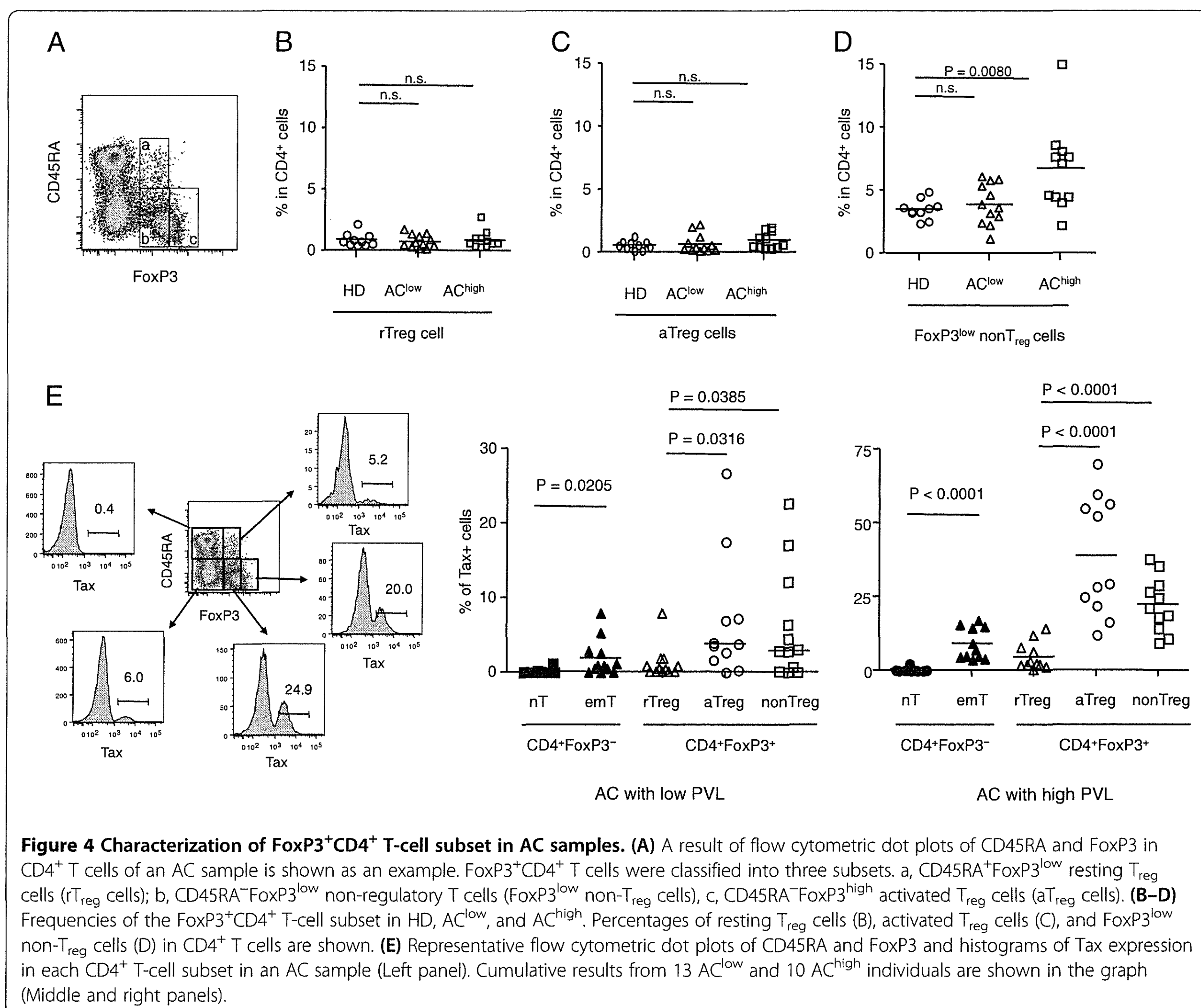

Figure 4 Characterization of FoxP3$^+$CD4$^+$ T-cell subset in AC samples. (A) A result of flow cytometric dot plots of CD45RA and FoxP3 in CD4$^+$ T cells of an AC sample is shown as an example. FoxP3$^+$CD4$^+$ T cells were classified into three subsets. a, CD45RA$^+$FoxP3^{low} resting T_{reg} cells (rT_{reg} cells); b, CD45RA$^-$FoxP3^{low} non-regulatory T cells (FoxP3^{low} non-T_{reg} cells), c, CD45RA$^-$FoxP3^{high} activated T_{reg} cells (aT_{reg} cells). **(B–D)** Frequencies of the FoxP3$^+$CD4$^+$ T-cell subset in HD, AC^{low}, and AC^{high}. Percentages of resting T_{reg} cells (B), activated T_{reg} cells (C), and FoxP3^{low} non-T_{reg} cells (D) in CD4$^+$ T cells are shown. **(E)** Representative flow cytometric dot plots of CD45RA and FoxP3 and histograms of Tax expression in each CD4$^+$ T-cell subset in an AC sample (Left panel). Cumulative results from 13 AC^{low} and 10 AC^{high} individuals are shown in the graph (Middle and right panels).

was significantly lower than that in HD ($p = 0.0328$ or 0.00002, respectively, Figure 5C). On the contrary, CD25 expression was high in HAM/TSP patients ($p = 0.0099$, Figure 5C). We further evaluated FoxP3$^+$CD4$^+$ T-cell subset in HAM/TSP patients. The frequencies of rT_{reg} were not significantly different from that in HD ($p = 0.9096$, Figure 5D), but aT_{reg} cells or FoxP3^{low} non-T_{reg} cells were remarkably increased ($p = 0.0250$ or 0.0004, Figure 5E and 5F). Furthermore, aT_{reg} cells or FoxP3^{low} non-T_{reg} cells showed a high frequency of Tax + cells compared with rT_{reg} cells ($p = 0.0069$ or 0.0069, respectively, Figure 5G) as observed in ACs (Figure 4E). These data indicated that HTLV-1 infection significantly influenced not only the frequency but also the phenotype of CD4$^+$FoxP3$^+$ T cells in an inflammatory disease HAM/TSP.

Phenotypical analyses of ATL cells

Previous studies reported that some ATL cells express FoxP3 or CD25 [30,31,33], but the precise information about FoxP3$^+$CD4$^+$ T-cell subset of ATL cells remains unknown. We, therefore, analyzed CD4$^+$ T-cell subsets for ATL cases. FoxP3 positivity was 80% in ATL cases; yet the expression level was different among the cases (Figure 6A), which is consistent with previous reports [30,31]. In line with the finding in asymptomatic HTLV-1-infected carriers that the percentage of HTLV-1 in FoxP3^{low} non-T_{reg} cells or aT_{reg} cells was high (Figure 4E), ATL cells analyzed in this study did not express CD45RA, suggesting that FoxP3-expressing ATL cells might be derived from FoxP3^{low} non-T_{reg} or aT_{reg} cells. CD25 expression on ATL cells was generally high, but there was also much variation among the cases (Figure 6B).

Discussion

FoxP3$^+$ T_{reg} cells play a crucial role in persistent infection and pathogenesis of chronic viral infection. Previous studies have suggested that T_{reg} cells suppress virus-

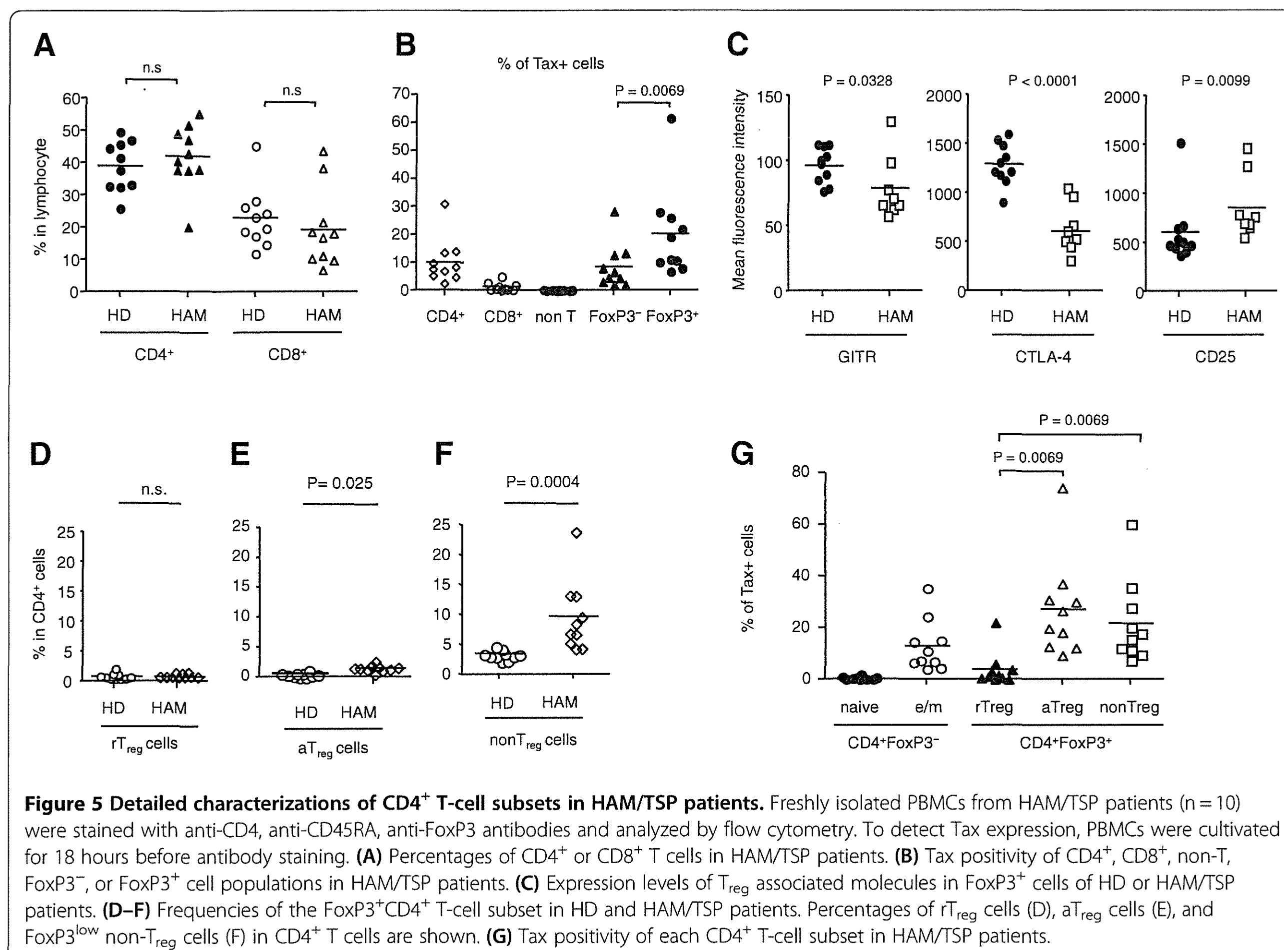

Figure 5 Detailed characterizations of CD4$^+$ T-cell subsets in HAM/TSP patients. Freshly isolated PBMCs from HAM/TSP patients (n = 10) were stained with anti-CD4, anti-CD45RA, anti-FoxP3 antibodies and analyzed by flow cytometry. To detect Tax expression, PBMCs were cultivated for 18 hours before antibody staining. **(A)** Percentages of CD4$^+$ or CD8$^+$ T cells in HAM/TSP patients. **(B)** Tax positivity of CD4$^+$, CD8$^+$, non-T, FoxP3$^-$, or FoxP3$^+$ cell populations in HAM/TSP patients. **(C)** Expression levels of T_{reg} associated molecules in FoxP3$^+$ cells of HD or HAM/TSP patients. **(D–F)** Frequencies of the FoxP3$^+$CD4$^+$ T-cell subset in HD and HAM/TSP patients. Percentages of rT_{reg} cells (D), aT_{reg} cells (E), and FoxP3^{low} non-T_{reg} cells (F) in CD4$^+$ T cells are shown. **(G)** Tax positivity of each CD4$^+$ T-cell subset in HAM/TSP patients.

specific CD8$^+$ T-cell effector functions in chronic human viral infections such as human immunodeficiency virus, hepatitis C virus and cytomegalovirus [34,35]. Regarding this point, FoxP3$^+$ T_{reg} cells play a role in facilitating viral persistence. In HTLV-1 infection, the frequency of FoxP3$^+$ cells is indeed correlated with the impairment of CTL activity against the viral antigen Tax in HAM/TSP patient [36]. On the other hand, FoxP3$^+$ T_{reg} cells could prevent tissue damage caused by excessive immune response triggered by viral infection. In addition to these general roles of FoxP3$^+$ T_{reg} cells in chronic viral infection, FoxP3$^+$ T_{reg} cells should have some specific role in HTLV-1 infection, because FoxP3$^+$ T_{reg} cells are comprised in CD4$^+$ T cells, which are a main host cell population of HTLV-1. Here we performed a comprehensive analysis of CD4$^+$ T-cell subsets in individuals naturally infected with HTLV-1 and revealed that the frequency of HTLV-1 infection is positively correlated with the frequency of FoxP3$^+$ T cells (Figure 1E). The increased FoxP3$^+$ T cells themselves are frequently infected with HTLV-1 (Figure 3B), suggesting that HTLV-1 utilizes the FoxP3$^+$ T cells as a host cell. What is the advantage for HTLV-1 to exist in FoxP3$^+$ T cells? There are two possibilities for this preference. First, FoxP3$^+$ T cells are known as hyper-proliferating cells *in vivo* with a doubling time of 8 days [37], which could contribute to clonal expansion of infected cells. Second, HTLV-1 can evade the host immune system by directly infecting this potentially immuno-suppressive cell population. Thus, HTLV-1-infection of FoxP3$^+$ T cells should enable the virus to increase or maintain proviral load and achieve persistent infection.

How then does HTLV-1 infection target FoxP3$^+$ T cells? This could be explained by the following two mechanisms. First, FoxP3$^+$ T cells are known to contact with dendritic cells (DCs) frequently [38], which could increase the chance of *de novo* viral infection between DCs and FoxP3$^+$ T cells. A recent study demonstrated that cell-free HTLV-1 efficiently infects DCs, and the infected DCs promote *de novo* infection of CD4$^+$ T cells [39]. This notion is consistent with the finding that effector/memory-type CD45RA$^-$ T_{reg} cells, including FoxP3^{low} non-T_{reg} cells and FoxP3^{high} aT_{reg} cells, are more frequently infected with HTLV-1 than CD45RA$^+$ rT_{reg} cells (Figure 4E). Second, once FoxP3$^-$ T cells are infected with HTLV-1, HBZ should be expressed in the host cells. Since HBZ is recently

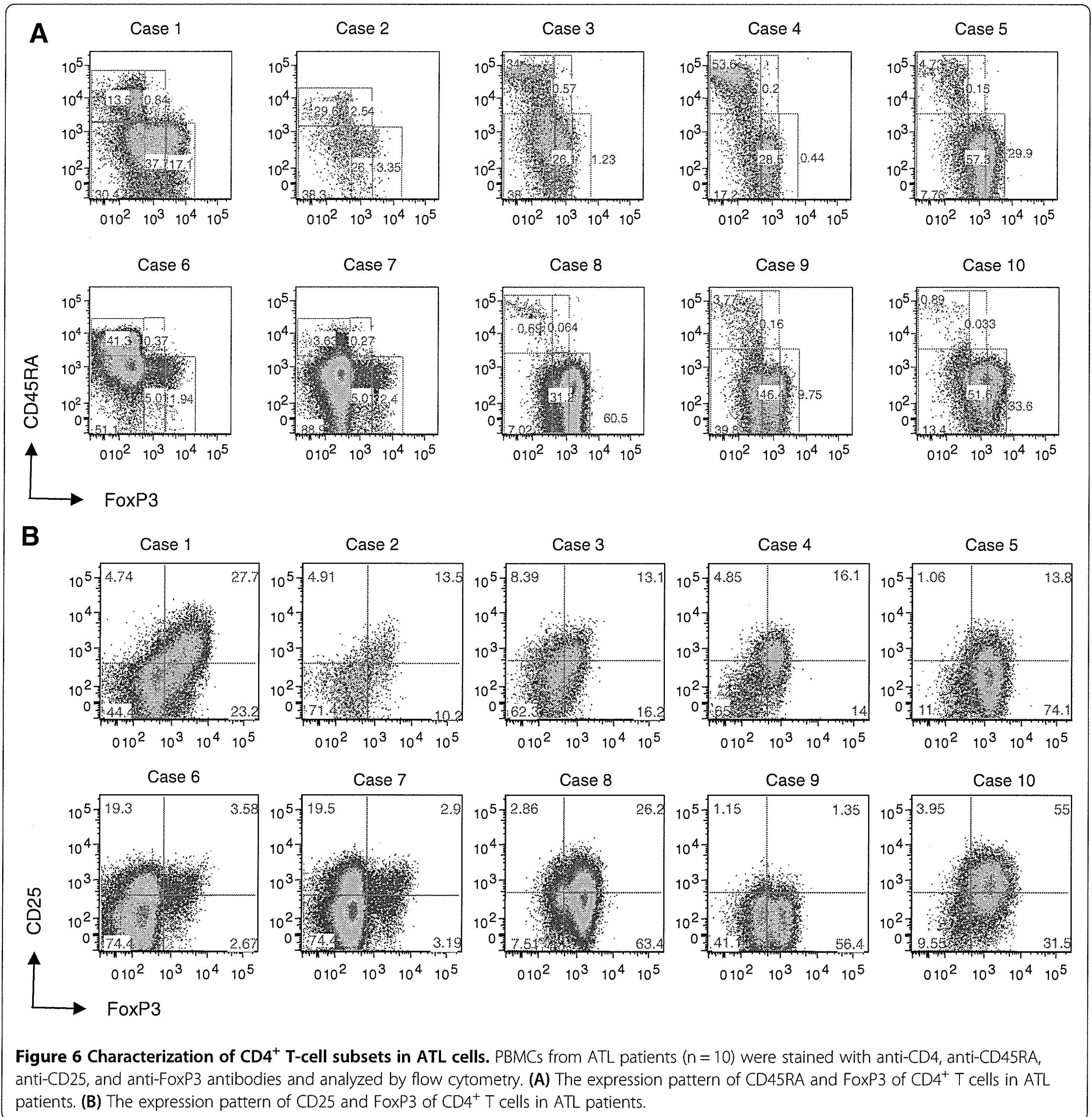

Figure 6 Characterization of CD4⁺ T-cell subsets in ATL cells. PBMCs from ATL patients (n = 10) were stained with anti-CD4, anti-CD45RA, anti-CD25, and anti-FoxP3 antibodies and analyzed by flow cytometry. **(A)** The expression pattern of CD45RA and FoxP3 of $CD4^+$ T cells in ATL patients. **(B)** The expression pattern of CD25 and FoxP3 of $CD4^+$ T cells in ATL patients.

reported to induce FoxP3 expression via enhancing TGF-β signaling pathway [17,40], HTLV-1 infection is likely to convert $FoxP3^-$ cells into $FoxP3^+$ cells. In addition, HTLV-1 has a cell-extrinsic effect on $FoxP3^+$ cell generation. HTLV-1 infected cells secrete CCL22 via expression of Tax, which indirectly contributes to the generation and maintenance of HTLV-1 uninfected $FoxP3^+$ cells [41,42]. This would contribute to an increased number of HTLV-1-uninfected $FoxP3^+$ cells.

Since $FoxP3^+$ T_{reg} cells play a crucial role in suppressing immune response, the increase of $FoxP3^+$ cells observed in HTLV-1 infection may contribute to immunodeficiency, which is frequently observed in HTLV-1 infection [43]. On the other hand, the high frequency of $FoxP3^+$ T cells observed in HAM/TSP patients is paradoxical, because the pathogenesis of HAM/TSP is believed to be inflammatory. Therefore, we analyzed the phenotype of the increased $FoxP3^+$ cells and observed that CTLA-4 and GITR expression of $FoxP3^+$ T cells in HAM/TSP patient was significantly reduced compared to uninfected individuals (Figure 5C). A similar observation was reported previously that the expression level of

FoxP3, GITR, or CTLA-4 mRNA in $CD4^+CD25^+$ T cells of HAM/TSP patients is lower than that of HD [44]. That report used $CD4^+CD25^+$ as a marker of T_{reg} cells, but $CD4^+CD25^+$ T cells contain not only $FoxP3^+$ T_{reg} cells but also $FoxP3^-$ activated T cells. Particularly the proportion of $CD4^+CD25^+FoxP3^-$activated T cells is upregulated in HAM/TSP patients, which is likely to reduce the proportion of $FoxP3^+$ T_{reg} cells in $CD4^+CD25^+$ T cells of HAM/TSP patients. Thus, the expression level of GITR or CTLA-4 in $FoxP3^+$ T cells of HAM/TSP patients has not been elucidated yet. To avoid this concern, we utilized the multicolor flow cytometry, which enabled us to show that CTLA-4 and GITR were clearly down regulated in $FoxP3^+$ T cells of HAM/TSP patients.

Then what is the underlying mechanism of this phenomenon? We reported recently that HBZ-Tg mice showed a pro-inflammatory phenotype in spite of the increase of $Foxp3^+$ T cells [17], which is similar to HAM/TSP patients (Figure 1D). T_{reg} associated molecules were also down regulated in $Foxp3^+$ T cells of HBZ-Tg mice. Thus, HBZ-mediated FoxP3 dysfunction may play a role in the abnormality regarding $FoxP3^+$ cells in HAM/TSP patients. It has been reported that Tax also contributes to the dysregulation of $FoxP3^+$ T_{reg} cells. Tax suppresses FoxP3 expression at transcriptional level [45], which alternatively or additionally could contribute to the abnormal phenotype of $FoxP3^+$ cells. These findings collectively indicate that the increased $FoxP3^+$ T_{reg} cells were functionally impaired in HAM/TSP patients. Furthermore, $FoxP3^+$ $CD4^+$ T cells in HAM/TSP patient contain an increased $FoxP3^+$ non-Treg population (Figure 5F), which would contribute to the inflammatory phenotype of HAM/TSP via generation of pro-inflammatory cytokine-producing $CD4^+$ T cells such as T_{HAM} cells [46] or exFoxp3 cells [47].

In the current study, we did not observed FoxP3 repression during Tax expression by *ex vivo* cultivation. This result seems to be inconsistent with a previous report that Tax represses FoxP3 expression [45]. There are two possible explanation of this inconsistency. First, there is the difference of the ways to express Tax. In the previous study, the authors used transfection of plasmid that induces Tax expression by the CMV promoter. We used endogenous HTLV-1 provirus to express Tax. Therefore, the expression level of Tax in our current study should be much lower than that of the previous study. In addition, Tax expression was induced in a proportion of $FoxP3^+$ cell in our current study. Second, there are differences in incubation time for Tax expression. In the previous study, the authors evaluated FoxP3 expression after 48 hours of transfection, whereas we evaluated FoxP3 expression within 24 hours after Tax expression.

High expression levels of CD25 are also well documented in HTLV-1 infection [33]. Consistent with previous findings, CD25 expression is upregulated in $FoxP3^+$ cells of HAM/TSP patient (Figure 5C). One determinant of the susceptibility to HAM/TSP is host genetic polymorphism such as MHC class 1, which influences the efficiency of CTL against HTLV-1 [48,49]. HTLV-1-infected individuals who have HLA class I susceptible for HAM/TSP may allow high expression of Tax and/or HBZ, which could cause upregulation of CD25 molecules in the $FoxP3^+$ cell population (Figure 5C).

It is controversial whether ATL is a leukemia of $FoxP3^+$ T_{reg} cells or not. However, there is no *a priori* reason to assume that ATL cells must be exclusively derived from $FoxP3^+$ T_{reg} cells or non-T_{reg} cells. Indeed, there are previous reports to support both possibilities. Some studies have reported that ATL cells have regulatory functions [50,51], whereas other studies reported no regulatory function in ATL [52,53]. We showed here that HTLV-1 is frequently detectable in $CD4^+FoxP3^+$ T cells (Figure 3B) in AC. More than half of ATL cells express FoxP3 (Figure 6), even though FoxP3 expression in ATL cells is variable as shown in the present and previous studies [30,31]. These findings prompt us to propose an idea that more than a half of ATL cells are possibly derived from $FoxP3^+$ T_{reg} cells. We reported previously that HBZ expression is constitutively active but Tax expression is frequently silenced in ATL cells, which possibly contributes to high frequency of $FoxP3^+$ ATL.

Conclusion

This study demonstrated that HTLV-1 infection induced the abnormality of frequency and phenotype of $FoxP3^+$ T cells, suggesting that HTLV-1 has evolved a sophisticated strategy to achieve persistent infection by directly affecting the central regulator of the host immune system. HTLV-1-mediated dysregulation of $FoxP3^+$ T cells is likely to be a critical cellular mechanism for the understanding HTLV-1 pathogenicity.

Methods

Clinical samples and ethics statement

PBMCs were obtained from asymptomatic HTLV-1 infected carriers (n = 23), HAM/TSP patients (n = 10), ATL patients (n = 10), and age-matched healthy controls (n = 10). Characteristics of each group are presented in Table 1. ATL patients consist of 2 acute, 4 smoldering and 4 chronic types of ATL cases. Genomic DNA extracted from PBMCs was used to determine proviral load (PVL) as described previously [29]. Briefly, PVL was quantified by real time PCR and calculated by using genomic DNA of TL-Om1, an ATL cell line with one copy of complete HTLV-1 provirus, as a standard of 100%. We defined AC with less than 2% of proviral load as AC^{low} and AC with more than 2% of proviral load as AC^{high}. This study was conducted according to the

principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of Kyoto University (844 and E-921). All patients provided written informed consent for the collection of samples and subsequent analysis.

Antibodies

The following antibodies were purchased from BD PharMingen; purified monoclonal antibody (mAb) for human CD3 (UCHT1), CD4 (RPA-T4), CD8a (RPA-T8), CD45RA (NI100) and CTLA-4 (BNI3). Purified mAbs for human CD25 (BC96), GITR (eBio AITR) and FoxP3 (236A/E7) were purchased from eBioscience.

Flow cytometric analysis

PBMCs were isolated with Ficoll-Isopaque (GE Healthcare) gradient centrifugation. Flow cytometric analyses were carried out using a FACS CantoII with Diva Software (BD Pharmingen), and the data were analyzed by FlowJo software (Treestar). To discriminate dead cells, we used LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). For cell surface staining, 10^6 cells were incubated with mAbs for 30 minutes at 4°C, and then analyzed. For intracellular staining, we used a human FoxP3 staining kit according to the manufacture's protocol (eBioscience). To distinguish FoxP3$^+$ and FoxP3$^-$ cell population clearly, we used isotype control according to the manufacture's recommendation. To detect the viral antigen Tax, we cultured PBMCs from ACs or HAM/TSP patients for 12–18 hours and stained with monoclonal antibodies against FoxP3 or Tax (MI-73) [54], and then analyzed by flow cytometry.

Statistical analysis

To compare 2 groups when data were determined to have a Gaussian distribution, the Student *t* test was used. If data did not have a Gaussian distribution, the Mann–Whitney *U* test was used for unpaired data, and the Wilcox signed-ranks test was used for paired data. The AC group and HD did not differ significantly in sex or age, using chi-squared test and Mann–Whitney *U* test. Differences with $P < 0.05$ were considered to be statistically significant. Correlations were evaluated using Spearman's rank correlation.

Additional files

Additional file 1: Figure S1. Absolute cell numbers of each CD4$^+$T-cell subset in HTLV-1 infected individuals. (A) Absolute cell numbers of CD4$^+$ T cells in 4 distinct subjects. Data shown are gated on lymphocyte fraction based on the dot plot pattern of SSC and FSC. (B and C) Absolute cell numbers of FoxP3$^-$CD45RA$^+$ naïve CD4$^+$ T cells (B) or FoxP3$^-$CD45RA$^-$ effector/memory CD4$^+$ T cells (C). (D) Absolute cell numbers of FoxP3$^+$ cells in CD4$^+$ T cells.

Additional file 2: Figure S2. Effect of *ex vivo* cultivation on FoxP3 and CD45RA expression. The percentages of FoxP3 and CD45RA expression in CD4$^+$ T cells both before and after *ex vivo* culture are shown from 5 distinct ACs.

Additional file 3: Figure S3. Frequency of each CD4 T-cell subset in Tax-expressing cell population in AC. Cumulative results from 23AC individuals are shown in the graph.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by Grant-in-Aid for Scientific Research from Japanese Society for the Promotion of Science and Ministry of Health Labor and Welfare, a grant from Takeda Science Foundation, a grant from Naito Foundation. We thank Prof. Charles R.M. Bangham for critical reading of the manuscript and Ms. M. Nakashima for preparation of peripheral blood of patients. We are most grateful to the patients and healthy donors who participated in this study.

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Authors' contributions

This study was designed by YS and MM. Laboratory analysis was performed by YS and JT. Data analysis was performed by YS, AU, JT and MM. Clinical samples and data were provided by AU, MN and KN. YS and MM wrote the paper. All authors read and approved the final manuscript.

Received: 24 December 2011 Accepted: 30 May 2012
Published: 30 May 2012

References

1. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H: **Adult T-cell leukemia: clinical and hematologic features of 16 cases.** *Blood* 1977, 50:481–492.
2. Gallo RC: **The discovery of the first human retrovirus: HTLV-1 and HTLV-2.** *Retrovirology* 2005, 2:17.
3. Takatsuki K: **Discovery of adult T-cell leukemia.** *Retrovirology* 2005, 2:16.
4. Matsuoka M, Jeang KT: **Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation.** *Nat Rev Cancer* 2007, 7:270–280.
5. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G: **Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.** *Lancet* 1985, 2:407–410.
6. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M: **HTLV-I associated myelopathy, a new clinical entity.** *Lancet* 1986, 1:1031–1032.
7. Sugimoto M, Nakashima H, Watanabe S, Uyama E, Tanaka F, Ando M, Araki S, Kawasaki S: **T-lymphocyte alveolitis in HTLV-I-associated myelopathy.** *Lancet* 1987, 2:1220.
8. Milagres SP, Sanches JA Jr, Milagres AC, Valente NY: **Histopathological and immunohistochemical assessment of acquired ichthyosis in patients with human T-cell lymphotropic virus type I-associated myelopathy.** *Br J Dermatol* 2003, 149:776–781.
9. Etoh K, Tamiya S, Yamaguchi K, Okayama A, Tsubouchi H, Ideta T, Mueller N, Takatsuki K, Matsuoka M: **Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells in vivo.** *Cancer Res* 1997, 57:4862–4867.
10. Cavrois M, Leclercq I, Gout O, Gessain A, Wain-Hobson S, Wattel E: **Persistent oligoclonal expansion of human T-cell leukemia virus type 1-infected circulating cells in patients with Tropical spastic paraparesis/HTLV-1 associated myelopathy.** *Oncogene* 1998, 17:77–82.
11. Yoshida M: **Multiple viral strategies of htlv-1 for dysregulation of cell growth control.** *Annu Rev Immunol* 2001, 19:475–496.

12. Giam CZ, Jeang KT: **HTLV-1 Tax and adult T-cell leukemia.** *Front Biosci* 2007, **12**:1496–1507.
13. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM: **The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription.** *J Virol* 2002, **76**:12813–12822.
14. Satou Y, Yasunaga J, Yoshida M, Matsuoka M: **HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells.** *Proc Natl Acad Sci U S A* 2006, **103**:720–725.
15. Usui T, Yanagihara K, Tsukasaki K, Murata K, Hasegawa H, Yamada Y, Kamihira S: **Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells.** *Retrovirology* 2008, **5**:34.
16. Arnold J, Zimmerman B, Li M, Lairmore MD, Green PL: **Human T-cell leukemia virus type-1 antisense-encoded gene, Hbz, promotes T-lymphocyte proliferation.** *Blood* 2008, **112**:3788–3797.
17. Satou Y, Yasunaga J, Zhao T, Yoshida M, Miyazato P, Takai K, Shimizu K, Ohshima K, Green PL, Ohkura N, *et al*: **HTLV-1 bZIP factor induces T-Cell lymphoma and systemic inflammation in vivo.** *PLoS Pathog* 2011, **7**:e1001274.
18. Saito M, Matsuzaki T, Satou Y, Yasunaga J, Saito K, Arimura K, Matsuoka M, Ohara Y: **In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP).** *Retrovirology* 2009, **6**:19.
19. Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S: **Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease.** *Nature* 1990, **348**:245–248.
20. Bangham CR, Osame M: **Cellular immune response to HTLV-1.** *Oncogene* 2005, **24**:6035–6046.
21. Kannagi M, Harada S, Maruyama I, Inoko H, Igarashi H, Kuwashima G, Sato S, Morita M, Kidokoro M, Sugimoto M, *et al*: **Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8+ cytotoxic T cells directed against HTLV-I-infected cells.** *Int Immunol* 1991, **3**:761–767.
22. Fontenot JD, Gavin MA, Rudensky AY: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.** *Nat Immunol* 2003, **4**: 330–336.
23. Hori S, Nomura T, Sakaguchi S: **Control of regulatory T cell development by the transcription factor Foxp3.** *Science* 2003, **299**:1057–1061.
24. Khattri R, Cox T, Yasayko SA, Ramsdell F: **An essential role for Scurfin in CD4+CD25+ T regulatory cells.** *Nat Immunol* 2003, **4**:337–342.
25. Sakaguchi S, Yamaguchi T, Nomura T, Ono M: **Regulatory T cells and immune tolerance.** *Cell* 2008, **133**:775–787.
26. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: **CTLA-4 control over Foxp3+ regulatory T cell function.** *Science* 2008, **322**:271–275.
27. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, *et al*: **Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor.** *Immunity* 2009, **30**:899–911.
28. Richardson JH, Edwards AJ, Cruickshank JK, Rudge P, Dalgleish AG: **In vivo cellular tropism of human T-cell leukemia virus type 1.** *J Virol* 1990, **64**:5682–5687.
29. Yasunaga J, Sakai T, Nosaka K, Etoh K, Tamiya S, Koga S, Mita S, Uchino M, Mitsuya H, Matsuoka M: **Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state.** *Blood* 2001, **97**:3177–3183.
30. Karube K, Ohshima K, Tsuchiya T, Yamaguchi T, Kawano R, Suzumiya J, Utsunomiya A, Harada M, Kikuchi M: **Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells.** *Br J Haematol* 2004, **126**:81–84.
31. Abe M, Uchihashi K, Kazuto T, Osaka A, Yanagihara K, Tsukasaki K, Hasegawa H, Yamada Y, Kamihira S: **Foxp3 expression on normal and leukemic CD4 + CD25+ T cells implicated in human T-cell leukemia virus type-1 is inconsistent with Treg cells.** *Eur J Haematol* 2008, **81**:209–217.
32. Hanon E, Hall S, Taylor GP, Saito M, Davis R, Tanaka Y, Usuku K, Osame M, Weber JN, Bangham CR: **Abundant tax protein expression in CD4+ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes.** *Blood* 2000, **95**:1386–1392.
33. Hattori T, Uchiyama T, Toibana T, Takatsuki K, Uchino H: **Surface phenotype of Japanese adult T-cell leukemia cells characterized by monoclonal antibodies.** *Blood* 1981, **58**:645–647.
34. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF: **Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens.** *J Virol* 2004, **78**:2454–2459.
35. Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR: **An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection.** *Hepatology* 2004, **40**:1062–1071.
36. Toulza F, Heaps A, Tanaka Y, Taylor GP, Bangham CR: **High frequency of CD4 + FoxP3+ cells in HTLV-1 infection: inverse correlation with HTLV-1-specific CTL response.** *Blood* 2008, **111**:5047–5053.
37. Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC, Akbar AN: **Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo.** *J Clin Invest* 2006, **116**:2423–2433.
38. Yamazaki S, Steinman RM: **Dendritic cells as controllers of antigen-specific Foxp3+ regulatory T cells.** *J Dermatol Sci* 2009, **54**:69–75.
39. Jones KS, Petrow-Sadowski C, Huang YK, Bertolette DC, Ruscetti FW: **Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells.** *Nat Med* 2008, **14**:429–436.
40. Zhao T, Satou Y, Sugata K, Miyazato P, Green PL, Imamura T, Matsuoka M: **HTLV-1 bZIP factor enhances TGF-beta signaling through p300 coactivator.** *Blood* 2011, **118**:1865–1876.
41. Hieshima K, Nagakubo D, Nakayama T, Shirakawa AK, Jin Z, Yoshie O: **Tax-inducible production of CC chemokine ligand 22 by human T cell leukemia virus type 1 (HTLV-1)-infected T cells promotes preferential transmission of HTLV-1 to CCR4-expressing CD4+ T cells.** *J Immunol* 2008, **180**:931–939.
42. Toulza F, Nosaka K, Tanaka Y, Schioppa T, Balkwill F, Taylor GP, Bangham CR: **Human T-lymphotropic virus type 1-induced CC chemokine ligand 22 maintains a high frequency of functional FoxP3+ regulatory T cells.** *J Immunol* 2010, **185**:183–189.
43. Tachibana N, Okayama A, Ishizaki J, Yokota T, Shishime E, Murai K, Shioiri S, Tsuda K, Essex M, Mueller N: **Suppression of tuberculin skin reaction in healthy HTLV-I carriers from Japan.** *Int J Cancer* 1988, **42**:829–831.
44. Ramirez E, Cartier L, Rodriguez L, Alberti C, Valenzuela MA: **In vivo fluctuation of Tax, Foxp3, CTLA-4, and GITR mRNA expression in CD4(+) CD25(+) T cells of patients with human T-lymphotropic virus type 1-associated myelopathy.** *Braz J Med Biol Res* 2010, **43**:1109–1115.
45. Yamano Y, Takenouchi N, Li HC, Tomaru U, Yao K, Grant CW, Maric DA, Jacobson S: **Virus-induced dysfunction of CD4 + CD25+ T cells in patients with HTLV-I-associated neuroimmunological disease.** *J Clin Invest* 2005, **115**:1361–1368.
46. Yamano Y, Araya N, Sato T, Utsunomiya A, Azakami K, Hasegawa D, Izumi T, Fujita H, Aratani S, Yagishita N, *et al*: **Abnormally high levels of virus-infected IFN-gamma + CCR4+ CD4+ CD25+ T cells in a retrovirus-associated neuroinflammatory disorder.** *PLoS One* 2009, **4**:e6517.
47. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA: **Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo.** *Nat Immunol* 2009, **10**:1000–1007.
48. Jeffery KJ, Usuku K, Hall SE, Matsumoto W, Taylor GP, Procter J, Bunce M, Ogg GS, Welsh KI, Weber JN, *et al*: **HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy.** *Proc Natl Acad Sci U S A* 1999, **96**:3848–3853.
49. Macnamara A, Rowan A, Hilburn S, Kadolsky U, Fujiwara H, Suemori K, Yasukawa M, Taylor G, Bangham CR, Asquith B: **HLA class I binding of HBZ determines outcome in HTLV-1 infection.** *PLoS Pathog* 2010, **6**: e1001117.
50. Chen S, Ishii N, Ine S, Ikeda S, Fujimura T, Ndhlovu LC, Soroosh P, Tada K, Harigae H, Kameoka J, *et al*: **Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells.** *Int Immunol* 2006, **18**:269–277.
51. Yano H, Ishida T, Inagaki A, Ishii T, Kusumoto S, Komatsu H, Iida S, Utsunomiya A, Ueda R: **Regulatory T-cell function of adult T-cell leukemia/lymphoma cells.** *Int J Cancer* 2007, **120**:2052–2057.
52. Shimauchi T, Kabashima K, Tokura Y: **Adult T-cell leukemia/lymphoma cells from blood and skin tumors express cytotoxic T lymphocyte-associated antigen-4 and Foxp3 but lack suppressor activity toward autologous CD8+ T cells.** *Cancer Sci* 2008, **99**:98–106.

53. Toulza F, Nosaka K, Takiguchi M, Pagliuca T, Mitsuya H, Tanaka Y, Taylor GP, Bangham CR: **FoxP3+ regulatory T cells are distinct from leukemia cells in HTLV-1-associated adult T-cell leukemia.** *Int J Cancer* 2009, **125**:2375–2382.
54. Mori K, Sabe H, Siomi H, Iino T, Tanaka A, Takeuchi K, Hirayoshi K, Hatanaka M: **Expression of a provirus of human T cell leukaemia virus type I by DNA transfection.** *J Gen Virol* 1987, **68**(Pt 2):499–506.

doi:10.1186/1742-4690-9-46
Cite this article as: Satou *et al.*: HTLV-1 modulates the frequency and phenotype of $FoxP3^{+}CD4^{+}$ T cells in virus-infected individuals. *Retrovirology* 2012 **9**:46.

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Mod Rheumatol (2013) 23:133–139
DOI 10.1007/s10165-012-0641-x

ORIGINAL ARTICLE

HTLV-I virological and histopathological analysis in two cases of anti-centromere-antibody-seropositive Sjögren's syndrome

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Received: 27 January 2012 / Accepted: 22 March 2012 / Published online: 18 April 2012
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Abstract
Introduction The aim of this study was to show the clinical and pathological characteristics of anti-centromere-antibody (ACA)-seropositive Sjögren's syndrome (SS) in two anti-human T-cell leukemia virus type I (HTLV-I)-seropositive patients.
Methods One patient was an HTLV-I carrier whereas the other was diagnosed with HTLV-I-associated myelopathy (HAM). Background data including serum HTLV-I titers, viral loads, and cytokine profiles were recorded. Azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry of the labial salivary glands (LSGs) and a muscle biopsy specimen from the HAM patient were performed.
Results Serum transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), and HTLV-I viral load were high in the HAM-SS patient compared with the HTLV-I carrier. Fibrous change in LSG was prominent in the HAM-SS patient. Although TGF-β expression was similar in the two patients, expression of HTLV-I-related proteins including p12, p28, group-specific antigen (GAG), and nuclear factor kappa-B (NF-κB) in the LSG were dominantly detected in the HAM-SS patient. Frequency of TGF-β staining in HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA was lower than that of the previous two patients.
Conclusion A high HTLV-I viral load in situ is supposed to promote the production of cytokines, especially TGF-β, resulting in the fibrous change of LSG in ACA-seropositive SS patients.

Keywords HTLV-I infection · Anti-centromere antibody · Sjögren's syndrome · Cytokine

Abbreviations

ACA	Anti-centromere antibody
ANA	Anti-nuclear antibody
CSF	Cerebrospinal fluid
HAM	HTLV-I-associated myelopathy
HTLV-I	Human T-cell leukemia virus type I
IFN-γ	Interferon gamma
MNC	Mononuclear cell
LSG	Labial salivary gland
SS	Sjögren's syndrome
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha

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Introduction

Human T-cell leukemia virus type I (HTLV-I) is known to be one of the causative agents of Sjögren's syndrome (SS) [1, 2]. Our previous epidemiologic studies show a close association between HTLV-I and SS [3, 4]. In addition, we found a significantly high prevalence of SS in patients with HTLV-I-associated myelopathy (HAM) [3, 5]. On the other hand, anti-centromere antibody (ACA) is known as a second class of autoantibodies in SS patients [6, 7]. Our previous report revealed that ACA is detected in only 4 % of HTLV-I-seropositive SS cases, demonstrating that HTLV-I might not be involved in the pathogenesis in ACA-seropositive SS patients [8]. However, if HTLV-I infection coincidently occurs in ACA-seropositive SS patients, the influence of ACA on HTLV-I-associated SS might become obvious. In this study, we report two cases of ACA-seropositive SS patients who were also seropositive for anti-HTLV-I antibody. One patient was complicated with HAM, whereas the other was an HTLV-I carrier. The variation in HTLV-I viral road in these patients appears to explain the differences in labial salivary gland (LSG) histopathology and cytokine profile.

Patients and methods

Patients

Case 1

This was a 61-year-old female patient who complained of sicca symptoms. Both ACA and anti-HTLV-I antibody measured by chemiluminescent enzyme immunoassay (CLEIA) were highly positive, as shown in Table 1. As no other symptoms or signs, including in the neuromuscular systems, were found in this patient, she was classified as an HTLV-I carrier.

Case 2

A 57-year-old female patient who complained of sicca symptoms and myalgia was diagnosed with HAM based on the diagnostic guidance for HAM determined by the Ministry of Health, Labour and Welfare. She had slowly progressive and symmetrical pyramidal tract damage with positive anti-HTLV-I antibody in both serum and cerebrospinal fluid (CSF). Antibodies against gp46, p53, p24, and p19 of HTLV-I in CSF were all positive. Serum ACA was also positive at a high titer (Table 1). She also suffered from inflammatory myopathy as evidenced by the elevation of muscle enzymes and by magnetic resonance imaging and muscle biopsy findings.

Both patients were diagnosed with SS according to the revised criteria [9], as proposed by the American–European Consensus Group. In both cases, HTLV-I viral roads in sera and serum cytokines including tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and transforming growth factor beta (TGF-β) were measured. For comparison, we studied the three groups of patients: (1) HTLV-I-seropositive SS patients without ACA, (2) HTLV-I-seronegative SS patients with ACA, and (3) HTLV-I-seronegative SS patients without ACA with respect to TGF-β immunostaining of LSG (four patients each in three groups).

LSG biopsy

LSG biopsy from the lower lip was performed under local anesthesia in SS patients. Informed consent to use biopsy samples was obtained from all participating patients at the commencement of the study. The study was conducted with the approval of the human ethical committee of our institution. The classifications of Chisholm and Mason [10] were used to determine the severity of mononuclear cell (MNC) infiltration.

Azan–Mallory staining and immunohistochemistry of labial salivary glands

Formalin-fixed, paraffin-embedded sections (3-µm thick) from the LSGs of these ACA-seropositive SS patients were used for azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan) with mouse anti-human CD4, CD8, CD20, and CD68 antibodies (DakoCytomation, Glostrup, Denmark), mouse anti-HTLV-I [p19, p28, and group-specific antigen (GAG)] antibody (Chemicon International Inc., Temecula, CA, USA), mouse anti-nuclear factor kappa B (NF-κB) p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse anti-TGF-β antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA). Briefly, endogenous peroxidase was inactivated in a 3 % hydrogen peroxide (H_2O_2) solution after microwave epitope retrieval. These sections were then blocked with 5 % normal horse serum, followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min. The color was developed by soaking the sections in 3.3′-diaminobenzidine (DAB) and H_2O_2 for 10 min, followed by counterstaining by soaking the sections in hematoxylin solution. Negative

Table 1 Background information and serum data of the human T-cell leukemia virus type I (HTLV-I)-associated anti-centromere antibody (ACA)-seropositive patients

	Case 1 HTLV-I carrier with ACA-seropositive SS	Case 2 HAM with ACA-seropositive SS
Age and gender	61 years old, female	57 years old, female
Xerostomia	Positive	Positive
Xerophthalmia	Positive	Negative
Schirmer test (right/left mm; <5 mm: positive)	5/4	11/11
Saxon test (g/2 min; <2 g: positive)	1.47	2.7
ANA: pattern	160×, centromere	640×, centromere
Anti-SS-A antibody: normal 10–30 U/ml	0.7	0.9
Anti-SS-B antibody: normal 15–25 U/ml	0.9	0.5
ACA: normal <16 index	172.8	165.0
IgG: normal 870–1,700 mg/dl	1,712	1,623
Rheumatoid factor: normal <15 IU/ml	11.4	17.0
Sialography[a] (Rubin and Holt)	Stage 1	Stage 2
Lip biopsy grade[b] (Chisholm and Mason)	3	3
LST (cpm) PHA(+)/no stimulation	105,936/617	184,859/19,319
LST (cpm) ConA(+)/no stimulation	160,934/617	102,299/19,319
Serum anti-HTLV-I antibody: normal <1.0 COI	>45	>45
Serum viral load (copies/10^4 cells)	<53	373
Serum TNF-α: normal 0.6–2.8 pg/ml	1.0	2.9
Serum IFN-γ: normal <0.1 IU/ml	<0.1	<0.1
Serum TGF-β: normal 1.56–3.24 ng/ml	2.76	12.6

Anti-SS-A Ab and anti-SS-B Ab (Mesacup SS-A/Ro test and SS-B/La test; Medical and Biological Laboratories, Nagoya, Japan) and ACA (Mesacup-2 test CENP-B; Medical and Biological Laboratories, Nagoya, Japan) were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Serum anti-HTLV-I antibody was measured by chemiluminescent enzyme immunoassay, and HTLV-I viral load was measured by the FastStart DNA Master Hybridization probe method. Serum TNF-α and TGF-β were measured by ELISA. Serum IFN-γ was measured by enzyme immunoassay. Data shown represent the period before treatments with agents such as glucocorticoids or immunosuppressive agents

SS Sjögren's syndrome, *ANA* anti-nuclear antibody, *COI* cutoff index, *ConA* concanavalin A, *cpm* count per minute, *HAM* HTLV-I-associated myelopathy, *Ig-G* immunoglobulin G, *LST* lymphocyte stimulation test, *PHA* phytohemagglutinin, *TNF* tumor necrosis factor, *IFN* interferon *TGF* transforming growth factor

[a] Sialography grading was determined by Rubin and Holt. Stages 1 and 2 represent punctate and globular patterns, respectively

[b] Grading defined by Chisholm and Mason: the presence of at least one focus of mononuclear cells per 4 mm^2 section = grade 3

control sections were treated with mouse immunoglobulin (Ig)G1.

Results

Clinical and serological data with cytokine profile

As shown in Table 1, a high ACA titer was detected in both patients. Serum IgG was almost normal, which is characteristic in ACA-seropositive SS patients [6]. As patient 2 was diagnosed with HAM, spontaneous proliferation of MNCs was significantly higher than in patient 1. Serum HTLV-I viral load was 373 copies/10^4 cells in patient 2, which is obviously higher than in patient 1 (<53 copies/10^4 cells). Serum TNF-α and TGF-β levels in patient 2 were increased compared with those in patient 1, although serum IFN-γ in both patients was within normal limits.

Azan–Mallory staining and immunohistochemical analysis

MNC infiltration was similar in both patients; however, Azan–Mallory staining showed a stronger fibrosis in patient 2 than in patient 1 (Fig. 1). In patient 2, TGF-β was highly stained in infiltrating MNCs and vessels, except in ductal and acinar cells. TGF-β staining, although weaker than MSG, was also performed in the muscle in patient 2. Accordingly, infiltration of CD4+ lymphocytes, which were dominant compared with CD20 and CD68, was shown in the LSGs of both patients (Fig. 2). Although CD8+ lymphocytes were also scattered in LSGs, CD4+

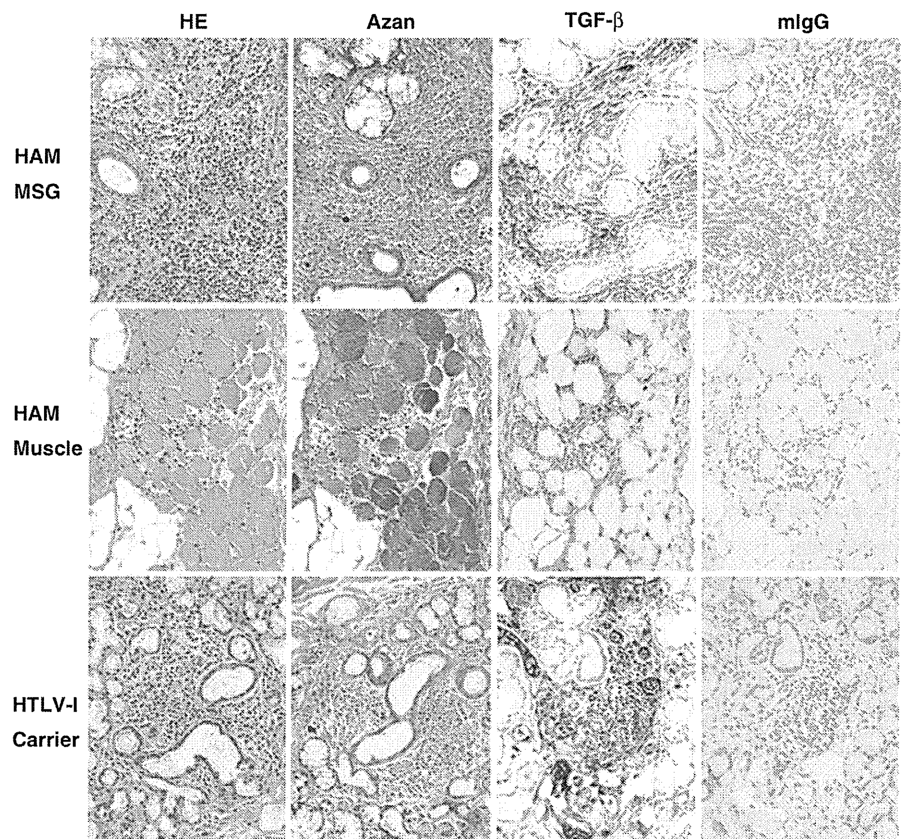

Fig. 1 Azocarmine with aniline blue (Azan)–Mallory staining and transforming growth factor beta (TGF-β) immunostaining in the labial salivary gland (LSG). Azan–Mallory staining and immunohistochemistry after epitope retrieval were performed for formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSG using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). The primary antibodies used for immunohistochemistry were TGF-β and mouse immunoglobulin (Ig)G1 (×200). Hematoxylin was used as a counterstain

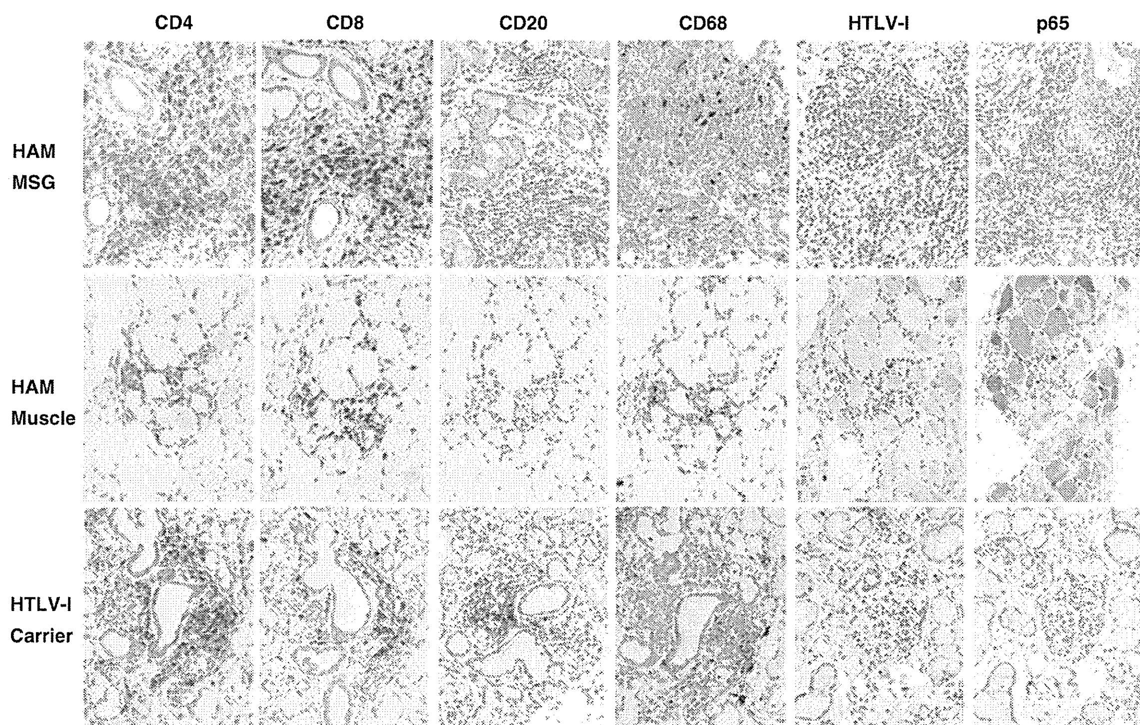

Fig. 2 Immunohistochemistry in the labial salivary gland (LSG). Immunohistochemistry after epitope retrieval was performed for formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSG using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). The primary antibodies used for immunohistochemistry were CD4, CD8, CD20, nuclear factor kappa B (NF-κB) (p65), and human T-cell leukemia virus type I (HTLV-I) [p19, p28, group-specific antigen (GAG)]. Lymph node from a patient with adult T-cell leukemia was used as a positive control for staining HTLV-I-related proteins (data not shown) (×200). Hematoxylin was used as a counterstain

and CD8+ lymphocytes were found in a muscle specimen from patient 2. It is interesting to note that HTLV-I-related proteins including p19, p28, and GAG were detected in the nuclei of a large percentage of infiltrating MNCs in LSGs and in the muscle specimen in patient 2, which was in accordance with the distribution of NF-κB p65.

TGF-β immunostaining in SS in the presence or absence of anti-HTLV-I antibody or ACA

We finally showed TGF-β immunostaining according to the presence of anti-HTLV-I antibody or ACA (Fig. 3). We performed these experiments in four patients each in three groups and show representative results (Fig. 3). In the HTLV-I-seropositive SS patients without ACA, TGF-β was dominantly found in vascular endothelial cells or fibrous tissues in LSG; however, the frequency of TGF-β+ cells (patients A–D in Fig. 3) appeared to be lower than the patients in cases 1 and 2 in Fig. 1. In the HTLV-I-seronegative SS patients with ACA, TGF-β was seen in infiltrating MNCs, vascular endothelial cells, and fibrous tissues in LSG. Then, in the HTLV-I-seropositive SS patients without ACA, TGF-β expression was similar to HTLV-I-seronegative SS patients with ACA (patients E–H in Fig. 3). In contrast, TGF-β expression was less in HTLV-I-seronegative patients without ACA (patients I, K, L) compared with other groups. In a HTLV-I-seronegative SS patient without ACA (as in patient J), TGF-β was not found in fibrous cells but in MNCs.

Discussion

Both HTLV-I and ACA are known to contribute to SS [1–8]; however, this coincidence of HTLV-I and ACA is supposed to occur with low frequency [8]. Our two cases presented here are rare but may illustrate the in vivo role of HTLV-I in patients with ACA-seropositive SS. Although both patients showed grade 3 MNC infiltration in LSGs, results from exocrine function tests, including Schirmer test and Saxon test in patient 1, were worse than those in patient 2. Except for the degree of MNC infiltration in LSGs, other factors such as aquaporin-5 distribution or type 3 muscarinic receptors [11, 12] might affect lacrimal and salivary secretion. With respect to MNC infiltration into the LSG, both cases showed similar findings. However, there were significant differences in fibrosis determined by Azan–Mallory staining and cytokine profiles.

As patient 2 was diagnosed with HAM, the HTLV-I viral load was high in comparison with patient 1, a finding that is consistent with previous reports [13]. Striking differences were observed in the Azan–Mallory staining findings; however, both patients showed high TGF-β expression in LSGs. TGF-β is a key cytokine for promoting the fibrotic process; thus, the prominent fibrosis of LSG is believed to be driven by TGF-β. Fibrosis was found in the LSG of both patients, which might be explained to some extent by the presence of ACA, as we previously reported [6]. However, a recent report found that HTLV-I basic-leucine zipper (bZIP) factor enhances TGF-β signaling through the p300 coactivator [14]. As strong expression of HTLV-I-related proteins was found in the LSG of patient 2, the TGF-β signaling pathways were suggested to be promoted in situ by HTLV-I, resulting in marked fibrosis. A similar phenomenon might occur in the muscle of patient 2, resulting in inflammatory myopathy. We previously reported that myopathy or uveitis was one characteristic of HTLV-I-seropositive SS patients [15]. With respect to a low level of IFN-γ, Santos et al. [16] demonstrated that administration of exogenous TGF-β induced a decrease of IFN-γ in cells from HTLV-I carriers, suggesting the possibility of the modulation of IFN-γ by TGF-β in HTLV-I-seropositive individuals. The high TNF-α level in patient 2 may also be driven by HTLV-I, as indicated for TGF-β.

To show the involvement of HTLV-I and ACA toward TGF-β expression, we examined TGF-β immunostaining for HTLV-I-seropositive patients without ACA, HTLV-I-seronegative patients with ACA, and HTLV-I-seronegative without ACA (Fig. 3). Although the precise quantitative analysis was not performed in this study, it may demonstrate that TGF-β expression in vascular endothelial cells and fibrous tissues of LSGs is more prominent in SS patients positive for both anti-HTLV-I antibody and ACA (two cases in Fig. 1) compared with SS patients positive for either one alone [two groups (patients A–H in Fig. 3)]. Accordingly, TGF-β expression in the above-mentioned sites was less in SS patients who were not positive for either anti-HTLV-I antibody or ACA (patients I–L in Fig. 3) in comparison with other groups. Therefore, we speculate that the synergistic effect of HTLV-I infection with ACA-carrying status induces the expression of TGF-β in LSGs, especially in vascular endothelial cells and fibrous tissue of SS patients (Fig. 4). However, we also found intense expression of TGF-β in MNCs even in HTLV-I-seronegative patients without ACA. As fibrous change determined by Azan–Mallory staining was not so significant in these patients, TGF-β in MNCs of LSGs may not be directly associated with the fibrotic process. In fact, TGF-β is known to be produced by CD4+ T lymphocytes [17] and influenced by other cytokines, such as IFN-γ [18]. Therefore, the two phenomena—Azan–Mallory-stain-proven fibrosis and TGF-β expression—should be carefully determined in patients with SS. Further studies with a larger number of participants and more definitive qualification approaches are necessary to prove our hypothesis.

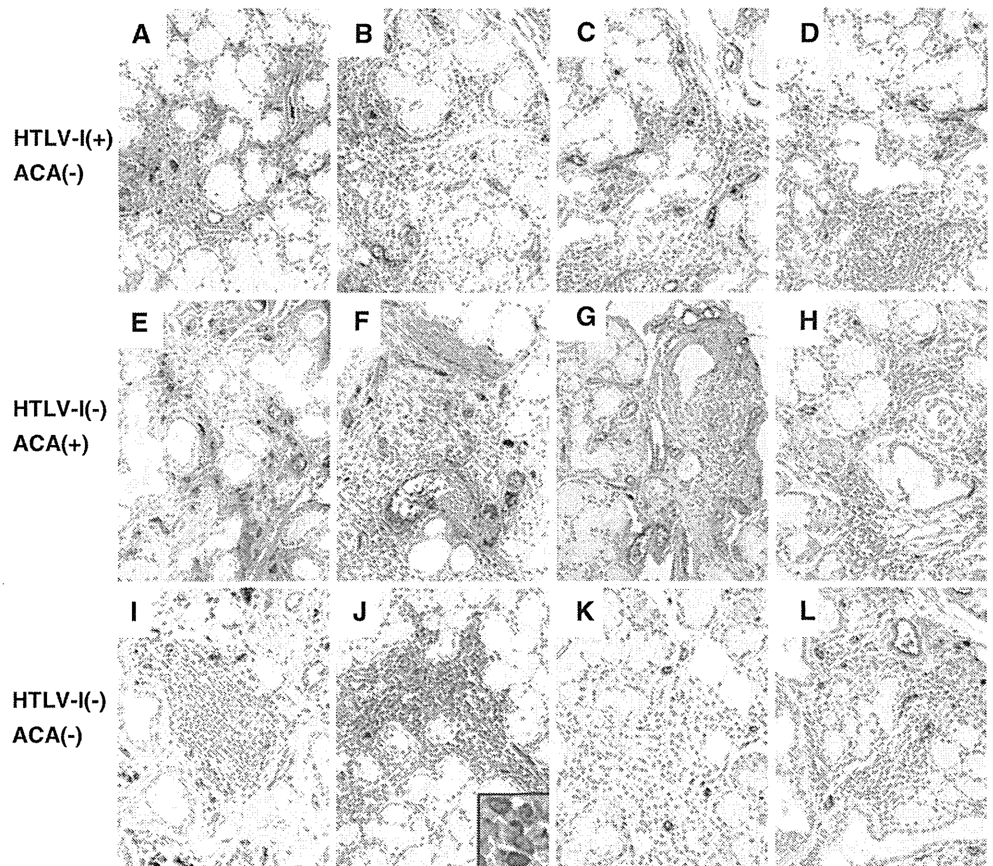

Fig. 3 Expression of transforming growth factor beta (TGF-β) in human T-cell leukemia virus type I (HTLV-I)-seropositive Sjögren's syndrome (SS) patients without anti-centromere-antibody (ACA), HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA. Immunohistochemistry for TGF-β after epitope retrieval was performed for formalin-fixed, paraffin-embedded sections (3-μm thick) from the labial salivary gland (LSGs) using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan). Staining was performed for four HTLV-I-seropositive SS patients without ACA (patients A–D), four HTLV-I-seronegative SS patients with ACA (patients E–H), and four HTLV-I-seronegative SS patients without ACA (patients I–J). For patient J, TGF-β-positive MNCs are shown in the *inset* (×200). Hematoxylin was used as a counterstain

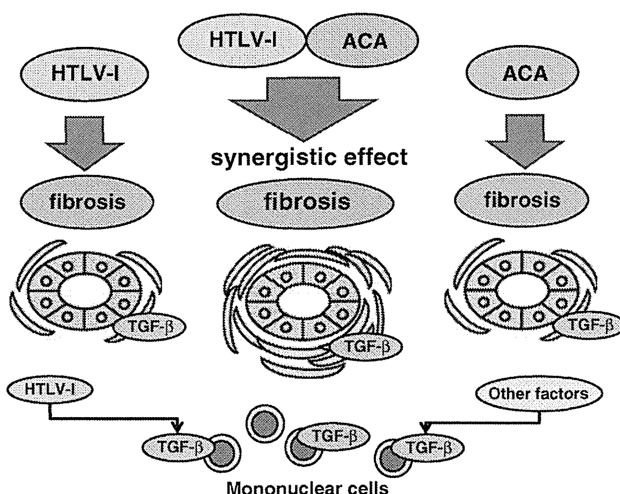

Fig. 4 Hypothesis for fibrotic alternation of salivary glands in Sjögren's syndrome (SS) patients through human T-cell leukemia virus type I (HTLV-I) infection and anti-centromere-antibody (ACA)-carrying status. From the results of the this study, HTLV-I- and ACA-carrying status induce fibrosis in labial salivary glands (LSGs). Furthermore, synergistic effects of HTLV-I infection with ACA-carrying status are assumed from the results of azocarmine with aniline blue (Azan)–Mallory staining. However, transforming growth factor beta (TGF-β), especially in mononuclear cells (MNCs), is also induced in HTLV-I infection and ACA-carrying status

In summary, we report two cases of ACA-seropositive SS found in HTLV-I-seropositive individuals and compared these patients with HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA. The predominant characteristics were found in a patient with HAM, which was believed to have been caused by elevated HTLV-I viral load and subsequent cytokine production. Elements other than TGF-β are also suggestive of influencing fibrotic alternation of LSGs in patients with SS.

Conflict of interest None.

References

1. Terada K, Katamine S, Eguchi K, Moriuchi R, Kita M, Shimada H, et al. Prevalence of serum and salivary antibodies to HTLV-1 in Sjögren's syndrome. Lancet. 1994;344(8930):1116–9.
2. Nakamura H, Kawakami A, Eguchi K. Mechanisms of autoantibody production and the relationship between autoantibodies and the clinical manifestations in Sjögren's syndrome. Transl Res. 2006;148:281–8.
3. Nakamura H, Eguchi K, Nakamura T, Mizokami A, Shirabe S, Kawakami A, et al. High prevalence of Sjögren's syndrome in patients with HTLV-I associated myelopathy. Ann Rheum Dis. 1997;56:167–72.

4. Hida A, Imaizumi M, Sera N, Akahoshi M, Soda M, Maeda R, et al. Association of human T lymphotropic virus type I with Sjogren syndrome. Ann Rheum Dis. 2010;69:2056–7.
5. Nakamura H, Kawakami A, Tominaga M, Hida A, Yamasaki S, Migita K, et al. Relationship between Sjögren's syndrome and human T-lymphotropic virus type I infection: follow-up study of 83 patients. J Lab Clin Med. 2000;135:139–44.
6. Nakamura H, Kawakami A, Hayashi T, Iwamoto N, Okada A, Tamai M, et al. Anti-centromere antibody-seropositive Sjögren's syndrome differs from conventional subgroup in clinical and pathological study. BMC Musculoskelet Disord. 2010;11:140.
7. Katano K, Kawano M, Koni I, Sugai S, Muro Y. Clinical and laboratory features of anti-centromere antibody positive primary Sjögren's syndrome. J Rheumatol. 2001;28:2238–44.
8. Hida A, Kawabe Y, Kawakami A, Migita K, Tominaga M, Nakamura H, et al. HTLV-I associated Sjögren's syndrome is aetiologically distinct from anti-centromere antibodies positive Sjögren's syndrome. Ann Rheum Dis. 1999;58:320–2.
9. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002;61:554–8.
10. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjögren's disease. J Clin Pathol. 1968;21:656–60.
11. Beroukas D, Hiscock J, Jonsson R, Waterman SA, Gordon TP. Subcellular distribution of aquaporin 5 in salivary glands in primary Sjögren's syndrome. Lancet. 2001;358:1875–6.
12. Tsuboi H, Matsumoto I, Wakamatsu E, Nakamura Y, Iizuka M, Hayashi T, et al. New epitopes and function of anti-M3 muscarinic acetylcholine receptor antibodies in patients with Sjögren's syndrome. Clin Exp Immunol. 2010;162:53–61.
13. Nagai M, Usuku K, Matsumoto W, Kodama D, Takenouchi N, Moritoyo T, et al. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. J Neurovirol. 1998;4:586–93.
14. Zhao T, Satou Y, Sugata K, Miyazato P, Green PL, Imamura T, et al. HTLV-1 bZIP factor enhances TGF-β signaling through p300 coactivator. Blood. 2011;118:1865–76.
15. Eguchi K, Matsuoka N, Ida H, Nakashima M, Sakai M, Sakito S, et al. Primary Sjögren's syndrome with antibodies to HTLV-I: clinical and laboratory features. Ann Rheum Dis. 1992;51:769–76.
16. Santos SB, Porto AF, Muniz AL, Luna T, Nascimento MC, Guerreiro JB, et al. Modulation of T cell responses in HTLV-1 carriers and in patients with myelopathy associated with HTLV-1. Neuroimmunomodulation. 2006;13:145–51.
17. Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. J Clin Invest. 2000;105:61–70.
18. McCartney-Francis NL, Wahl SM. Dysregulation of IFN-gamma signaling pathways in the absence of TGF-beta 1. J Immunol. 2002;169:5941–7.

Enose-Akahata et al. Retrovirology 2012, 9:16
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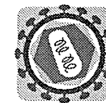

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Minocycline modulates antigen-specific CTL activity through inactivation of mononuclear phagocytes in patients with HTLV-I associated neurologic disease

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Abstract

Background: The activation of mononuclear phagocytes (MPs), including monocytes, macrophages and dendritic cells, contributes to central nervous system inflammation in various neurological diseases. In HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), MPs are reservoirs of HTLV-I, and induce proinflammatory cytokines and excess T cell responses. The virus-infected or activated MPs may play a role in immuneregulation and disease progression in patients with HTLV-I-associated neurological diseases.

Results: Phenotypic analysis of $CD14^+$ monocytes in HAM/TSP patients demonstrated high expression of CX_3CR1 and HLA-DR in $CD14^{low}CD16^+$ monocytes, compared to healthy normal donors (NDs) and asymptomatic carriers (ACs), and the production of TNF-α and IL-1β in cultured $CD14^+$ cells of HAM/TSP patients. $CD14^+$ cells of HAM/TSP patients also showed acceleration of HTLV-I Tax expression in $CD4^+$ T cells. Minocycline, an inhibitor of activated MPs, decreased TNF-α expression in $CD14^+$ cells and IL-1β release in PBMCs of HAM/TSP patients. Minocycline significantly inhibited spontaneous lymphoproliferation and degranulation/IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients. Treatment of minocycline also inhibited IFN-γ expression in $CD8^+$ T cells of HAM/TSP patients after Tax11-19 stimulation and downregulated MHC class I expression in $CD14^+$ cells.

Conclusion: These results demonstrate that minocycline directly inhibits the activated MPs and that the downregulation of MP function can modulate $CD8^+$ T cells function in HAM/TSP patients. It is suggested that activated MPs may be a therapeutic target for clinical intervention in HAM/TSP.

Keywords: HTLV-I, HAM/TSP, monocyte, CTL, minocycline

Background

The human T cell lymphotropic virus I (HTLV-I) infects 20 million people worldwide of which the majority of infected individuals are asymptomatic carriers (AC) of the virus [1]. However, in a small percentage of infected individuals, HTLV-I is the etiologic agent of adult T cell leukemia/lymphoma (ATL) [2] and a chronic, progressive neurological disease termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Patients with HAM/TSP demonstrate high HTLV-I proviral DNA load, high HTLV-I Tax mRNA load, and high virus-specific immune responses, including increased production of inflammatory cytokines and expansion of Tax-specific $CD8^+$ T cells [5-9]. A high frequency of $CD4^+$ T cells is persistently infected and exhibits high expression of Tax protein [10]. These infected cells are responsible for the increased lymphocyte proliferation in patients with HAM/TSP [11]. High frequency of activated $CD8^+$ T cells in peripheral blood and even higher in cerebrospinal fluid has been reported [12]. In addition to these strong HTLV-I-associated T cell responses, it has been suggested that mononuclear phagocytes (MPs; monocytes, dendritic cells, tissue macrophages and microglia) are also involved in the

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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

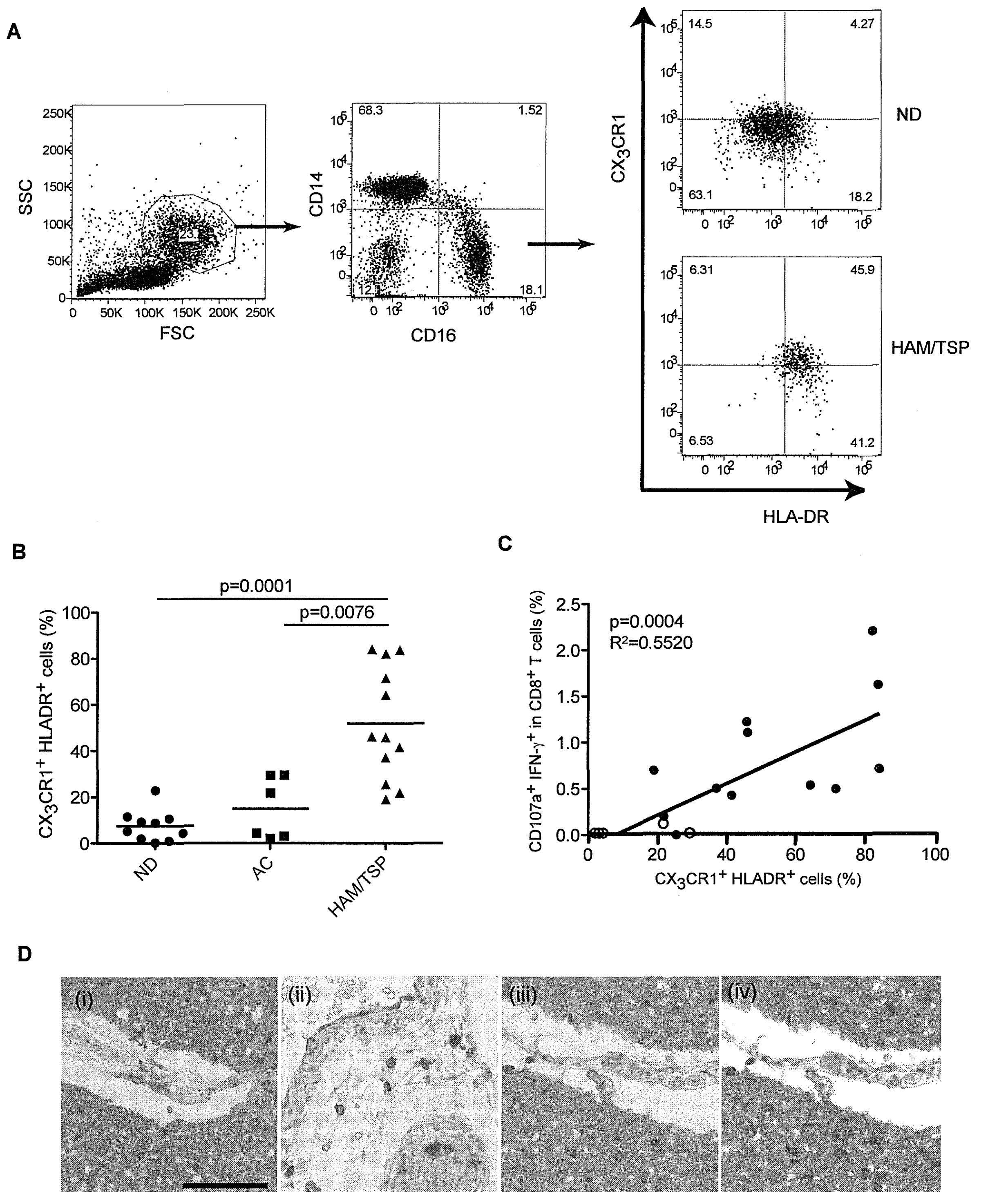

Figure 1 Characterization of mononuclear phagocytes in patients with HAM/TSP. (A) Representative dot plots of CX_3CR1 and HLA-DR expression in $CD14^{low}$ $CD16^+$ cells of a ND and a HAM/TSP patient. (B) Comparison of frequencies of CX_3CR1^+ HLA-DR^+ cells in $CD14^{low}$ $CD16^+$ mononuclear phagocytes 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

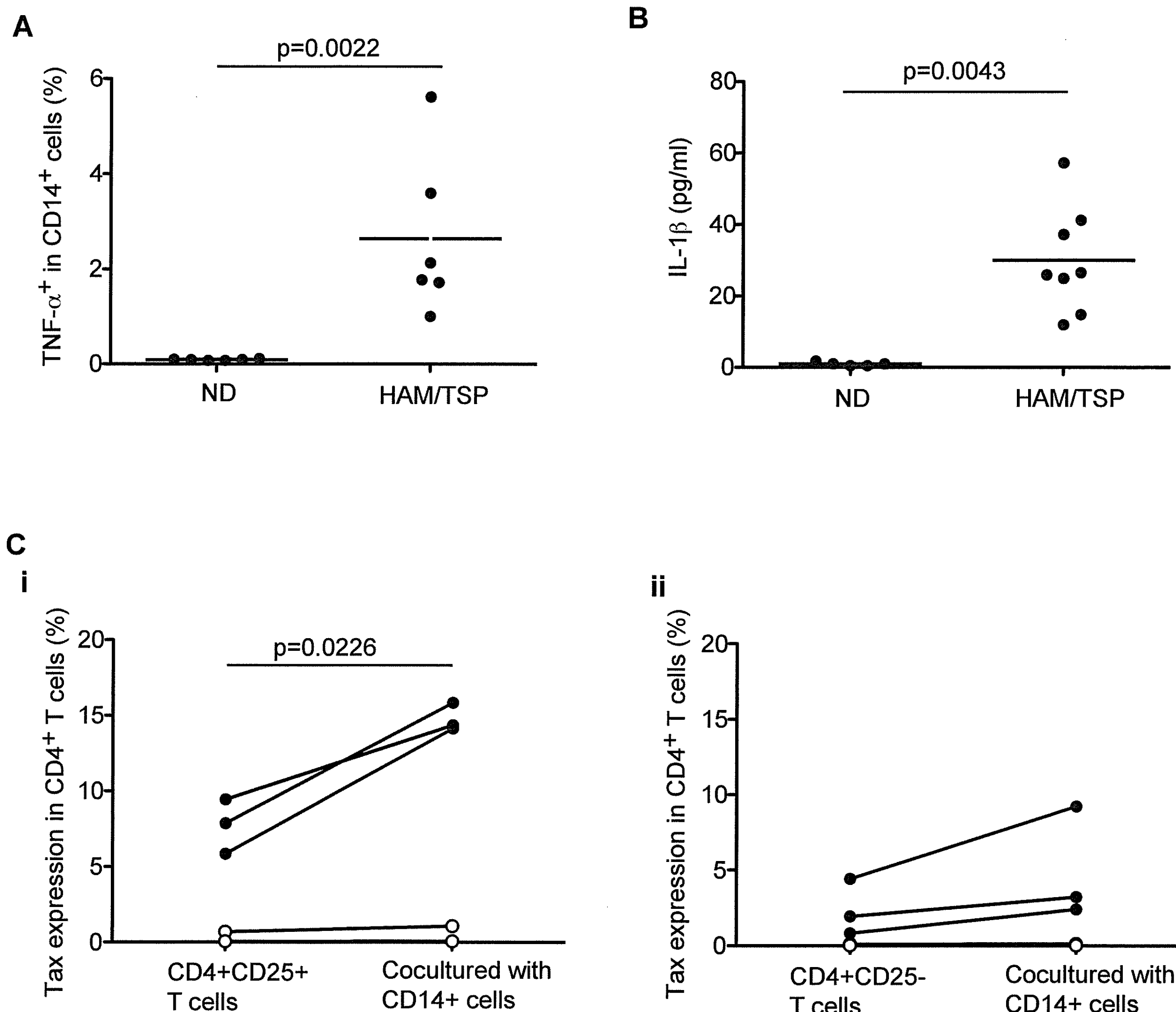

Figure 2 Activated CD14+ cells in patients with HAM/TSP. (A) TNF-α expression in CD14+ cells of NDs and HAM/TSP patients after culture for 24 hours. The data were obtained from six NDs and six HAM/TSP patients. CD14+ cells expressing TNF-α was significantly elevated in HAM/TSP patients, compared to NDs 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 $CD8^+$

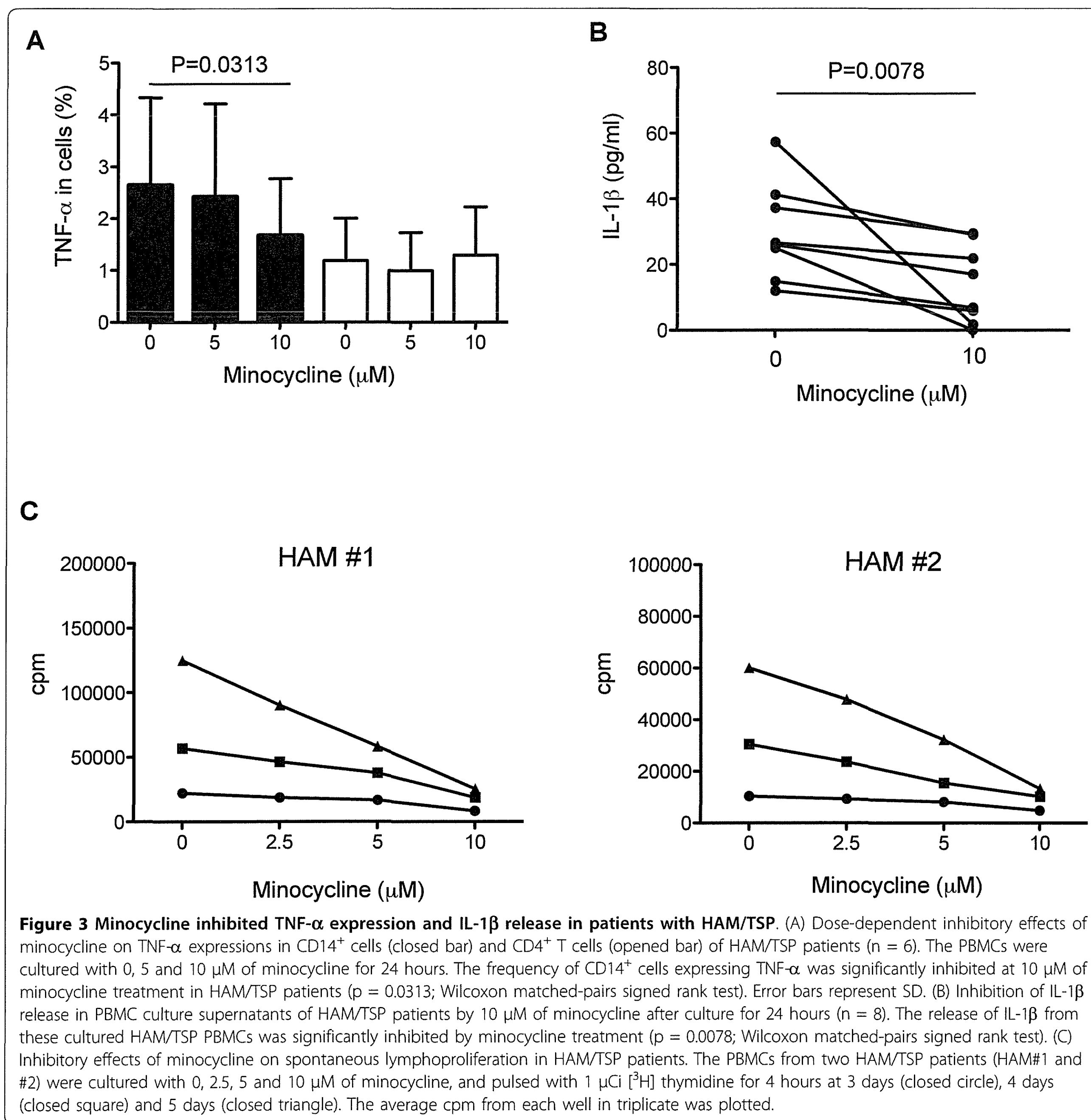

Figure 3 Minocycline inhibited TNF-α expression and IL-1β release in patients with HAM/TSP. (A) Dose-dependent inhibitory effects of minocycline on TNF-α expressions in $CD14^+$ cells (closed bar) and $CD4^+$ T cells (opened bar) of HAM/TSP patients (n = 6). The PBMCs were cultured with 0, 5 and 10 μM 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 [^{3}H] 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