Tax-Inducible Production of CC Chemokine Ligand 22 by Human T Cell Leukemia Virus Type 1 (HTLV-1)-Infected T Cells Promotes Preferential Transmission of HTLV-1 to CCR4-Expressing CD4$^+$ T Cells[1]

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Adult T cell leukemia is a mature CD4$^+$ T cell malignancy which predominantly expresses CCR4 and is etiologically associated with human T cell leukemia virus type 1 (HTLV-1). Because HTLV-1 transmission depends on close cell-cell contacts, HTLV-1-infected T cells may preferentially interact with CCR4$^+$CD4$^+$ T cells for efficient viral transmission. In terms of gene expression and protein secretion, we found a strong correlation between HTLV-1 Tax oncoprotein and CCL22, a CCR4 ligand, in HTLV-1-infected T cells. Transient Tax expression in an HTLV-1-negative T cell line activated the CCL22 promoter and induced CCL22. Additionally, *tax* gene knockdown by small interference RNA reduced CCL22 expression in the infected T cells. These findings indicate that CCL22 is a cellular target gene of Tax. In chemotaxis assays, the culture supernatants of HTLV-1-infected T cells selectively attracted CCR4$^+$CD4$^+$ T cells in PBMCs. This was blocked by pretreating the supernatants with anti-CCL22 Ab or PBMCs with a synthetic CCR4 antagonist. In coculture experiments, primary CCR4$^+$CD4$^+$ T cells significantly adhered to Tax-expressing cells. This adhesion was blocked by the CCR4 antagonist or pertussis toxin. Interestingly, CCR4 was redistributed to the contact region, and in some cases, this was accompanied by a polarized microtubule-organizing center, which is an indicator of virological synapse formation, in the infected T cells. Finally, anti-CCL22 Ab treatment also blocked HTLV-1 transmission to primary CD4$^+$ T cells in coculture experiments with HTLV-1 producer cells. Thus, HTLV-1-infected T cells produce CCL22 through Tax and selectively interact with CCR4$^+$CD4$^+$ T cells, resulting in preferential transmission of HTLV-1 to CCR4$^+$CD4$^+$ T cells. ***The Journal of Immunology,*** **2008, 180: 931–939.**

Human T cell leukemia virus type 1 (HTLV-1)[3] is an exogenous retrovirus that infects 10~20 million people worldwide (1–4). Although the majority of infected individuals remain lifelong asymptomatic carriers, HTLV-1 is also etiologically associated with adult T cell leukemia (ATL) and a range of inflammatory diseases, including HTLV-1-associated myelopathy/tropical spastic paraparesis, which is a chronic disease of the CNS (1–3). HTLV-1-infected lymphocytes produce very few infectious cell-free virions, and the virus is mainly transmitted via cell-cell contacts (2, 4, 5). Therefore, HTLV-1 transmission between individuals occurs by the transfer of infected lymphocytes through breast milk, semen, or blood (4, 6). In vitro, HTLV-1 is capable of transforming CD4$^+$ T cells into continuously growing T cell lines (2, 3). The potent viral transactivator Tax is known to activate both the HTLV-1 long terminal repeat and the promoters of various cellular genes, leading to strong promotion of cell proliferation and activation (7). However, ATL develops only after a long period of latency, usually after several decades, and during this period, tumor progression occurs through the accumulation of multiple genetic alterations (1, 3, 4). Thus, ATL cells usually do not express the *tax* gene and are considered to be independent of the growth-promoting effects of Tax (3, 4). This suggests that Tax is mainly involved in virus replication and transmission as well as in the early stages of tumorigenesis.

ATL is typically a malignancy of mature CD4$^+$ T cells (1–3). In addition, studies by our group and others have shown that most ATL cases are strongly positive for CCR4 (8, 9), the chemokine receptor known to be selectively expressed by Th2 cells, regulatory T cells, and skin-homing memory/effector T cells (10). Thus, ATL may be preferentially derived from any one of these T cell subsets. Indeed, several recent studies have demonstrated that FOXP3, a forkhead/winged helix transcription factor and a specific marker of regulatory T cells (11), is expressed in a fraction of ATL cases (12–14).

Although a number of cell surface molecules have been reported to play a role in HTLV-1 envelope (Env)-mediated syncytium formation (15), glucose transporter-1 (GLUT-1) and heparan sulfate proteoglycan (HSPG) appear to be of primary importance in HTLV-1 infection (16–18). Additionally, neuropilin-1, a member

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[3] Abbreviations used in this paper: HTLV-1, human T cell leukemia virus type 1; ATL, adult T cell leukemia; Env, envelope; GLUT-1, glucose transporter-1; HSPG, heparan sulfate proteoglycan; MTOC, microtubule organizing center; VS, virological synapse; siRNA, small interference RNA; PTX, pertussis toxin; MMC, mitomycin C; RT, room temperature.

of the immune synapse, forms ternary complexes with GLUT-1 and Env, indicating that neuropilin-1 is also involved in Env-mediated cell fusion and viral infection (19). Because GLUT-1 and HSPG are reported to be expressed on the surface of many cell types, it has been found that not only CD4$^+$ T cells but also CD8$^+$ T cells and other cell types can be infected by HTLV-1; however, CD4$^+$ T cells constitute the major population of HTLV-1-infected cells in vivo with frequent clonal expansion (15, 20–23). Furthermore, CD4$^+$ and CD8$^+$ T cells specific for HTLV-1 are more frequently infected than T cells with unrelated specificities (5), suggesting that HTLV-1 transmission is assisted by close cell-cell interactions between the viral Ag-specific T cells and HTLV-1-infected cells. Similarly, there may be a mechanism for a preferential interaction between HTLV-1-infected T cells and CD4$^+$ T cells expressing CCR4.

Recently, Igakura et al. (24) have reported the formation of a highly organized structure at the cell-cell junction, termed "the virological synapse (VS)," between HTLV-1-infected CD4$^+$ T cells and uninfected autologous or allogeneic CD4$^+$ T cells. The adhesion adaptor protein talin and the microtubule organizing center (MTOC) are polarized to the cell-cell junction in HTLV-1-infected T cells together with an accumulation of the HTLV-1 Gag protein and the HTLV-1 genome. This leads to the transfer of both the Gag protein and the HTLV-1 genome to uninfected T cells through VS (5, 24). However, it is still unknown whether the attraction and initial interaction of target CD4$^+$ T cells to HTLV-1-infected T cells are mostly random processes.

Chemokines are a group of cytokines that regulate lymphocyte migration and cell-cell interaction via G protein-coupled receptor-type receptors (10). HTLV-1-infected T cells have been reported to produce various chemokines mostly through transcriptional activation by Tax (25–29). Therefore, HTLV-1-infected T cells may use the chemokine-chemokine receptor systems for promoting cell-cell interactions with target CD4$^+$ T cells. In this study, we demonstrate that HTLV-1-infected T cells abundantly produce CCL22 (also known as macrophage-derived chemokine) through induction by Tax and selectively attract and interact with CCR4$^+$CD4$^+$ T cells in PBMCs, thereby resulting in the preferential transmission of HTLV-1 to primary CCR4$^+$CD4$^+$ T cells.

Materials and Methods

Reagents

AZ no. 112, a small molecule CCR4-specific antagonist, was synthesized from patent information (international application no. PCT/SE/03/00041) and with the help of the NARD Institute. We confirmed that AZ no. 112 specifically inhibits CCR4-mediated chemotaxis by using a panel of cell lines, including murine L1.2 cells, murine B300.19 cells, or Jurkat cells stably expressing the chemokine receptors for human CC (CCR1–10), CXC (CXCR1–4), CX3C (CX3CR1), and XC (XCR1) chemokines (30). All the chemokines used were purchased from R&D Systems.

Cells

C8166, C91/PL, ILT8M2, and TCL-Kan cells are HTLV-1-transformed human T cell lines. H582, KOB, KK1, SO4, and ST1 cells are ATL-derived T cell lines (31). Jurkat and Molt-4 cells are HTLV-1-negative human T cell lines. JPX-9 and JPX-M cells are Jurkat cell sublines that express either wild-type Tax or a nonfunctional Tax mutant (Tax-Arg^{63}), respectively, under the control of the metallothionein promoter (32). JPX-9 and JPX-M cells were provided by M. Nakamura (Tokyo Medical and Dental University, Tokyo, Japan). Murine L1.2 cells stably expressing human CCR4 (L-CCR4) were prepared as described previously (30). All the cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME (complete medium). For ATL-derived cell lines, the culture medium was further supplemented with 100 U/ml IL-2. For induction of wild-type and mutant *tax*, JPX-9 and JPX-M cells were treated with 20 μM Cd^{2+}, as described previously (32). PBMCs were isolated from heparinized venous blood using Ficoll-Paque Plus (Pharmacia). All blood donors provided written informed consent, and the study was conducted according to the principles of the Helsinki declaration.

Semiquantitative RT-PCR

Total RNA was prepared from cells in culture by using TRIzol reagent (Invitrogen Life Technologies) and the RNeasy kit (Qiagen). Reverse transcription of total RNA (1 μg) was conducted using the oligo(dT)$_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen Life Technologies). First-strand DNA (20 ng of total RNA equivalent) and original total RNA (20 ng) were amplified in a final volume of 20 μl containing 10 pM of each primer and 1 U of Ex-*Taq* polymerase (Takara), in accordance with the manufacturer's protocol. The primers used were as follows: +5′-AGGACAGAGCATGGCTCGCCTACAGA-3′ and −5′-TAATGGCAGGGAGGTAGGGCTCCTGA-3′ for CCL22; +5′-ACTGCTCCAGGGATGCCATCGTTTTT-3′ and −5′-ACAAGGGGATGGGATCTCCCTCACTG-3′ for CCL17; +5′-CCGGCGCTGCTCTCATCCCGGT-3′ and −5′-GGCCGAACATAGTCCCCCAGAG-3′ for Tax; +5′-AAAAAGCGGGTCACTCTATATGCTC-3′ and −5′-CCACTGCTACCTGGTACTCTGTTGT-3′ for CD25; and +5′-GCCAAGGTCATCCATGACAACTTTGG-3′ and −5′-GCCTGCTTCACCACCTTCTTGATGTC-3′ for GAPDH. The amplification conditions, which were carefully chosen to obtain signals in a linear amplification range, were as follows: denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 30 s (5 min for the last cycle). There were 36 cycles for CCL22, CCL17, and CD25; 33 cycles for Tax; and 27 cycles for GAPDH. Amplification products (10 μl each) were loaded onto 2% agarose, electrophoresed, and visualized by ethidium bromide staining.

ELISA

Chemokines present in the culture supernatants were measured using commercial ELISA kits (purchased from R&D Systems). For each sample, two serial 5-fold dilutions were analyzed in triplicates, and the mean values within the limits of the standard curve were presented along with the SD.

Luciferase reporter assay

The CCL22 promoter-luciferase construct (pGL3-CCL22(−722/−11)) has been described previously (33). Transient transfection was performed using the DMRIE-C transfection reagent (Invitrogen Life Technologies), in accordance with the manufacturer's instructions. Briefly, Jurkat cells ($3\sim4 \times 10^5$) were mixed with 3 μl of DMRIE-C reagent, 0.5 μg of phRL-TK, and 1 μg of pGL3-CCL22(−722/−11) or pGL3-Basic (Promega) either with or without 0.5 μg of pHβPr.1-TaxMT-2 (provided by M. Fujii, Niigata University, Niigata, Japan). After 5 h of incubation at 37°C, RPMI 1640 and 15% FBS were added, and the cells were further incubated in a 24-well plate at 37°C. After 24 h, the cells were lysed with passive lysis buffer (Promega), and the luciferase activity was measured on a Wallac 1420 ARVOsx multilabel counter (Amersham Biosciences) using a Dual-Luciferase Reporter Assay kit (Promega). The activities of the reporter vectors were indicated by the ratio of the activity of firefly luciferase to that of the control vector *Renilla* luciferase. All studies were performed at least three times with three independent transfections each time.

Small interference RNA (siRNA) and nucleofection

Synthetic *tax* siRNA oligonucleotides were purchased from Qiagen and designed in the coding region of Tax using multiple HTLV-1 complete genomic sequences (GenBank accession numbers AF033817, AF042071, AF139170, AY563953, D13784, J02029, L03561, and NC_001436). The *tax* siRNA duplex sequences are as follows: Tax sense was 5′-rGGCCUUAUUUGGACAUUUAdTdT-3′ and Tax antisense was 5′-rUAAAUGUCCAAAUAAGGCCdTdT-3′. The GFP-22 siRNA (catalog no. 1022064; Qiagen) was used as a negative control. C8166 cells or TCL-Kan cells (1×10^6) were resuspended in 100 μl of nucleofector solution (Cell Line Nucleofector kit T: VCA-1002; Amaxa). Four micrograms of siRNA was added and mixed well. The cell-siRNA mixture was transferred to an electroporation cuvette and placed in the Nucleofector II device (Amaxa). Nucleofection of the cells was accomplished using the O-17 program. Immediately after nucleofection, 500 μl of prewarmed medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, and 50 μM 2-ME) was added to the cuvette, and the samples were transferred to 12-well plates containing 1.5 ml of prewarmed medium. The cells were incubated for 48 h in a 37°C incubator containing 5% CO_2 and then harvested and subjected to RT-PCR analysis.

Chemotaxis assays

Chemotaxis assays were performed using 96-well ChemoTx microplates of pore size 5-μm (NeuroProbe), as described previously (33). Ten million L-CCR4 cells or PBMCs from healthy donors were resuspended in 1 ml of

phenol red-free RPMI 1640 containing 1 mg/ml BSA (Sigma-Aldrich) and 20 mM HEPES (pH 7.4) (chemotaxis buffer). The cells were suspended in chemotaxis buffer and added to the upper wells in a volume of 25 μl. Recombinant CCL22 (R&D Systems) in the chemotaxis buffer or the culture supernatants from C8166 or TCL-Kan cells diluted with the chemotaxis buffer were added to the lower wells in a volume of 27.5 μl. In some experiments, the culture supernatants were pretreated with anti-CCL22 mAb (clone 57226.11; R&D Systems) or control IgG for 30 min at 4°C. Otherwise, the cells were pretreated with 20 μM AZ no. 112 at 37°C for 30 min before the migration assay. Cell viability was not adversely affected by AZ no. 112 or its vehicle DMSO. After 2 h at 37°C, cells that had migrated to the lower wells were counted by flow cytometry immediately or after staining with anti-CD4 and anti-CCR4 (see below). Cell migration was expressed as a percentage of input cells. All assays were performed in triplicates.

Flow cytometry

Cells were pretreated with human AB serum for 20 min at 4°C to block the FcRs. For detection of CCR4 or CD4, the cells were stained with anti-human CCR4-allophycocyanin (clone 205410; R&D Systems), anti-human CD4-PE (clone MT310; DakoCytomation), or appropriately labeled control IgG for 30 min at 4°C in PBS containing 3% FBS and 0.05% sodium azide. For detection of HTLV-1 Env gp46, the cells were stained with either mouse anti-HTLV-1 gp46 Ab (clone 67/5.5.13.1; Abcam) or mouse IgG1 as the primary Ab and with goat anti-mouse IgG-PE as the secondary Ab. Cells were further stained with mouse anti-CD4-allophycocyanin (clone 13B8.2; Beckman Coulter) and propidium iodide. The stained cells were immediately analyzed on a FACSCalibur system (BD Biosciences).

Cell-cell interaction assay

PBMCs were stained to detect CCR4 and CD4, as described above. After two washes with complete medium, the cells were resuspended in complete medium, and $CCR4^+CD4^+$ cells in the lymphocyte gate were immediately sorted using FACSVantage SE (BD Biosciences). The purity of sorted $CCR4^+CD4^+$ T cells was 80~85%. The sorted cells (1×10^4) were then cocultured with Tax-positive (C8166 and TCL-Kan) or Tax-negative (ST-1) HTLV-1-infected cells (3×10^3) on Lab-Tek chamber slides (Nalge Nunc International) either with or without AZ no. 112 (20 μM) or pertussis toxin (PTX, 100 ng/ml; Invitrogen Life Technologies) at 37°C. After 30–60 min, the cells were pipetted vigorously and observed under a light microscope without fixation and photographed by the KEYENCE BZ-8000 system. We confirmed that the staining of CCR4 and CD4 had no adverse effect on cell migration or adhesion.

For immunofluorescence staining, the Lab-Tek chamber slides were pretreated for 30 min at room temperature (RT) with 100 μg/ml poly-L-lysine (200 μl/well; Sigma-Aldrich) and allowed to dry before use. PBMCs were first stained with mouse anti-human CCR4 Ab (clone 1B1; BD biosciences) followed by sheep $F(ab')_2$ anti-mouse IgG-FITC (Sigma-Aldrich). The cells were further stained with anti-human CD4-allophycocyanin and anti-human CD45RA-PE (clone HI100; BD Biosciences). The cells were resuspended in complete medium, and the $CD45RA^-CCR4^+CD4^+$ T cells were sorted immediately by the FACSVantage SE system. The sorted cells were resuspended in complete medium and kept at 37°C before use. The purity of sorted $CCR4^+CD4^+$ T cells was ~99%. TCL-Kan cells (1×10^4) were added to a poly-L-lysine-coated chamber slide and left for 30 min at 37°C to allow adherence. The sorted $CCR4^+CD4^+$ T cells (1×10^5) were then added onto TCL-Kan cells that had been adhered to slides at a ratio of 10:1. After 1 h at 37°C, the cells were fixed with 4% paraformaldehyde/PBS for 30 min at RT, permeabilized with 1% Triton X-100/PBS for 10 min at RT, blocked with 1% BSA/PBS for 10 min at RT, and stained with anti-β-tubulin-Cy3 (clone TUB2.1; Sigma-Aldrich) for detecting the microtubule network. Cells were mounted with fluorescent mounting medium (DakoCytomation) and observed under a confocal microscope (LSM-510META; Carl Zeiss). We considered that the MTOC was reoriented when it was present within the cell-cell contact site. At least 30 events were counted per experiment.

HTLV-1 infection of PBMCs

PBMCs from healthy donors were treated with PHA for 3 days before cocultivation. HTLV-1-immortalized C91/PL cells were pretreated with 50 μg/ml mitomycin C (MMC) for 60 min at 37°C, pipetted vigorously, washed with complete medium four times, and further cultured for 12 h in the presence of 20 μg/ml anti-CCL22 neutralizing Ab or control IgG2b before cocultivation. PHA-treated PBMCs (1×10^4/well) and MMC-treated C91/PL cells (3×10^2/well) were cocultured in a flat-bottom 96-well plate in the presence of 100 U/ml IL-2. The culture medium was half-changed with fresh medium supplemented with anti-CCL22 Ab or control IgG2b at 20 μg/ml every 12 h for 6 days, and IL-2 was added every 5 days. Ten days after cocultivation, PBMCs from each well were harvested and stained for HTLV-1 gp46 and CD4, as described above.

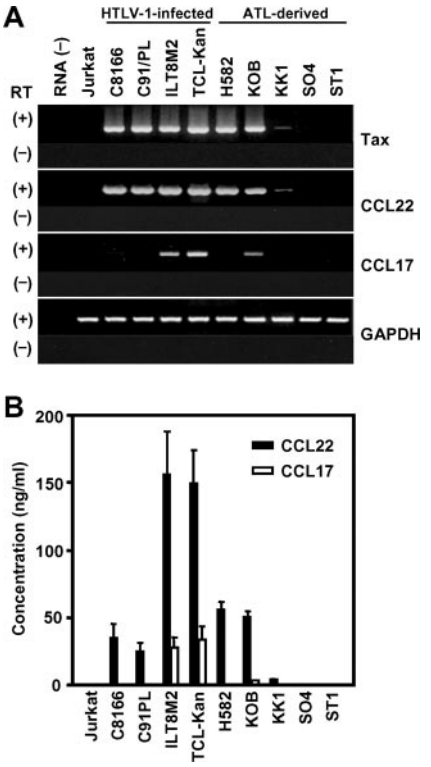

FIGURE 1. Strong expression of CCL22 in Tax-expressing HTLV-1 transformants and ATL-derived cell lines. *A*, Gene expression of CCL22 and CCL17 together with that of HTLV-1 Tax in HTLV-1 transformants and ATL-derived cell lines was analyzed by RT-PCR. GAPDH served as the loading control. *B*, Secretion of CCL22 and CCL17 by Tax-expressing HTLV-1 transformants and ATL-derived cell lines. All cell lines shown were seeded in 6-well plates at 10^6 cells/well and cultured for 3 days. The amounts of CCL22 and CCL17 in culture supernatants were measured using ELISA kits. Data from three separate experiments are presented as mean ± SD.

Statistical analyses

The Student *t* test (unpaired, two-tailed) was used to determine the level of significance using the WinSTAT software. A p value <0.05 was considered to be significant.

Results

Production of CCL22 in Tax-expressing HTLV-1-infected T cell lines

We first examined the gene expression of CCL22 and CCL17 (also known as thymus and activation-regulated chemokine) in HTLV-1-infected T cell lines by semiquantitative RT-PCR. As shown in Fig. 1*A*, we found a striking correlation between Tax and CCL22 in terms of gene expression. Consistent with this observation, all Tax-expressing cell lines secreted a large amount of CCL22 in the culture supernatants (30~150 ng/ml) (Fig. 1*B* and Table I). Only a fraction of Tax-expressing cell lines with higher CCL22 production also expressed and secreted CCL17 (Fig. 1). Because Tax has also been shown to induce CCL3/MIP-1α and CCL5/RANTES (the CCR5 ligands) as well as CXCL10/IFN-γ-inducible protein 10 (the CXCR3 ligand) (25), we also measured the levels of these chemokines in the culture supernatants. As summarized in Table I, CCL3, CCL5, and CXCL10 were also secreted in Tax-expressing

Table I. *Chemokine secretion levels in HTLV-1-infected cell lines (ng/ml)*[a]

Cell Line	Tax	CCL22	CCL17	CCL3	CCL5	CXCL10
C8166	+	32.9 ± 11.3	0.04 ± 0.007	2.7 ± 0.4	1.7 ± 0.3	0.05 ± 0.02
C91/PL	+	25.1 ± 6.0	0.05 ± 0.003	2.3 ± 0.2	2.4 ± 0.1	2.8 ± 0.3
ILT8M2	+	155.4 ± 31.5	43.5 ± 12.9	11.7 ± 2.2	NT	NT
TCL-Kan	+	140.0 ± 22.6	33.3 ± 10.1	25.3 ± 2.2	6.2 ± 2.0	8.3 ± 1.1
KOB	+	50.7 ± 3.7	1.5 ± 0.1	29.9 ± 1.3	33.6 ± 5.9	32.5 ± 10.9
KK1	±	2.0 ± 0.6	Nd	32.6 ± 2.2	23.5 ± 4.6	0.2 ± 0.03
ST1	−	Nd	Nd	1.6 ± 0.2	3.1 ± 0.3	1.9 ± 0.3
SO4	−	Nd	Nd	Nd	3.1 ± 0.3	Nd

[a] The values are indicated as mean ± SD; $n = 3$ for each experiment. Nd, Not detectable; NT, not tested.

cells; however, their levels did not correlate well with Tax expression. Furthermore, the levels of CCL22 secreted by Tax-expressing cells were often more noticeable than those of the other chemokines measured.

Tax-dependent expression of CCL22 mRNA

The above results suggested that HTLV-1 Tax induces CCL22 expression. To test this possibility, we first used JPX-9 and JPX-M cells; these are the Jurkat sublines that carry the wild-type and mutant *tax* genes, respectively, under the control of the metallothionein gene promoter (32). These matched pair cell lines have been widely used to examine the effect of Tax on the expression of various cellular genes (25, 29, 32). As shown in Fig. 2*A*, Cd^{2+} treatment of JPX-9 and JPX-M cells rapidly induced the expression of wild-type and mutant Tax, respectively. Thus, as expected, *CD25*, one of the known target genes of Tax (34), was selectively induced in JPX-9 but not in JPX-M cells. Furthermore, CCL22 mRNA was also selectively induced in Cd^{2+}-treated JPX-9 cells, and its amount steadily increased with time during the 5-day observation period. CCL17 was also weakly induced in Cd^{2+}-treated JPX-9 cells. We also confirmed the secretion of CCL22 by Cd^{2+}-treated JPX-9 cells (Fig. 2*B*). To test the effect of Tax on CCL22 expression at the transcriptional level, we further performed the luciferase reporter assays in Jurkat cells using a CCL22 promoter-luciferase reporter plasmid (33). As shown in Fig. 2*C*, cotransfection of an expression vector for Tax strongly activated the CCL22 promoter in Jurkat cells after 24 h, indicating that Tax is capable of directly activating the CCL22 promoter. Finally, we also tested the effect of Tax siRNA on the expression of CCL22 in the Tax-expressing cell lines C8166 and TCL-Kan, which were representative moderate and high CCL22 producers, respectively. We synthesized double-stranded siRNA to target Tax mRNA, although it is not strictly *tax*-specific because the Tax mRNA sequence is shared with other HTLV-1-related genes. As shown in Fig. 2*D*, introduction of the Tax siRNA, not the control siRNA, strongly reduced Tax mRNA as well as CCL22 mRNA in both cells tested. Because both C8166 and TCL-Kan cells do not express accessory genes, including $p12^I$, $p13^{II}$, and $p30^{II}$ (data not shown), we consider that the knockdown of Tax mRNA contributed to the decrease in CCL22 mRNA. Collectively, these results clearly demonstrated that CCL22 is a new bona fide target gene of Tax.

Preferential attraction of CCR4$^+$CD4$^+$ T cells by the supernatants of Tax-expressing HTLV-1-infected T cell lines

We next examined the chemotactic activity of CCL22 in the culture supernatants of Tax-expressing T cell lines (C8166 and TCL-Kan). As shown in Fig. 3*A*, both supernatants robustly induced the migration of a murine L1.2 cell line that stably expressed CCR4 (L-CCR4). Furthermore, L-CCR4 migration was effectively blocked by pretreating the supernatants with anti-CCL22 Ab or L-CCR4 cells with a synthetic CCR4 antagonist AZ no. 112. We next examined the chemotactic activity of the culture supernatants using PBMCs from healthy donors. As shown in Fig. 3*B*, the supernatants selectively attracted cells in the CCR4$^+$CD4$^+$ fraction (G_1). In contrast, the supernatants showed little increase in cell migration in the CCR4$^-$CD4$^+$ fraction (G_2) or CCR4$^-$CD4$^-$ fraction (G_3) in comparison with the control medium. As further shown in Fig. 3, *B* and *C*, the migration of CCR4$^+$CD4$^+$ cells was effectively inhibited by pretreatment of the supernatants with anti-CCL22 Ab or PBMCs with AZ no. 112. Thus, Tax-expressing HTLV-1-infected T cells did indeed selectively attract CCR4$^+$CD4$^+$ T cells by producing large amounts of CCL22. It was also noteworthy that although the supernatants also contained CCL3, CCL5, and CXCL10 in significant amounts (Table I), these chemokines apparently had minor roles in the attraction of primary CD4$^+$ T cells in fresh PBMCs.

The CCR4 antagonist AZ no. 112 as well as PTX blocks cell-cell interactions between CCR4$^+$CD4$^+$ T cells and Tax-expressing HTLV-1-infected T cell lines

Chemokines are also known to rapidly increase the binding avidity of LFA-1 to ICAM-1 by inside-out signaling pathways (35). Furthermore, it is also known that ICAM-1 is strongly up-regulated by Tax in HTLV-1-infected T cells (36). Therefore, it is possible that CCL22 produced by HTLV-1-infected T cells induces not only the migration of CCR4$^+$CD4$^+$ T cells but also their firm adhesion to HTLV-1-infected T cells expressing ICAM-1 at high levels via LFA-1 activation. To test this possibility, we next conducted coculture experiments of sorted primary CCR4$^+$CD4$^+$ T cells and HTLV-1-infected T cell lines (CCL22-producing C8166 or TCL-Kan and CCL22-negative ST-1 cells). After 30–60 min at 37°C, CCR4$^+$CD4$^+$ T cells efficiently adhered to C8166 or TCL-Kan cells but not to ST-1 cells (Fig. 4*A*, *left column*, arrowheads; Table II). Given that CCL22-negative ST-1 cells did not induce cell adhesion, we attributed this phenomenon to the CCL22-CCR4 pathway. To confirm this, we pretreated CCR4$^+$CD4$^+$ T cells with the CCR4 antagonist AZ no. 112. Indeed, AZ no. 112 potently inhibited the adhesion of CCR4$^+$CD4$^+$ T cells to CCL22-positive C8166 or TCL-Kan cells (Fig. 4*A*, *middle column*; Table II). We also tested PTX, which inhibits heterotrimeric $G\alpha_i$ protein signaling, because all chemokine receptors including CCR4 require the $G\alpha_i$ protein for signaling (10). Pretreatment of CCR4$^+$CD4$^+$ T cells with PTX also inhibited their adhesion to C8166 cells or TCL-Kan cells (Fig. 4*A*, *right column*; Table II). These results indicated that the CCL22-CCR4 pathway is indeed involved in the firm adhesion of CCR4$^+$CD4$^+$ T cells to HTLV-1-infected T cells.

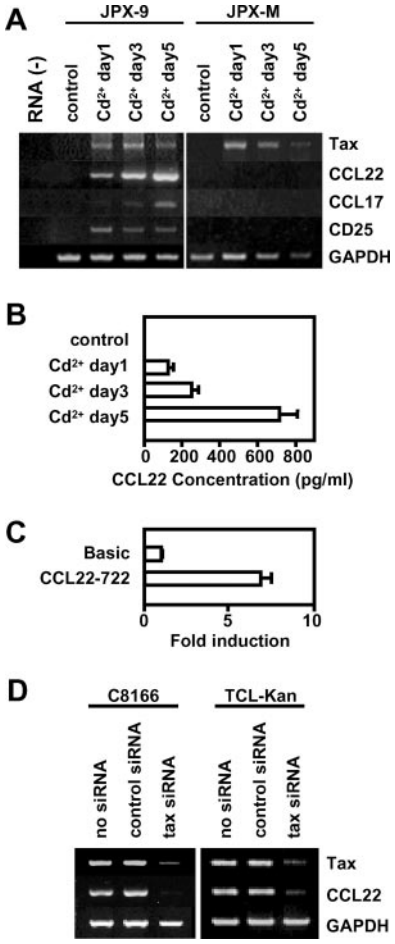

FIGURE 2. Induction of *CCL22* gene expression by HTLV-1 Tax. *A*, Induction of CCL22 mRNA in JPX-9 but not in JPX-M by Cd^{2+}. Cells (5×10^6/flask) were treated with 20 μM Cd^{2+} for 1, 3, and 5 days. RT-PCR analyses were performed for Tax, CCL22, CCL17, CD25, and GAPDH. The representative results from three individual experiments are shown. *B*, CCL22 secretion by Cd^{2+}-treated JPX-9 cells. CCL22 secreted in the culture supernatants of the Cd^{2+}-treated JPX-9 cells used in *A* was measured using an ELISA kit. The results of three independent experiments are presented as mean ± SD. *C*, Luciferase reporter assay using pGL3-CCL22 (−722/−11), a human CCL22 promoter construct containing −722 to −11 bp upstream from the transcriptional initiation site. Transient transfection of pGL3-Basic (Basic) or pGL3-CCL22 (−722/−11) (CCL22–722) in Jurkat cells was conducted either with or without a Tax-expressing vector. Luciferase assays were performed 24 h after transfection. Luciferase activity was calculated as the fold induction in comparison with the control value (mean ± SD, $n = 3$). One set of representative data is shown from three individual experiments. *D*, Knockdown of the *tax* gene. C8166 and TCL-Kan cells were transfected either with or without Tax or control siRNAs (4 μg each), cultured for 2 days, and subsequently harvested for mRNA preparation. Semiquantitative RT-PCR analysis was performed for Tax, CCL22, and GAPDH. Tax siRNA markedly reduced the steady-state level of CCL22 mRNA as well as Tax mRNA in both cell lines. Representative data from three separate experiments are shown.

Redistribution of CCR4 in primary $CCR4^+CD4^+$ T cells and reorientation of MTOC in HTLV-1-infected T cells toward the contact region

It has been shown that contact with target $CD4^+$ T cells rapidly induces the formation of VS with reorientation of MTOC in HTLV-1-infected T cells toward a cell-cell junction (24). Therefore, the orientation of MTOC toward the contact interface is considered as an indicator of VS formation. To test whether

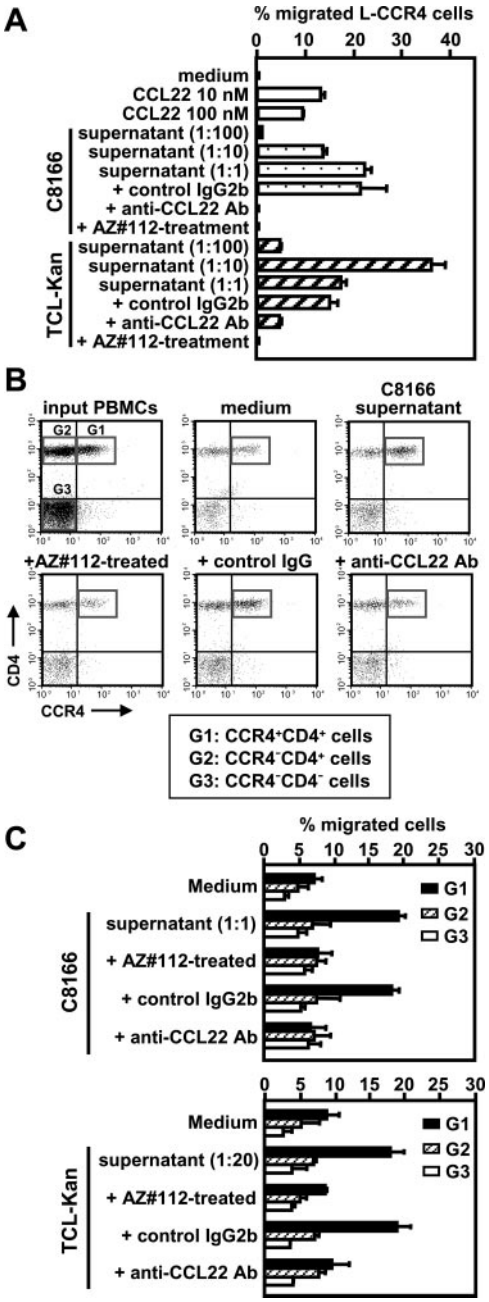

FIGURE 3. Selective attraction of $CCR4^+CD4^+$ T cells in PBMCs from healthy donors by culture supernatants from HTLV-1-infected T cells. *A*, Chemotaxis assays using L-CCR4 cells. Murine L1.2 cells stably expressing human CCR4 (L-CCR4) were pretreated either with or without a CCR4 inhibitor, i.e., AZ no. 112 (20 μM). rCCL22 at the indicated concentrations or culture supernatants from C8166 and TCL-Kan at the indicated dilutions pretreated either with or without a neutralizing Ab against CCL22 (20 μg/ml) or control IgG (20 μg/ml) were used. Cell migration was expressed as a percentage of input cells. Data from three independent experiments are shown as mean ± SD. *B*, Chemotaxis assays using PBMCs, which were pretreated either with or without AZ no. 112 (20 μM), as shown. The culture supernatant of C8166 was pretreated with a neutralizing Ab against CCL22 (20 μg/ml) or control IgG (20 μg/ml), as indicated. Migrated cells were stained for CD4 and CCR4 and evaluated by flow cytometry. Representative data from three independent experiments are shown. *C*, Chemotaxis assays using PBMCs. The chemotaxis assays and staining of migrated cells for CD4 and CCR4 were conducted as described in *B*. Cell migration was expressed as a percentage of input cells. Data from three independent experiments are shown as mean ± SD.

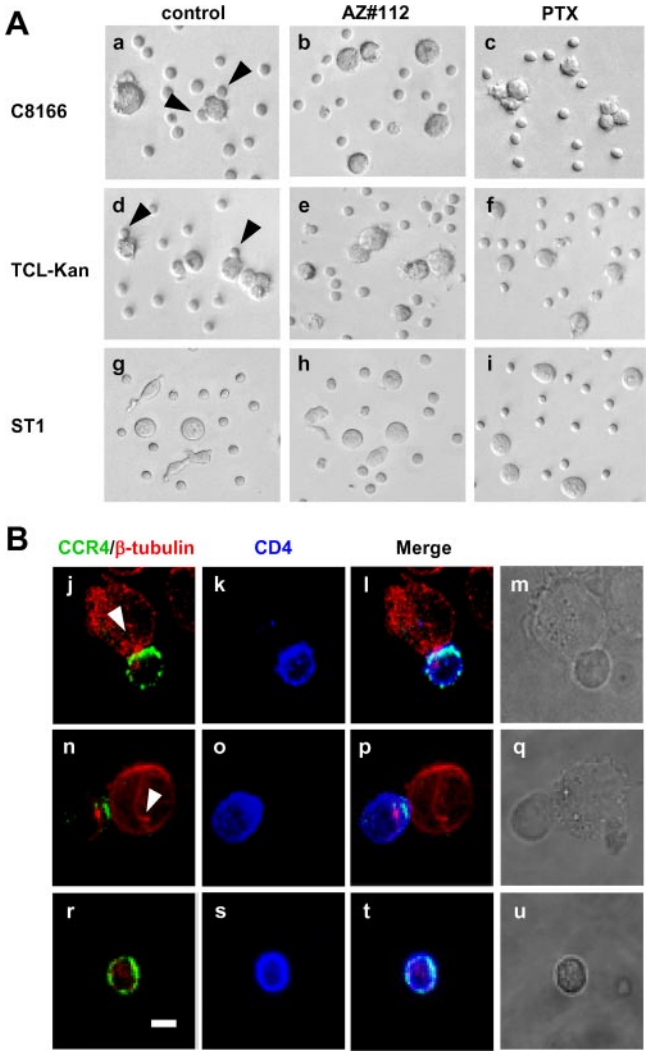

FIGURE 4. Adhesion of CCR4$^+$CD4$^+$ T cells to HTLV-1-infected T cells. *A*, Inhibition of cell adhesion by a CCR4 inhibitor, i.e., AZ no. 112 and PTX. CCR4$^+$CD4$^+$ T cells that were sorted from PBMCs were treated either with or without 20 μM AZ no. 112 or 100 ng/ml PTX for 30 min and mixed with C8166 cells (*a–c*), TCL-Kan cells (*d–f*), or ST1 cells (*g–i*). After 30–60 min, the cells were resuspended vigorously and observed under a light microscope. CCR4$^+$CD4$^+$ T cells attached to tumor cells are shown by the arrowheads. Original magnification: ×400. *B*, Redistribution of CCR4 on CCR4$^+$CD4$^+$ T cells at the cell-cell junction with TCL-Kan cells. CCR4$^+$CD4$^+$ T cells were sorted from PBMCs and cocultured with TCL-Kan cells for 60 min. After fixing, the cells were further stained for β-tubulin. Distribution of CCR4 (green), β-tubulin (red), and CD4 (blue) was observed under a confocal microscope. *r–u* correspond to isolated CCR4$^+$CD4$^+$ T cells, while *j–q* correspond to cell conjugates. Redistributed CCR4 in CCR4$^+$CD4$^+$ T cells conjugated with TCL-Kan cells with (*j* and *l*) or without (*n* and *p*) reoriented MTOC, and redistributed CD4 in the CCR4$^+$CD4$^+$ T cells (*k*, *l*, *o*, and *p*) are shown. Subparts *m*, *q*, and *u* show transmission light images of *j–l*, *n–p*, and *r–t*, respectively. Arrowheads, MTOC. Scale bar, 5 μm.

CCL22-induced cell adhesion also induced VS formation, we next examined the physical relationship between CCR4 and MTOC. Sorted CCR4$^+$CD4$^+$ T and TCL-Kan cells were cocultured for 60 min, fixed with 4% paraformaldehyde, stained for β-tubulin, and observed under a confocal microscope. As shown in Fig. 4*B*, *r–u*, the isolated CCR4$^+$CD4$^+$ T cells exhibited relatively homogeneous distribution of CCR4 over the cell membrane (Fig. 4*B*, *r* and *t*). In contrast, in cell conjugates, CCR4 was predominantly redistributed to the contact region between CCR4$^+$CD4$^+$ T cells and TCL-Kan cells (Fig. 4*B*, *j*, *l*, *n*, and *p*); ~70% of the adhered CCR4$^+$CD4$^+$ T cells exhibited concentrated CCR4 staining at the cell-cell junction. Interestingly, CD4 also redistributed toward the contact region, suggesting the colocalization of CCR4 and CD4 (Fig. 4*B*, *l* and *p*). In contrast to the redistribution of CCR4 to the contact site, only ~10% of TCL-Kan cells bound by CCR4$^+$CD4$^+$ T cells showed reorientation of MTOC at the cell contact site (at the junction (Fig. 4*B*, *j* and *l*) and at random (Fig. 4*B*, *n* and *p*)). These results suggested that, even though the efficiency was relatively low, VS formation was induced between CCR4$^+$CD4$^+$ T cells and TCL-Kan cells.

Table II. *Effect of CCR4-specific inhibitor*

Cell Line	% Adhered CCR4$^+$CD4$^+$ T Cells[a]		
	Control	AZ No. 112	PTX
C8166	11.9 ± 3.3	1.7 ± 0.4*	0.5 ± 0.8*
TCL-Kan	14.2 ± 4.9	3.9 ± 0.7*	2.1 ± 2.8*
ST-1	0.8 ± 2.1	0.5 ± 1.1	0.4 ± 0.6

[a] Percent of primary CCR4$^+$CD4$^+$ T cells adhered to the indicated HTLV-1-infected cell lines in Fig. 4 were enumerated in the CCR4$^+$CD4$^+$ T cell population; the data are expressed as mean ± SD per 10 high-power fields in duplicate wells; *, $p < 0.0001$ when compared with control.

Anti-CCL22 Ab inhibits HTLV-1 infection to primary CD4$^+$ T cells

If the CCL22-CCR4 pathway plays an important role in initial cell-cell interactions between primary CD4$^+$ T cells and HTLV-1 infected T cells, blockade of this pathway would suppress HTLV-1 transmission from HTLV-1-infected T cells to primary CD4$^+$ T cells. To test this hypothesis, we cocultured PHA-treated PBMCs (1×10^4/well) and MMC-treated C91/PL cells (3×10^2/well) in a 96-well flat-bottom plate. Anti-CCL22 Ab or control IgG2b was added to MMC-treated C91/PL cells 12 h before cocultivation and

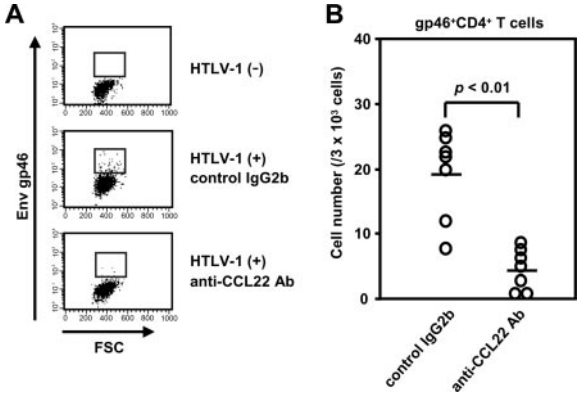

FIGURE 5. Inhibition of HTLV-1 transmission to CD4$^+$ T cells by anti-CCL22 Ab. MMC-treated C91/PL cells were also pretreated with 20 μg/ml anti-CCL22 Ab or control IgG2b for 12 h. PHA-treated PBMCs from healthy donors (1×10^4/well) and MMC-treated C91/PL cells (3×10^2/well) were cocultured in a 96-well flat-bottom plate in the presence of 20 μg/ml anti-CCL22 Ab or control IgG2b. The Abs were added every 12 h for 6 days to maintain sufficient concentrations. The culture medium that was supplemented with IL-2 (100 U/ml) was changed on day 5. Ten days after cocultivation, cells from each well were stained for HTLV-1 Env gp46 and CD4 and analyzed by flow cytometry. *A*, Expression of gp46 in cells gated for scatter characteristics, CD4 expression, and exclusion of propidium iodide. *B*, The numbers of gp46$^+$ cells per 3×10^3 CD4$^+$ cells in each experimental group are shown ($n = 7$). Representative data from three independent experiments are shown.

every 12 h postcocultivation for 6 days. On day 10, when no viable C91/PL cells were observed, PBMCs were collected and costained for CD4 and HTLV-1 Env gp46 to assess HTLV-1 transmission to primary $CD4^+$ T cells. Gag Ags were not used as the viral Ags since at least 3 wk are required for HTLV-infected PBMCs to produce viral particles after cocultivation (23). As shown in Fig. 5*A*, the anti-CCL22 Ab significantly reduced the number of $gp46^+CD4^+$ T cells in comparison with control IgG2b. These data are summarized in Fig. 5*B* (4.7 ± 3.2 vs 19. 2 ± 6.6 (per 3×10^3 cells), respectively, $p < 0.01$ ($n = 7$)). We also found that anti-CCL22 Ab significantly reduced the intensities of gp46 in $gp46^+CD4^+$ T cells in comparison with control IgG2b (63.0 ± 14.3 vs 189.0 ± 44.2, respectively, $p < 0.01$ ($n = 7$)). These results support the hypothesis that blockade of the CCL22-CCR4 pathway suppresses transmission of HTLV-1 to $CCR4^+CD4^+$ T cells by inhibiting initial cell-cell interactions.

Discussion

It is now known that most ATL cases are strongly positive for CCR4 (8, 9), which is known to be selectively expressed by Th2 cells, regulatory T cells, and CLA^+ skin-homing memory/effector T cells (10). Therefore, frequent expression of CCR4 may partly explain the high frequency of skin involvement in ATL patients (8, 9). Furthermore, HTLV-1 may have a mechanism that allows it to be preferentially transmitted to $CD4^+$ T cells expressing CCR4. Given that HTLV-1 is mainly transmitted via close cell-cell contacts (2, 4, 5), HTLV-1-infected T cells may preferentially attract and interact with $CCR4^+CD4^+$ T cells. In the present study, we demonstrated that HTLV-1 Tax is a strong inducer of CCL22, which is a CCR4 ligand (Figs. 1 and 2). Accordingly, HTLV-1-infected T cells expressing Tax produce CCL22 in large amounts (Table I) and preferentially attract $CCR4^+CD4^+$ T cells in PBMCs (Fig. 3). Furthermore, HTLV-1-infected T cells engage in close cell-cell contacts with $CCR4^+CD4^+$ T cells resulting in the formation of VS (Fig. 4) and eventually transmit HTLV-1 to $CCR4^+CD4^+$ T cells (Fig. 5). Because $CCR4^+CD4^+$ T cells migrated toward HTLV-1-infected T cells more robustly than other T cell subsets in PBMCs (Fig. 3, *B* and *C*), this initial selectivity at the time of primary infection may partly explain the strong bias toward the $CCR4^+$ phenotype in HTLV-1-infected $CD4^+$ T cells and eventually ATL cells (8). In contrast, we previously reported that Tax does not induce CCR4 expression in JPX-9 cells upon Cd^{2+} stimulation (8). Therefore, we think that it is unlikely that Tax induces CCR4 in primary $CD4^+$ T cells.

Previously, Shimauchi et al. (37) reported the production of CCL17 and CCL22 by fresh ATL cells upon costimulation with anti-CD3 and -CD28 Abs for 96 h and even without costimulation depending on the case. Given that Tax is a potent inducer of CCL22 and probably of CCL17 (Fig. 2), it may be possible that costimulation of fresh ATL cells strongly induced Tax, which in turn induced the expression of these chemokines. Furthermore, it is well-known that fresh ATL cells from a substantial fraction of cases rapidly express the *tax* gene upon brief in vitro culture (38). Therefore, even without costimulation, fresh ATL cells may express CCL22 and/or CCL17 upon Tax induction by in vitro culture. Indeed, we detected frequent expression of CCL22 and also weakly CCL17 in fresh ATL cells only after overnight culture in parallel with Tax induction (data not shown).

Previously, Igakura et al. (24) demonstrated that HTLV-1-infected T cells from a patient with HTLV-1-associated myelopathy/tropical spastic paraparesis and uninfected $CD4^+$ T cells from autologous and allogeneic donors formed cell-cell conjugates leading to the formation of VS within 40 min. This is in good agreement with our data where the firm adhesion of primary $CCR4^+CD4^+$ T cells to HTLV-1-infected T cells occurred in ~30–60 min (Fig. 4). Furthermore, the fact that the CCR4 antagonist AZ no. 112 as well as PTX potently inhibited cell adhesion between $CCR4^+CD4^+$ T cells and HTLV-1-infected T cells strongly suggested that CCL22 produced by HTLV-1-infected cells is the major facilitator of cell-cell contacts at least in the case of $CD4^+$ T cells. However, we do not exclude the possibility that chemokines other than CCL22 produced by HTLV-1-infected T cells may also play roles in cell-cell interactions between HTLV-1-infected T cells and target cells. This is because in contrast to PTX treatment, AZ no. 112 treatment could not completely inhibit cell adhesions between TCL-Kan and $CCR4^+CD4^+$ T cells (Table II). So far, HTLV-1 Tax has been shown to induce a number of chemokines, including CCL3, CCL5, and CXCL10 (Table I) (25–29). Thus, we may speculate that chemokine systems other than the CCL22-CCR4 system may also promote cell-cell contacts between HTLV-1-infected T cells and target cells expressing appropriate chemokine receptors. In fact, apart from $gp46^+CD4^+$ T cells, we observed significant numbers of $gp46^+CD8^+$ T cells after 10 days of coculture of PHA-activated PBMCs and MMC-treated HTLV-1-infected T cells (data not shown). Although the immortalization of $CD4^+$ T cells is more common, HTLV-1 can infect and immortalize $CD8^+$ T cells as well as $CD4^+$ T cells (39–41). However, $CD8^+$ T cells do not express CCR4; other chemokine systems may facilitate the transmission of HTLV-1 to $CD8^+$ T cells.

The engagement of ICAM-1 promotes the polarization of the MTOC in HTLV-1-infected T cells toward the cell-cell junction with target $CD4^+$ T cells (42), leading to VS formation. Conversely, blocking ICAM-1 on HTLV-1-infected cells abolished microtubule polarization and VS formation (42). These findings indicate that the interaction between LFA-1 on target $CD4^+$ T cells and ICAM-1 on HTLV-1-infected T cells is of prime importance for HTLV-1 transmission. In this context, Tax is known to up-regulate ICAM-1 expression in HTLV-1-infected T cells (36). Furthermore, Tax seems to directly promote VS formation by associating with polarized MTOC and accumulating at the cell-cell junction of HTLV-1-infected T cells conjugated with target $CD4^+$ T cells (43). Thus, the expression of Tax in HTLV-1-infected T cells at the time of transfer into new host individuals or by other stimuli may facilitate HTLV-1 transmission in multiple ways: Tax induces ICAM-1 up-regulation (36) and CCL22 production; CCL22 attracts uninfected host $CCR4^+CD4^+$ T cells and activates their LFA-1; the activated LFA-1 in turn binds with high affinity to ICAM-1 on HTLV-1-infected T cells, thus allowing firm adhesion to the cell surface; the engagement of ICAM-1 by LFA-1 promotes MTOC polarization in HTLV-1-infected T cells and VS formation at the cell junction with target $CD4^+$ T cells (42); and Tax also directly promotes MTOC formation and VS (43). However, our results showed a relatively low frequency of reorientation of MTOC in HTLV-1-infected T cells attached to $CCR4^+CD4^+$ T cells. The reason for the poor induction of MTOC polarization in the present study is not known but it might be due to the use of HTLV-1-infected T cell lines instead of fresh HTLV-1-infected T cells from patients.

The importance of milk-borne infection of HTLV-1 is supported by the presence of infected cells in the milk from carrier mothers (44) and by the experimental transmission of the virus to animal models by oral administration of carrier mother's milk (45, 46). The intervention study that has restrained breast feeding has blocked >80% of the mother-to-infant transmission of HTLV-I in Nagasaki, an endemic area in southwestern Japan (6). These studies clearly indicate that breast milk is an important vehicle of HTLV-1 transmission in human populations, and the results may also imply that certain components in breast milk help in virus

transmission. In this context, lactoferrin, a milk protein that has a variety of antimicrobial and immunomodulatory activities, has been shown to promote *HTLV-1* gene expression in lymphocytes derived from asymptomatic carriers, and it also promotes HTLV-1 transmission to cord blood lymphocytes in vitro (47). Therefore, it is probable that lactoferrin present in the carrier mother's milk promotes HTLV-1 transmission by inducing HTLV-1-infected T cells to express Tax, which in turn induces CCL22 and up-regulates ICAM-1 in HTLV-1-infected T cells. In contrast, it has also been shown that although breast-feeding infants of HIV-infected women ingest large amounts of HIV-1, they generally escape infection. This is partly because of the presence of HIV-1-specific CD8$^+$ CTL in breast milk (48, 49). Given that the CTL response to HTLV-1 is mainly directed against Tax, and Tax-specific CTL circulate in the blood of the majority of healthy carriers (5), CTL present in the breast milk of a carrier mother may reduce Tax-expressing cells. Therefore, HTLV-1-infected T cells in breast milk may have a narrow margin for evading Tax-specific CTL and Tax-promoted interactions with host target cells.

In conclusion, we have demonstrated a new mechanism by which HTLV-1-infected T cells preferentially transmit HTLV-1 to CD4$^+$ T cells where the CCL22-CCR4 pathway plays an important role in promoting cell-cell interactions. Even though HTLV-1 infection would be critically dependent on the expression of HTLV-1 Env receptors such as GLUT-1 and HSPG, we may also include the CCL22-CCR4 system as an important biological factor that promotes HTLV-1 tropism for CCR4$^+$CD4$^+$ T cells.

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Disclosures

The authors have no financial conflict of interest.

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