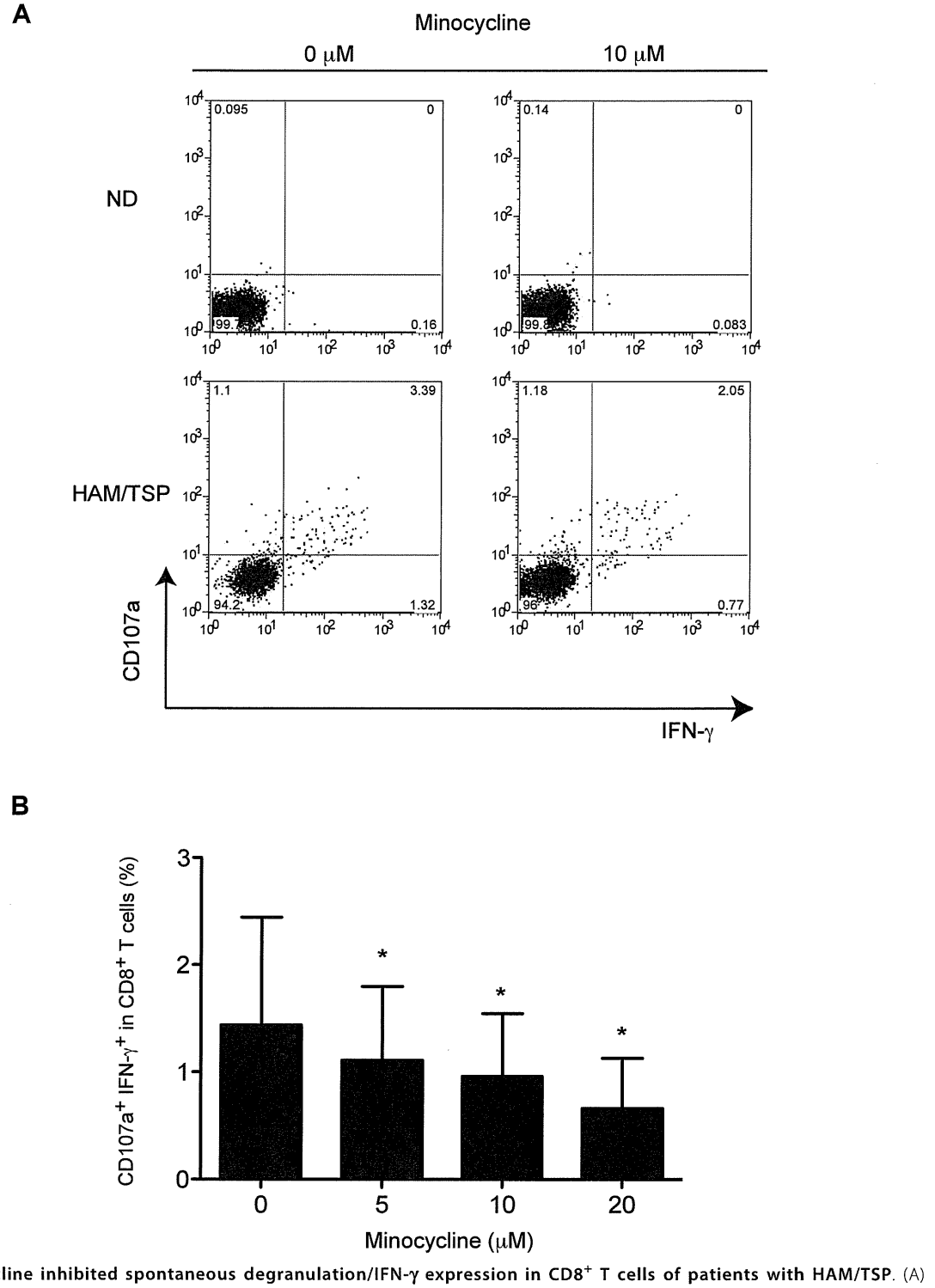

Figure 4 Minocycline inhibited spontaneous degranulation/IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107a and IFN-γ expression in $CD8^+$ T cells of a ND and a HAM/TSP patient after culture for 24 hours with or without 10 μM of minocycline. (B) Inhibitory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

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

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

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

Discussion

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

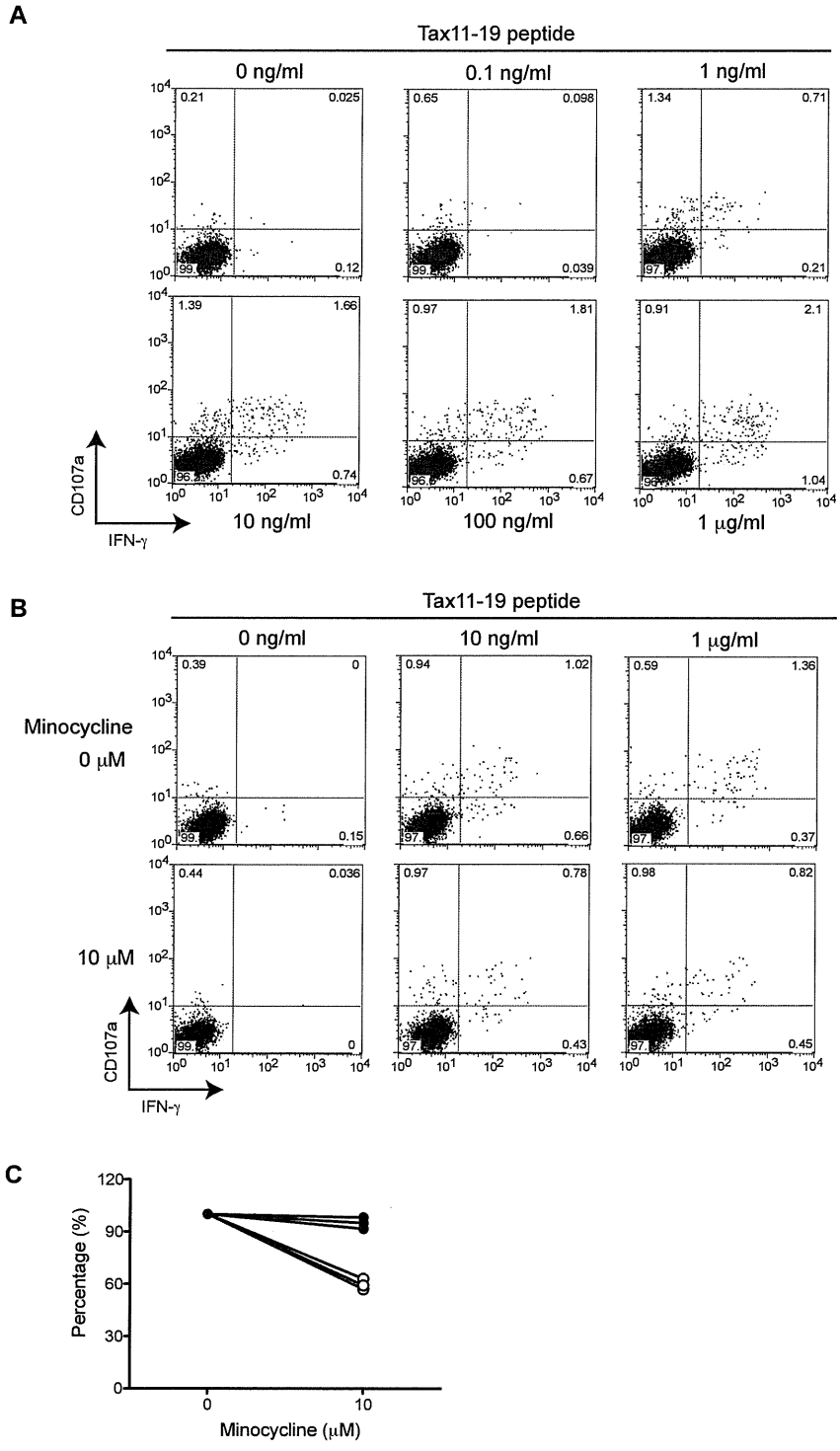

Figure 5 Minocycline inhibited Tax11-19-specific IFN-γ expression in CD8$^+$ T cells of patients with HAM/TSP. (A) Representative dot plots of CD107 and IFN-γ expression in CD8$^+$ T cells of a HLA-A*201$^+$ HAM/TSP patients, stimulated with Tax11-19 peptides. PBMCs were stimulated with Tax 11-19 peptide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

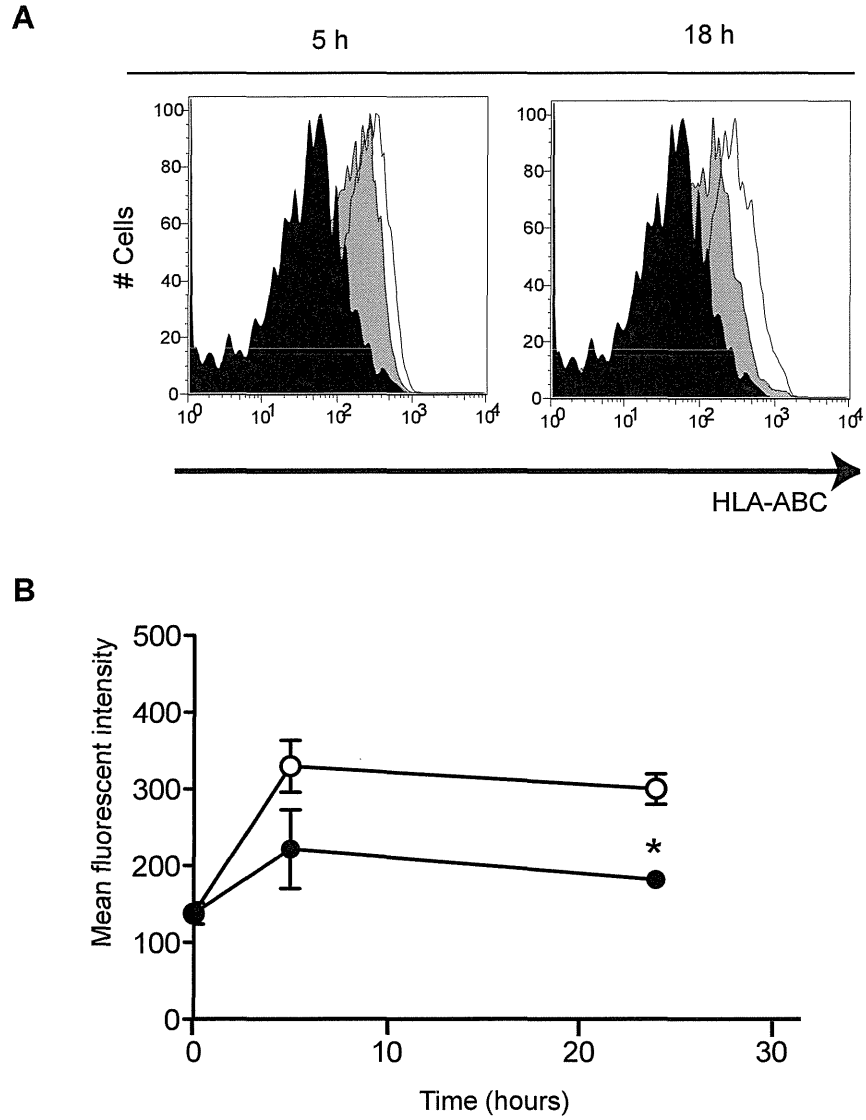

Figure 6 Minocycline downregulated HLA-ABC expressions in CD14$^+$ cells of patients with HAM/TSP. (A) Representative histograms of HLA-ABC expression on CD14$^+$ cells of a HAM/TSP patient. Staining on CD14$^+$ cells before culture (closed histogram) and after culture for 5 hours and 18 hours, with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

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

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

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

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

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

Conclusions

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

Methods

Patient samples

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

Antibodies and reagents

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

Cell culture

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

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

ELISA

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

CD107a mobilization assay

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

Flow cytometry

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

Lymphoproliferation assay

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

Immunohistochemistry

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

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

Statistical analysis

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

Acknowledgements

This research was supported by the Intramural Research Program of the NINDS, NIH.

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Authors' contributions

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

Competing interests

The authors declare that they have no competing interests.

Received: 17 November 2011 Accepted: 15 February 2012
Published: 15 February 2012

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doi:10.1186/1742-4690-9-16
Cite this article as: Enose-Akahata *et al.*: Minocycline modulates antigen-specific CTL activity through inactivation of mononuclear phagocytes in patients with HTLV-I associated neurologic disease. *Retrovirology* 2012 9:16.

Adachi et al. Retrovirology 2011, 8:84
http://www.retrovirology.com/content/8/1/84

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Identification of an unique CXCR4 epitope whose ligation inhibits infection by both CXCR4 and CCR5 tropic human immunodeficiency type-I viruses

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Abstract

Background: Small chemical compounds which target chemokine receptors have been developed against human immunodeficiency virus type 1 (HIV-1) and are under investigation for use as anti-HIV-1 microbicides. In addition, monoclonal antibodies (mAbs) against chemokine receptors have also been shown to have anti-HIV-1 activities. The objective of the present study was to screen a panel of three anti-CXCR4 specific monoclonal antibodies (mAbs) for their ability to block the HIV-1 infection using *in vitro* activated primary peripheral blood mononuclear cells (PBMCs).

Results: PBMCs from normal donors were pre-activated with anti-CD3 and anti-CD28 mAbs for 1 day, and aliquots were infected with a low dose of CCR5-tropic (R5), CXCR4 tropic (X4) or dual tropic (X4R5) HIV-1 isolates and cultured in the presence of a panel of anti-CXCR4 mAbs. The panel included clones A145 mAb against the N-terminus, A120 mAb against a conformational epitope consisting of extracellular loops (ECL)1 and ECL2, and A80 mAb against ECL3 of CXCR4. Among these mAbs, the A120 mAb showed the most potent inhibition of infection, by not only X4 but surprisingly also R5 and X4R5 HIV-1. The inhibition of R5 HIV-1 was postulated to result from the novel ability of the A120 mAb to induce the levels of the CCR5-binding β-chemokines MIP-1α, MIP-1β and/or RANTES, and the down modulation of CCR5 expression on activated $CD4^+$ T cells. Neutralizing anti-MIP-1α mAb significantly reversed the inhibitory effect of the A120 mAb on R5 HIV-1 infection.

Conclusions: The **d**ata described herein have identified a unique epitope of CXCR4 whose ligation not only directly inhibits X4 HIV-1, but also indirectly inhibits R5 HIV-1 infection by inducing higher levels of natural CCR5 ligands.

Background

CXCR4 and CCR5 belonging to the family of G-protein coupled receptors (GPCR) serve as receptors for the CXC-chemokine stromal derived factor 1 (SDF-1) and the CC-chemokines MIP-1α, MIP-1β and RANTES, respectively. The ligation of these chemokine receptors transmits a number of intracellular signals, and the receptors also serve as co-receptors for HIV-1 [1-5]. Under normal physiological conditions, CXCR4 molecules form closely linked dimers [6] and heterodimers with other chemokine receptors including CCR5 [7]. CXCR4 is expressed extracellularly, consisting of an N-terminal (NT) region and extracellular loops (ECL) 1, ECL2 and ECL3. Several lines of evidence indicate that the interaction between CXCR4 and SDF-1 or HIV-1 involves multiple domains of the receptor. For example, while the NT and the ECL2 domains appear to be critical for SDF-1 binding and signaling, the regions contiguous to the ECL2 and ECL3 have been implicated in HIV-1 co-receptor activity and homologous cell adhesion [8-11]. Studies with CXCR4 mutants have revealed that the HIV-1 co-receptor activity of CXCR4 is

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independent of its ability to function as a chemokine receptor and/or transduce intracellular signaling [11,12].

Current and prospective anti-HIV-1 therapy includes the use of small chemical compounds which target chemokine receptors that are termed viral occupancy inhibitors (VIROC) [13]. In addition, mAbs against chemokine receptors have also been shown to have a potential for HIV-1 inhibition. For example, an anti-human CCR2 mAb that is neither an agonist nor an antagonist blocks both X4 and R5 HIV-1, due to oligomerization of CCR2 with CCR5 and CXCR4, but not receptor down-modulation [14]. In addition, an unique mAb with specificity for the N-terminus region of CCR5 that does not block the interaction between HIV-1 gp120 and CCR5, blocks R5 HIV-1 infection by inducing CCR5 dimerization [15].

Herein, we examined a series of three rat IgG anti-human CXCR4 mAbs made by our laboratory [16], and we demonstrate that clone A120, that recognizes a conformational epitope encompassing the ECL1 and ECL2 domains of CXCR4, has a unique functional property. Thus, the interaction of the A120 mAb with CXCR4 inhibits not only X4, but also R5 HIV-1 infection of in vitro activated PBMCs, via mechanisms detailed herein. The novel anti-CXCR4 mAb function described in this study potentially provides a unique adjunct to conventional anti-HIV-1 chemotherapy with activity against not only CXCR4 but also CCR5 and dual tropic HIV-1.

Results

Suppressive effects of anti-CXCR4 mAbs on HIV-1 infection in primary activated PBMCs

We first tested our 3 different anti-CXCR4 mAb clones (A145, A120 and A80) for their potential to inhibit the infection of the prototype X4 HIV-1_{NL4-3} and for purposes of controlling the prototype R5 HIV-1_{JR-FL} in *in vitro* activated primary PBMC cultures. None of these anti-human CXCR4 mAbs cross-reacts with human CCR5, and only the A120 mAb can block the SDF-1-mediated Ca^{2+} influx [16]. Thus, the PBMCs infected with low levels of HIV-1 (at a multiplicity of infection of lower than 0.01) were cultured for 5 days in the presence or absence of 10 μg/ml of either anti-CXCR4 mAb or isotype control. As shown in Figure 1a, while the A145 mAb had minimal inhibitory effect, the A120 and A80 mAbs markedly inhibited the infection of the

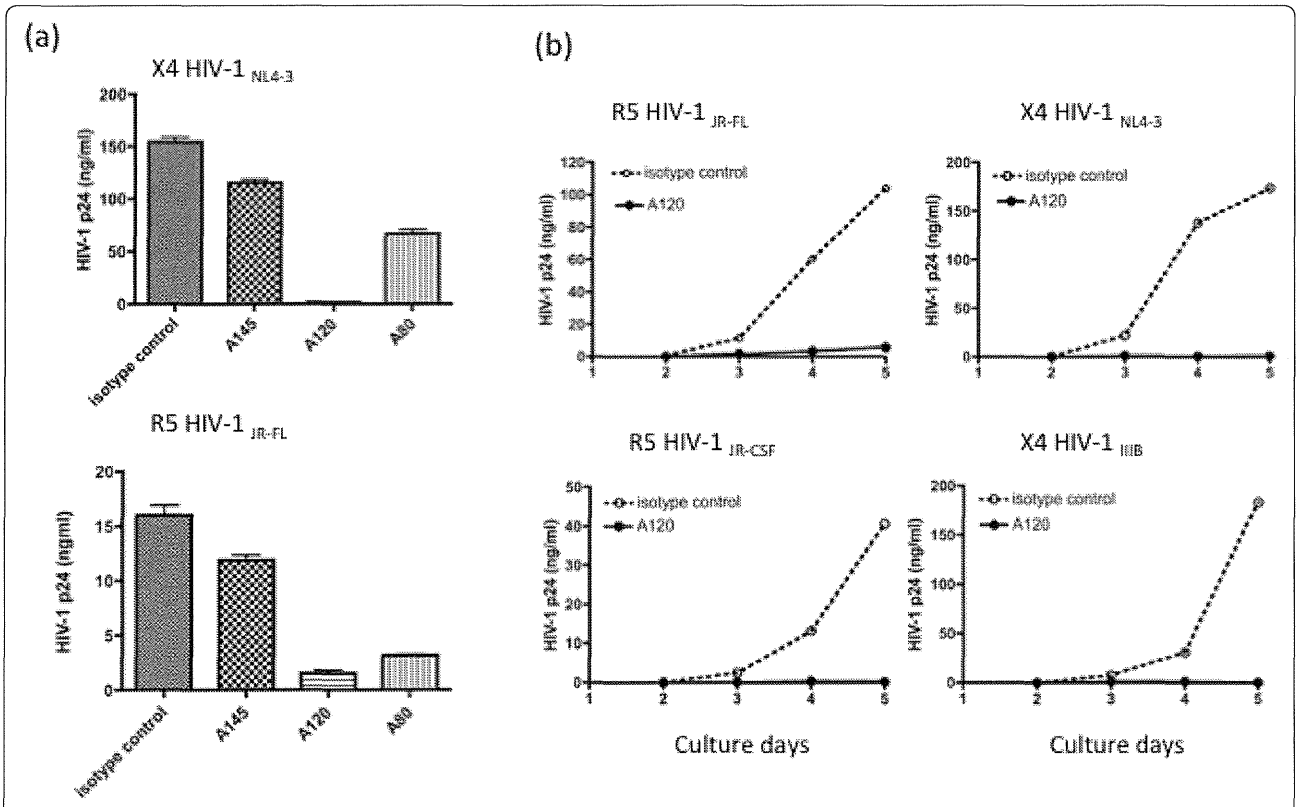

Figure 1 Inhibition of HIV-1 infection in activated PBMCs by anti-CXCR4 mAbs. (a) PBMCs activated with anti-CD3/CD28 for 1 day were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours, washed and then cultured in the presence of 10 μg/ml of the A145, A120, A80 rat IgG mAbs or isotype control rat IgG mAb mixture. After 5 days, virus production in the culture supernatants was determined by p24 ELISA. (b) Activated PBMCs infected with R5 HIV-1_{JR-FL}, R5 HIV-1_{JR-CSF}, X4 HIV-1_{NL4-3} or X4 HIV-1_{IIIB} were aliquoted and cultured in the presence of 10 μg/ml of the A120 mAb or isotype control mAb. The p24 levels in the culture supernatants were monitored daily by ELISA. Data shown for both (a) and (b) are representative of 3 independent experiments using PBMCs from different donors.

X4, but to our surprise, also the R5 HIV-1 strain. Since the inhibitory potential of the A120 mAb was the highest among these mAbs, we selected the A120 mAb for further characterization. Although the production of HIV-1 from activated PBMCs was influenced by culture conditions, mostly cell concentration at time of infection and cultivation steps, as shown in Figure 1b, the inhibitory effect of A120 mAb was further confirmed using an additional R5 (JR-CSF) and X4 (IIIB) HIV-1 strains.

To examine tPBMC donor variabilities, the ability of the A120 mAb to inhibit R5 HIV-1_{JR-FL} and X4 HIV-1_{NL4-3} in activated PBMCs from 6 different unrelated donors was also studied. Viral production was quantitated by measuring both the levels of p24 and the frequency of infected cells using flow cytometry as outlined in the methods section. As seen in Figure 2a, whereas there was indeed considerable variability in the relative susceptibility of *in vitro* activated PBMCs from different donors to support R5 and X4 HIV-1 infection, the addition of the A120 mAb to the cultures showed variable levels of moderate to significant inhibition in each case (differences in the ability of PBMCs from different donors to support R5 versus X4 HIV-1 is an interesting subject that is currently under study). In addition, the fact that the addition of the A120 mAb also inhibited the increase in the frequency of infected

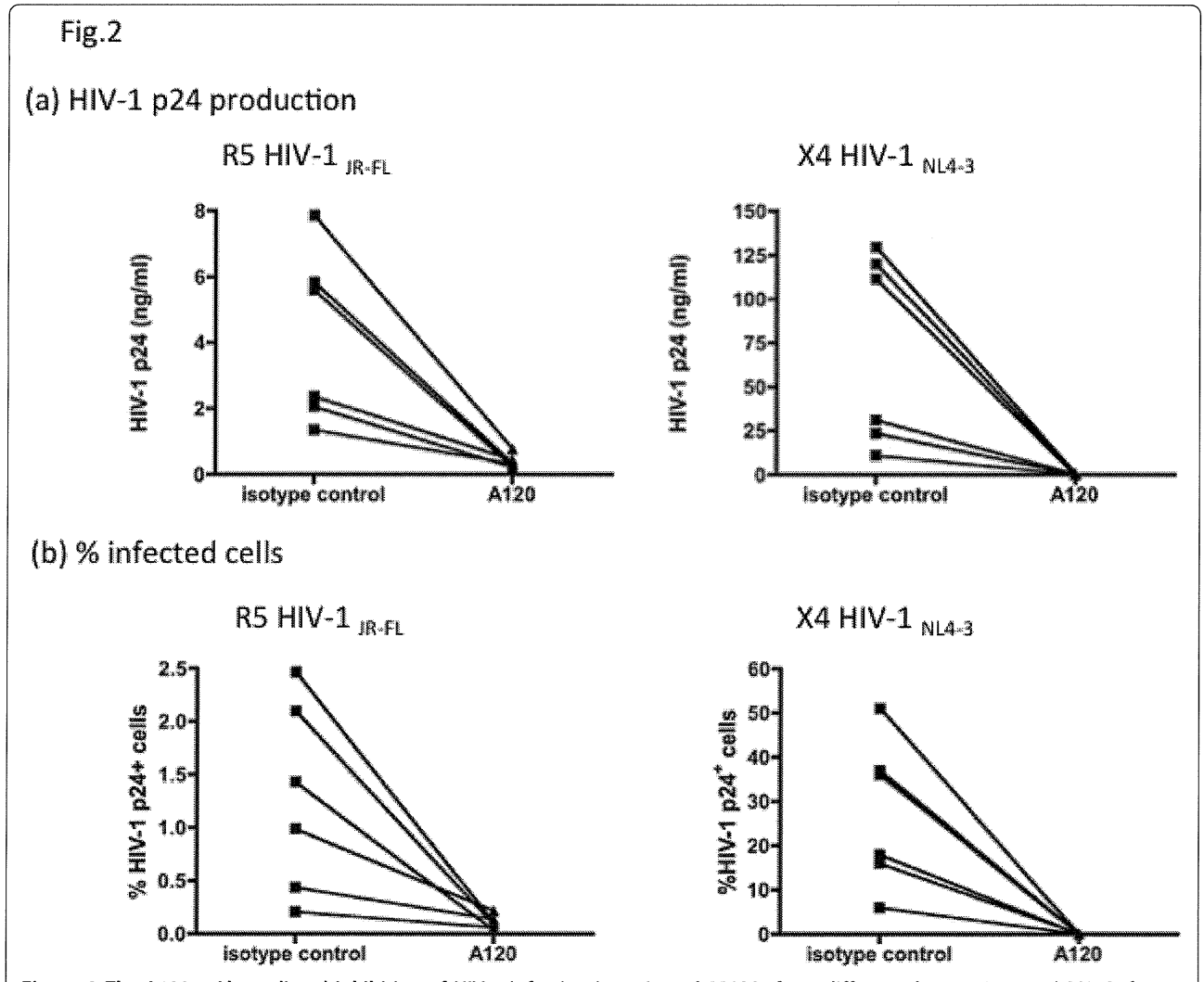

Figure 2 The A120 mAb-mediated inhibition of HIV-1 infection in activated PBMCs from different donors. Activated PBMCs from 6 different donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours. After extensive washing, the PBMCs were aliquoted and cultured in the presence of A120 or isotype control IgG at 10 μg/ml. (a) After 3~5 days, virus production was determined by p24 ELISA in the culture supernatants, and values obtained on day 4 are shown as representative. P values were 0.007 and 0.032 for R5 HIV-1 and X4 HIV-1, respectively. (b) The PBMC samples obtained on day 4 after infection were fixed and permeabilized, and then stained with anti-HIV-1 p24 mAb labeled with Alexa Fluor 488 and examined by flow cytometry. The frequencies (percentages) of $p24^+$ cells were plotted. P values were 0.026 and 0.031 for R5 HIV-1 and X4 HIV-1, respectively. Representative data from 3 independent experiments are shown.

cells as determined by flow cytometry (Figure 2b) suggests that the A120 mAb inhibits new infection in the cultures. To our knowledge, this is the first report of an anti-CXCR4 mAb that inhibits infection of both X4 and R5 HIV-1 strains in activated PBMCs.

Dose response studies were conducted next to determine whether differences exist in the inhibition of R5 as compared with X4 HIV-1. As seen in Figure 3, maximum inhibition was achieved at a concentration of more than 5 μg/ml and 0.6 μg/ml for R5 and X4 HIV-1, respectively. The difference noted in the titration curves indicates that the potential mechanisms for A120 mAb-mediated R5 and X4 HIV-1 suppression are likely to be distinct from each other. The inhibition of virus replication by the addition of the A120 mAb in these cultures was not secondary to the presence of non-specific inhibitors in the A120 mAb preparation since the addition of the same A120 mAb preparation to the CXCR4 expressing HIV-1 producing Molt-4/IIIB cell line and the HTLV-1 producing MT-2 cell line had no detectable effect on virus production (Figure 4). Because the two cell lines express high levels of CXCR4 that readily binds the A120 mAb, it appears that the mere ligation of CXCR4 via A120 mAb epitope does not interfere with the virus production from these cell lines.

One of the trivial explanations for the R5 HIV-1 suppression by the anti-CXCR4 mAb could be ascribed to the potential presence of LPS in the A120 mAb preparation. However, it is highly unlikely, because (1) the A120 mAb preparation contained little LPS since it was repeatedly passed through a polymyxin B column to eliminate possible LPS contamination, (2) exogenously added LPS at 0.1 μg/ml did not inhibit R5 HIV-1 infection in the same culture conditions, and (3) the inclusion of anti-human CD14 mAb that blocks the binding of LPS failed to interfere with the A120 mAb-mediated R5 HIV-1 inhibition (Figure 5). As seen in Figure 5a, while the addition of the A120 mAb clearly inhibited the generation of syncytia by R5 HIV-1_{JR-FL} and p24 production, there was no detectable inhibition with the addition of LPS. The facts that LPS at 0.1 μg/ml failed to inhibit HIV-1 production (unlike the A120 mAb) and that the addition of anti-CD14 mAb (which blocks LPS binding to its receptor, CD14) did not reverse the inhibition of R5 HIV-1 infection suggest that the activity of the A120 mAb is not due to LPS contamination.

Altogether, these data document that the anti-human CXCR4 mAb, clone A120, which ligates CXCR4 molecules via the ECL1/ECL2 domains potently inhibited not only X4 but also R5 HIV-1 strains in freshly *in vitro* activated primary PBMC cultures.

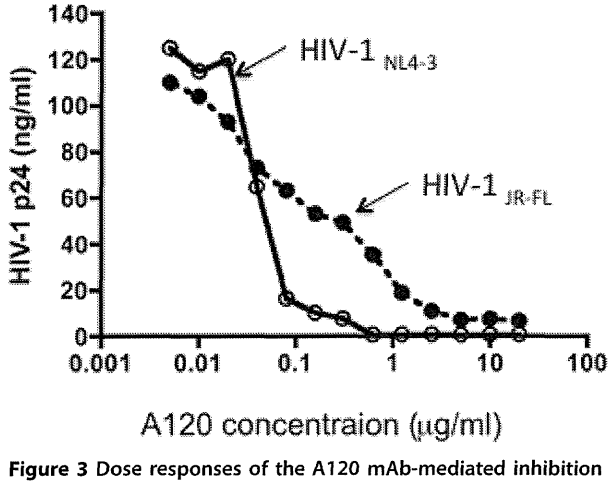

Figure 3 Dose responses of the A120 mAb-mediated inhibition of R5 and X4 HIV-1 infection in activated PBMCs. Activated PBMCs from the donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3}. After washing, the PBMCs were aliquoted and cultured in the presence of graded concentrations of the A120 mAb for 4 days. Virus production in the culture supernatant was determined by p24 ELISA. Representative data from 3 independent experiments using 3 different donors' PBMCs are shown.

Enhancement of the production of the CCR5 binding β-chemokines and reduction of CCR5 expression by A120 mAb treatment

The present observations that the anti-CXCR4 A120 mAb inhibited the production of R5 HIV-1 in *a*ctivated PBMCs prompted us to examine whether CCR5 binding β-chemokines were involved. Thus, we tested whether neutralizing mAbs against human MIP-1α, MIP-1β and RANTES could reverse the effects of the A120 mAb on virus infection. As shown in Figure 6, indeed the A120 mAb-mediated inhibition of R5 HIV-1 infection was significantly reversed by anti-MIP-1α mAb and partially by anti-MIP-1β but not anti-RANTES mAb. These data suggest that MIP-1α and possibly MIP-1β were likely the major factors involved in the inhibition of R5 HIV-1 infection. As expected, the addition of these anti-β-chemokine mAbs did not reverse A120 mAb-mediated blocking of X4 HIV-1 infection (data not shown). However, this β-chemokine dependent mechanism for the inhibition of R5 HIV-1 by the addition of the A120 mAb is donor-dependent. Notably, the addition of the anti-chemokine mAbs failed to reverse the A120 mAb mediated inhibition of R5 HIV-1 in cultures of PBMCs from 2 of the 6 donors. The reason(s) for this resistance in these donors remains to be studied.

To confirm that the β-chemokines were indeed produced by the ligation of CXCR4 by the A120 mAb in activated PBMCs, we quantitated the concentration of these chemokines. Figure 7a shows that the A120 mAb enhanced the synthesis of MIP-1α and MIP-1β in most if not all the cases. Although enhanced RANTES production was seen in 3 out of the 6 donors, it is unlikely

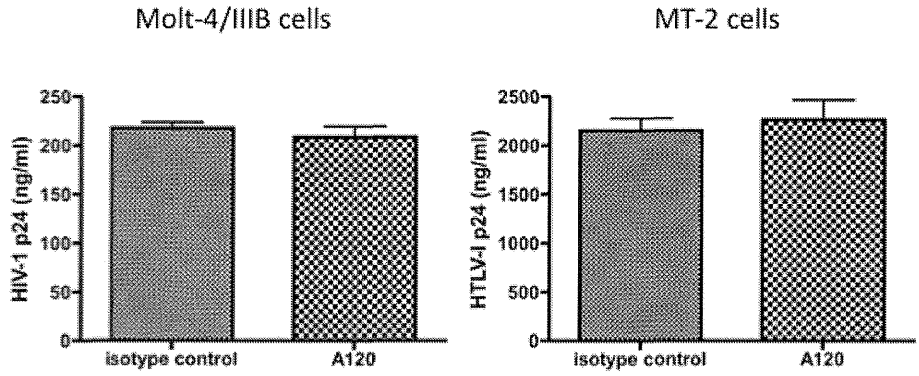

Figure 4 The A120 mAb does not affect HIV-1 and HTLV-I production from producer cell lines. The X4 HIV-1_{IIIB} producer cell line (Molt-4/IIIB) and the HTLV-I producer cell line (MT-2) cells were cultured in the presence of 10 μg/ml of A120 or control mAb for 3 days. The culture supernatants were assayed for HIV-1 p24 and HTLV-I p24 by standard ELISA.

that RANTES is involved in the A120 mAb-mediated R5 HIV-1 inhibition as shown in Figure 6. As expected, treatment of activated PBMCs with the A120 mAb led to a significant reduction in the frequency of cells expressing CCR5 (Figure 7b and 7c). In contrast, there appeared to be a slight increase in the frequency of CXCR4 expressing $CD4^+$ T cells (Figure 7b and 7c). Therefore, these results indicate that the incubation of

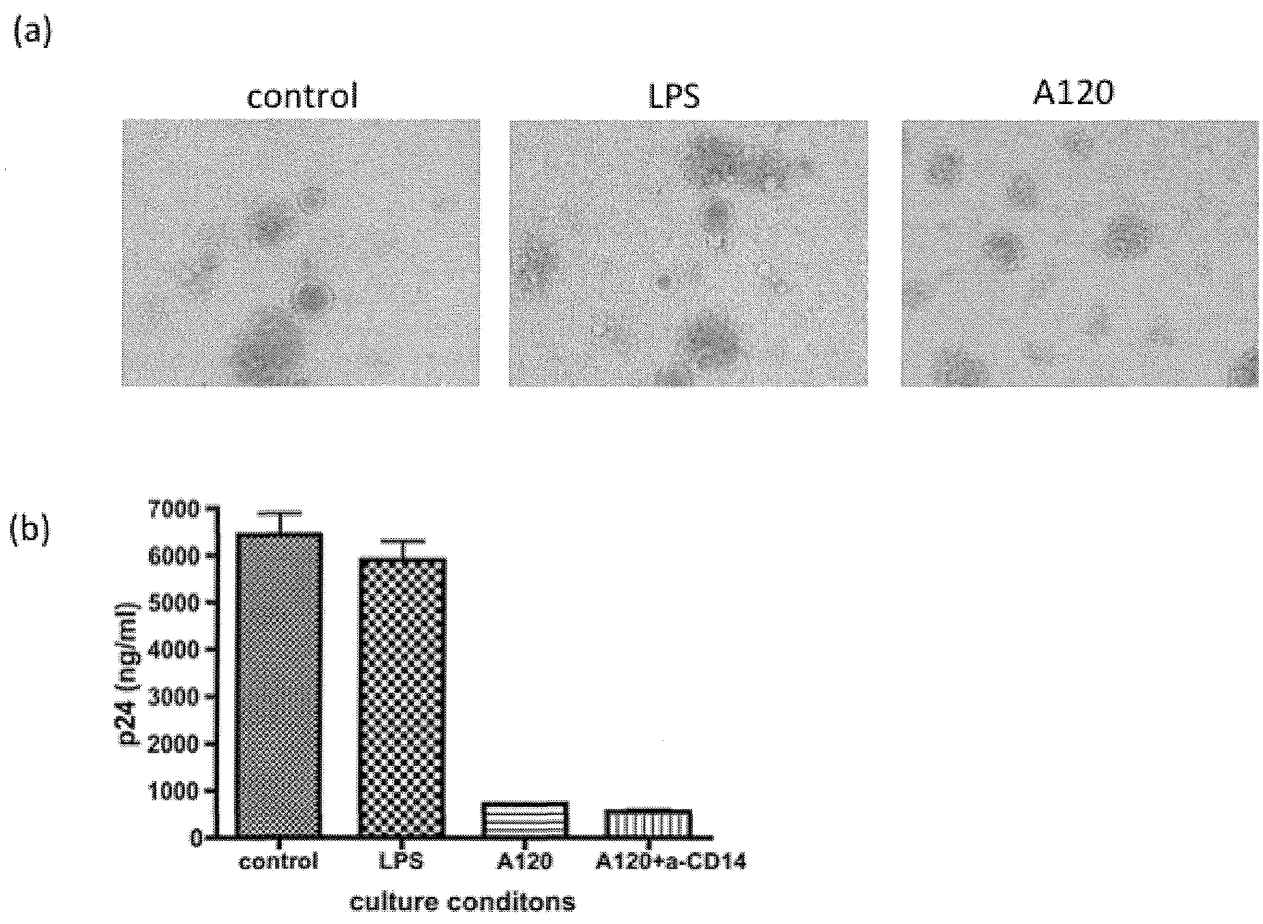

Figure 5 LPS is not involved in the A120 mAb-mediated inhibition of HIV-1 infection. Activated PBMCs infected with R5 HIV-1_{JR-FL} were cultured in the presence or absence of LPS (0.1 μg/ml) or the A120 mAb with or without anti-CD14 mAb. After 4 days, syncytium formation and virus production in the culture supernatants were determined microscopically (a) and using a p24 ELISA kit (b), respectively.

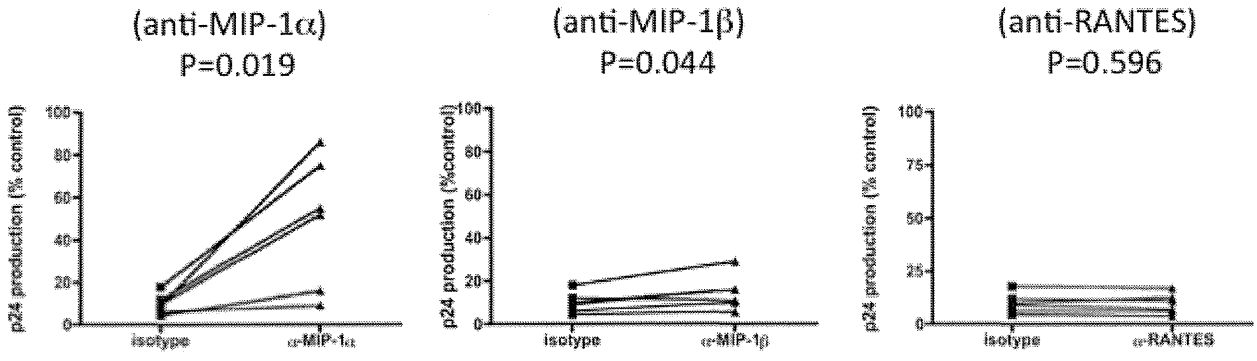

Figure 6 Reversal of the A120 mAb-mediated inhibition of R5 HIV-1 infection in activated PBMCs with anti-CCR5 ligand-neutralizing mAbs. Activated PBMCs from 6 donors were infected with R5 HIV-1_{JR-FL} and cultured in the presence of 10 μg/ml A120 mAb or isotype control mAb together or without anti-chemokine mAbs including anti-MIP-1α, anti-MIP-1β or anti-RANTES at 10 μg/ml for 4 days. Virus production in the culture supernatants was determined by p24 ELISA. The p24 levels were plotted as percent of control values obtained in cultures incubated in each anti-β-chemokine mAb for each donor.

activated PBMCs in the presence of the A120 mAb inhibited R5 HIV-1 infection primarily via the blockade of the co-receptor function of CCR5, most likely due to its ability to induce the synthesis of CCR5-binding β-chemokines. It is important to note that the levels of MIP-1α induced by the A120 mAb showed a typical dose response curve (Additional file 1), and the level of R5 HIV-1 inhibition was inversely-correlated with levels of MIP-1α detected.

Cell populations that produce the β-chemoknes

In an effort to identify the cell lineage that was involved in the synthesis of the β-chemokines following incubation of the activated PBMCs in the presence of the

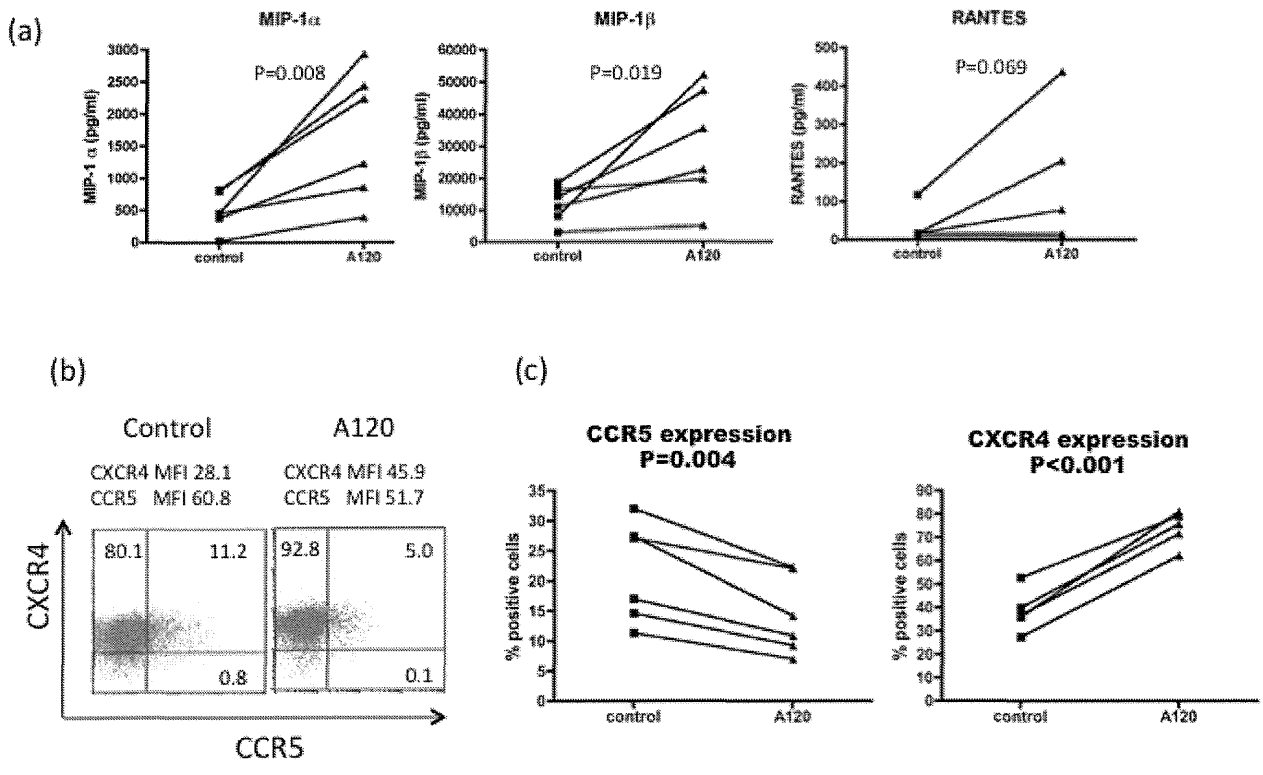

Figure 7 The A120 mAb-treatment induces the production of CCR5 binding β-chemokines and the down-modulation of CCR5 expression. PBMCs from 6 donors were activated with anti-CD3/28 mAbs for 1 day, washed, aliquoted and then incubated in the presence of 10 μg/ml A120 mAb or isotype control mAb for an additional day. (a) Changes in the concentrations of MIP-1α, MIP-1β and RANTES in the culture supernatants were assayed by ELISA. (b and c) Cells were analyzed for changes in the cell surface expression of CCR5 and CXCR4 on gated populations of $CD4^+$T cells (MFI denotes mean fluorescence intensity). A representative flow cytometry dot blot profile is shown.

A120 mAb, cell depletion experiments were carried out. Thus, aliquots of activated PBMCs were depleted of $CD19^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells or $CD14^+$ monocytes utilizing immune-beads conjugated with the appropriate lineage specific mAbs. Non-depleted (mock) and each cell lineage depleted PBMCs were cultured for 24 hours in the presence or absence of 10 μg/ml of A120 mAb. As shown in Figure 8, the most marked reduction in β-chemokine levels in the culture supernatants was noted in cultures depleted of $CD14^+$ monocytes followed by those depleted of $CD4^+$ T cells and $CD8^+$ T cells. However, B-cell depletion had minimal if any effect on the levels of β-chemokines synthesized. These results suggest that activated T cells along with monocytes were responding to the A120 mAb by secreting β-chemokines.

A120 mAb exhibits broad HIV-1 clade inhibition

Finally, the unique availability of a panel of HIV-1 with distinct co-receptor usage and clades prompted us to examine the breadth of inhibitory activity of the A120 mAb. Once again, aliquots of 1-day anti-CD3/28 activated PBMCs were infected with 15 different HIV-1 strains and then cultured in the presence of 10 μg/ml of the A120 mAb or control IgG, and the levels of p24 in the supernatant fluids were quantitated on day 5 after infection. As shown in Table 1, incubation of the cultures in the presence of the A120 mAb uniformly led to a marked decrease in the levels of p24 for all ten R5 HIV-1 strains, three X4 HIV-1 strains, and two dual R5/X4 tropic HIV-1 strains ($p = 0.0065$).

Discussion

The present study is the first report that documents the unique property of an anti-humanCXCR4 mAb (clone A120) which upon ligation of CXCR4 via the ECL1/ECL2 domains strongly blocks the infection of not only X4 but also R5 and dual tropic HIV-1 strains in freshly *in vitro* activated PBMC cultures. The mechanism for the inhibition of the X4 HIV-1 is likely due to direct interference and binding of gp120 to CXCR4 as reported previously. In addition, since A120 mAb treatment increases CXCR4 expression on $CD4^+$ T cells (Figure 7), it may also be possible that the A120 mAb may block X4 HIV-1 infection by interfering with CXCR4 trafficking. By contrast, the predominant mechanism for the inhibition of the R5 HIV-1 infection by the A120 mAb is most likely due to the production of the CCR5-binding β-chemokines, especially MIP-1α, from activated T cells and monocytes leading to down-modulation of CCR5 expression on $CD4^+$ T cells. The observations that the anti-CXCR4 N-terminus mAb (clone A145) showed little or no inhibition, and the anti-CXCR4 ECL3 mAb (clone A80) was not as potent in inhibiting HIV-1 infection, as compared with the A120 mAb, indicate that the ligation via the ECL1 and/or ECL2 domains is critical for the inhibition of R5 and X4 HIV-1 infection. This view is supported by the finding that a panel of commercially available murine mAbs, whose reactive sites were localized to the ECL1/ECL2 domains or the single ECL2 domain of CXCR4, also showed similar, but less effective suppressive effects on infection with both the X4 and R5 HIV-1 and enhanced MIP-1α and β production under the same culture conditions presented herein (data not shown).

Preliminary data indicate that chemically inactivated X4 HIV-1 (HIV-1IIIB) and recombinant SDF-1 did not induce the synthesis of such β-chemokines or inhibit R5 HIV-1 infection in activated PBMCs (data not shown). Thus, it is important to point out that ligation of CXCR4 by its natural ligand SDF-1 or HIV-1 gp120 is not sufficient for generating signals suitable for the synthesis of the CCR5 ligands, and that ligation of CXCR4 via specific domains is required for these unique anti-HIV-1 activities.

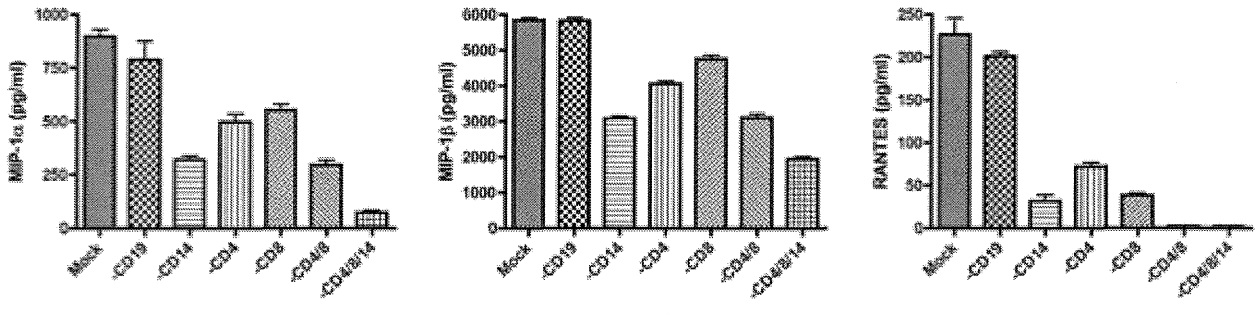

Figure 8 The A120 mAb stimulates T cells and monocytes to produce β-chemokines in activated PBMCs. One day-activated PBMCs were depleted of $CD19^+$B cells, $CD4^+$T cells, $CD8^+$T cells and/or $CD14^+$monocytes using immunobeads conjugated with appropriate lineage specific mAbs, and then cultured in the presence of A120 mAb or isotype control mAb for one day. Concentrations of MIP-1α, MIP-1β and RANTES in the culture supernatants were assayed by ELISA. Representative data from three independent experiments are shown.

Table 1 Suppressive effect of the A120 mAb on various clades of HIV-1 strains.

Member	HIV-1 Subtype	Isolate	Country of Origin	Syncytium	Co-receptor Usage	Percent inhibition of p24 production
PRD320-01	A	UG275	Uganda	NSI	CCR5	88.3%
PRD320-02	A	I-2496	Ghana	NSI	CCR5	99.8%
PRD320-03	CRF02_AG	DJ263	Djibouti	NSI	CCR5	94.7%
PRD320-04	CRF02_AG	POC44951	Liberia	NSI	CCR5	99.7%
PRD320-06	B	BZ167	Brazil	SI	CXCR4	97.2%
PRD3200-7	C	DJ259	Djibouti	NSI	CCR5	91.5%
PRD320-08	C	ZAM18	Zambia	NSI	CCR5	93.7%
PRD320-09	D	SE365	Senegal	SI	CXCR4	98.5%
PRD320-10	D	UG270	Uganda	SI	CXCR4	99.7%
PRD320-11	CRF01_AE	ID17	Indonesia	NSI	CCR5	81.0%
PRD320-12	CRF01_AE	NP03	Thailand	SI	CXCR4	94.5%
PRD320-14	F	BCI-R07	Romania	SI	CXCR4/CCR5	99.4%
PRD320-15	G	BCF-DIOUM	Zaire	NSI	CCR5	99.9%
PRD320-16	G	HH8793	Kenya	NSI	CCR5	83.3%
PRD320-17	H	BCF-KITA	Zaire	NSI	CCR5	92.5%
PRD320-18	O	BCF06	Cameroon	SI	CXCR4/CCR5	98.3%
PRD320-19	O	I-2478B	US	NSI	CCR5	65.6%

Anti-CD3/CD28 activated PBMCs were infected with each of 15 different HIV-1 strains belonging to various clades and with previously defined different CXCR4 and CCR5 usages. HIV-1 dose of 10 ng p24 value was added to 1×10^6 cells for infection. After washing, PBMCs were aliquoted and cultured in triplicate in the presence of 10 μg/ml of the A120 mAb or isotype control IgG for 5 days. Virus production was determined by quantitation of p24 in the culture supernatants by ELISA and the mean values calculated. Percent inhibition was calculated relative to the values obtained with the isotype control mAb alone. Representative data from three independent experiments are shown.

So far, similar suppression of both X4 and R5 HIV-1 infection has also been reported in a study utilizing anti-human CCR2 mAb that is neither agonistic nor antagonistic [14]. It was reasoned that this anti-CCR2 mAb functions by the induction of hetero-oligomerization of CCR2 with CCR5 and CXCR4, but not receptor down-modulation. Another report showed that a non-agonistic/antagonistic anti-CCR5 N-terminus specific mAb that is unable to block the binding of R5 HIV-1 gp120 to CCR5 interferes with R5 HIV-1 infection by induction of CCR5 dimerization rather than down-modulation of CCR5 [16]. It is of interest to note that this anti-CCR5 mAb does not inhibit X4 HIV-1. Thus, our finding that ligation of CXCR4 via the ECL1/ECL2 region on activated PBMCs results in the production of CCR5-binding β-chemokines followed by down-modulation of CCR5 expression is unique. However, it remains to be determined whether the ligation of CXCR4 with the A120 mAb similarly induces hetero-dimerization of CXCR4 with CCR5 or the other chemokine receptors or CCR5 homo-dimerization. Further studies are in progress using immunoprecipitation and Western blot techniques utilizing appropriate mAbs.

It is important to note that the addition of anti-chemokine mAbs did not show the same degree of reversal of the A120 mAb-induced inhibition of R5 HIV-1 infection in the cultures from 2 out of the 6 PBMC donors (Figure 6). In addition, there was a lack of correlation between enhanced β-chemokine levels and the reversing effects of the anti-β-chemokine antibodies on the A120-mediated R5 HIV-1 inhibition. We assume that the concentration of the β-chemokine antibodies (10 μg/ml) was sufficient to neutralize endogenously produced β-chemokines as the antibodies at this concentration could neutralize > 100 ng/ml of each of the recombinant β-chemokines (data not shown). While resistance of these donors was not due to the production of some other anti-HIV-1 factor such as $CD8^+$T lymphocyte antiviral factor (CAF) [17], it may be possible that treatment with the A120 mAb might induce the hetero-dimerization of CXCR4 and CCR5 which results in resistance to R5 HIV-1 infection. Further studies are in progress to address this issue. It is interesting to note that among the neutralizing mAbs against the β-chemokines, the anti-MIP-1α mAb was the most effective in reversing the A120 mAb-induced R5 HIV-1 inhibition. Since all the available anti-MIP-1α mAbs at present do not distinguish MIP-1α (LD78α) from its homologue CCR3L1 product (LD78β) [18], it is possible that CCR3L1 protein is also produced upon A120 mAb treatment and involved in the R5 HIV-1 inhibition. As CCR3L1 is known to be a potent factor that may delay the progression to clinical AIDS [19], it will be important to determine whether A120 mAb stimulates the production of CCR3L1 proteins. Such studies are also in progress.

The generation of resistance to CCR5 inhibitors involving either the selection of pre-existing CXCR4 tropic HIV-1 and/or due to the evolution of Env variants has been well documented [20]. Thus, in such cases, the availability of a reagent like the A120 mAb that has inhibitory properties for both CCR5 and CXCR4 tropic HIV-1 may provide a unique therapeutic tool worthy of consideration. Since the A120 mAb also inhibits the SIV-1 infection in activated PBMCs from rhesus macaques (Takahashi et al., unpublished), this hypothesis is currently being investigated using the nonhuman primate model.

Conclusions

Data described herein have identified a unique epitope of CXCR4 whose ligation not only directly inhibits CXCR4 tropic HIV-1, but also indirectly inhibits the infection of R5 tropic HIV-1 via the synthesis of natural CCR5 ligands.

Methods

Reagents

RPMI 1640 medium (Sigma-Aldrich. Inc. St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (hereinafter called RPMI medium) was utilized for the described studies. Anti-human CD3 (clone OKT-3) and anti-CD28 (clone 28.2) were obtained from the American Type Culture Collection (Rockville, MD) and BioLegend (San Diego, CA), respectively. Neutralizing mAbs against human RANTES, MIP-1α and MIP-1β were purchased from R&D systems (Minneapolis, MN). The rat anti-CXCR4 mAbs used were produced in our laboratory and included clones A145 (IgG1), A120 (IgG2b) and A80 (IgG1) [16]. Mapping of the epitopes recognized by these mAbs was reported previously [16]. Other rat mAbs used were IgG1 anti-CCR5, IgG2b anti-HTLV-I gp46 and IgG1 anti-HCV produced in our laboratory [16,21,22]. These mAbs were purified from CB.17-SCID mouse ascites fluids by ammonium sulfate precipitation followed by gel filtration using Superdex G-200 (GE), and passed through a polymyxin B column to remove potential LPS contamination. The fluorescent dye-labeled anti-human CD4, CD8, CD14 and CD19 mAbs were purchased from Beckman-Coulter or BioLegend. The anti-HIV-1 p24 mAbs used were also produced in our laboratory. Magnetic beads conjugated with mAbs against human CD4, CD8, CD14 or CD19 were purchased from Dynal and used according to the manufacturer's recommendation. Low endotoxin murine anti-CXCR4 mAbs including clone 12G5 and the other anti-CXCR4 ECL2 mAbs were purchased from BioLegend and R&D.

HIV-1 preparation

Virus stocks of R5 HIV-1_{JR-FL}, R5 HIV-1_{JR-CSF} and X4 HIV-1_{NL4-3} were produced by transfection of the 293T cells with the appropriate HIV-1 infectious plasmid DNAs utilizing the calcium phosphate method as described previously [23]. X4 HIV-1_{IIIB} was produced in the Molt-4/IIIB cell line. The other HIV-1 isolates used were from the HIV subtype infectivity panel PRD320 (BBI Diagnostics, West Bridgewater, MA, USA) which included clade A R5 HIV-1 (UG275, I-2496 isolates), clade CRF02AG R5 HIV-1 (DJ263, POC44951 isolates), clade B R5 (US2 isolate) and X4 HIV-1 (BZ167 isolate), clade C R5 HIV-1 (DJ259, ZAM18 isolates), clade D X4 HIV-1 (SE365, UG270 isolates), clade CRF01AE R5 (ID17 isolate) and X4 HIV-1 (NP03 isolate), clade F R5 (BZ163 isolate) and X4/R5 HIV-1 (BCI-R17 isolate), clade G R5 HIV-1 (BCF-DIOUM, HH8793 isolates), clade H R5 HIV-1 (BCF-KITA isolate), clade O R5 (I-2478B isolate) and X4/R5 HIV-1(BCF06 isolate). Each of these panel HIV-1 strains was grown in primary PHA-activated PBMCs and the levels of p24 determined and 10 ng of p24 used to infect PBMCs. These HIV-1 stocks were aliquoted and stored at -80°C until used.

In vitro stimulation of PBMCs and infection with HIV-1

PBMCs from healthy donors were obtained by density gradient centrifugation on HistoPAQUE-1077 (Sigma-Aldrich), suspended at 2×10^6 cells/ml in RPMI medium, dispensed into individual wells of 24-well plates (BD) (1 ml/well) pre-coated with 5 μg/ml anti-CD3 mAb (OKT-3) and cultured in the presence of soluble 0.1 μg/ml anti-CD28 mAb at 37°C in a 5% CO_2 humidified atmosphere for 24 hours. The activated PBMCs were collected, washed once and infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.005~0.01 or at 10 ng p24 per $1\sim2 \times 10^6$ cells for 2 hours. Infected PBMCs were washed three times, re-suspended at $0.5\sim1 \times 10^6$ cells/ml in RPMI medium containing 20 U/ml recombinant human IL-2 containing RPMI medium, dispensed into individual wells of 48-well plates (BD) (0.5 ml/well) and then cultured in the presence or absence of various concentrations of the anti-CXCR4 or control mAbs. Production of HIV-1 was determined by the measurement of HIV-1 core p24 levels by ELISA, and the number of HIV-1 $p24^+$ cells were determined by FCM as described previously [24]. For select experiments, activated PBMCs were cultured at 1×10^6 cells/ml in RPMI medium containing 20 U/ml IL-2 in the presence or absence of 10 μg/ml of A120 mAb for 24 hours, and the culture supernatants were collected, and the levels of β-chemokines were determined by ELISA. All the experiments in this study were performed in triplicate wells.

Cell lines

Molt-4/IIIB [25] and MT-2 [26] cells that were productively infected with HIV-1IIIB (Molt-4/IIIB) and human T cell leukemia virus type-I (HTLV-I), respectively, were cultured in RPMI medium. HIV-1 and HTLV-I production were determined by our in-house HIV-1 p24 and HTLV-I p24 sandwich ELISA kits (Tanaka et al., unpublished).

Flow Cytometry (FCM)

Cells to be analyzed were Fc-blocked with 2 mg/ml normal human pooled IgG on ice for 15 minutes, and aliquots of these cells were subjected to staining using pre-determined optimum concentrations of fluorescent dye-conjugated mAbs for 30 minutes on ice. The cells were then washed using FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), fixed in 1% paraformaldehyde (PFA) in FACS buffer and analyzed using a FACS Calibur. The data obtained were analyzed using the Cell Quest software (BD). For detection of HIV-1 infected cells, cells were fixed with 4% PFA-containing PBS for 5 min at room temperature followed by washing with 0.1% Saponin-containing FACS buffer. These cells were then Fc-blocked with 2 mg/ml normal human pooled IgG on ice for 15 min, and aliquots of these cells were stained with Alexa Fluor 488-conjugated anti-HIV-1 p24 mAb (clone 2C2) for 30 min on ice. The cells were then washed using FACS buffer and the frequency and the absolute number of p24+ cells determined by FCM using a cell counting kit (BD) according to the manufacturer's protocol.

Statistical analysis

Data were tested for significance using the Student's *t* test using the Prism software (GraphPad Software).

Additional material

Additional file 1: Dose response of the A120 mAb-mediated MIP-1α production in activated PBMCs. As described in the legend for Figure 7, activated PBMCs were incubated in the presence of graded concentrations of the A120 mAb or isotype control mAb for an additional day. Changes in the concentrations of MIP-1α in the culture supernatants were assayed by ELISA. Isotype control mAbs did not enhance MIP-1α production at 0.5~20 μg/ml in these culture conditions (data not shown). Representative data are from 3 independent experiments using PBMCs from a single donor.

Lists of abbreviations used

HIV: human immunodeficiency virus; PBMC: peripheral blood mononuclear cells; mAb: monoclonal antibody; X4: CXCR4-tropic; R5: CCR5-tropic; ECL: extra-cellular loop.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Welfare of Japan. We are grateful to Prof. Koyanagi of Kyoto University for providing stocks of HIV-1.

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Authors' contributions

TA and RT performed research, analyzed data, and wrote the manuscript. AK, SM, and Takahashi contributed to experiments and analyzed data. AAA contributed to designing research and wrote the manuscript. YT designed and preformed research, wrote the manuscript and provided funding for this study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 7 September 2011 Accepted: 22 October 2011
Published: 22 October 2011

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doi:10.1186/1742-4690-8-84
Cite this article as: Adachi *et al.*: Identification of an unique CXCR4 epitope whose ligation inhibits infection by both CXCR4 and CCR5 tropic human immunodeficiency type-I viruses. *Retrovirology* 2011 8:84.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 259045, 12 pages
doi:10.1155/2012/259045

Review Article

Immunopathogenesis of Human T-Cell Leukemia Virus Type-1-Associated Myelopathy/Tropical Spastic Paraparesis: Recent Perspectives

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Received 1 August 2011; Revised 30 September 2011; Accepted 9 October 2011

Academic Editor: Pooja Jain

Human T-cell leukemia virus type-1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease only in a minority of infected individuals: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities. Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence from host population genetics, viral genetics, DNA expression microarrays, and assays of lymphocyte function suggests that complex virus-host interactions and the host immune response play an important role in the pathogenesis of HAM/TSP. Especially, the efficiency of an individual's cytotoxic T-cell (CTL) response to HTLV-1 limits the HTLV-1 proviral load and the risk of HAM/TSP. This paper focuses on the recent advances in HAM/TSP research with the aim to identify the precise mechanisms of disease, in order to develop effective treatment and prevention.

1. Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is a human retrovirus etiologically associated with adult T-cell leukemia (ATL) [1–3] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4, 5]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. Cases of HAM/TSP have been reported throughout the HTLV-1 endemic areas such as Southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [7]. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. In contrast to HIV-1 infection, few with HTLV-1 develop disease: approximately 2%-3% of infected persons develop ATL [8] and other 0.25%–3.8% develop HAM/TSP [9–12], while the majority of infected individuals remain lifelong asymptomatic carriers (ACs). However, the ability to evaluate the individual risk of HTLV-1-associated diseases in each AC would make a significant clinical impact, especially in HTLV-1 endemic areas. During the last three decades since the discovery of HTLV-1 as the first pathogenic human retrovirus, advances in HTLV-1 research have helped us to understand the clinical features of HTLV-1 associated diseases, the virological properties of HTLV-1, and the importance of the viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection. However, the precise mechanism of disease pathophysiology is still incompletely understood, and the treatment is still unsatisfactory, because good small-animal models for studying HTLV-1 infection and its associated diseases were unavailable until recently. In this paper, we summarize the recent developments of HTLV-1 research to try to identify more precisely the pathogenetic mechanisms