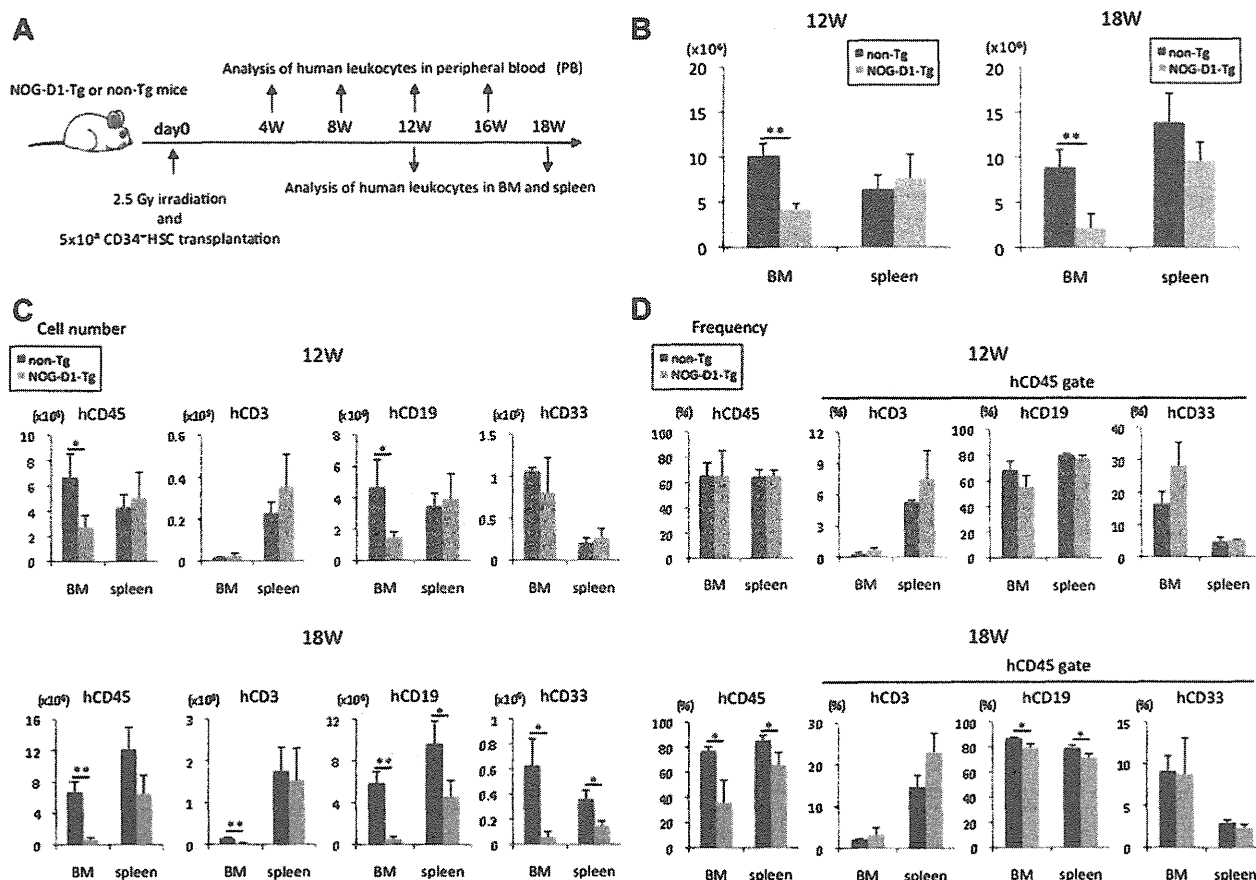

Figure 3. Development of human leukocytes in NOG-D1-Tg mice. (A) Schematic representation of transplantation of human $CD34^+$ HSCs into NOG-D1-Tg or non-Tg mice. (B) Twelve and 18 weeks after human HSC transplantation, total mononuclear cell numbers in the BM and spleen from NOG-D1-Tg or non-Tg mice were counted in two visual fields with 10× microscopic magnification (n = 3). An engrafted cell number (C) and frequency (D) of human CD45, CD3, CD19, and CD33 cells in the BM and spleen at 12 and 18 weeks after transplantation were analyzed by flow cytometry (n = 3). $*p < 0.05$ and $**p < 0.005$.

82% in the intracellular domain, and 95% in the N-terminal plus the conserved DSL [Delta, Serrate, and Lag-2] domain sequences), and demonstrated that human DLL1 protein inhibits myeloid lineage cell differentiation from murine progenitor cells in vitro. These results indicated functional cross-reactivity between human DLL1 and murine Notch receptors. Therefore, human DLL1 may stimulate mouse osteoblasts in NOG-D1-Tg mice and promote osteogenic differentiation in mice.

Several researchers have investigated the relationship between Notch signaling and osteosclerosis [26,27]. Engin et al. [26] generated Notch1 intracellular domain (NICD) Tg mice, which gained Notch function under the control of the *Col1a1* promoter. These mice showed an increase in bone mass and enhanced early osteoblastic proliferation. They demonstrated that NICD induces expansion of early osteoblastic precursors that produce an anti-osteoclastic differentiation factor, osteoprotegerin, and decreasing osteoclastic activity. Hence, these mutant mice showed enhancement of bone formation that greatly outweighed bone resorption, leading to an osteosclerotic phenotype. The enhanced bone matrix production might depend on activation of Notch signaling by DLL1 because the bones in NICD Tg mice and our NOG-D1-Tg mice showed a similar osteosclerotic phenotype.

In the present study, human hematopoiesis, especially B-cell development, from transplanted HSCs was strongly suppressed in NOG-D1-Tg mice. Similar observations have been reported in various osteopetrotic mice lacking molecules essential for osteoclast differentiation or function [28–32]. Blin-Wakkach et al. found that B lymphopoiesis was blocked in ossified bone of oc/oc mice that underwent osteopetrosis [29]. They argued that the blockage in B-cell differentiation was not due to a cell autonomous effect of the oc mutation, but was rather the consequence of altered BM architecture. Although our studies also showed decreased numbers of B cells in NOG-D1-Tg mice, a similar differentiation rate in B-cell subsets was observed for both NOG-D1-Tg and non-Tg mice. In general, human B lymphopoiesis predominates over T lymphopoiesis and

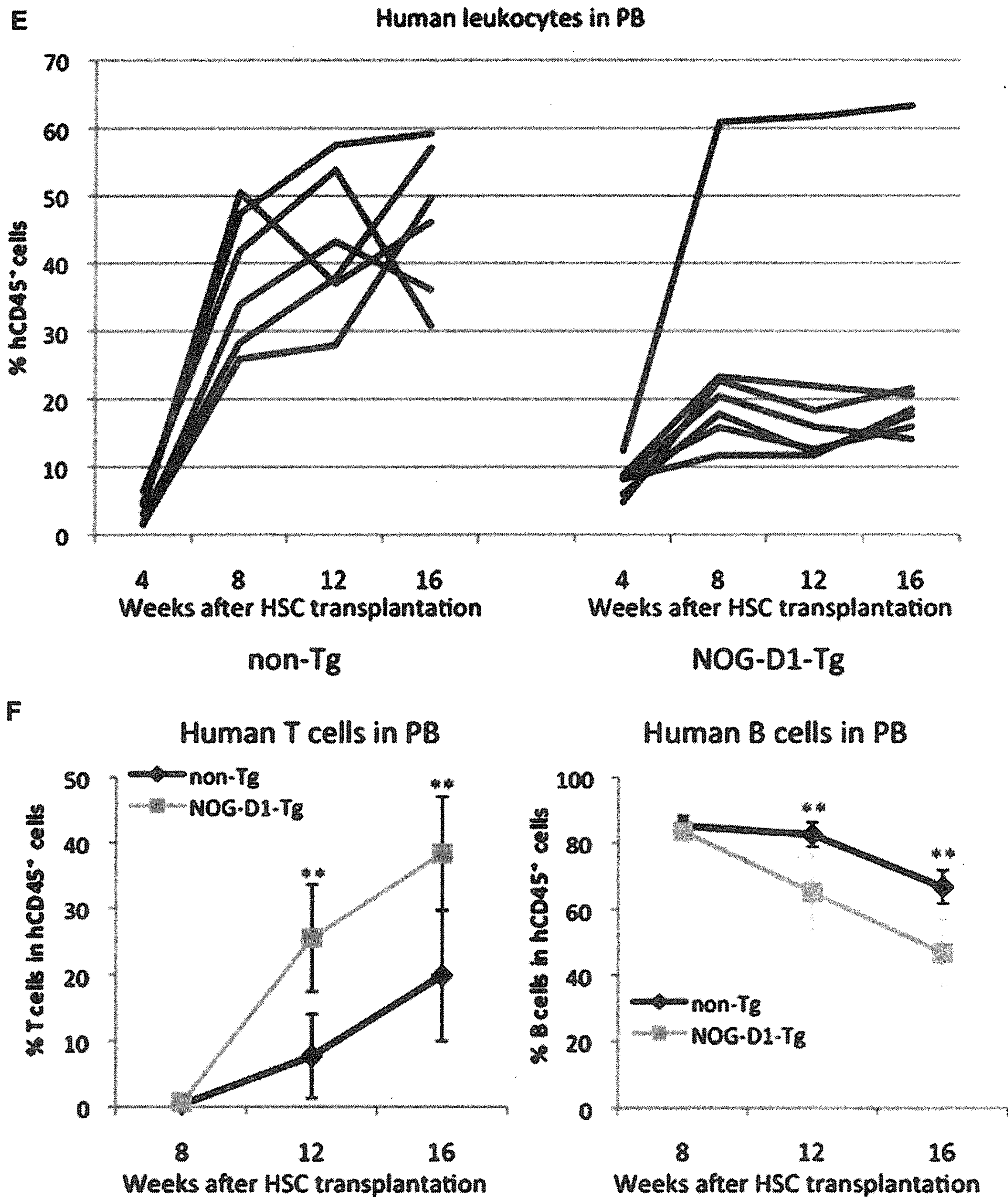

Figure 3. (*continued*). Time courses of the frequency of human CD45 (**E**), CD3, and CD19-positive cells (**F**) in human CD45-positive cells from NOG-D1-Tg (n = 7) or non-Tg (n = 6) mice were also analyzed by flow cytometry.

myelopoiesis in HSCs of humanized mice. B cells might accordingly be more susceptible than T or myeloid cells when HSC maintenance is impaired by an altered BM architecture. Another possibility is that human DLL1 may directly bind to HSCs or B cells expressing the Notch receptor and might induce specific apoptosis of B-lineage cells. Rangarajan et al. reported constitutive activation of Notch1-induced cell-cycle arrest and apoptotic cell death in a chicken B-cell line [33]. Pecci et al. also reported that DLL1 induces apoptosis in monocytic cells [34]. These

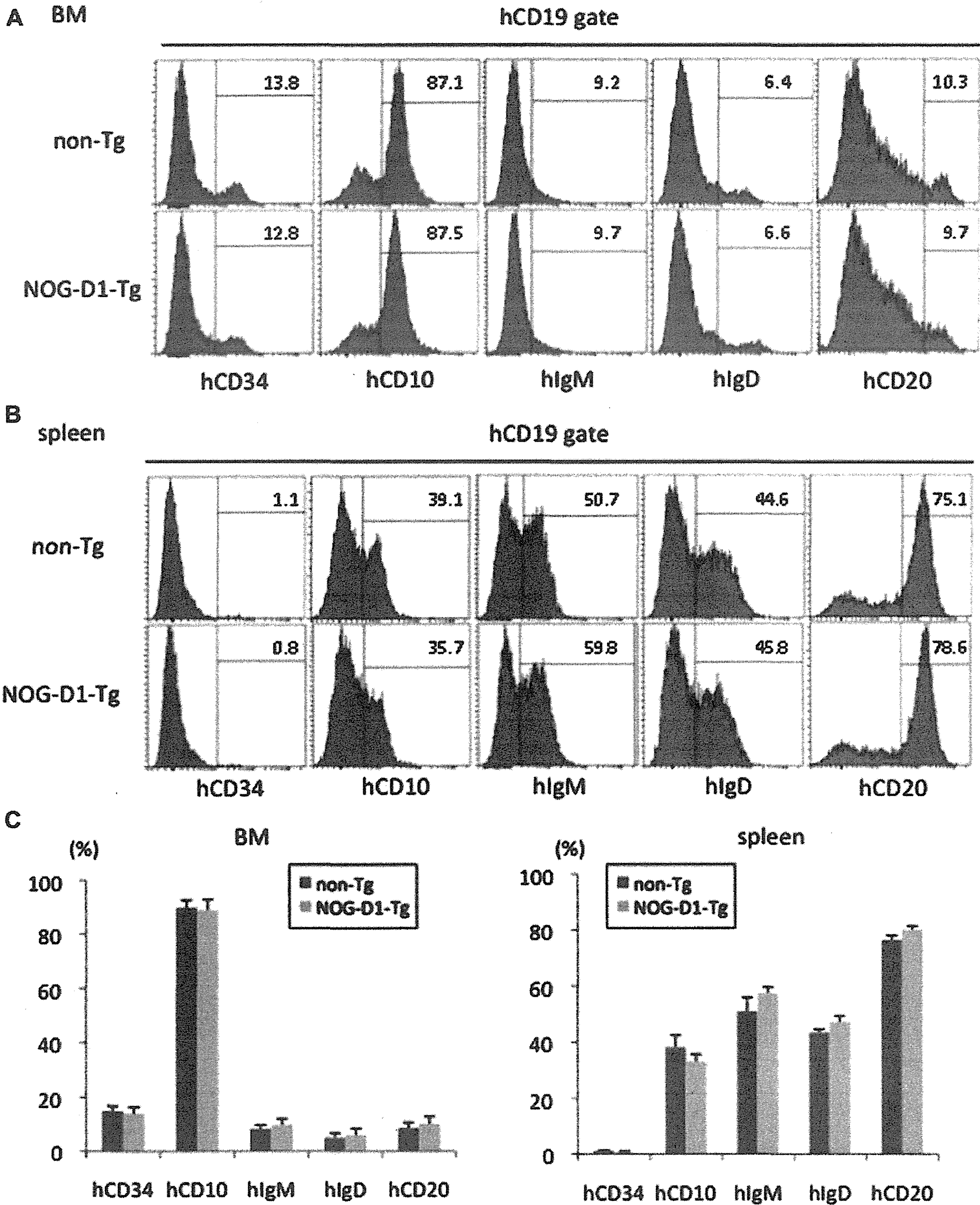

Figure 4. Human B-cell differentiation in NOG-D1-Tg mice. Twelve weeks after human HSC transplantation, human leukocytes were isolated from the BM and spleen of NOG-D1-Tg mice or littermates of non-Tg mice and stained with anti-hCD34, hCD10, hIgM, hIgD, hCD20, and hCD19 monoclonal antibodies. The histogram shows the frequencies of these markers in $hCD19^{+}$ cells in BM (A) and spleen (B). (C) Cumulative frequencies of hCD34, hCD10, hIgM, hIgD, and hCD20 cells in human $CD19^{+}$ cells from (A) and (B). Data represent mean ± standard deviation.

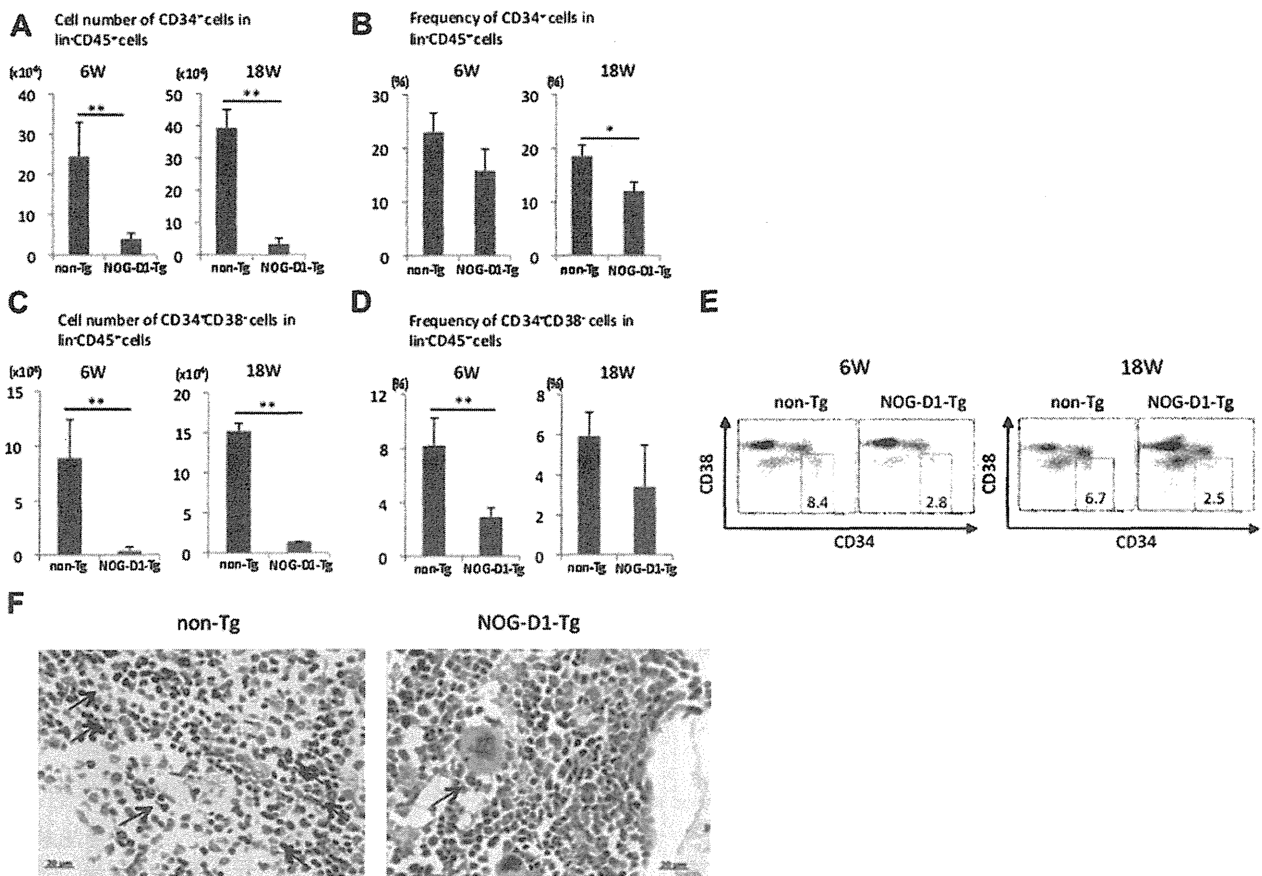

Figure 5. Maintenance of human HSCs in bone marrow of NOG-D1-Tg mice. At 6 or 18 weeks after human HSC transplantation, BM mononuclear cells were obtained from femurs of NOG-D1-Tg or non-Tg mice, and an engrafted number and ratio of lineage$^-$CD34$^+$ (A, B), and lineage$^-$CD34$^+$CD38$^-$ HSCs (C, D, and E), were analyzed by flow cytometry. (F) In immunohistochemistry, femur sections at 18 weeks after HSC transplantation and engrafted human HSCs were detected by anti-hCD34 antibody. Red arrows showed human CD34$^+$ HSCs represented by brown coloration. (B) and (C) show representative data. $^*p < 0.05$ and $^{**}p < 0.005$.

reports are consistent with our observation that the CD33$^+$ myeloid lineage cells was slightly decreased in the BM of NOG-D1-Tg mice at 18 weeks after human HSC transplantation (Fig. 3C). It is also known that Notch activation provides anti-apoptotic signals in developing T cells [35]. Jaleco et al. reported that B-cell differentiation from human CD34$^+$ cells is completely blocked when cultured with human DLL1-expressing stromal cells [36]. In their system, DLL1-expressing stroma cells could induce HSCs to T/ natural killer cell precursors, whereas they did not interfere with the developmental potential of B cells in a culture with Jagged1-expressing stromas. They explained that the differential effect by alternative Notch signals in human lymphopoiesis might be mediated by two candidate downstream molecules of Notch-signaling: HES-1, which is upregulated when undergoing T-cell lineage specification and is required for the development of normal T-cell numbers, and E47, a product of the E2A gene that is essential for B-cell specification. DLL1 can induce transactivation of HES-1, resulting in the accumulation of T/natural killer progenitors when cultured with DLL1-expressing stromas. On the other hand, DLL1 suppresses E47 activity, and mice lacking E2A showed complete inhibition of B-cell development [37]. Furthermore, de la Coste et al. [9] and Pui et al. [8] reported that ectopic T-cell differentiation in BM was induced by Notch signaling. Our results may appear inconsistent with previous findings in that, in the BM, thymus and periphery of NOG-D1-Tg mice, human T-cell differentiation was not enhanced (Fig. 3C and Supplementary Figure E2; online only, available at www.exphem.org) and CD7$^+$ T-cell progenitors did not increase (Supplementary Figure E3; online only, available at www.exphem.org). This inconsistency has two possible explanations: cytokines support T-cell development and maturation might have occurred in previous mouse studies. However, murine cytokines cannot always react to human cells in humanized mice systems [38]. Ohishi et al. reported that while Delta-1 promotes early T-cell differentiation from human HSC, but pre-exposure to several human cytokines (stem cell factor, fms-like tyrosine kinase receptor-3 ligand, thrombopoietin, and IL-6) was

necessary for human T-cell differentiation [12]. A reduced number of HSCs was observed in the BM of NOG-D1-Tg mice because of osteosclerosis, as shown in Figure 5. Such microenvironments may be suboptimal for supporting hematopoiesis because HSCs have less opportunity to encounter stromal cells in ossified bone. Thus, DLL1-expressing stromal cells may not efficiently interact with HSCs and differentiate into T lineage cells. It is necessary to further analyze whether human DLL1 can directly affect transplanted HSCs in NOG-D1-Tg mice.

Chadwick et al. [39] reported a negative influence of Notch signaling on HSCs. They clearly demonstrated that constitutive activation of the Notch signaling pathway via ectopic expression of NICD induces apoptosis of primary human $CD34^+$ cells [39]. Their findings may support our results showing that human HSCs were not maintained in the BM of NOG-D1-Tg mice, as measured in terms of cell number or cell ratio. These results suggest that transplanted human HSCs may come into contact with DLL1-expressing osteoblastic stromal cells, and the subsequent DLL1-dependent Notch signaling may induce apoptotic signals in HSCs.

Conclusions

NOG-D1-Tg mice are unique and interesting models of osteosclerosis and can mimic the state of ossification in the human patient. These mice may also be appropriate models to understand human hematological defects in patients with osteosclerosis and to discover new principles for therapeutic intervention.

Funding disclosure

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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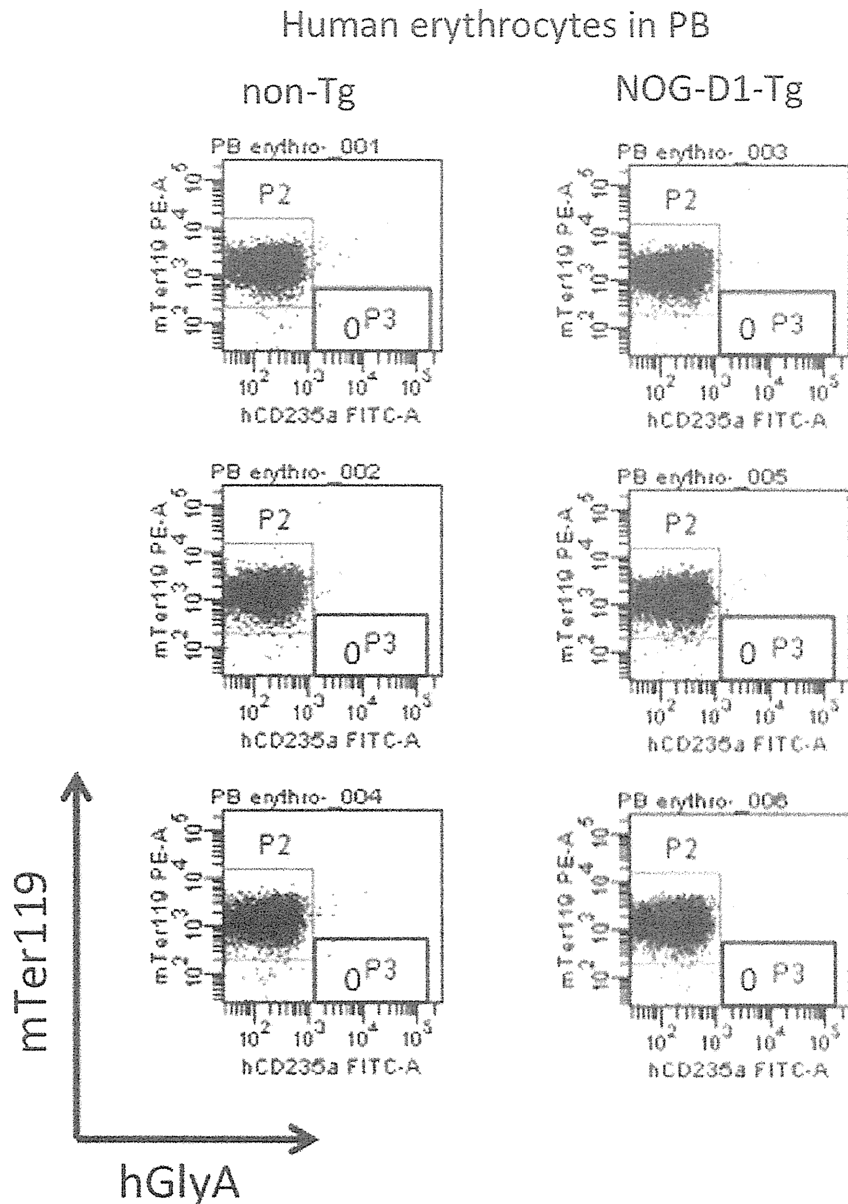
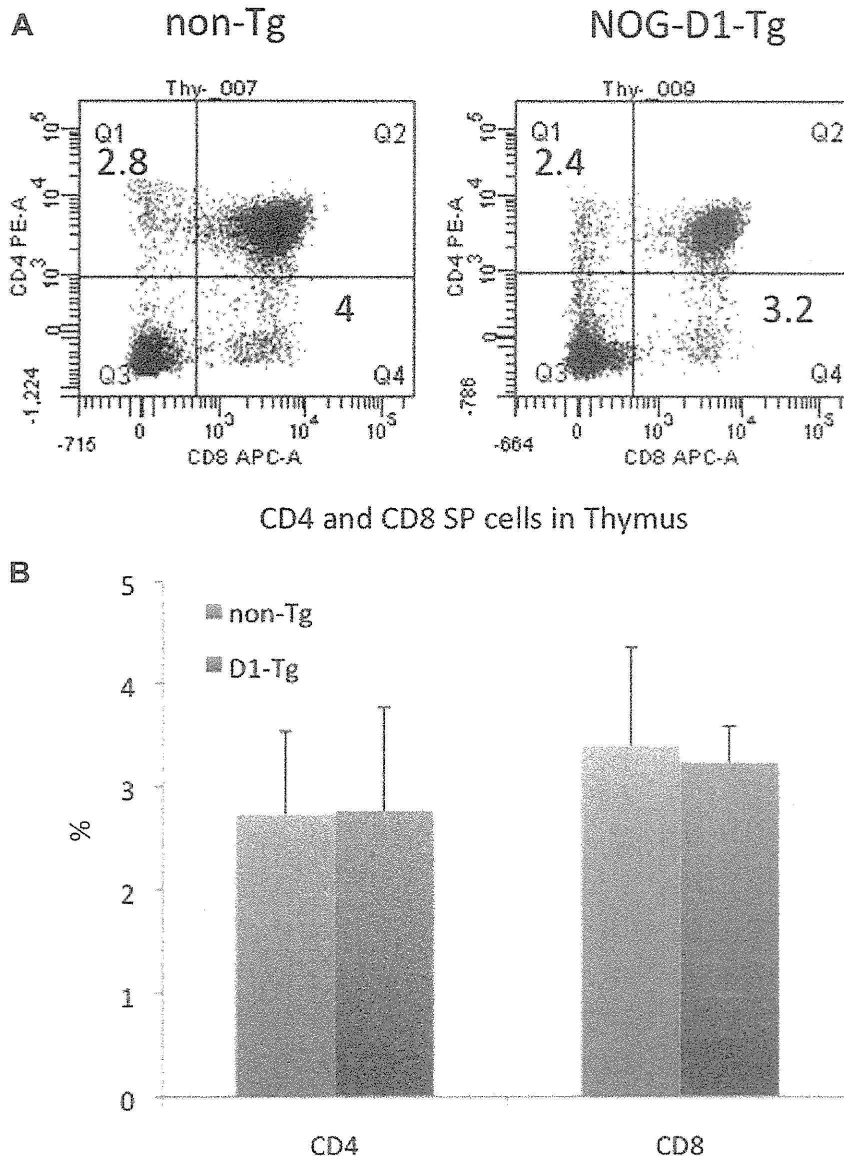
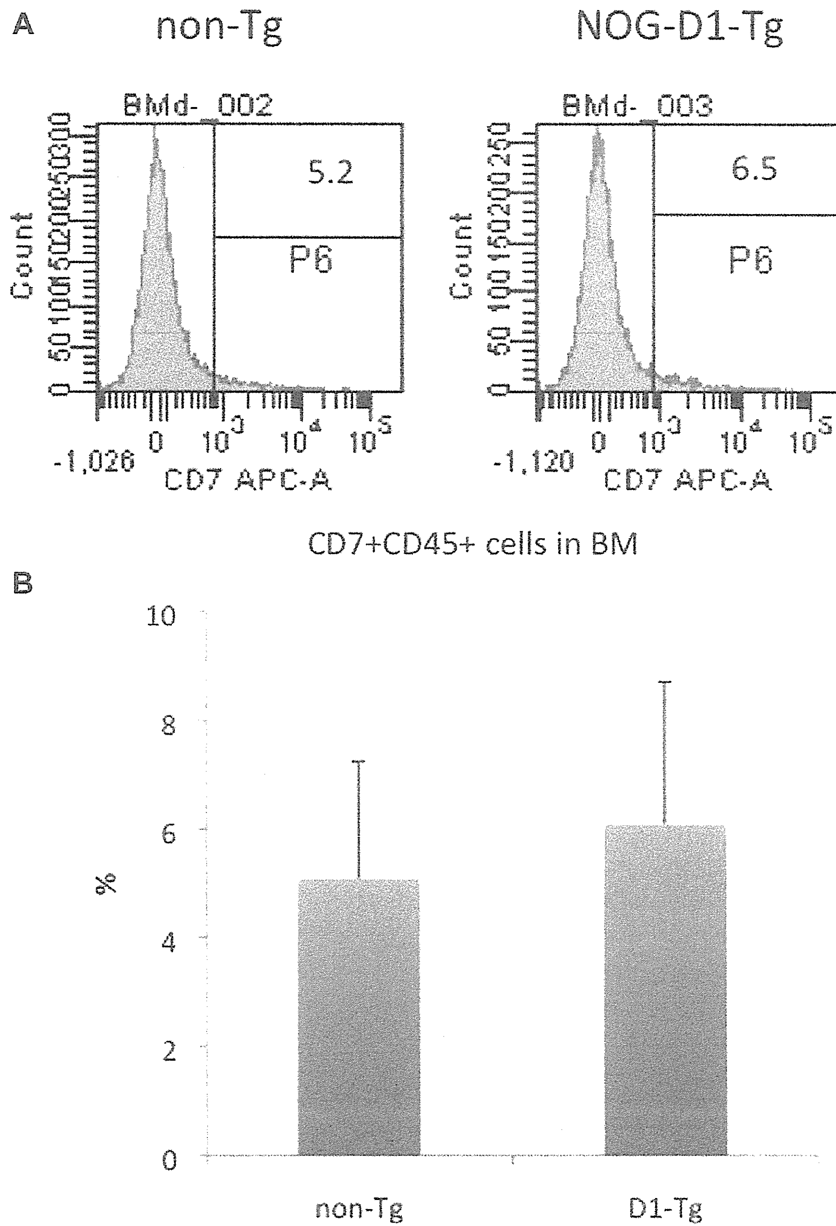


Supplementary Figure E1. Human erythrocytes in PB. Twelve weeks after human HSC transplantation, PB samples were collected from each 3 of NOG-D1-Tg and non-Tg mice, and were stained with anti-mouse Ter119 (BD Biosciences) and anti-human glycopholin A (BioLegend) antibodies. The data were analyzed by flow cytometry.



Supplementary Figure E2. Differentiation of human CD4 and CD8 single positive (SP) cells in the thymus. Thymocytes from NOG-D1-Tg and non-Tg mice (n = 3 each) were isolated at 12 weeks after human HSC transplantation, and were stained with anti-human CD4 (BioLegend), CD8 (BioLegend) and CD45 antibodies. Typical flow cytometric patterns of CD4 and CD8 positive cells in CD45 positive gating were showed in **A**, and accumulative data in the frequencies of CD4 and CD8 positive cells was showed in **B**.



Supplementary Figure E3. Human T cell progenitors in BM. Twelve weeks after human HSC transplantation, BM mononuclear cells were collected from NOG-D1-Tg and non-Tg mice, respectively, and were stained with anti-human CD7 (eBioscience) and CD45 antibodies. The histograms showed the frequency of CD7 positive cells in CD45 positive gating in **A**. These accumulative data were showed in **B**.

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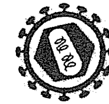

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Minocycline modulates antigen-specific CTL activity through inactivation of mononuclear phagocytes in patients with HTLV-I associated neurologic disease

Yoshimi Enose-Akahata[1], Eiji Matsuura[1,2], Yuetsu Tanaka[3], Unsong Oh[1,4] and Steven Jacobson[1*]

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

* Correspondence: JacobsonS@ninds.nih.gov
[1]Viral Immunology Section, Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892 USA
Full list of author information is available at the end of the article

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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

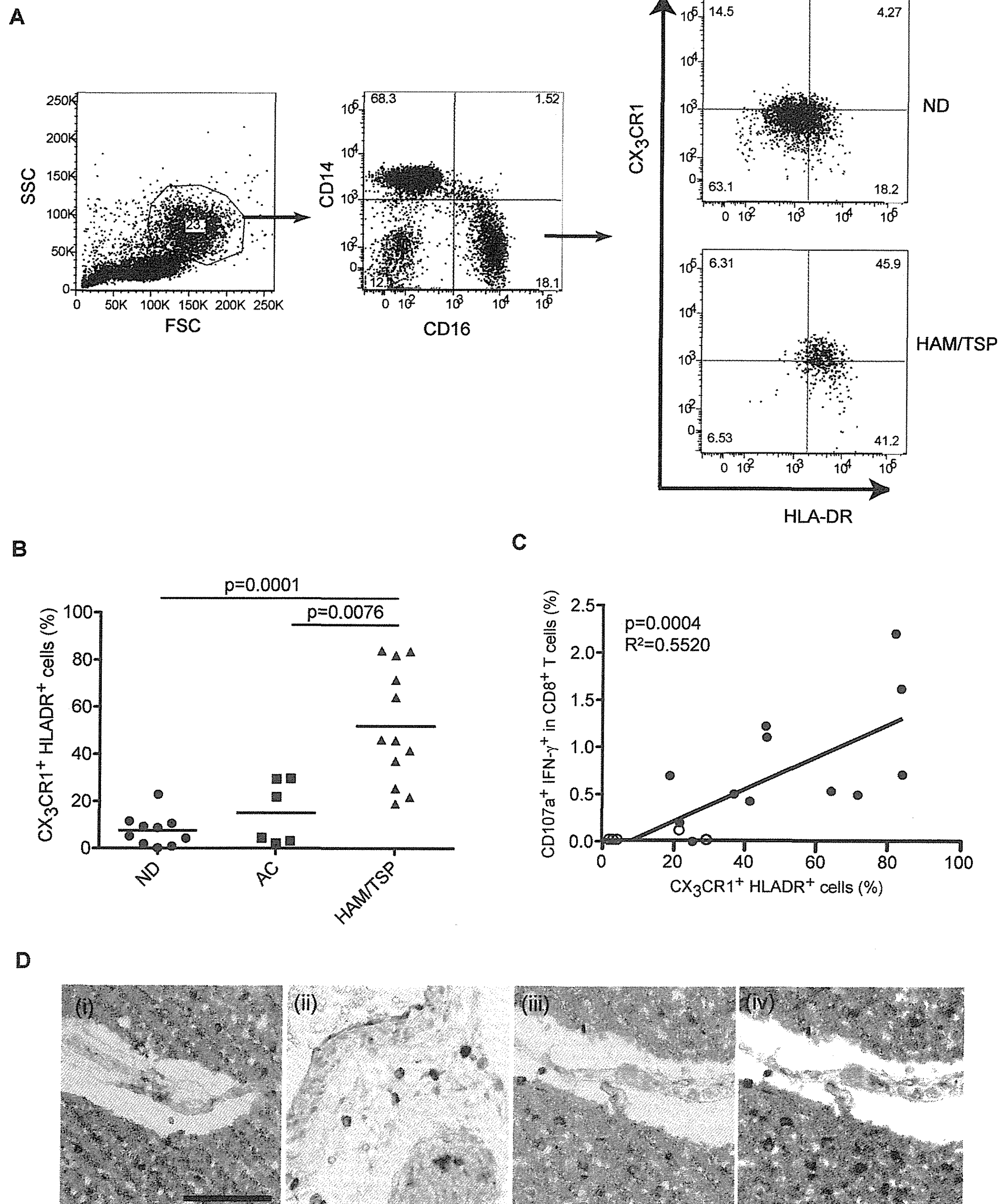

Figure 1 Characterization of mononuclear phagocytes in patients with HAM/TSP. (A) Representative dot plots of CX_3CR1 and HLA-DR expression in $CD14^{low}$ $CD16^+$ cells of a ND and a HAM/TSP patient. (B) Comparison of frequencies of CX_3CR1^+ HLA-DR^+ cells in $CD14^{low}$ $CD16^+$ mononuclear phagocytes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

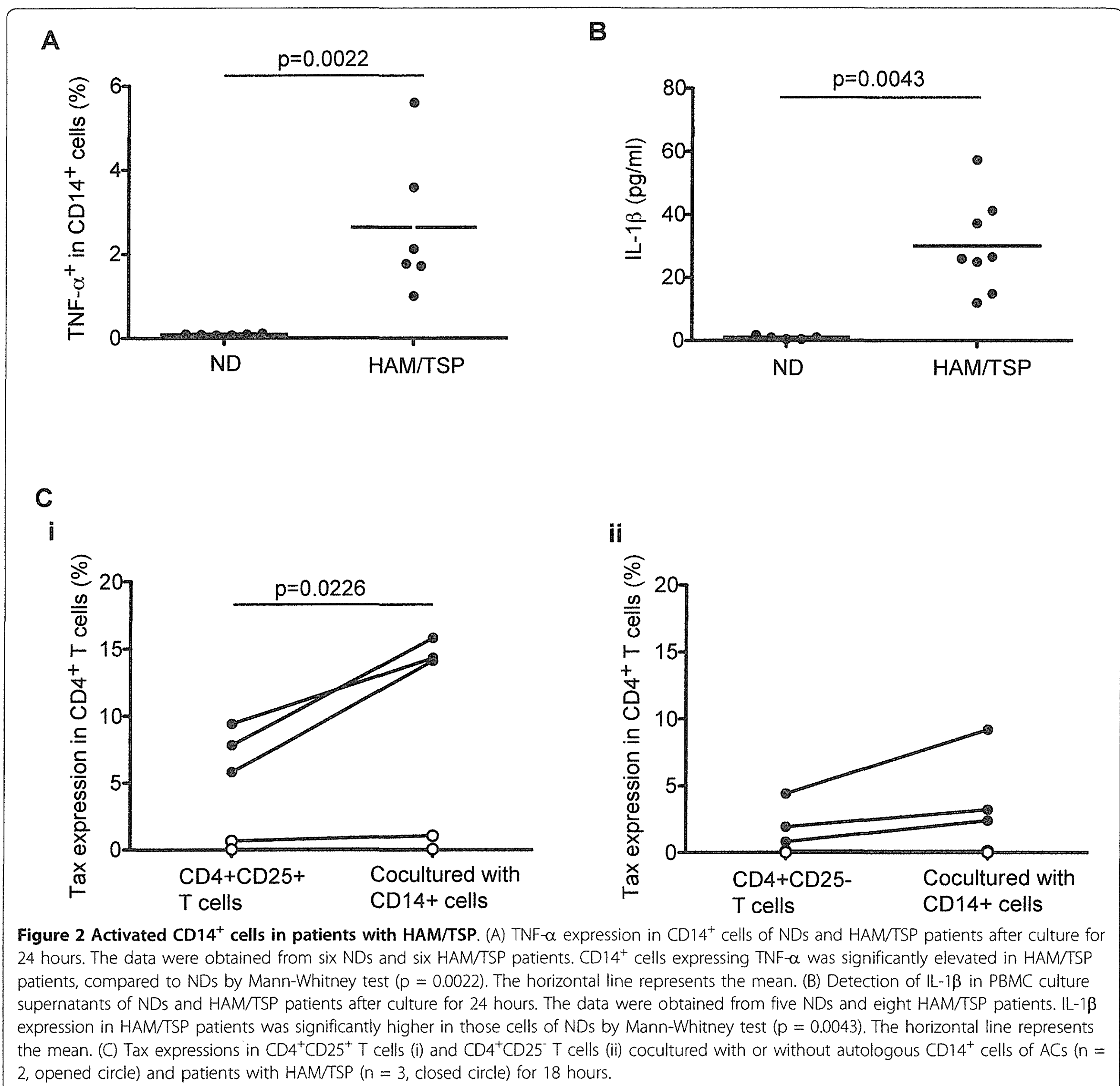

Figure 2 Activated CD14$^+$ cells in patients with HAM/TSP. (A) TNF-α expression in CD14$^+$ cells of NDs and HAM/TSP patients after culture for 24 hours. The data were obtained from six NDs and six HAM/TSP patients. CD14$^+$ cells expressing TNF-α was significantly elevated in HAM/TSP patients, compared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

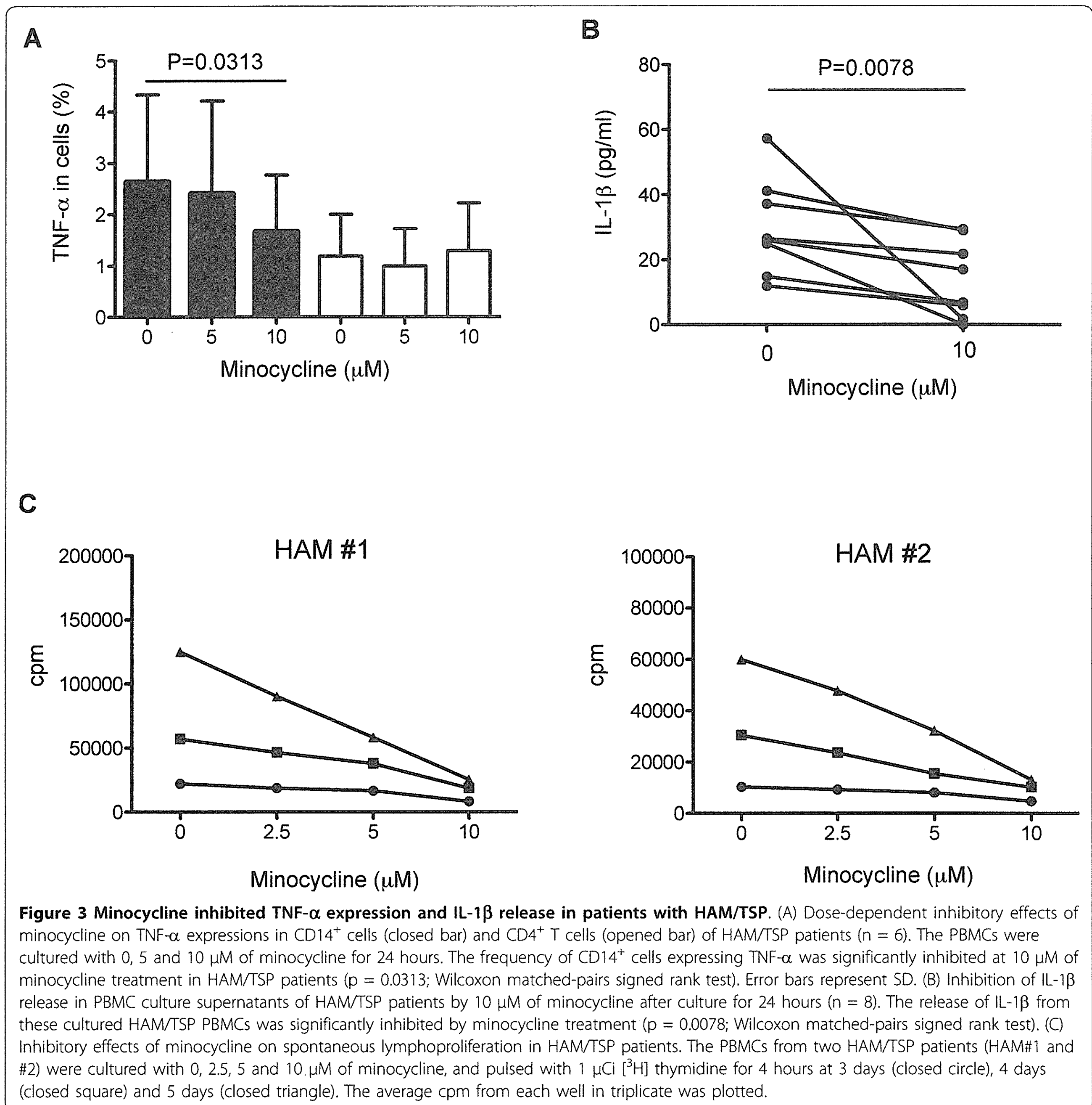

Figure 3 Minocycline inhibited TNF-α expression and IL-1β release in patients with HAM/TSP. (A) Dose-dependent inhibitory effects of minocycline on TNF-α expressions in CD14$^+$ cells (closed bar) and CD4$^+$ T cells (opened bar) of HAM/TSP patients (n = 6). The PBMCs were cultured with 0, 5 and 10 μM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

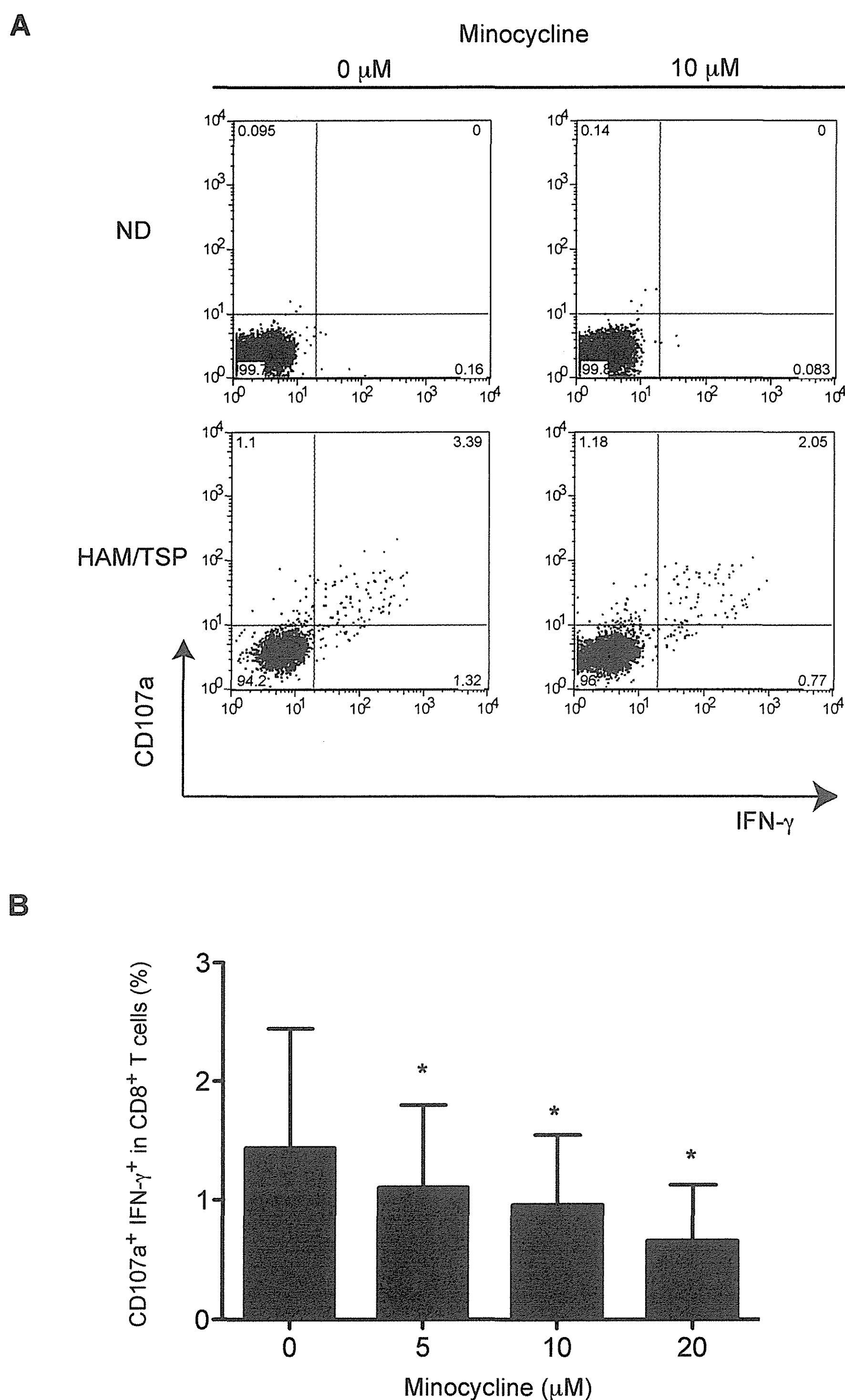

Figure 4 Minocycline inhibited spontaneous degranulation/IFN-γ expression in CD8+ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107a and IFN-γ expression in CD8+ T cells of a ND and a HAM/TSP patient after culture for 24 hours with or without 10 μM of minocycline. (B) Inhibitory 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.

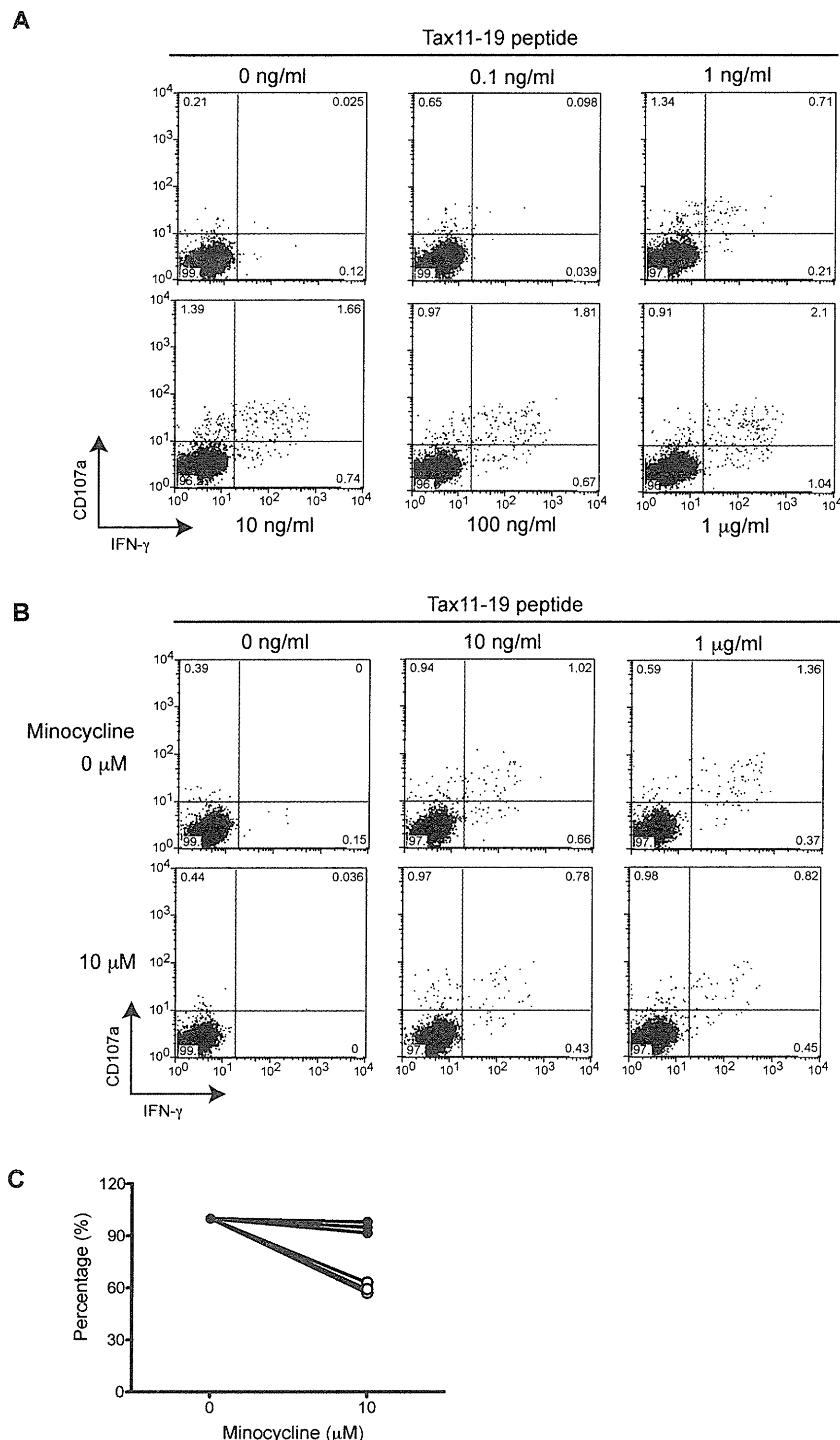

Figure 5 Minocycline inhibited Tax11-19-specific IFN-γ expression in CD8⁺ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107 and IFN-γ expression in CD8$^+$ T cells of a HLA-A*201$^+$ HAM/TSP patients, stimulated with Tax11-19 peptides. PBMCs were stimulated with Tax 11-19 peptide 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.

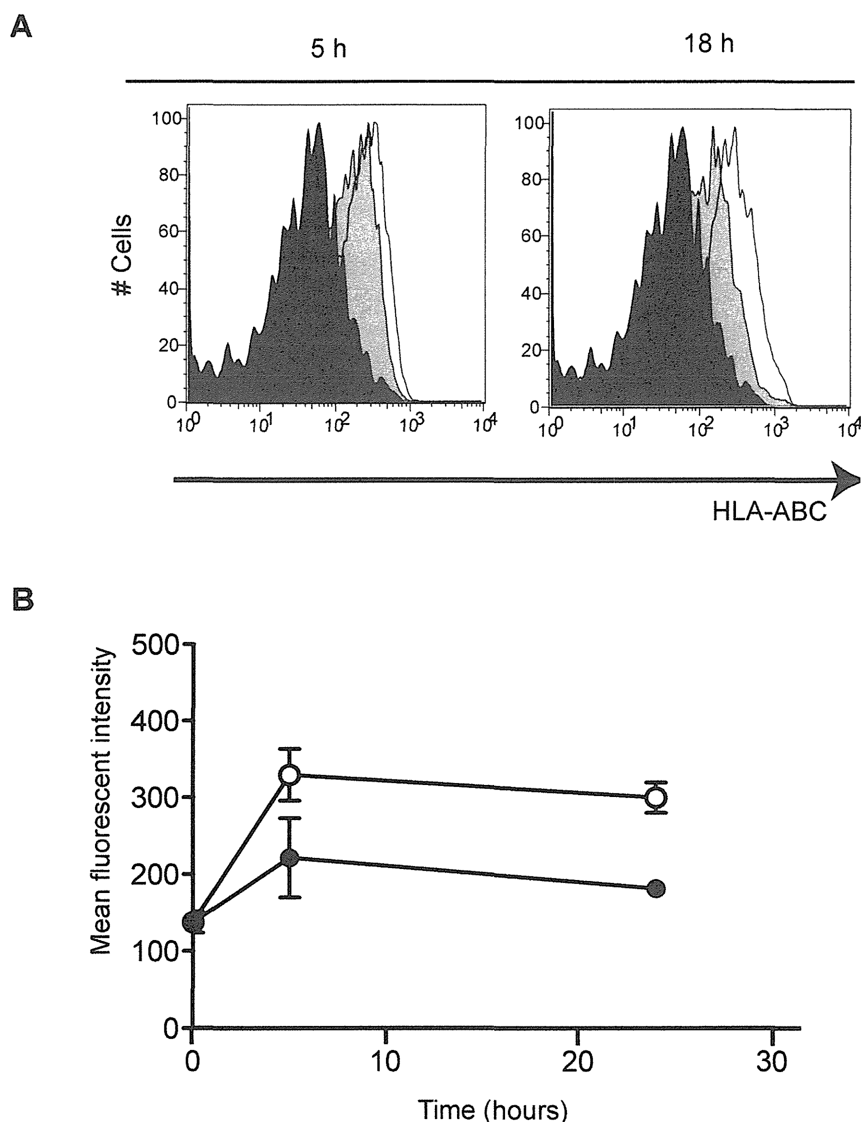

Figure 6 Minocycline downregulated HLA-ABC expressions in CD14$^+$ cells of patients with HAM/TSP. (A) Representative histograms of HLA-ABC expression on CD14$^+$ cells of a HAM/TSP patient. Staining on CD14$^+$ cells before culture (closed histogram) and after culture for 5 hours and 18 hours, with 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.