I expression on $CD14^+$ cells were significantly inhibited by treatment with minocycline after 18 hours culture (Figure 6B). These results demonstrated that minocycline down-modulated MHC class I expression on activated HAM/TSP MPs, suggesting that the inflammatory response of $CD8^+$ T cells in patients with HAM/TSP was suppressed through down-regulation of MP activation by minocycline.

Discussion

MPs play pivotal roles in antigen capture and presentation, pathogen and tissue debris clearance, and cellular secretory functions. However, activated MPs can infiltrate through the blood brain barrier and contribute to the CNS inflammation by secreting various inflammatory cytokines and growth-inhibiting proteins. In HAM/TSP, MPs are reservoirs of HTLV-I, induce proinflammatory cytokines and excessive antigen-specific T cell responses, and can also infiltrate the CNS. In our study, we analyzed $CD14^+$ cell subpopulation in PBMCs of patients with HAM/TSP and demonstrated that $CD14^{low}CD16^+$ subset of patients with HAM/TSP showed significantly higher CX_3CR1 and HLA-DR expression, compared to NDs and ACs. Since it has been reported that CX_3CR1 expression is regulated by IL-2 and IL-15 [37], activated T cells expressing these cytokines might affect CX_3CR1 expression on monocytes in patients with HAM/TSP [19,38,39]. In mice, $GR1^-$ CX_3CR1^{high} monocytes (homolog of human $CD16^+$ monocytes) patrol vascular endothelium by mechanisms involving LFA-1 and CX_3CR1 and are rapidly recruited into inflamed tissues, such as spleen, gut, lung and brain, where they differentiate into macrophage [23,40]. In humans, $CD16^+$ monocytes that have the potential to migrate preferentially in response to fractalkine, a ligand of CX_3CR1, have more Fc receptor mediated phagocytosis function and are at a more advanced stage of differentiation to macrophage and dendritic cell [41-43]. These findings suggest that $CD14^{low}CD16^+$ and $CD14^+CD16^-$ cells are recruited into different anatomic sites under constitutive or inflammatory conditions and play distinct functional roles in immunity and disease pathogenesis.

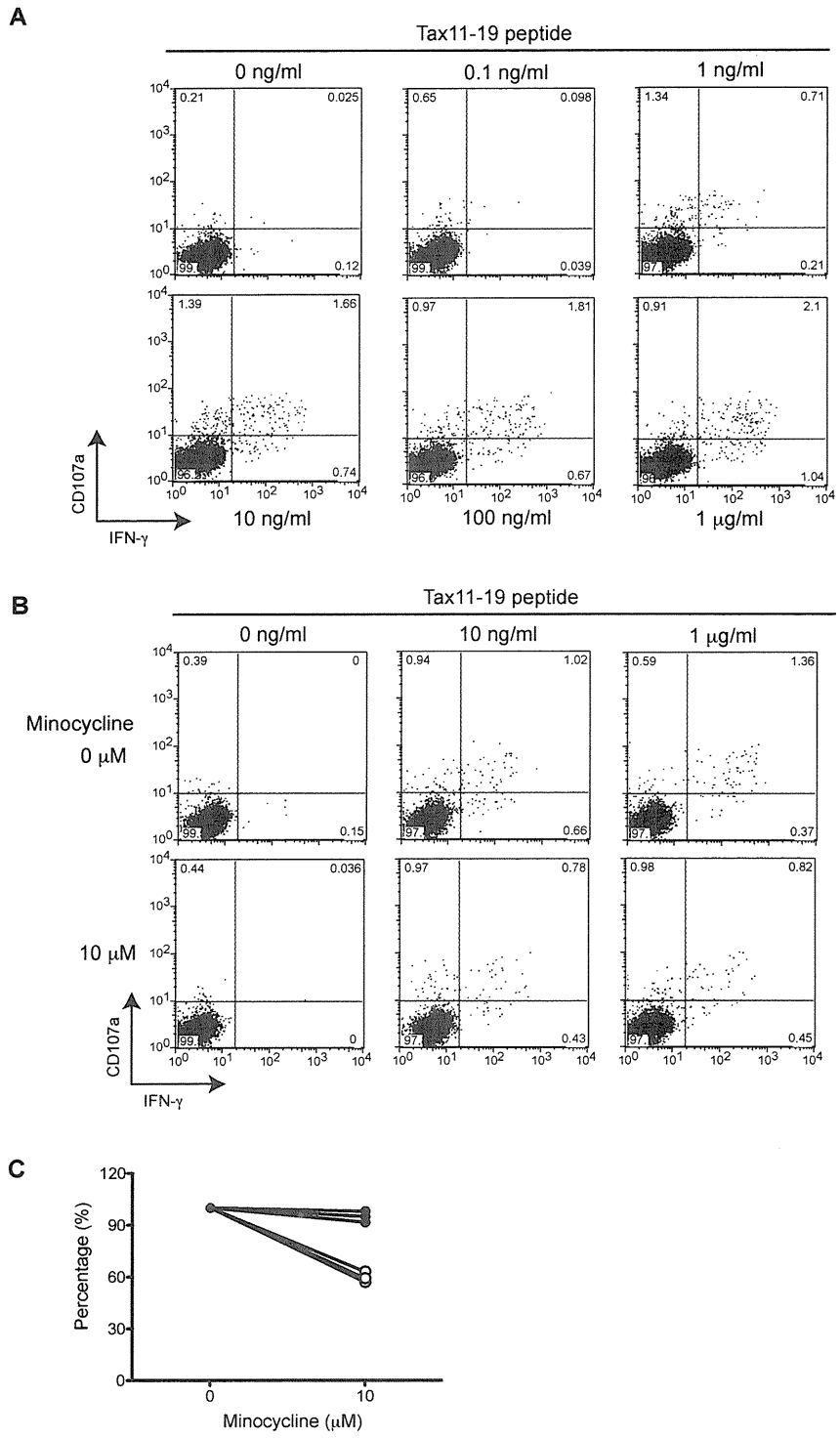

Figure 5 Minocycline inhibited Tax11-19-specific IFN-γ expression in CD8$^+$ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107 and IFN-γ expression in CD8$^+$ T cells of a HLA-A*201$^+$ HAM/TSP patients, stimulated with Tax11-19 peptides. PBMCs were stimulated with Tax 11-19 peptide at concentration of 0, 0.1, 1, 10, 100 ng/ml, and 1 μg/ml for 5 hours. (B) Representative dot plots of Tax11-19 specific CD107 and IFN-γ expression in CD8$^+$ T cells of a HAM/TSP patient after treatment with or without 10 μM of minocycline. PBMCs were stimulated with Tax 11-19 peptide at concentration of 0, 10 ng/ml and 1 μg/ml for 5 hours. (C) Inhibitory effects of minocycline on IFN-γ expression, but not degranulation, in CD8$^+$ T cells of HAM/TSP patients after stimulation with 1 μg/ml of Tax11-19 peptides. The amounts of CD107a$^+$ (closed circles) and CD107a$^+$IFN-γ$^+$ (opened circles) cells in CD8$^+$ T cells cultured without minocycline were normalized to 100%, and then, those in CD8$^+$ T cells cultured with minocycline were calculated. The graph was prepared from data obtained from three HLA-A*201$^+$ HAM/TSP patients. Tax11-19-specific IFN-γ expression, but not degranulation, in CD8$^+$ T cells of HAM/TSP patients was inhibited 40% by minocycline treatment.

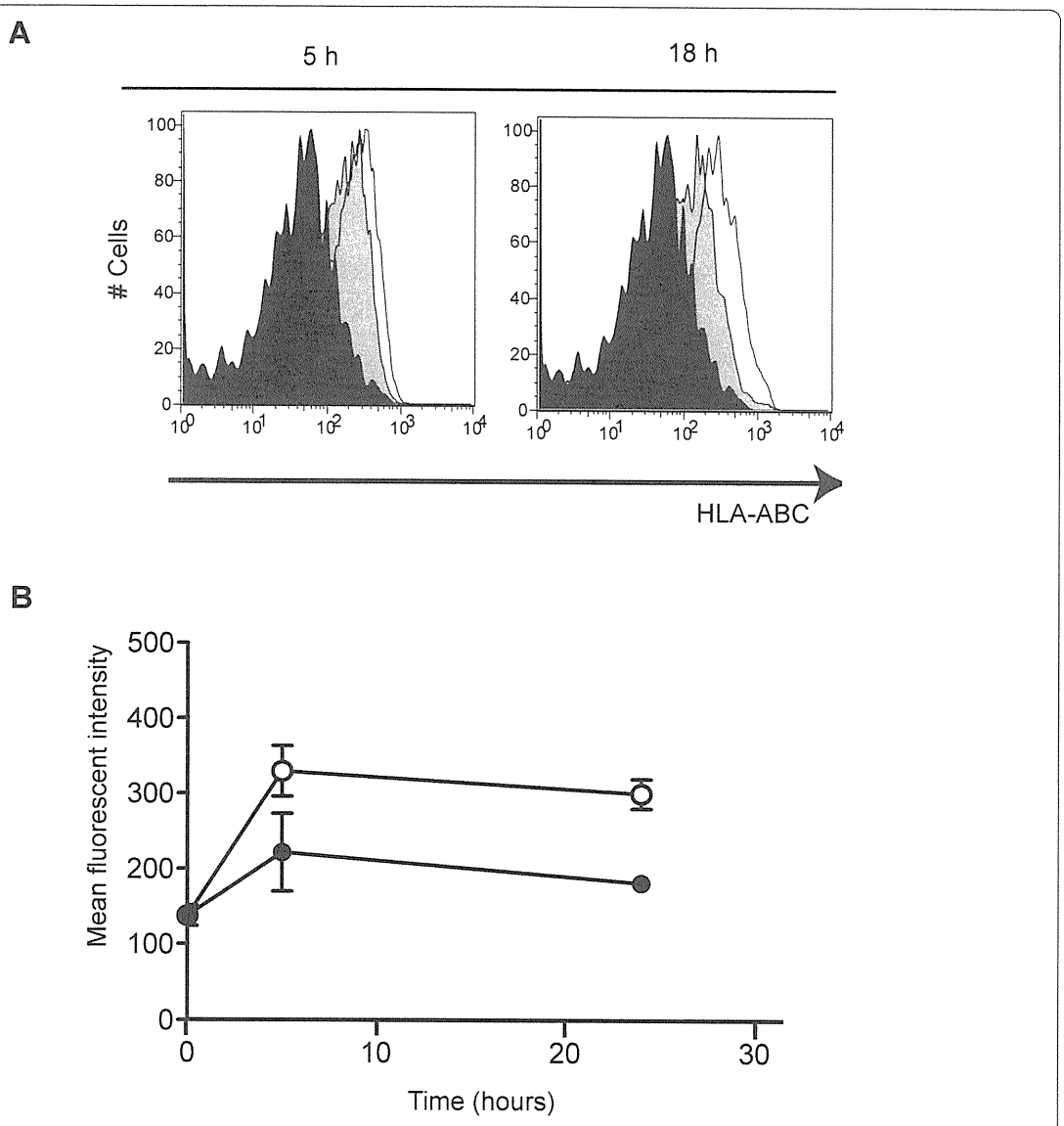

Figure 6 Minocycline downregulated HLA-ABC expressions in CD14$^+$ cells of patients with HAM/TSP. (A) Representative histograms of HLA-ABC expression on CD14$^+$ cells of a HAM/TSP patient. Staining on CD14$^+$ cells before culture (closed histogram) and after culture for 5 hours and 18 hours, with minocycline (grayed histogram) and without minocycline (opened histogram), were shown. (B) Comparison of HLA-ABC expression in CD14$^+$ cells of HAM/TSP patients after 18 hours culture with minocycline (closed circle) or without minocycline (opened circle). The mean fluorescent intensities of MHC class I expression on CD14$^+$ cells were significantly inhibited by treatment with minocycline at 18 hours culture (*p = 0.0382). Error bars represent SD.

Fractalkine is expressed on activated endothelial cells [44], neuron [45], apoptotic cells [46], and brain with inflammation [47]. Therefore, HTLV-I-activated or infected cells might induce fractalkine expression at the site of inflammation such as the spinal cord to recruit and adhere CX_3CR1^+ cells. The hypothesis was supported by the accumulation of CX_3CR1^+ cells immunohistochemically detected in the meninges and parenchyma of HAM/TSP spinal cords as well as around blood vessels (Figure 1D). The CX_3CR1^+ cells were CD68$^+$ and also morphologically consistent with MPs. Therefore, these results suggested that CX_3CR1^+ MPs could accumulate in spinal cords of patients with HAM/TSP. Moreover, the increase of degranulation and IFN-γ expression in CD8$^+$ T cells were significantly correlated with the increase of CX_3CR1 and HLA-DR expression in $CD14^{low}CD16^+$ subset of HTLV-I-infected patients. These results support the hypothesis that strong correlation between CD8$^+$ T cell activation and MP activation contribute to the pathogenesis of HAM/TSP. These differential changes in peripheral MP

subpopulations *in vivo* may also be associated with the infiltration of MPs into the CNS and $CD8^+$ T cell activation in patients with neurologic inflammatory disease.

MP activation in patients with HAM/TSP was also suggested by TNF-α and IL-1β expression in $CD14^+$ cells. Expression of IL-1β and TNF-α was detected in perivascular infiltrating macrophages and microglia in the spinal cords of patients with HAM/TSP and in infiltrating macrophage in the muscle of patients with HTLV-I-related myositis [27,48]. Thus, the proinflammatory cytokine expression in peripheral MPs might be related to the infiltration of MPs into the inflammatory site of patients with HTLV-I-related diseases. Moreover, $CD14^+$ cells accelerated HTLV-I Tax expression of autologous $CD4^+CD25^+$ T cells in patients with HAM/TSP, which was dependent on cell-cell contact. In patients with HAM/TSP, high HTLV-I Tax expression is mainly detected in $CD4^+$ T cells after *ex vivo* culture, but dendritic cells and $CD14^+$ cells can also express HTLV-I Tax, consistent with the observation that HTLV-I infects dendritic cells to effectively transfer cell-free virus to $CD4^+$ T cells [18,19]. In HIV, human $CD16^+$ monocytes have been shown to be more susceptible to infection than $CD16^-$ monocytes, to preferentially harbor the virus over the long-term, and to promote high levels of HIV replication upon differentiation into macrophages and interaction with activated T cells [30,49]. Therefore, HTLV-I infected and activated MP might likewise contribute to T cell activation and virus dissemination in HTLV-I associated disease.

Minocycline is a well known as inhibitor of MP activation and has been reported to have beneficial effects on inflammation, microglial activation, matrix metalloproteinases, nitric oxide production, and apoptotic cell death [29]. Furthermore, minocycline has been suggested to have neuroprotective effects in human as well as in animal models of a number of neurologic diseases including stroke, multiple sclerosis, and Parkinson's disease [29]. In our study, minocycline treatment significantly inhibited proinflammatory cytokine expression (TNF-α and IL-1β) in $CD14^+$ cells of patients with HAM/TSP, while TNF-α expressions in $CD4^+$ T cells of patients with HAM/TSP did not change. These results suggest that the effects of minocycline may act through inhibition of MP activation rather than HTLV-associated T cell activation. Unexpectedly, minocycline treatment also effectively inhibited spontaneous lymphoproliferation and IFN-γ expression of $CD8^+$ T cells, which are well-described observations of T cell activation in patients with HAM/TSP. While these T cell responses have been reported to be due to IL-2/IL-2 receptor and IL-15/IL-15 receptor autocrine loop following expression of HTLV-I Tax in T cells [32,38], a number of studies have demonstrated that non-T cells and $CD14^+$ cells can also play a stimulatory role in HTLV-I-associated T cell activation [5,19,38]. Therefore, our results support the view that T cell responses in patients with HAM/TSP are due, in part, to the activation of MPs.

Inhibition of MPs resulted in the suppression of $CD8^+$ T cell dysregulation (degranulation and IFN-γ expression). Elevated IFN-γ expression is an important immunological marker in the pathogenesis of HAM/TSP [50], and $CD8^+$ T cell dysregulation was mediated by various factors, including virus infection, enhanced IL-2/IL-15, and expression of cellular molecules [19,51-54]. Unexpectedly, minocycline inhibited spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients as well as HTLV-I Tax11-19-specific $CD8^+$ T cell responses. Antiviral $CD8^+$ T cells can elaborate at least two effector functions, cytotoxicity and inflammatory cytokine production, which are determined primarily by antigen concentration [36]. Interestingly, minocycline treatment suppressed inflammatory IFN-γ production, but not total cytotoxicity (CD107a expression) in Tax-specific $CD8^+$ T cells of patients with HAM/TSP. Moreover, after the treatment with minocycline, MHC class I expression on $CD14^+$ cells of patients with HAM/TSP was gradually suppressed in cultured cells, compared to untreated MPs. These results suggested that the activation of $CD8^+$ T cells was inhibited through MHC class I downregulation on $CD14^+$ cells after minocycline treatment. This may be one mechanism involved in the reduction of $CD8^+$ T cell inflammatory IFN-γ production in the presence of minocycline. Moreover, minocycline significantly inhibited spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients. As previously reported, the spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients was mediated by various factor(s) [19,52]. To evaluate regulatory effects of $CD8^+$ T cell by minocycline, further analysis would be needed. In addition, even though minocycline down-modulates the capacity of antigen-presenting cells to trigger $CD8^+$ T cell effector responses, the cytotoxic function of Tax-specific $CD8^+$ T cells might be still maintained and continue to provide control of virus-infected cells. This may have a positive clinical consequence for use of minocycline in treatment of HTLV-I-associated disease.

Conclusions

Collectively, these results suggest that minocycline does not only inhibit the activation of MPs of patients with HAM/TSP, but also HTLV-I-associated T cell activation such as lymphoproliferation and inflammatory cytokine production of $CD8^+$ T cells through the downregulation of MP function. Thus, the inhibition of HTLV-I-infected or activated MPs may be of clinical use in the treatment of patients with HTLV-I-associated neurological disease.

Methods

Patient samples

Blood samples were obtained from twelve patients with HAM/TSP (HAM#1-12), six HTLV-I-seropositive asymptomatic carriers (AC#1-6), and ten HTLV-I-seronegative healthy donors (ND#1-10). Diagnosis of HAM/TSP was based on WHO diagnostic criteria. Three patients with HAM/TSP were HLA-A*201$^+$. PBMCs were isolated by Ficoll-Hypaque (Lonza Walkersville, Walkersville, MD) centrifugation. The PBMCs obtained from HTLV-I-infected patients or ND were cryopreserved in liquid nitrogen until use. Informed consent was obtained from each subject. The study was reviewed and approved by the National Institute of Neurological Disorders and Stroke (NINDS) Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki.

Antibodies and reagents

For flow cytometry, antibodies for human CD3, CD4, CD8, CD14, CD16, CD28, CD49d, CD107a, IFN-γ, TNF-α, and HLA-DR (all from BD Biosciences, San Jose, CA), CX_3CR1 (Medical and Biological laboratories, Nagoya, Japan), HLA-ABC (AbD Serotec, Oxford, UK), and anti-Tax monoclonal antibody (Lt-4) were used. For immunohistochemistry, rabbit polyclonal anti-human CX_3CR1 (abcam, Cambridge, MA) was used as primary antibody. Minocycline was purchased from Sigma (St. Louis, MO).

Cell culture

PBMCs of NDs or patients with HAM/TSP were suspended at 1 × 10^6 cells/mL in RPMI media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine), and cultured for 24 hours either with or without minocycline in 24 well plate in 5% CO_2 incubator at 37°C. The culture supernatants were collected, centrifuged at 2000 g for 10 min to remove cellular debris and stored at -80°C until use. The cultured cells were collected for immunofluorescence staining or stored at -80°C until use. For immunofluorescence staining of MHC class I on MPs, PBMCs were collected after culture for 5 hours or 18 hours.

To examine Tax expression in $CD4^+$ T cells cocultured with or without $CD14^+$ cells, $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells and $CD14^+$ cells were magnetically isolated from PBMCs of HTLV-I-infected patients by $CD4^+CD25^+$ Regulatory T cell Isolation Kit and CD14 MicroBeads (both from Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacture's instruction, and 2 × 10^5 cells of each $CD4^+$ T cells were cocultured with or without the same amount of autologous $CD14^+$ cells for 18 hours in 48 well plate in 5% CO_2 incubator at 37°C.

ELISA

IL-1β was detected in the PBMC culture supernatants of NDs and patients with HAM/TSP using Human IL-1β Quantikine ELISA (R & D systems), according to the manufacturer's instructions

CD107a mobilization assay

CD107a mobilization assay was performed as previously described [19]. To detect spontaneous degranulation and IFN-γ expression in $CD8^+$ T cells, PBMCs of patients with HAM/TSP were cultured for 24 hours. To detect Tax11-19 specific responses, PBMCs were stimulated with an appropriate concentration of HTLV-I Tax11-19 LLFGYPVYV and 1 μg/mL each of CD28 and CD49d for 5 hours. In treatment of minocycline, appropriate minocycline was added into the culture. Conjugated CD107a antibody, 0.7 μL/mL of GoldiStop™ (BD Biosciences), and 1 μg/mL of brefeldin A (Sigma) were added into the culture for 5 hours before the time point for detection.

Flow cytometry

For analysis of peripheral blood monocyte populations, patients' PBMCs were stained with CD3, CD4, CD8, CD14, CD16, HLA-DR and CX_3CR1. Expression of CD107a, IFN-γ, TNF-α and intracellular Tax in the cultured or uncultured PBMCs was examined by flow cytometoric analysis. First, PBMCs were surface-stained with specific antibodies. After fixation and permeabilization with Fixation/Permeabilization solution (BD Biosciences) according to the manufacturer's instructions, the cells were intracellularly stained with anti-IFN-γ, anti-TNF-α or anti-Tax for each experiment. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) or LSR II (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Lymphoproliferation assay

Lymphoproliferation assay was performed as previously described [55]. PBMCs were suspended in RPMI medium supplemented with 5% human AB serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine, and plated in triplicate on a round bottom 96-well plate at a concentration of 3 × 10^5 cells/well with or without minocycline. The cells were cultured in 5% CO_2 incubator at 37°C, and pulsed after 3 to 5 days of culture for 4 h with 1 μCi [^{3}H] thymidine. The average cpm from each of the wells was plotted.

Immunohistochemistry

Spinal cord tissues from a patient with HAM/TSP were fixed with buffered formalin and embedded in paraffin wax. Microtome sections were cut 10 μm thick. Sections

were deparaffinized with xylene, rehydrated and immersed in Target Retrieval Solution, pH6.0, (Dako, Carpinteria, CA) at 121°C for 10 min. After blocking of endogenous peroxide with 3% hydrogen peroxide for 10 min, the sections were incubated with a rabbit anti-CX_3CR1 antibody (1 μg/ml) for one hour at room temperature. Reactivity was visualized with diaminobenzidine (DAB) using Envision™+system (Dako), followed by counterstaining with hematoxylin. The stained sections were visualized with Zeiss 200M Axiovert inverted microscope (Carl Zeiss MicroImaging Inc, Thornwood, NY). The image data of each section were created using Volocity imaging analysis software (Improvision, Waltham, MA).

Statistical analysis

Mann-Whitney test and Wilcoxon matched-pairs signed rank test were used for comparison between groups. Simple linear regression analysis was used for explaining a relationship between groups, respectively. All statistical analysis was performed using Prism (GraphPad software).

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Authors' contributions

YE-A designed the research, performed most of the experiments, analyzed results, made the figures and wrote the manuscript; EM analyzed immunohistochemical image, analyzed results, made the figures and wrote the manuscript; UO coordinated clinical work, analyzed results and wrote the manuscript; YT contributed reagents for analysis. SJ designed the research, analyzed results and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Activation of mTOR by human T-cell leukemia virus type 1 Tax is important for the transformation of mouse T cells to interleukin-2-independent growth

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Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia, and it immortalizes and transforms human T cells in both an interleukin (IL)-2-dependent and -independent manner. HTLV-1 encodes Tax, which plays crucial roles in HTLV-1-mediated immortalization and transformation of human T cells. A previous study showed that Tax can transform a mouse T-cell line, CTLL-2, from having IL-2-dependent growth to IL-2-independent growth. Given that the Akt/mTOR pathway is essential for IL-2-induced cell growth in T cells, we examined whether the Akt/mTOR pathway is involved in Tax-induced transformation to IL-2-independent growth. The stable and transient expression of Tax in CTLL-2 induced the phosphorylation of p70S6 kinase and ribosomal protein S6, downstream targets of the mTOR kinase, whereas that of Akt was only minimally induced. Studies with Tax mutants indicated that the activation of mTOR by Tax was correlated with the transformation of CTLL-2 cells to IL-2-independent growth. Rapamycin, an inhibitor of mTOR kinase, reduced the growth of Tax-transformed CTLL-2 cells. Moreover, the transduction of a constitutively active form of Akt in the CTLL-2 cells also induced IL-2-independent growth. Like CTLL-2/Tax, constitutive phosphorylation of p70S6 kinase was detected in the absence of IL-2 in all of the HTLV-1-infected human T-cell lines. These results suggest that Tax activates the mTOR pathway in T cells, and that this activation plays a crucial role in the growth of HTLV-1-infected T cells when a limited amount of IL-2 is available. (*Cancer Sci* 2012; 103: 369–374)

Human T-cell leukemia virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia (ATL),[1–4] which is characterized by monoclonal proliferation of HTLV-1-infected $CD4^+$ T cells. HTLV-1 is mainly transmitted from a mother to an infant through breast milk, but only 3–5% of infections develop ATL, with an average latency of 40–60 years.[4] HTLV-1 immortalizes $CD4^+$ T cells in an interleukin (IL)-2-dependent manner, although some cells acquire IL-2-independent growth properties.[5,6] Understanding how HTLV-1 immortalizes and transforms $CD4^+$ T cells is an important step to elucidate the molecular mechanism underlying the leukemogenesis.

In addition to its structural genes, HTLV-1 encodes several non-structural genes.[7] Of these, *tax1* is essential for the immortalization of $CD4^+$ T cells by HTLV-1.[8] For instance, recombinant HTLV-1 defective for the *tax1* gene does not immortalize human T cells and did not establish persistent infection in an animal model.[8] Moreover, Tax1 by itself immortalizes human $CD4^+$ T cells in an IL-2-dependent manner *in vitro*.[9,10] Therefore, Tax1 plays crucial roles in the IL-2-dependent immortalization of human T cells by HTLV-1.

HTLV-2, a close relative of HTLV-1, is not associated with ATL or any other types of leukemia.[11,12] HTLV-2 also encodes a transforming protein, Tax2, which has approximately 75% amino acid similarity to Tax1, and shares many functions, such as activation of NF-κB, CREB, and AP-1. Intriguingly, Tax1 has one dominant activity over Tax2; it converts a mouse T-cell line, CTLL-2, from IL-2-dependent growth to IL-2-independent growth much more efficiently than Tax2.[13,14] These results suggest that the activity of Tax1 for inducing the IL-2-independent growth of cells is associated with the HTLV-1-specific pathogenesis.

The Akt/mTOR kinase pathway is activated by IL-2 and transmits cell survival signals in T cells.[15,16] Moreover, aberrant activation of the Akt/mTOR pathway is frequently observed in leukemia and lymphoma.[17,18] Our data indicate that Tax1 activates the mTOR kinase in CTLL-2 cells and that this activation is important for the Tax1-induced transformation of CTLL-2 cells to IL-2-independent growth.

Materials and Methods

Cells and culture conditions. The cell lines used in this study have all been described previously.[19,20] SLB-1, MT-4, and HUT102 are HTLV-1-infected human T-cell lines. HUT78 and Jurkat are HTLV-1-negative human T-cell lines. These human T-cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL) (RPMI/10%FBS). Kit225 is an IL-2-dependent HTLV-1-negative human T-cell line originated from a patient with T-cell chronic lymphocytic leukemia,[21] and the cells were cultured in RPMI/10%FBS containing 0.5 nM IL-2 (RPMI/10%FBS/IL-2). ILT-Koy and ILT-Oot are IL-2-dependent HTLV-1-infected human T-cell lines, and they were cultured in RPMI/20%FBS/IL-2.[19] CTLL-2 is a mouse T-cell line that grows in an IL-2-dependent manner, and the cells were cultured in RPMI/10%FBS/IL-2 supplemented with 55 μM 2-mercaptoethanol (2-ME) (RPMI/10%FBS/IL-2/2-ME). CTLL-2 cells stably expressing Tax1 were cultured in RPMI/10%FBS/IL-2/2-ME containing 2 μg/mL puromycin.[14] Tax1-transformed IL-2-independent CTLL-2 cells were cultured in RPMI/10%FBS/2-ME. CTLL-2 cells stably expressing hAkt1mΔPH (CTLL-2/hAkt1mΔPH) were cultured in RPMI/10%FBS/IL-2/2-ME containing 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA). Human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL).

Plasmids. pHβPr-1-neo-Tax1, pHβPr-1-neo-TaxM22, and pHβPr-1-neo-Tax703 were expression plasmids encoding Tax1, TaxM22, and Tax703, respectively.[19,22] CS-EF-Tax1 is a lentiviral vector expressing Tax1.[23] To construct the expression

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vectors for Tax1 mutant genes, the TaxM22 and Tax703 cDNA fragments were amplified by PCR from the pHβPr-1-neo-TaxM22 and pHβPr-1-neo-Tax703 plasmids, respectively, and the fragments were inserted into the Gateway entry vector pENTR/D-TOPO (Invitrogen). These *tax* genes in the entry vectors were then transferred to the lentiviral vector CSII-EF-RfA (provided by Dr. H. Miyoshi, Riken Tsukuba Institute, Tsukuba, Japan) by an LR recombination reaction using LR clonase (Invitrogen), and they were designated CS-EF-TaxM22 and CS-EF-Tax703. The κB-Luc and WT-Luc plasmids are luciferase reporters regulated by the κB element of the IL-2 receptor α-chain gene and the Tax-inducible 21-bp sequence in the HTLV-1 long-terminal repeat, respectively.[13] The pGK/β-gal plasmid expresses β-galactosidase under the control of the phosphoglycerate kinase promoter and was used to normalize the transfection efficiency. The retroviral expression vector, pQCXIN-hAkt1mΔPH, was constructed by inserting the hAkt1mΔPH fragment from the pCS2-hAkt1mΔPH plasmid into the *Bam*HI site of pQCXIN (Clontech, Palo Alto, CA, USA). The pCS2-hAkt1mΔPH plasmid encoding a constitutively active human Akt1 (hAkt1mΔPH) was provided by Dr. Y. Gotoh, Institute of Molecular and Cellular Biosciences, University of Tokyo (Tokyo, Japan).[24]

Generation of lentiviruses and retroviruses. Recombinant lentiviruses were generated by transfecting pCAG-HIVgp, pCMV-VSV-G-RSV-Rev (provided by Dr. H. Miyoshi, Riken Tsukuba Institute) and the respective lentiviral vectors encoding GFP, Tax1, Tax703, and TaxM22 into 293T cells using FuGENE HD (Roche, Indianapolis, IN, USA). At 72 h after the transfection, the culture supernatants were collected, and the viral titers were measured by a Lenti-X qRT-PCR Titration Kit (Clontech). They were then infected into CTLL-2 cells (4×10^5) in 2 mL RPMI/10%FBS/IL-2/2-ME containing 8 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA). Recombinant retroviruses were generated by transfecting pGag-pol-IRES-bs^r (a gift from Dr. T. Kitamura, Institute of Medical Science, University of Tokyo),[25] pMD.G, an expression vector for VSV-G, and the retroviral vector plasmid encoding hAktmΔPH into 293T cells using FuGENE 6 (Roche). At 72 h after transfection, the culture supernatant was collected and used to infect the CTLL-2 cells (4×10^5) in a final volume of 2 mL RPMI/10%FBS/IL-2/2-ME containing 8 μg/mL polybrene. At 48 h post-infection, the cells infected with lentiviruses or retroviruses were cultured in RPMI/10%FBS/IL-2/2-ME supplemented with 0.5 mg/mL G418 for more than 7 days, and the expression of hAkt1mΔPH in the established cells was measured by Western blot analysis.

Western blot analysis. The cell lysates were prepared by treating the cells with 1× SDS sample buffer (2% SDS, 62.5 mM Tris–HCl [pH 6.8], 10% glycerol, 0.01% bromophenol blue, 50 mM DTT). The protein concentrations of the cell lysates were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates (15–20 μg) were size-separated by SDS-PAGE, and electronically transferred onto a PVDF membrane. The membrane was incubated with 5% skim milk/TBS-T (20 mM Tris–HCl [pH7.6], 150 mM NaCl, 0.1 % Tween 20) at room temperature for 1 h, and further incubated with the primary antibodies. After washing with TBS-T, the membranes were incubated with an anti-rabbit or anti-mouse antibody or Protein A conjugated with HRP. The proteins recognized by the antibodies on the membrane were visualized using the ECL Western Blotting Detection System (GE Healthcare Bioscience, Little Chalfont, UK). The primary antibodies used in this study were an rabbit anti-Akt mAb, rabbit anti-phospho-Akt mAb, rabbit anti-p70 ribosomal S6 kinase (p70S6K) antibody, rabbit anti-phospho-p70S6K antibody, rabbit anti-ribosomal protein S6 (RPS6) antibody, rabbit anti-phospho-RPS6 antibody (Cell Signaling Technology, Beverly, MA, USA), a mouse anti-PTEN mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-Tubulin antibody (Calbiochem, La Jolla, CA, USA), and a mouse anti-Tax mAb (Taxy7).[26]

Cell growth assay. CTLL-2 cells expressing Tax1 or parental CTLL-2 cells were cultured at 1×10^5 cells/mL in 1 mL RPMI/10%FBS/2-ME together with either 100 nM rapamycin or its solvent (DMSO) on a 24-well plate. CTLL-2/hAkt1mΔPH cells were washed once with RPMI 1640 medium, and were cultured at 2×10^5 cells/mL in 1 mL RPMI/10%FBS/2ME in the presence of either 100 nM rapamycin or its solvent (DMSO) on a 24-well plate. The number of viable cells was counted by a Trypan blue dye exclusion method using light microscopy. CTLL-2/hAkt1mΔPH cells were also cultured in 96-well plates at a cell density of 1000, 3000, or 9000 cells/well for 3 weeks without IL-2. The number of wells containing outgrowing cells was counted under a light microscope.

Luciferase assay. CTLL-2 cells (2.5×10^6) were transfected with pHβPr-1-neo-Tax1, pHβPr-1-neo-Tax703, or pHβPr-1-neo-TaxM22, together with κB-Luc or WT-Luc (CRE-Luc), and pGK/β-gal by using the DEAE dextran method as described previously.[19] At 48 h after transfection, the cell lysates were prepared, and the luciferase and β-galactosidase activities in the lysates were measured using the Luciferase Assay System (Promega, Fitchburg, WI, USA) and Galacto-Light System (Applied Biosystems, Foster City, CA, USA), respectively.

Results

Tax1 induces phosphorylation of S6K and RPS6 in a T-cell line. CTLL-2 is a mouse T-cell line, the growth of which is dependent on IL-2. We previously showed that HTLV-1 Tax1 transforms CTLL-2 cells to induce their IL-2-independent growth.[19] IL-2-induced growth in normal T cells requires activation of the Akt/mTOR pathway, so we examined the involvement of Akt/mTOR in Tax1-induced IL-2-independent cell growth of CTLL-2 cells. We previously established stable Tax1-expressing cells in the presence of IL-2 by transfecting them with a Tax1 expression plasmid.[14] Western blot analysis confirmed that two Tax1-transduced cell lines (Tax1-14, Tax1-18), but not the vector-only transduced cells, expressed the Tax1 protein (Fig. 1). These cells were then cultured with and without IL-2 for 18 h, and the status of the Akt/mTOR pathway was characterized by Western blot analysis. After IL-2 deprivation, two Tax1-transduced cell lines showed increased phosphorylation of S6K and RPS6, two well-known downstream targets of mTOR kinase, relative to the control cells, whereas they showed an equivalent level of phosphorylated Akt (Fig. 1). The CTLL-2/Tax1-14 cells showed stronger phosphorylation of S6K and RPS6 than CTLL-2/Tax1-18 cells, which correlated with the high Tax1 expression. Unlike Tax1, IL-2 induced the phosphorylation of Akt in CTLL-2 cells, indicating that Akt is able to be activated in CTLL-2 cells. To confirm the activity of Tax1 with regard to S6K and RPS6, it was transiently transduced into CTLL-2 cells by a lentiviral vector, and the cells were cultured in the absence of IL-2 for 18 h. The transient expression of Tax1 also induced the phosphorylation of S6K and RPS6, but not that of Akt (Fig. 2). Therefore, these results indicate that Tax1 induces the phosphorylation of S6K and RPS6 in CTLL-2 cells.

To obtain information about how Tax1 induces the phosphorylation of S6K and RPS6, we used two Tax1 mutants, Tax703 and TaxM22.[27,28] These *tax1* expression plasmids, together with luciferase reporter plasmids under the control of either NF-κB or CREB, were transfected into a mouse T-cell line (CTLL-2) using the DEAE–dextran method. At 48 h after transfection, the cell lysates were prepared, and the luciferase activities of the cells were measured. Tax703 efficiently activated NF-κB-dependent transcription, but the activity level of CREB was only half of that induced by the wild-type Tax.

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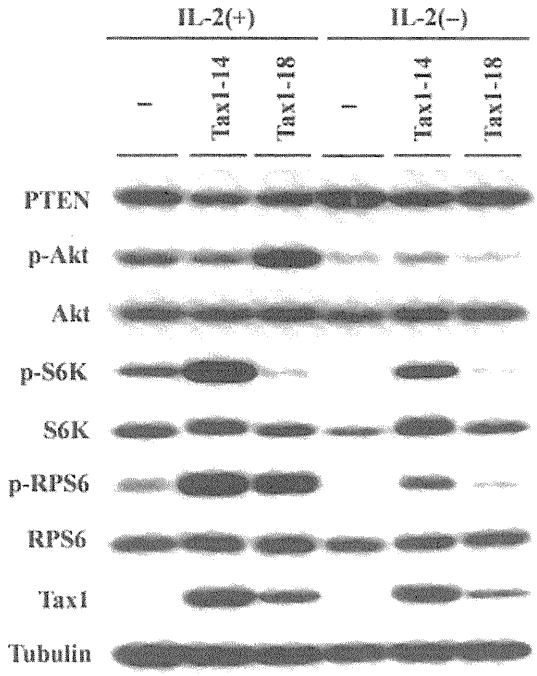

Fig. 1. Augmented phosphorylation of S6K and RPS6 is present in CTLL-2 mouse T cells expressing Tax1. CTLL-2 cells stably expressing Tax1 (Tax1-14, Tax1-18), maintained in the presence of 0.5 nM IL-2, were cultured with (+) or without (–) IL-2 for 18 h. Lysates prepared from the cells were then subjected to Western blot analysis with the indicated antibodies. The antibodies used were anti-Akt, anti-phospho-Akt, anti-p70S6K, anti-phospho-p70S6K, anti-RPS6, anti-phospho-RPS6, and anti-PTEN, anti-tubulin, and an anti-Tax mAb (Taxy7).

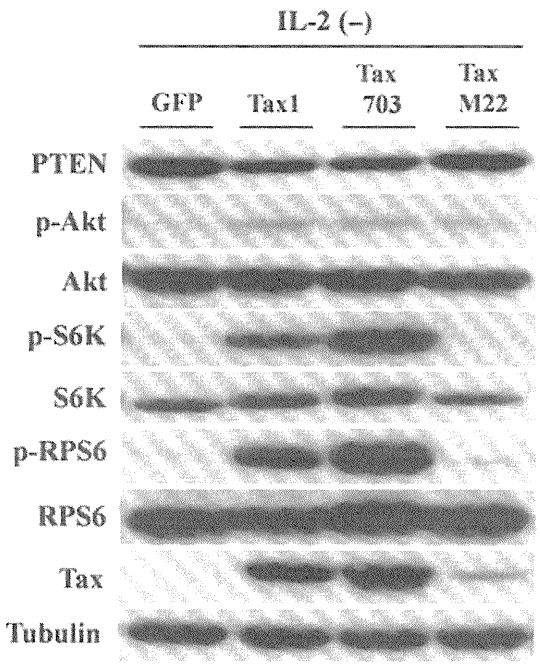

Fig. 2. Tax1 induces the phosphorylation of S6K and RPS6 in CTLL-2 mouse T cells. CTLL-2 cells (4×10^5) were infected with lentiviruses encoding Tax1, Tax703, or TaxM22. At 48 h after infection, the cells were washed with RPMI-1640 medium, and cultured without (–) IL-2 for 18 h. Lysates prepared from the cells were analyzed by Western blot analysis using the indicated antibodies.

TaxM22 only minimally activated NF-κB, and activated CREB more than did Tax703 (Fig. 3). Like Tax1, transduction of Tax703 into CTLL-2 cells induced the phosphorylation of S6K and RPS6, but not Akt. However, TaxM22 in the CTLL-2 cells did not induce any phosphorylation of S6K, RPS6, or Akt, although its inability to induce the phosphorylation of S6K and RPS6 may be due to the low protein expression in CTLL-2 cells. Collectively, these results suggest that the Tax1 functions associated with NF-κB activation play a major role in the phosphorylation of S6K and RPS6. We also noticed that stable, as well as transient, expression of Tax1 or Tax703 in CTLL-2 cells reduced the expression of PTEN, which is the main phosphatase of phosphorylated Akt. This is consistent with the results of a previous study indicating that Tax1 downregulates the expression of PTEN through the NF-κB pathway.[29]

mTOR plays a role in the IL-2-independent growth of CTLL-2/Tax1 cells. The above data suggested that Tax1 stimulates mTOR activity to phosphorylate S6K and RPS6 in CTLL-2 cells. To examine whether mTOR is involved in the Tax1-induced growth stimulation of CTLL-2 cells, Tax1-transformed IL-2-independent CTLL-2 cells were cultured in the presence of rapamycin, an mTOR kinase inhibitor,[30] and the number of viable cells was counted by light microscopy. Rapamycin inhibited the growth of Tax1-transformed CTLL-2 cells (Fig. 4A), which was associated with the decreased phosphorylation of RPS6 (Fig. 4B). Rapamycin also reduced the IL-2-induced growth of CTLL-2 cells (Fig. 4A), which was also associated with reduced phosphorylation of RPS6. These results suggest that mTOR activation plays a role in the Tax1-induced IL-2-independent growth of CTLL-2 cells.

Constitutively active Akt1 induces IL-2-independent growth of CTLL-2 cells. To further clarify the role of the Akt/mTOR pathway in the IL-2-independent growth of CTLL-2 cells, we established CTLL-2 cells expressing constitutively active Akt1 (hAkt1mΔPH) by retrovirus-mediated gene transfer in the presence of IL-2. Western blot analysis detected hAkt1mΔPH in the CTLL-2 cells (Fig. 5A). Next, the established cells were cultured without IL-2 for the indicated

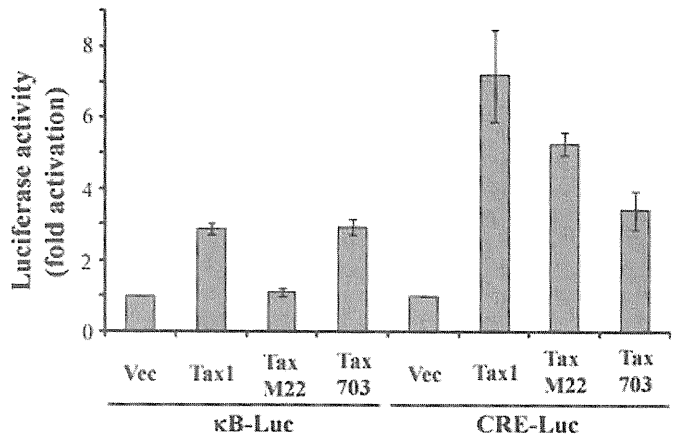

Fig. 3. CTLL-2 mouse T cells were transfected with pHβPr-1-neo-Tax1, pHβPr-1-neo-Tax703, or pHβPr-1-neo-TaxM22, together with κB-Luc or WT-Luc (CRE-Luc), and pGK/β-gal using the DEAE–dextran method. At 48 h after transfection, the cell lysates were prepared, and the luciferase and β-galactosidase activities in the lysates were determined using a luminometer. The activity of luciferase was normalized to that of β-galactosidase, and the fold activation was calculated as the ratio to that of the control transfection with the pHβPr-1-neo plasmid. The data shown are the averages of triplicate scores with standard deviations.

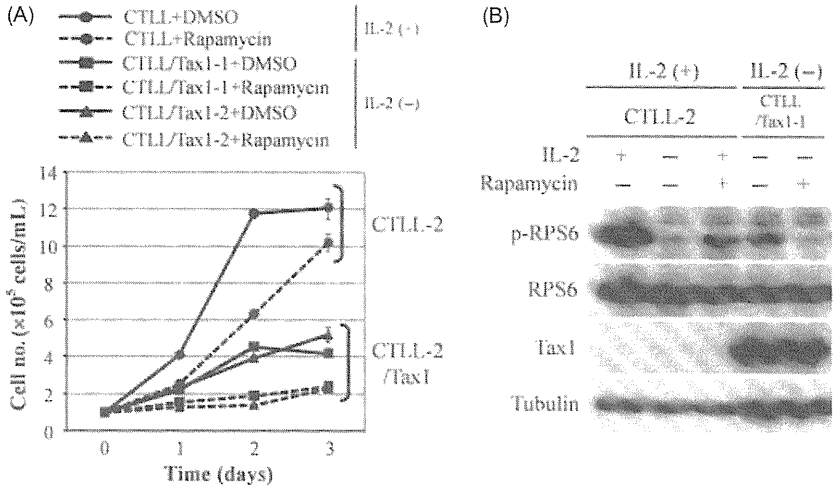

Fig. 4. Rapamycin suppresses the growth of Tax1-transformed CTLL-2 mouse T cells. (A) CTLL-2 cells and Tax1-transformed IL-2-independent CTLL-2 cells (Tax1-1 and Tax1-2, respectively) were cultured in the presence or absence of 100 nM rapamycin. The number of viable cells was counted by a Trypan blue dye exclusion method using a light microscope. (B) CTLL-2, Tax1-transformed IL-2-independent CTLL-2 cells (Tax1-1) were cultured in the presence or absence of 100 nM rapamycin. After 24 h in culture, cell lysates were prepared and subjected to Western blot analysis using the indicated antibodies. RPS6, ribosomal protein S6.

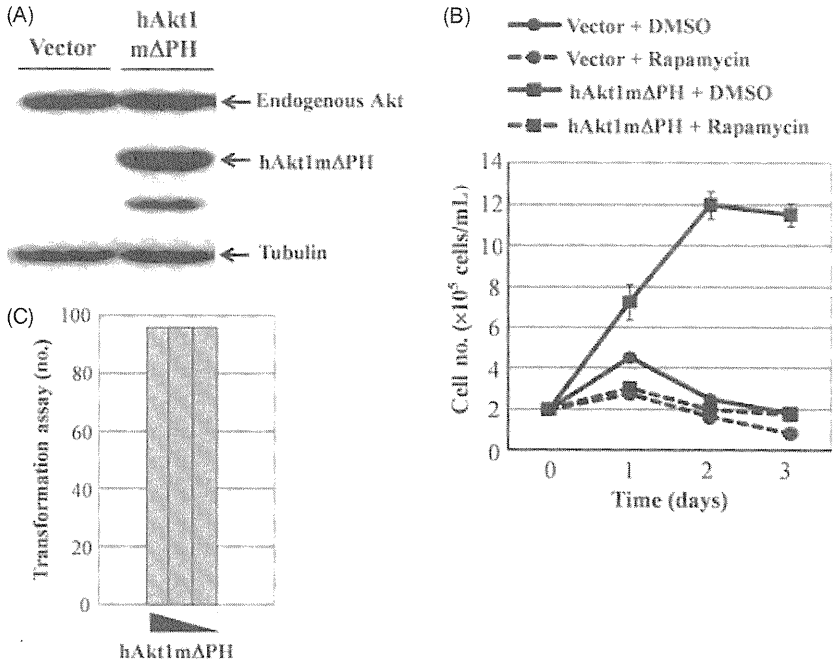

Fig. 5. Constitutively active Akt1 transforms CTLL-2 mouse T cells from IL-2-dependent growth to independent growth. (A) CTLL-2 cells were infected with retroviruses encoding hAktmΔPH, and the cells were cultured in medium containing IL-2 and G418 for 7 days. Cell lysates were prepared, and the expression of the hAktmΔPH protein was measured by Western blot analysis using an anti-Akt antibody. (B) CTLL-2 cells stably expressing hAkt1mΔPH were washed with RPMI-1640 medium, suspended in RPMI/10%FBS/2-ME in the presence or absence of 100 nM rapamycin, and seeded at 2×10^5 cells/mL in a 24-well plate. The number of viable cells was counted by a Trypan blue dye exclusion method using a light microscope. (C) CTLL-2 cells stably expressing hAkt1mΔPH were washed twice with PBS, and cultured without IL-2 at a cell density of 1000, 3000, or 9000 cells/well in 96-well plates for 3 weeks. The number of wells containing outgrowing cells was counted under a light microscope.

number of days, and the number of viable cells was counted using a Trypan blue exclusion method under a light microscope. Unlike the parental CTLL-2 cells, the cells expressing constitutively active Akt1 continuously grew in the absence of IL-2 (Fig. 5B). Moreover, the cell growth induced by constitutively active Akt1 was efficiently inhibited by rapamycin.

After obtaining these results, the CTLL-2/ hAkt1mΔPH cells were next cultured in the absence of IL-2 at a cell density of 1000, 3000, or 9000 cells/well in 96-well plates. After 3 weeks in culture, all the wells contained outgrowing cells (Fig. 5C). These results indicated that the activation of the Akt/mTOR pathway is sufficient to induce the IL-2-independent growth of CTLL-2 cells.

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Akt/mTOR pathway activated in HTLV-1-infected T-cell lines. There are two types of HTLV-1-infected human T-cell lines, one shows IL-2-dependent cell growth and the other shows IL-2-independent growth. For our subsequent study, we used two types of HTLV-1-negative human T-cell lines, IL-2-dependent (Kit225) and IL-2-independent (HUT78) cells. Jurkat cells are an HTLV-1-negative human T-cell line that has constitutively active Akt as a result of the deletion of the *PTEN* gene[31] (Fig. 6). The status of the Akt/mTOR pathway in these cell lines was characterized by Western blot analysis (Fig. 6). The phosphorylated forms of Akt, S6K, and RPS6 were higher in all the HTLV-1-infected human T-cell lines, except for MT-4 cells, than those in HUT78 or Kit225 cells in the absence of IL-2. These results indicated that the Akt/mTOR pathway is activated in most HTLV-1-infected human T-cell lines. MT-4 cells showed increased phosphorylation of S6K relative to the control cells, but no differences in the phosphorylation of RPS6 or Akt.

Discussion

After treatment with IL-2, the mTOR kinase is activated in T cells, which then induces the phosphorylation of its downstream targets, S6K and RPS6, the activation of which is essential for IL-2-induced cell growth.[32] In this study, we found that Tax1 induces the phosphorylation of S6K and RPS6 in the CTLL-2 T-cell line (Figs 1,2), and that the inhibition of mTOR by rapamycin reduced the growth of Tax1-transformed IL-2-independent CTLL-2 cells (Fig. 4A). Moreover, a constitutively active form of Akt1 transformed the CTLL-2 cells from having IL-2-dependent growth to have IL-2-independent growth (Fig. 5). Taken together, these results suggest that Tax1-mediated activation of mTOR kinase in CTLL-2 cells is crucial for their IL-2-independent growth.

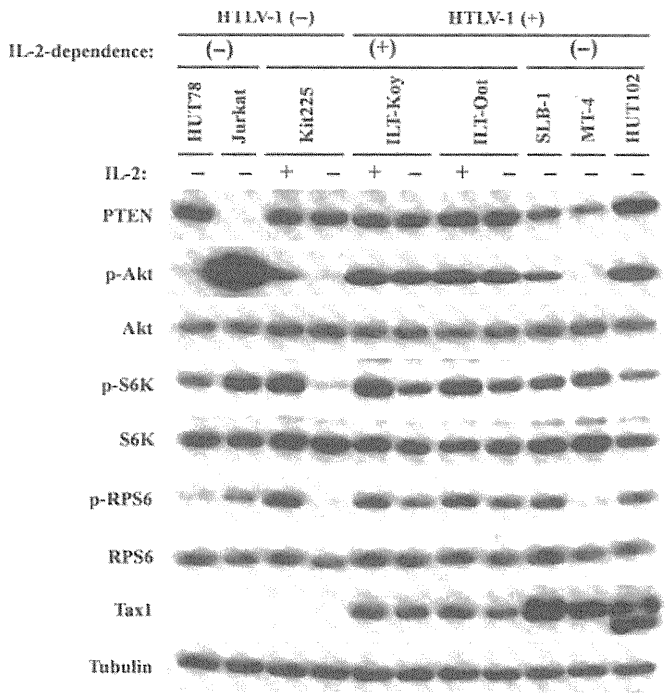

Fig. 6. Status of the Akt/mTOR pathway in HTLV-1 negative and positive human T-cell lines. IL-2-dependent human T-cell lines (Kit225, ILT-Koy, and ILT-Oot) were washed twice with PBS, and cultured in 5 mL of RPMI/10%FBS without IL-2 for 18 h. Cell lysates were prepared from these cells, as well as the indicated IL-2-independent HTLV-1 negative human T-cell lines (HUT78, Jurkat, and Kit225), and HTLV-1 positive human T-cell lines (SLB-1, MT-4, and HUT102), and their protein expression was assessed by Western blot analysis using the indicated antibodies. IL-2 dependence (+) indicates IL-2-dependent growth properties of the respective cells. HTLV-1(+), HTLV-1-infected cell lines.

Two previous studies showed that Tax1 activates Akt in a fibroblast cell line and T cells.[29,33] For instance, transient expression of Tax1 in primary human T cells purified from PBMC induced the phosphorylation of Akt through the activation of phosphatidylinositol 3-kinase (PI3K).[29] Although it is unclear why we could not detect the Tax1-induced phosphorylation of Akt in CTLL-2 cells, this might be due to the distinct experimental conditions used in the two studies, including the differences in cell types (purified primary human T cells versus an IL-2-dependent T-cell line). Therefore, the present study could not clarify whether the activation of mTOR by Tax1 is mediated by Akt or not.

Tax703 activated mTOR in CTLL-2 cells (Fig. 2). A previous study showed that Tax703 induced the IL-2-independent growth of CTLL-2 cells.[19] Taken together, these results support our hypothesis that mTOR activation by Tax1 is involved in the induction of IL-2-independent growth of CTLL-2 cells. It should be noted that Tax703 activates or represses several signaling pathways other than NF-κB-dependent transcriptional activation, such as transcriptional activation through E2F.[34] Therefore, the present data does not necessarily mean that mTOR is activated by Tax1 through NF-κB-inducible genes. As a result, a further analysis is required to elucidate how Tax1 activates mTOR.

All but one (MT-4) of the HTLV-1-infected T-cell lines that were examined possessed augmented phosphorylation of S6K and RPS6 relative to that in uninfected cells (Fig. 6). A previous study showed that rapamycin inhibits the growth of IL-2-independent HTLV-1-infected T-cell lines.[35] Taken together, the results of the present and previous studies suggest that Tax1 plays a role in mTOR activation in HTLV-1-infected T-cell lines, thereby mediating their IL-2-independent growth. Of the various HTLV-1-positive cell lines that were examined, MT-4 showed increased phosphorylation of S6K, but not of RPS6 nor Akt, relative to HTLV-1-negative cells. Therefore, S6K might be activated in MT-4 cells through an mTOR-independent mechanism.

The phosphorylation levels of S6K and RPS6 in IL-2-dependent HTLV-1-infected cells in the absence of IL-2 were closely equivalent to those of the IL-2-independent HTLV-1-infected cells (Fig. 6). These results suggest that mTOR activation is not sufficient for the IL-2-independent growth of HTLV-1-infected cells, and event(s) other than mTOR activation are required for their IL-2-independent growth. In this respect, some other viral protein different from Tax and/or the aberrant activation of cellular factor(s) is likely to be involved in the IL-2-independent growth of HTLV-1-infected T-cells, as Tax immortalizes human T cells only in an IL-2-dependent manner.

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Disclosure Statement

The authors have no conflict of interest.

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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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