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

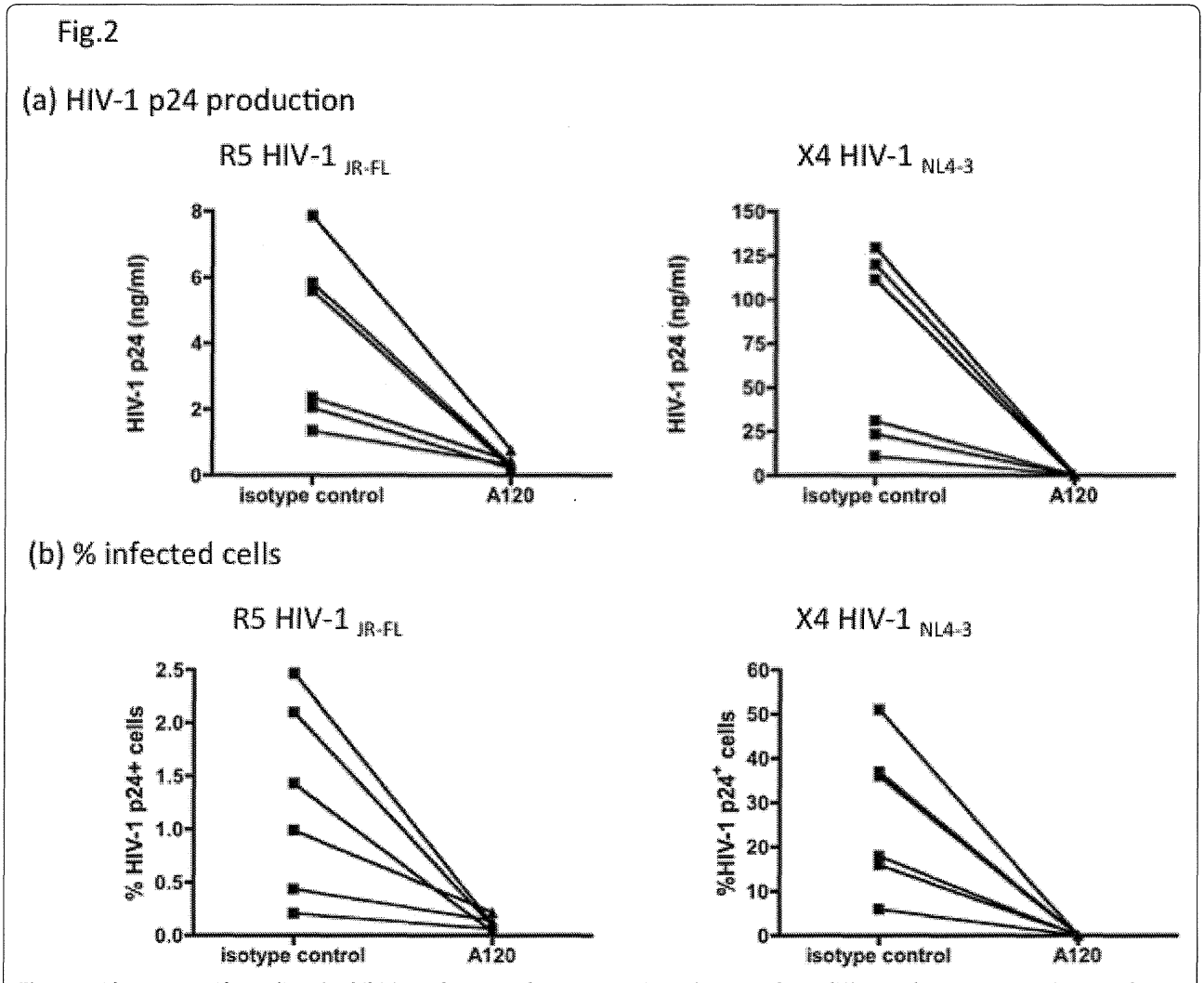

Figure 2 The A120 mAb-mediated inhibition of HIV-1 infection in activated PBMCs from different donors. Activated PBMCs from 6 different donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours. After extensive washing, the PBMCs were aliquoted and cultured in the presence of 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.

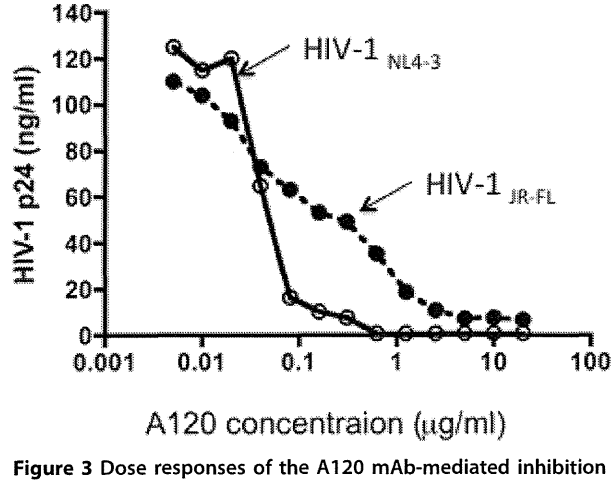

Figure 3 Dose responses of the A120 mAb-mediated inhibition of R5 and X4 HIV-1 infection in activated PBMCs. Activated PBMCs from the donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3}. After washing, the PBMCs were aliquoted and cultured in the presence of graded concentrations of 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

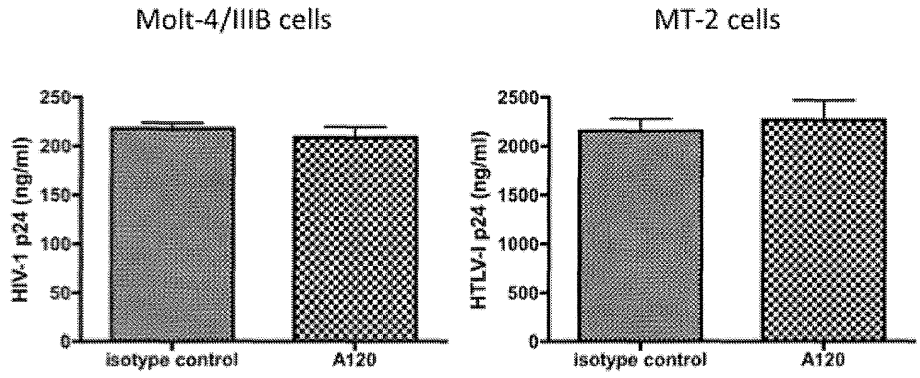

Figure 4 The A120 mAb does not affect HIV-1 and HTLV-I production from producer cell lines. The X4 HIV-1_{IIIB} producer cell line (Molt-4/IIIB) and the HTLV-I producer cell line (MT-2) cells were cultured in the presence of 10 μg/ml of A120 or control mAb for 3 days. The culture supernatants were assayed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

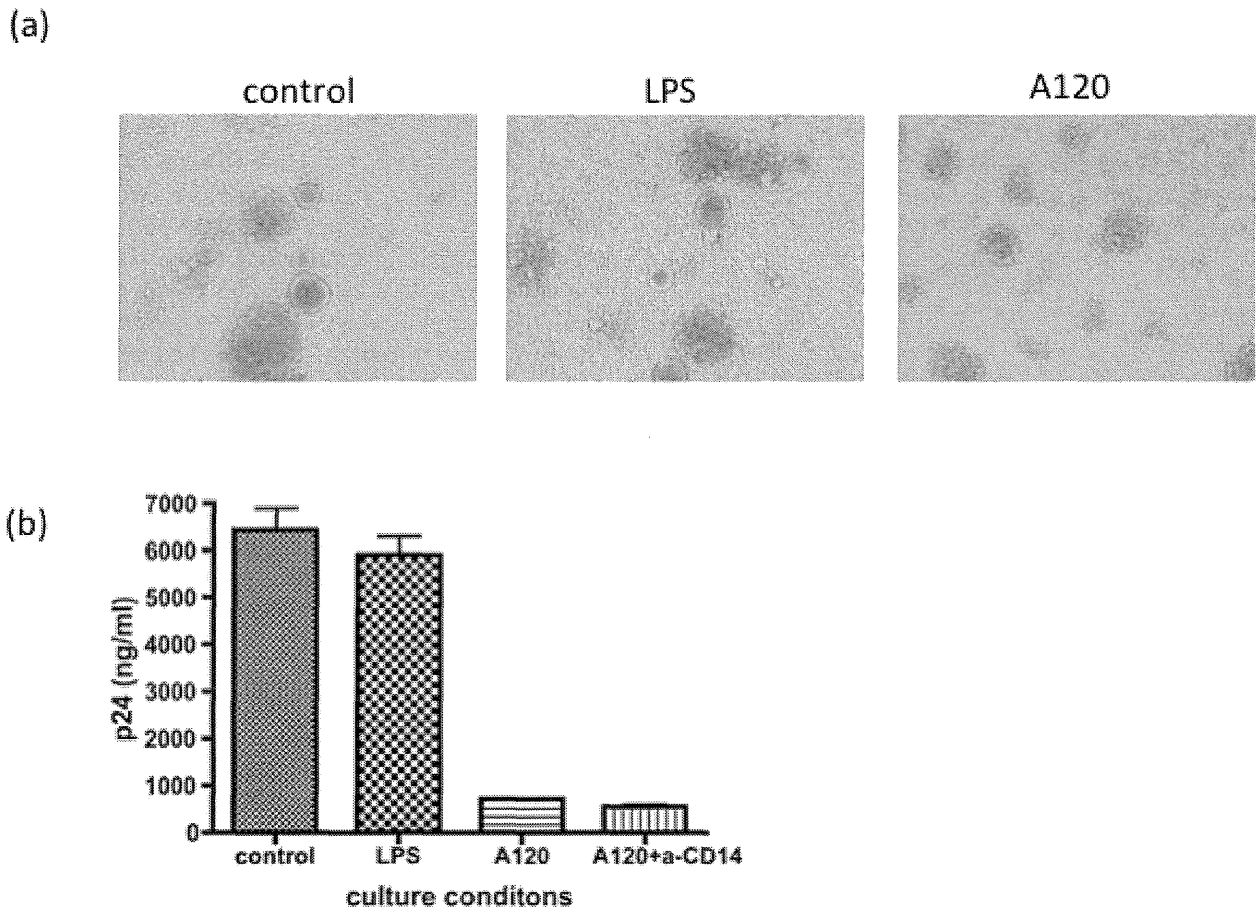

Figure 5 LPS is not involved in the A120 mAb-mediated inhibition of HIV-1 infection. Activated PBMCs infected with R5 HIV-1_{JR-FL} were cultured in the presence or absence of LPS (0.1 μg/ml) or the A120 mAb with or without anti-CD14 mAb. After 4 days, syncytium formation and virus production in the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

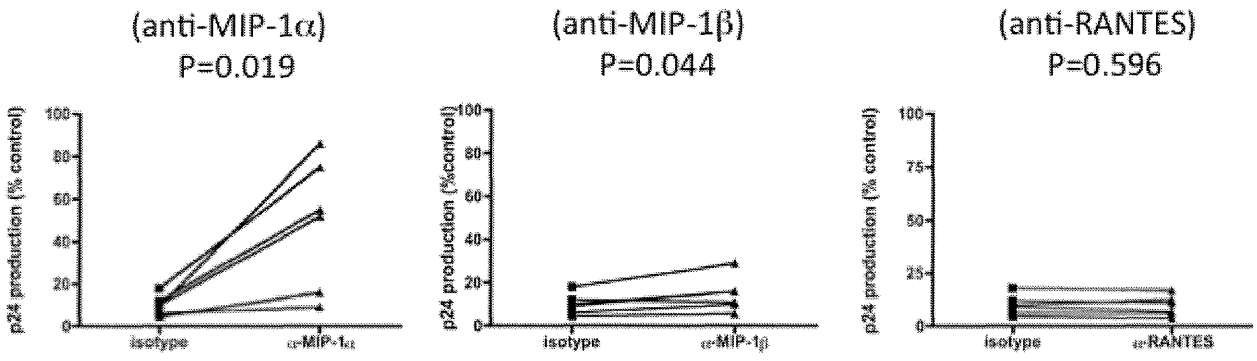

Figure 6 Reversal of the A120 mAb-mediated inhibition of R5 HIV-1 infection in activated PBMCs with anti-CCR5 ligand-neutralizing mAbs. Activated PBMCs from 6 donors were infected with R5 HIV-1_{JR-FL} and cultured in the presence of 10 μg/ml A120 mAb or isotype control mAb together or without anti-chemokine 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

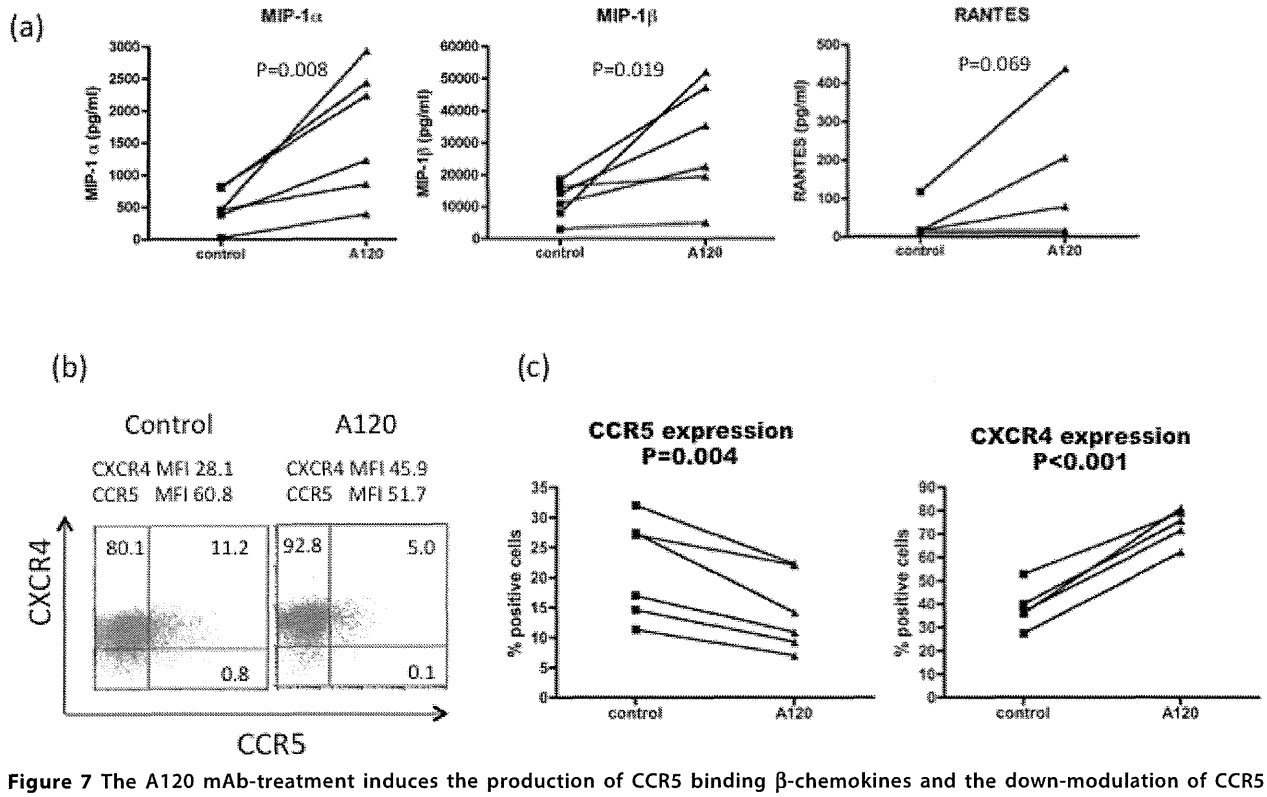

Figure 7 The A120 mAb-treatment induces the production of CCR5 binding β-chemokines and the down-modulation of CCR5 expression. PBMCs from 6 donors were activated with anti-CD3/28 mAbs for 1 day, washed, aliquoted and then incubated in the presence of 10 μg/ml A120 mAb or isotype control mAb for an additional 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.

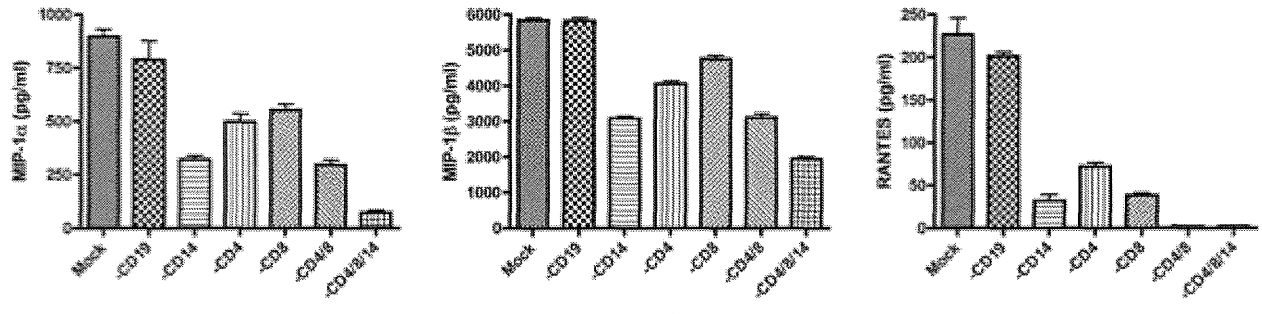

Figure 8 The A120 mAb stimulates T cells and monocytes to produce β-chemokines in activated PBMCs. One day-activated PBMCs were depleted of $CD19^+$B cells, $CD4^+$T cells, $CD8^+$T cells and/or $CD14^+$monocytes using immunobeads conjugated with appropriate lineage specific mAbs, and then cultured in 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.

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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.

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Review Article

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

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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

of the disease in order to develop effective treatment and prevention.

2. HTLV-1 Infection and Clinical Features of HAM/TSP

2.1. Virological Aspects of HTLV-1. HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [13–15]. HTLV-1 can be transmitted through sexual contact [16], injection drug use [15], and breastfeeding from mother to child [17, 18]. For over two decades, the investigation of HTLV-1-mediated pathogenesis has been focused on Tax, an HTLV-1 encoded viral oncoprotein, since Tax has been viewed as critical for leukemogenesis because of its pleiotropic effects on both viral and many cellular genes responsible for cell proliferation, genetic instability, dysregulation of the cell cycle, and apoptosis [19]. However, Tax expression is not detected in about 60% of freshly isolated samples from ATL cases [20]. In 2002, another regulatory protein encoded in the minus or antisense strand of the virus genome, named HTLV-1 basic leucine zipper factor (HBZ), was identified [21]. The spliced form of HBZ is expressed in all ATL [22] and HAM/TSP [23] cases, and its expression is strongly correlated with the HTLV-1 proviral load (PVL) in HTLV-1-infected individuals and with disease severity in HAM/TSP patients [23]. Also, HBZ protein promotes proliferation of ATL cells and induces T-cell lymphomas in $CD4^+$ T cells by transgenic expression, indicating the possible involvement of HBZ expression in the development of ATL [22, 24]. Moreover, among the HTLV-1 encoded viral genes, only the HBZ gene sequence remains intact, unaffected by nonsense mutations and deletion [25]. These findings indicate that HBZ expression is indispensable for proliferation and survival of ATL cells and HTLV-1 infected cells, and that Tax expression is not always necessary for the maintenance of ATL [26].

2.2. Clinical and Pathological Features of HAM/TSP. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. In addition to neurological symptoms, some HAM/TSP cases also exhibit autoimmune-like disorders, such as uveitis, arthritis, T-lymphocyte alveolitis, polymyositis, and Sjögren syndrome [14]. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [9–12]. The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than for ATL onset [11, 31]. HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life (i.e., through sexual contact [almost exclusively from male to female], intravenous drug use, contaminated blood transfusions, etc.). The mean age at onset is 43.8 years, and the frequency of HAM/TSP is higher in women than in men (the male to female ratio of occurrence is 1 : 2.3) [11].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [27, 32, 33]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. In the cases with active-chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain [28]. The peripheral nerve pathology of HAM/TSP patients with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis [29, 30]. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal immunoglobulin bands in the CSF, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-γ, and an increased intrathecal antibody (Ab) synthesis specific for HTLV-1 antigens have also been described [34]. Clinical progression of HAM/TSP is associated with an increase in the proviral load in individual patients, and a high ratio of proviral loads in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease [35]. The clinical and pathological characteristics of HAM/TSP described above are shown in Table 1.

3. Risk Factors for HAM/TSP

3.1. Host Genetic. A previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima prefecture, HTLV-1 endemic Southern Japan, revealed that one of the major risk factors is the HTLV-1 PVL. The median PVL was more than ten times higher in HAM/TSP patients than in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. A higher PVL in HAM/TSP patients than in ACs was observed in other endemic areas such as the Caribbean [38], South America [39], and the Middle East [40]. It was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in HAM/TSP patients and genetic relatives. In Southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population (Table 2) [37, 41]. Since the function of class I HLA proteins is to present antigenic peptides to CTL, these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may therefore be an important determinant of HTLV-1 PVL and the risk of HAM/TSP. In fact, it has been reported that CTL spontaneously kills autologous HTLV-1-infected

Table 1: Clinical and pathological characteristics of HAM/TSP.

Clinical characteristics		References
Onset	Insidious, slowly progressive	[11]
Major clinical symptoms	Spastic paraparesis Sphincter dysfunction Mild sensory disturbance in the lower extremities	[11]
Complications	Uveitis Arthritis T-lymphocyte alveolitis Polymyositis Sjögren syndrome	[14]
Mean age at onset	43.8 years	[11]
Male-to-female ratio	1 : 2.3 (male : female)	[11]
Laboratory data	Positive anti-HTLV-1 antibody in both serum and CSF Moderate pleocytosis and raised protein content in CSF	[11]
Pathological characteristics		References
Spinal cord	Loss of myelin and axons in the lateral, anterior, and posterior columns-predominantly at the thoracic level Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis-predominantly at the thoracic level	[27]
Brain	Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis Perivascular inflammatory infiltration and fibrosis only in the cases with active-chronic lesions in the spinal cord. The composition of cell subsets was similar both in the spinal cord and in the brain	[28]
Peripheral nerve	Varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis	[29, 30]

cells *ex vivo* [42], granzymes and perforin are more highly expressed in individuals with a low PVL [43], and the lytic efficiency of the $CD8^+$ T cell response, that is, the fraction of autologous HTLV-1-expressing cells eliminated per $CD8^+$ T cell per day, was inversely correlated with both PVL and the rate of spontaneous proviral expression [44]. These findings indicate that the CTL against HTLV-1 reduces PVL and risk of HAM/TSP. Moreover, using a combination of computational and experimental approaches, MacNamara et al. recently reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, determines the outcome of HTLV-1 infection [45].

Analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP (Table 2). For example, the TNF-α promoter-863 A allele [47] and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [48] predisposed to HAM/TSP, whereas IL-10-592 A [49], stromal-derived factor (SDF)-1 +801A, and IL-15 +191 C alleles [47] conferred protection against HAM/TSP. The polymorphisms in the MMP-9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene [48, 49]. However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown, and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in larger sample size may provide evidence for the association of non-HLA genes with HAM/TSP pathogenesis.

3.2. HTLV-1 Genotype and Genomic Integration Site. Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [46]. The *tax* subgroup A, which belongs to cosmopolitan subtype A, was more frequently observed in HAM/TSP patients, and this association was independent of the protective effect of the HLA allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B which belongs to cosmopolitan subtype B, but not against *tax* subgroup A in the Japanese population [46]. Interestingly, HLA-A*02 appeared not to give protection against infection with cosmopolitan subtype A in a population in Iran [40]. Moreover, the Iranian HTLV-1 strain has a Rex protein that is 20 amino acids longer than that of the Japanese strain that belongs to cosmopolitan subtype B. Experiments are now underway to compare the functions of these Rex proteins.

Table 2: Host genetic and viral factors associated with the risk of HAM/TSP.

Factor	Condition	Effect	Reference(s)
Viral factors	HTLV-1 *tax* subgroup A	Susceptible	[46]
	Proviral load	Susceptible	[36]
Host factors			
HLA	A*02	Protective	[37, 41]
	Cw*08	Protective	[41]
	B*5401	Susceptible	[41]
	DRB1*0101	Susceptible	[37]
Non-HLA	TNF-α promoter −863 A allele	Susceptible	[47]
	longer CA repeat alleles of MMP-9 promoter	Susceptible	[48]
	IL-10 promoter −592 A allele	Protective	[49]
	SDF-1 promoter +801 A allele	Protective	[47]
	IL-15 +191 C allele	Protective	[47]

Recently, to test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T-cell clone, Gillet et al. reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome [50]. They mapped >91,000 unique insertion sites (UISs) of the provirus from 61 HTLV-1-infected individuals in primary PBMCs and showed that a typical HTLV-1-infected host carries between 500 and 5000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index (OCI) to quantify the clonality of HTLV-1-infected cells *in vivo* and found that the OCI did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between OCI and HTLV-1PVL in either ACs or HAM/TSP patients. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, from a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion *in vivo* is favored by a proviral integration site near a region of host chromatin undergoing active transcription, or same-sense transcriptional orientation of the provirus. Negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T-cell clones with low pathogenic potential.

4. Immune Response to HTLV-1

4.1. Innate Immune Response

4.1.1. Natural Killer (NK) Cells. Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs although the results were not normalized with respect to PVL [51]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, a synthetic tetramer of HLA-E with the HLA-G signal sequence peptide was used to identify NK cells in HAM/TSP patients [52]. The results showed a significantly lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [51], this reduction in frequency was particularly notable in the $CD3^+$ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [52]. Recent data also suggest that the frequency of invariant NKT (iNKT) cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [53, 54]. These findings indicate that the activity of the NK or NKT cell response was associated with the absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of treatment of HAM/TSP with fermented milk containing viable *Lactobacillus casei* strain Shirota resulted in a significant increase in NK cell activity, with improvements in clinical symptoms [55]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP. Recently, it has been reported that in addition to the previously described $CD8^+$ T-cell spontaneous proliferation [56], $CD56^+$ NK cells also spontaneously proliferated *in vitro*, and spontaneous NK cell proliferation positively correlated with HTLV-1 PVL but not with the presence of HAM/TSP [57]. A hallmark of HTLV-1 infection is the *in vitro* proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [58, 59]. Most of the high SLP observed in PBMCs from HAM/TSP patients is likely to be explained by a greater spontaneous expression of the provirus and consequently a greater proliferation of responding $CD8^+$ T cells in culture [56]. The greater proviral expression may be partly attributable to the impaired function and decreased number of NK cells in HAM/TSP patients. Although further

studies are required to clarify the role of NK cells in HTLV-1 infection and HAM/TSP pathogenesis, NK cells might be also an interesting candidate for future immunotherapy.

4.1.2. Interferons. Type I interferon (IFN) is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of interferon-stimulated genes (ISGs), which execute the antiviral effects of IFN [60]. The ISGs then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself [61]. To date, IFN-α is not only one of the effective therapeutic agents for HAM/TSP, but also known as an only therapeutic agent whose efficacy was demonstrated in randomized placebo-controlled trials [62, 63]. However, the therapeutic benefit is small, and IFN-α is not in general use in the treatment of HAM/TSP. The combination of the antiretroviral agent zidovudine (AZT) and IFN-α is also beneficial for overall survival in smoldering and chronic (i.e. indolent) ATL [64] although its efficacy has not yet been confirmed in well-designed prospective studies. It might be interesting to analyse which ISGs are changed in the course of IFN-α treatment and the functional role of ISGs as potential targets for therapy. In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 mRNA is very low, and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture *in vitro* [42]. However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T-cells is suppressed by stromal cells, that is epithelial cells and fibroblasts, in culture through type I IFNs [65]. Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression *in vitro* and *in vivo*, at least through type I IFN.

4.2. Antibody Response to HTLV-1. In 2002, it was reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous-nuclear-riboprotein (hnRNP-) A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [66]. However, subsequent analysis using Japanese samples under fully masked conditions indicated that there was no difference in the incidence of anti-hnRNP A1 Abs between HAM/TSP and other neurological diseases [67]. It is unlikely that anti-Tax Ab explains the onset or initial tissue damage of HAM/TSP, as the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [68] and is not normally accessible to Ab attack. Anti-Tax Ab might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

In HTLV-1 infection, HAM/TSP patients generally have a higher anti-HTLV-1 Ab titer than ACs with a similar HTLV-1 proviral load [69–71]. These anti-HTLV-1 Abs often include IgM in both ACs and patients with HAM/TSP [70, 71]. These findings suggest that there was persistent expression of HTLV-1 proteins *in vivo* and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Although Ab responses to the immunodominant epitopes of the HTLV-1 envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was higher in HAM/TSP patients (71%–93%) than in ATL patients (4%–31%) or ACs (27%–37%) [72]. Among these anti-HTLV-1 antibodies, anti-EnvAb is particularly important since some anti-Env Abs have neutralizing activity against HTLV-1. Antisera raised against recombinant HTLV-1 Env polypeptides [73, 74], vaccinia virus containing HTLV-1 env gene [75, 76], immunization with neutralizing epitope peptides [77], and passive transfer of human IgG that has neutralizing activity [78, 79] were all shown to neutralize HTLV-1 infectivity. In HTLV-1 infection, the roles of HTLV-1 neutralizing Ab *in vivo* are still largely unknown. It will be interesting to examine whether HTLV-1 neutralizing Ab titres correlate with disease status and PVL in infected individuals. Since the mutation rate of HTLV-1 provirus is significantly lower than HIV-1, passive immunization with human monoclonal Ab may be beneficial and effective method to prevent HTLV-1 infection.

4.3. Cytotoxic T-Lymphocyte (CTL) Response to HTLV-1. Previous reports indicated that the HTLV-1-specific $CD8^+$ CTLs are typically abundant, chronically activated, and mainly targeted to the viral trans activator protein Tax [80]. Also, as already mentioned, the median PVL in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower PVL and a lower risk of HAM/TSP [37, 41], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [42]. These data have raised the hypothesis that the class I-restricted $CD8^+$ CTL response plays a critical part in limiting HTLV-1 replication *in vivo* and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. Indeed, as mentioned above (Section 3.1), MacNamara et al. [45] have shown that HLA class 1 alleles which strongly bind oligopeptides from the HBZ protein enable the host to make a more effective immune response against HTLV-1; therefore, such individuals have a lower PVL and are more likely to be asymptomatic. Moreover, another recent report showed the presence of HBZ-specific $CD4^+$ and $CD8^+$ cells *in vivo* in patients with HAM/TSP and in ACs and a significant association between the HBZ-specific $CD8^+$ cell response and asymptomatic HTLV-1 infection [81]. These findings provide strong evidence to support the hypothesis of the crucial role of CTLs and also confirm the importance of HBZ for persistent infection.

Since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly higher in HAM/TSP patients than ACs [82, 83], and these cells have the potential to produce

proinflammatory cytokines [84], there is a debate on the role of HTLV-1-specific-CD8$^+$ T cells, that is, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP, or whether the dominant effect of such cells *in vivo* is protective against disease. The analysis of gene expression profiles using microarrays in circulating CD4$^+$ and CD8$^+$ lymphocytes indicated that granzymes and perforin are more highly expressed in individuals with a low PVL [43], suggesting that a strong CTL response is associated with a low PVL and a low risk of HAM/TSP. Indeed, the lytic capacity of HTLV-1-specific CTL in patients with HAM/TSP and ACs, quantified by a CD107a mobilization assay, showed significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs [85]. Recently, it has been reported that the high CTL avidity, which is closely associated with the lytic efficiency of CTL, correlates with low PVL and proviral gene expression [44], indicating that the efficient control of HTLV-1 *in vivo* depends on the quality of CTL, which determines the position of virus-host equilibrium and also the outcome of persistent HTLV-1 infection. However, two caveats must be made here. First, a protective role and a pathogenic role of CTLs are not mutually exclusive. Indeed, there are other examples of viral infections in which the virus-specific CTLs exert both beneficial (antiviral) and detrimental (inflammatory) effects, such as lymphocytic choriomeningitis virus (LCMV) infection in the mouse [86]. Second, it is difficult to separate cause and effect in analyzing the association between T-cell attributes and the efficiency of viral control in a persistent infection at equilibrium.

4.4. CD4$^+$ Helper T-Cell Response to HTLV-1. Antiviral CD4$^+$ T-cell responses are of central importance in driving B-cell and CD8$^+$ T-cell responses *in vivo*. The most common HTLV-1 antigen recognized by CD4$^+$ T-cells is the Env protein [87, 88], in contrast with the immunodominance of Tax in the CD8$^+$ T-cell response [89–91]. At a similar PVL, patients with HAM/TSP had significantly increased frequency of virus-specific CD4$^+$ T cells compared to ACs [88, 92]. The antiviral T-helper (Th)1 phenotype is also dominant among HTLV-1-specific CD4$^+$ T cells in both ACs and patients with HAM/TSP [93], and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4$^+$ T cells in patients with HAM/TSP compared to AC of a similar PVL [93, 94]. A role for CD4$^+$ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in Southern Japan [37, 41] and Northeastern Iran [40]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4$^+$ T cells directly *ex vivo* [92]. The results showed that the frequency of tetramer$^+$CD4$^+$ T cells was significantly higher in HAM/TSP patients than ACs with similar PVL. Furthermore, direct ex vivo analysis of tetramer$^+$CD4$^+$ T cells from two unrelated DRB1*0101-positive HAM/TSP patients indicated that certain T-cell receptors (TCRs) Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These results suggest that the observed increase in virus-specific CD4$^+$ T cells in HAM/TSP patients, which may contribute to CD4$^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected CD4$^+$ T cells but was the result of *in vivo* selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 tetramer$^+$CD8$^+$ T cells [95] and muscle-infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [96].

4.5. Regulatory T Cells (Tregs) in HTLV-1 Infection. Regulatory T cells (Tregs) are important mediators of peripheral immune tolerance and also play an important role in chronic viral infections. In HTLV-1 infection, it has been reported that HTLV-1 preferentially and persistently infects CD4$^+$CD25$^+$ lymphocytes *in vivo* [97], which contain the majority of the Foxp3$^+$ Tregs [98]. In HAM/TSP patients, the frequency of Foxp3$^+$ expression in CD4$^+$CD25$^+$ cells is lower than that in ACs and uninfected healthy controls [97, 99]. This is probably due to the fact that CD25 is transcriptionally induced by HTLV-1 Tax [100], which may result in the reduced proportion of Foxp3$^+$ cells in the CD4$^+$CD25$^+$ population in HTLV-1-infected individuals, especially HAM/TSP patients. It is important to note that the CD4$^+$CD25$^+$ population contains a mixture of Tregs and activated non-Tregs. Therefore, it is inappropriate to use CD25 as a marker of Tregs in HTLV-1 infection: the best current working definition of Treg phenotype is CD4$^+$Foxp3$^+$. Reports from different geographic regions indicate that the percentage of CD4$^+$Foxp3$^+$ cells is higher in the HAM/TSP patients than in ACs [101–103]. It has been reported that the high frequency of CD4$^+$Foxp3$^+$T cells in HTLV-1-infected individuals is maintained by CCL22 produced by HTLV-1-infected PBMCs [104]. The frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells was positively correlated with the HTLV-1 proviral load [102, 105], and the CTL activity was negatively correlated with the frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells [102], suggesting that CD4$^+$Foxp3$^+$ Tregs may impair the CTL surveillance of HTLV-1. If this is the case, activity of CD4$^+$Foxp3$^+$ cells may also determine the risk of developing HAM/TSP via increasing the HTLV-1 PVL.

4.6. Dendritic Cells (DCs). Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [106], and the development of HAM/TSP is associated with rapid maturation of DCs [107]. As already mentioned, one of the hallmarks of HTLV-1 infection is the spontaneous lymphocyte proliferation (SLP). Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolished SLP, whereas supplementing DCs restores proliferation [106]; supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC

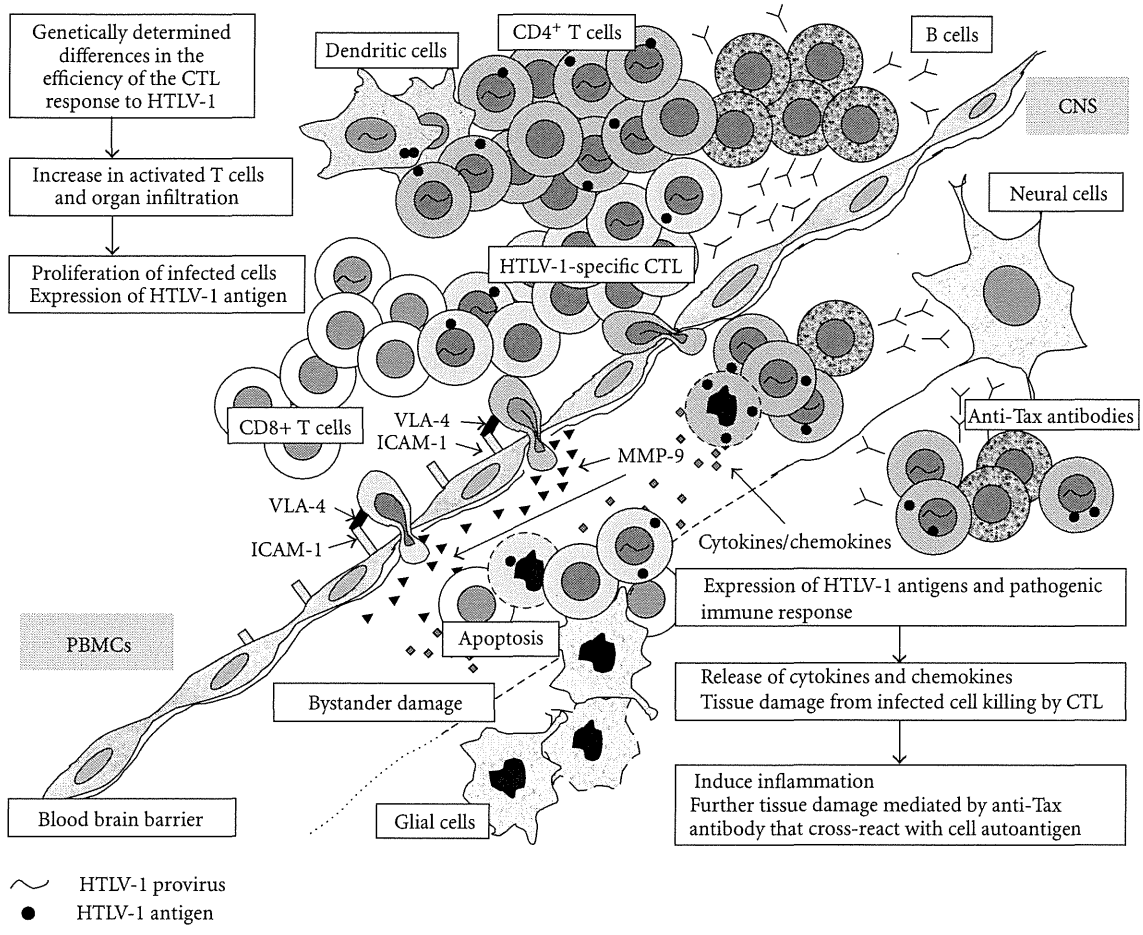

FIGURE 1: Hypothesis for the pathogenesis of human T-cell leukemia virus type-1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP). Accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-react with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

class II, CD86, and CD58 can block SLP [108]. Recently, it has been demonstrated that both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [109]. In addition, other groups have obtained evidence that HTLV-1 transmission from DCs to T cells was mediated primarily by DC-SIGN [110], and DCs play a major part in generating and maintaining the Tax-specific $CD8^+$ T cells both *in vitro* and *in vivo* [111]. Moreover, using transgenic mouse models that permit conditional transient depletion of $CD11c^+$ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection [112, 113]. Since HTLV-1 can impair the differentiation of monocytes into DCs [114], the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

5. Concluding Remarks

As shown in Figure 1, accumulating evidence suggests that the host immune response, especially the CTL response, plays a critical role in determining the risk of HAM/TSP. A less efficient CTL response against HTLV-1 may cause a higher PVL and higher antigen expression in infected individuals, which in turn lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression to HAM/TSP. Since HLA class 1 genotype determines only up to 50% of HAM/TSP risk in infected people [41], it is important to discover other factors that determine the efficiency of the CTL response

to HTLV-1 and the outcome of HTLV-1 infection. Studies of the HTLV-1 receptor and DCs are also critical in the development of vaccine approaches to elicit cellular immune responses to key viral proteins such as Tax and Env to ablate HTLV-1-infected T cells. Newer approaches using genetically engineered and/or humanized mouse models for HTLV-1 infection will help to develop effective treatment and prevention of HAM/TSP in the future.

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