Gain-of-function *CCR4* mutations in adult T cell leukemia/lymphoma

Masao Nakagawa,* Roland Schmitz,* Wenming Xiao, Carolyn K. Goldman, Weihong Xu, Yandan Yang, Xin Yu, Thomas A. Waldmann,** and Louis M. Staudt**

Lymphoid Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Adult T cell leukemia/lymphoma (ATLL) is an aggressive malignancy caused by human T cell lymphotropic virus type-I (HTLV-I) without curative treatment at present. To illuminate the pathogenesis of ATLL we performed whole transcriptome sequencing of purified ATLL patient samples and discovered recurrent somatic mutations in *CCR4*, encoding CC chemokine receptor 4. *CCR4* mutations were detected in 14/53 ATLL samples (26%) and consisted exclusively of nonsense or frameshift mutations that truncated the coding region at C329, Q330, or Y331 in the carboxy terminus. Functionally, the CCR4-Q330 nonsense isoform was gain-of-function because it increased cell migration toward the CCR4 ligands CCL17 and CCL22, in part by impairing receptor internalization. This mutant enhanced PI(3) kinase/AKT activation after receptor engagement by CCL22 in ATLL cells and conferred a growth advantage in long-term in vitro cultures. These findings implicate somatic gain-of-function *CCR4* mutations in the pathogenesis of ATLL and suggest that inhibition of CCR4 signaling might have therapeutic potential in this refractory malignancy.

CORRESPONDENCE
Louis M. Staudt:
lstaudt@mail.nih.gov

Abbreviations used: ATLL, adult T cell leukemia/lymphoma; CCR4, CC chemokine receptor 4; GPCR, G protein–coupled receptor; huKO, human Kusabira-Orange; PI3K, PI(3) kinase; PTX, pertussis toxin; SNV, single nucleotide variant.

Adult T cell leukemia/lymphoma (ATLL) is one of the most aggressive forms of peripheral T cell lymphoma, with a median survival of <1 yr with current therapy, which consists primarily of cytotoxic chemotherapy (Campo et al., 2011). Molecular analyses of ATLL cells revealed that high expression of CC chemokine receptor 4 (CCR4) is a hallmark of this disease (Yoshie et al., 2002; Ishida et al., 2003; Iqbal et al., 2010). Clinical trials in ATLL of a therapeutic monoclonal antibody directed against CCR4 (KW-0761) are ongoing, and promising early results have been reported (Yamamoto et al., 2010; Ishida et al., 2012).

CCR4 is a chemokine receptor that has a critical role in immune cell trafficking. T-helper type 2 cells (Th2), regulatory T cells (T_{reg}), interluekin-17–producing T-helper cells (Th17), and skin-homing memory T cells express CCR4 on their surface and migrate toward the chemokines CCL17 and CCL22 (Imai et al., 1997, 1998; Yoshie, 2005). The leukemic cells in 90% of ATLL cases express CCR4 on their surface (Ishida et al., 2003). Interestingly, the most frequent sites of ATLL involvement are lymph nodes and skin (Campo et al., 2011), where dendritic cells, M2-phenotype macrophages, Langerhans cells, and cutaneous venules can produce CCL17 and/or CCL22 (Campbell et al., 1999; Vissers et al., 2001; Vulcano et al., 2001; Chong et al., 2004). These observations suggest that CCR4 could have a role in ATLL biology, but it is still unclear whether dysregulation of CCR4 function contributes to ATLL pathogenesis.

Human T cell lymphotropic virus type-I (HTLV-I) is believed to be the causative agent for ATLL (Matsuoka and Jeang, 2007; Campo et al., 2011). However, only a small proportion of HTLV-I carriers (2–7%) develop ATLL with a long latency (40–50 yr; Arisawa et al., 2000; Campo et al., 2011). Thus, acquisition of somatic mutations in cellular genes is likely to be crucial for the development of ATLL. Identifying such somatic mutations is essential not only for understanding ATLL pathogenesis but also for defining molecular targets for therapy.

*M. Nakagawa and R. Schmitz contributed equally to this paper.
**T.A. Waldmann and L.M. Staudt contributed equally to this paper.

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Somatic mutations in *p53*, *NOTCH1*, *JAK3*, and *FAS* have been reported in ATLL (Elliott et al., 2011; Yamagishi and Watanabe, 2012), but our knowledge of genetic aberrations in this malignancy is nonetheless incomplete.

In the present study, we used whole transcriptome analysis (RNA-seq) to discover activating mutations in *CCR4*, which we found to be a frequent genetic event in this malignancy. Functional analysis clarified the gain-of function nature of these mutations, suggesting that dysregulation of CCR4 function is key to the pathogenesis of ATLL.

RESULTS AND DISCUSSION

Frequent mutation of *CCR4* in ATLL

We performed RNA-seq of peripheral blood leukemia samples from two ATLL patients, TW36R and TW51R, which allowed us to identify 85 and 127 genes with potential coding region mutations in these two samples, respectively. These candidates included two genes that were mutated in both samples: *CCR4* and *MICALL1*. High expression of *CCR4* is a well-known hallmark of ATLL, whereas *MICALL1* has not been implicated in this disease. Importantly, both ATLL samples had the same nonsense *CCR4* mutation affecting the Y331 codon (Y331*), suggesting that *CCR4* mutations might play a critical role in ATLL pathogenesis. The percentage of mutant *CCR4* sequencing reads was 39% in TW36R and 55% in TW51R. Because both blood samples had a high proportion of malignant cells (TW36R: 90%, TW51R: 84%), the *CCR4* mutations are likely to be heterozygous and potentially might exert a dominant functional effect.

By Sanger sequencing of genomic DNA, we confirmed the heterozygous nature of the *CCR4* nonsense mutations in TW36R and TW51R. We extended this analysis to an additional cohort of ATLL primary patient samples (n = 41) and ATLL cell lines (n = 12). *CCR4* mutations were detected in 26.4% (14/53) of ATLL samples (Fig. 1, A and B; and Table S1). In five cases for which paired normal DNA was available, three different *CCR4* mutations were detected only in the ATLL cells (Q330*, Q330 frameshift, and Y331*), demonstrating that they were acquired somatically during malignant transformation or progression (Fig. 1 C). *CCR4* mutations were identified in both primary ATLL patient samples (24%, 10/41) and in ATLL cell lines (33.3%, 4/12). Mutations were heterozygous in all samples except one cell line (ATL42T+). We also confirmed that mutant CCR4 mRNAs were heterozygously expressed in six primary ATLL samples (Table S1). These findings indicated that *CCR4* mutations in ATLL might be either gain-of-function or potentially dominant negative.

All *CCR4* mutations were either nonsense or frameshift in nature and affected the codons for four nearby amino acids (F326, C329, Q330, and Y331) that are located in an evolutionarily conserved region in the carboxy terminus of the protein (Fig. 1, A and B). The most frequent nonsense mutation affected codon Y331 (Y331*; 7/53, 13%). Four cell lines (ED40515(+), ED40515(−), ED41214(+), and ED41214 C(−)) had Q330* nonsense mutations, which is understandable given that these four lines were established from the same patient at different clinical time points.

In cancer genome studies cataloged in the COSMIC database (Catalogue of somatic mutations in cancer) or conducted by The Cancer Genome Atlas (TCGA) initiative, we found only three primary human cancer samples with CCR4 nonsense mutations, and these mutations did not target the carboxy-terminal cytoplasmic domain. Thus, truncation of the CCR4 cytoplasmic domain by somatic mutation appears to be a specific and frequent genetic event to ATLL.

Mutant CCR4 enhances chemotaxis toward CCR4 ligands

Chemokine receptors, including CCR4, belong to the seven-transmembrane G protein–coupled receptor (GPCR) family. All *CCR4* mutations in ATLL encode truncated receptors that lack most of the carboxy-terminal cytoplasmic domain, which typically serves a regulatory role in GPCRs (Fig. 1, A and B; Luttrell and Lefkowitz, 2002). The loss of this region of CCR4 might deregulate its activity, potentially altering the migration of ATLL cells in response to CCR4 ligands. The *CCR4* mutations in ATLL are reminiscent of mutations affecting the chemokine receptor CXCR4 in WHIM syndrome, a human immunodeficiency disease (Diaz, 2005). Most *CXCR4* mutations in WHIM syndrome are nonsense or frameshift mutations that truncate the carboxy-terminal cytoplasmic domain of the protein, conferring a gain-of-function phenotype with respect to chemotaxis toward the CXCR4 ligand SDF1 (Balabanian et al., 2005; Diaz, 2005; Kawai et al., 2005).

We therefore hypothesized that mutant CCR4 isoforms might enhance chemotaxis of the affected cells to CCR4 ligands. To study this, we infected a mouse myeloid cell line, 32Dβ, with retroviral vectors expressing WT CCR4 (CCR4-WT) or CCR4-Q330* coding regions and tested the migration of the transduced cells toward CCL22, the most potent CCR4 ligand (Fig. 2 A; D'Ambrosio et al., 2002). Surface CCR4 levels were similar between CCR4-WT– and CCR4-Q330*–transduced 32Dβ cells, whereas mock vector–transduced 32Dβ cells did not have detectable CCR4 expression (Fig. 2 B). Mock vector–infected 32Dβ cells did not migrate toward CCL22 (Fig. 2, A and B). CCR4-WT–transduced 32Dβ cells migrated with a typical bell-shaped dose–response curve (Fig. 2, A and B). Compared with CCR4-WT, CCR4-Q330*–transduced 32Dβ cells migrated to a significantly greater extent (Fig. 2, A and B). These results support the view that the CCR4 mutants in ATLL are gain-of-function with respect to ligand-directed chemotaxis.

Next we evaluated the ability of CCR4 isoforms to mediate chemotaxis in ATLL cell lines (Fig. 2 C). Most ATLL cell lines express CCR4 on their surface (not depicted), reflecting the high frequency (~90%) of CCR4 expression in primary ATLL cases (Ishida et al., 2003). To evaluate CCR4-WT and CCR4-Q330* in ATLL cells, we first knocked down the endogenous expression of CCR4 in ED40515(+) cells using an shRNA targeting the 3′ untranslated region (UTR) of the CCR4 mRNA. This resulted in a >90% reduction of surface CCR4 expression (Fig. 2 D) and substantially

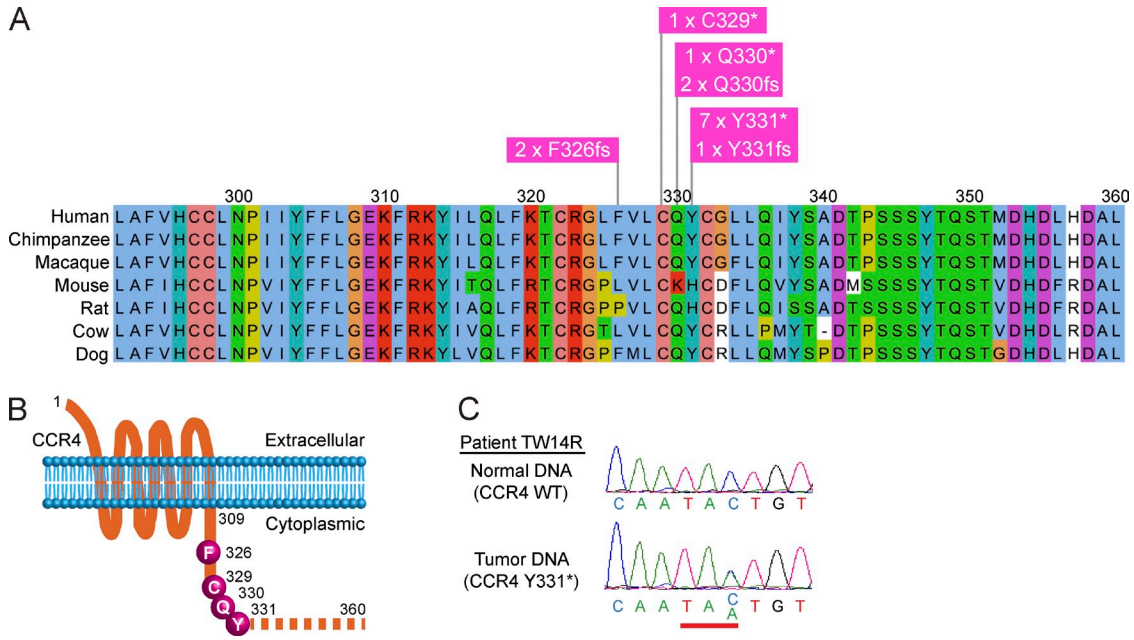

Figure 1. CCR4 mutations in ATLL. (A) Amino acid residues in the carboxy-terminal region of CCR4. (B) Schematic of CCR4 mutant isoforms in ATLL. (C) DNA sequence of the CCR4-Y331* mutation in the TW14R ATLL biopsy sample by Sanger sequencing (bottom). The WT sequence of normal DNA obtained from the same patient is shown in the top.

decreased chemotaxis of the cells (Fig. 2 C). Cells were then transduced with retroviruses expressing CCR4-WT or CCR4-Q330* coding regions, resulting in cell populations with equivalent expression of CCR4 on the cell surface (Fig. 2 D). In chemotaxis assays with CCL17, CCR4-WT–reconstituted cells exhibited greater dose-dependent migration than mock vector–reconstituted cells, but migration of CCR4-Q330*–reconstituted cells was significantly greater than either of the other populations (Fig. 2 C, left). In response to CCL22, the most potent CCR4 ligand (D'Ambrosio et al., 2002), CCR4-Q330*–reconstituted ED40515(+) cells again displayed significantly greater chemotaxis than CCR4-WT–reconstituted cells and responded better to lower concentrations of the ligand (Fig. 2 C, right). Because CCR4 mutations in ATLL samples were heterozygous, we also analyzed whether these mutations could enhance chemotaxis in the presence of WT CCR4. To this end, we transduced the shCCR4-ED40515(+) line with one retroviral vector that coexpresses CCR4-WT and the human Kusabira-Orange (huKO) fluorescent protein and another vector that coexpresses CCR4-Q330 and GFP. This strategy allowed us to compare the phenotype of GFP/huKO double-positive cells that expressed CCR4-WT and Q330* with GFP or huKO single-positive cells that only ectopically expressed one CCR4 isoform (Fig. 2, E and F). Cells expressing both CCR4-WT and CCR4-Q330* showed greater CCL22-directed migration than cells expressing CCR4-WT alone, which was similar to the phenotype of cells expressing CCR4-Q330* alone (Fig. 2 F). These results suggest that ATLL cells may acquire *CCR4* mutations to migrate more effectively toward their ligands.

Mutant CCR4 impairs receptor internalization after CCL22 binding

The carboxy-terminal region of CCR4 that is truncated by mutations in ATLL contains a serine- and threonine-rich motif that is shared by many GPCRs (Fig. 1 A, amino acid positions 342–351). These serine and threonine residues become rapidly phosphorylated in response to ligand, which results in receptor internalization and contributes to "desensitization" of the cells to ligand (Luttrell and Lefkowitz, 2002). Carboxy-terminal truncation of *CXCR4* in WHIM syndrome impairs receptor internalization, contributing to the enhanced migration of these cells in response to ligand (Balabanian et al., 2005; Kawai et al., 2005).

We therefore studied the change in surface CCR4 levels after CCL22 exposure in CCR4-WT– and CCR4-Q330*–reconstituted ED40515(+) cells. In CCR4-WT–reconstituted cells, surface CCR4 levels declined rapidly, with a 58% reduction at 5 min after CCL22 exposure and a maximum reduction of 75% at 20 min (Fig. 2 G). In comparison, CCR4 internalization in CCR4-Q330*–reconstituted cells was significantly impaired, with a 27% of reduction at 5 min and reaching only a 54% reduction at 20 min (Fig. 2 G). These results suggest that the ATLL CCR4 mutants impair desensitization by ligand, which likely contributes to the enhanced chemotaxis of cells bearing these mutants.

Mutant CCR4 enhances PI(3) kinase (PI3K)/AKT signaling in response to ligand

We explored the influence of the ATLL CCR4 mutants on PI3K–dependent activation of AKT because it has been

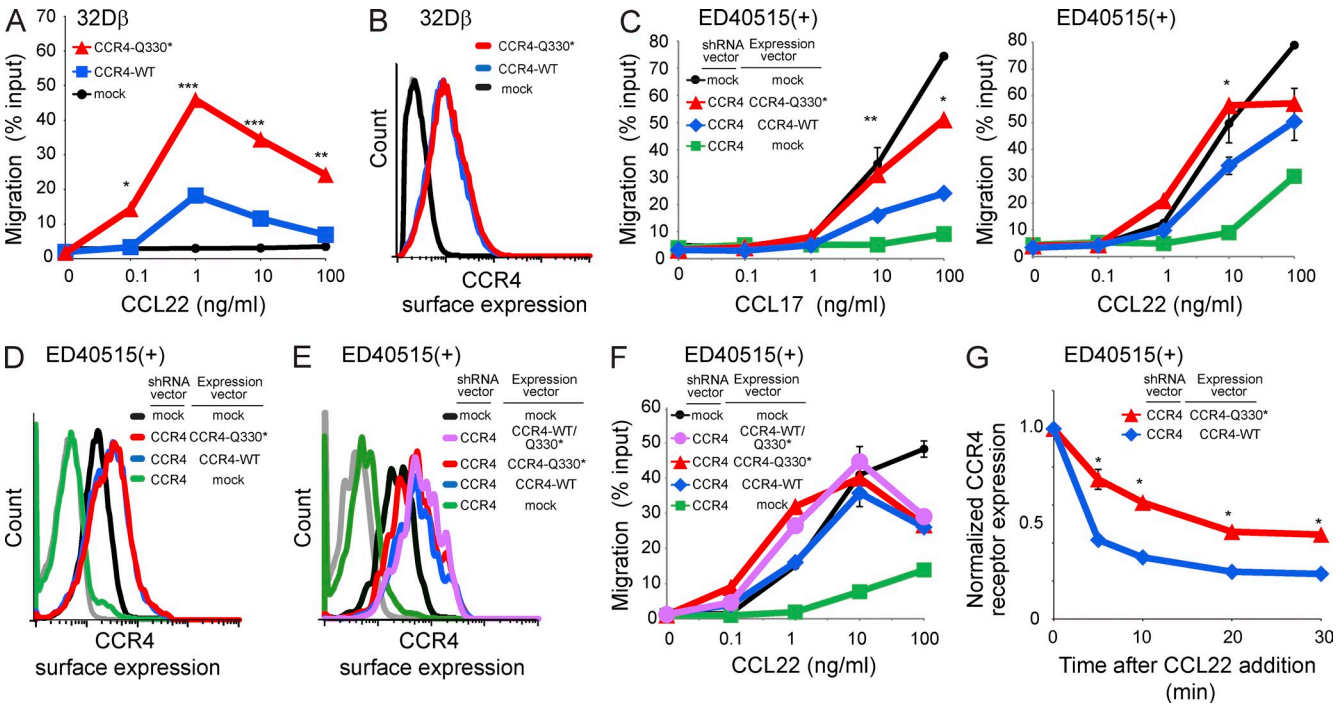

Figure 2. CCR4 mutant isoforms enhance chemotaxis and impair receptor internalization. (A) CCL22-mediated chemotaxis of mouse 32Dβ cells ectopically expressing CCR4-WT or CCR4-Q330*. In these Transwell assays, the lower chamber contained the indicated amount of CCL22. After a 2-h incubation, the number of cells migrating from the upper to lower chamber was determined and plotted as a percentage of the input cell number. (B) Surface CCR4 expression levels of 32Dβ cells ectopically expressing CCR4-WT or CCR4-Q330* analyzed by FACS. (C) Chemotactic ability of CCR4-WT– or CCR4-Q330*–reconstituted ED40515(+) ATLL cells toward CCL17 and CCL22. (D) Surface CCR4 expression levels in CCR4-WT– or CCR4-Q330*–reconstituted ED40515(+) ATLL cells analyzed by FACS. (E) Surface CCR4 expression levels analyzed by FACS in ED40515(+) ATLL cells ectopically expressing CCR4-WT and/or CCR4-Q330*. (B, D, and E) Isotype control IgG staining is indicated in gray. (F) CCL22-induced chemotaxis of ED40515(+) ATLL cells ectopically expressing CCR4-WT and/or CCR4-Q330*. (G) Time course of surface CCR4 levels after CCL22 exposure in CCR4-WT– or CCR4-Q330*–reconstituted ED40515(+) ATLL cells. Surface CCR4 levels were analyzed by FACS and normalized to the levels at time 0. Data in all panels are presented as mean ± SEM of technical duplicates representative of at least two biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for a comparison between CCR4-WT and CCR4-Q330*.

reported that binding of CCL22 to CCR4 activates AKT in CEM leukemic T cells and in human Th2 cells (Cronshaw et al., 2004). We first studied two ATLL cell lines: ED40515(+), which bears a *CCR4-Q330** mutant allele, and KOB, in which *CCR4* is WT. Immunoblot analysis revealed that baseline levels of phospho-AKT (P-AKT), a measure of AKT activation, were much lower in ED40515(+) than in KOB (Fig. 3 A, lane 2 vs. lane 6). However, ED40515(+) showed stronger induction of AKT phosphorylation at 10 min after CCL22 exposure than KOB (Fig. 3 A, lane 3 vs. lane 7). The activation of AKT was transient in both cell lines, decreasing by 30 min after stimulation (Fig. 3 A, lanes 4 and 8). These findings indicated that AKT activation is one of the signaling pathways downstream of CCR4 in ATLL cells and suggested that CCR4 mutations might affect the magnitude of AKT activation. To accurately evaluate the relative ability of CCR4 isoforms to activate AKT, we studied ED40515(+) cells that were transduced with a CCR4 shRNA to knock down endogenous CCR4 expression and were reconstituted with CCR4-WT or CCR4-Q330*. Cells were treated with CCL22, and P-AKT levels were measured by FACS at various time points after exposure (Fig. 3 B). Knockdown of endogenous CCR4 reduced AKT activation by CCL22 compared with mock-infected cells, confirming that AKT activation was dependent on CCR4. Ectopic provision of CCR4-WT did not restore AKT activation despite expression of CCR4 at a higher level than in mock-transduced ED40515(+) cells (Fig. 2 D), presumably because the endogenous *CCR4* locus in ED40515(+) encodes the CCR4-Q330* isoform. In contrast, cells reconstituted with CCR4-Q330* restored robust AKT activation in response to CCL22 (Fig. 3 B). In KOB cells, reconstitution with CCR4-WT modestly increased P-AKT activation in response to CCL22, but cells reconstituted with CCR4-Q330* again responded with a significantly greater rise in P-AKT levels (Fig. 3, C and D). The effect of heterozygous mutation of CCR4 on AKT activation was analyzed using the dual fluorescence strategy described above for experiments depicted in Fig. 2 (E and F). After CCL22 treatment, ED40515(+) cells expressing both CCR4-WT and CCR4-Q330* or CCR4-Q330* alone had elevated p-AKT levels compared with cells expressing only CCR4-WT (Fig. 3 E). Together, these data demonstrate that *CCR4* mutations in ATLL enhance AKT activation in response to ligand engagement.

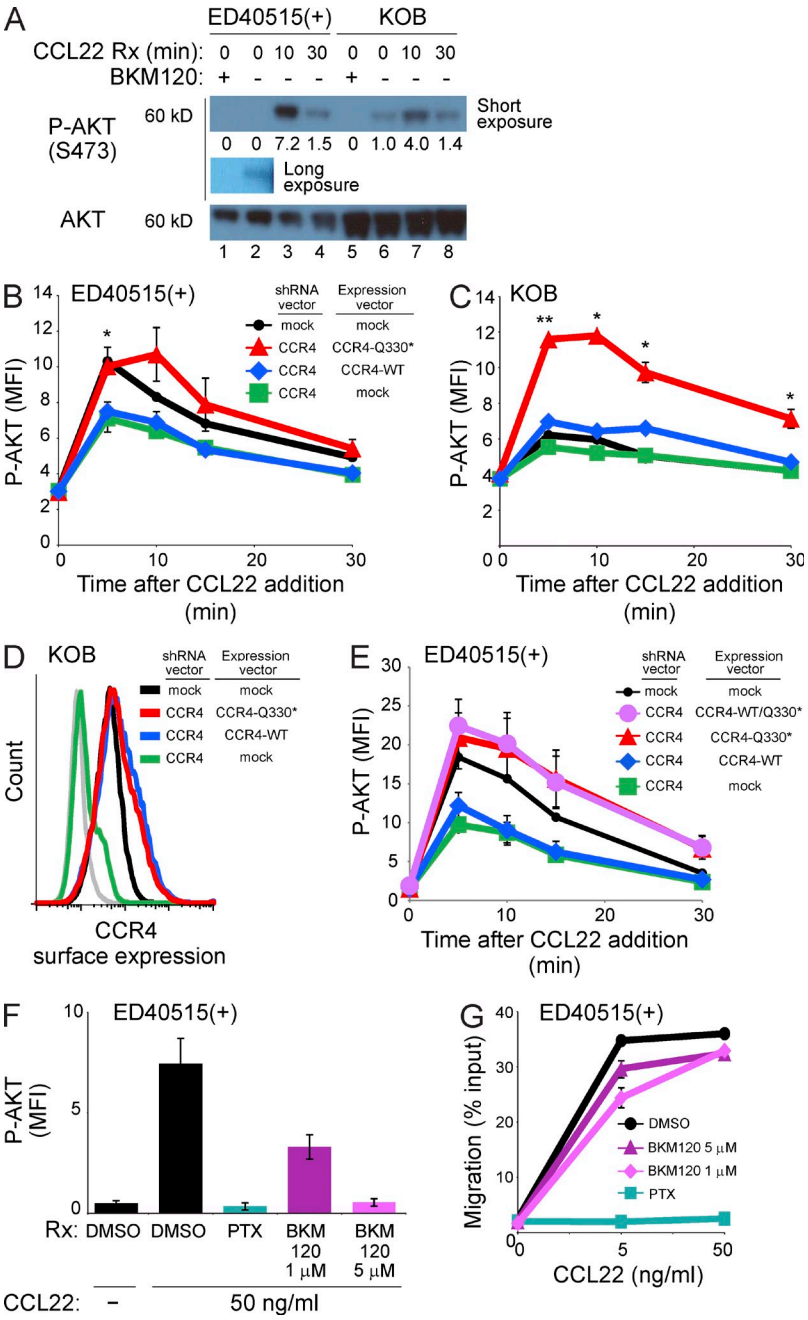

Figure 3. Enhanced PI3K/AKT activation by mutated CCR4. (A) Immunoblot analysis for P-AKT levels in ED40515(+) and KOB ATLL cells after CCL22 exposure (50 ng/ml). Blots using P-AKT (S473) antibody are shown with short and long exposure times. Relative scanning densitometry estimates of P-AKT levels are depicted under the panel showing the short exposure. The pan-PI3K inhibitor BKM120 was used at 1 μM. (B and C) Time course experiment of AKT activation after CCL22 exposure (50 ng/ml) using CCR4-reconstituted ED40515(+) cells (B) and KOB cells (C), transduced as indicated with shRNA and cDNA expression vectors. P-AKT (S473) levels were analyzed by FACS at the indicated times after CCL22 exposure. MFI, mean fluorescence intensity. (D) Surface CCR4 expression levels in CCR4-WT– or CCR4-Q330*–reconstituted KOB cells. Isotype control IgG staining is indicated in gray. (E) Time course experiment of AKT activation after CCL22 exposure (50 ng/ml) in ED40515(+) ATLL cells ectopically expressing CCR4-WT and/or CCR4-Q330*. (F) P-AKT (S473) levels of ED40515(+) treated with inhibitors. Cells were pretreated with 100 ng/ml PTX for 16 h or the indicated amount of the pan-PI3K inhibitor BKM120 for 1 h and then incubated with 50 ng/ml CCL22 for 5 min. P-AKT (S473) was analyzed by FACS. (G) Chemotaxis of ED40515(+) cells treated with inhibitors. Cells were pretreated with PTX or BKM120 as in F and then used in a CCL22-mediated chemotaxis assay. Data in all panels are presented as mean ± SEM of technical duplicates representative of at least two biological replicates. *, $P < 0.05$; **, $P < 0.01$ for a comparison between CCR4-WT and CCR4-Q330*.

We next investigated whether AKT activation is involved in CCR4-mediated chemotaxis in ATLL. The pan-PI3K inhibitor BKM120 abrogated CCR4-mediated AKT activation in ED40515(+) cells in a dose-dependent manner, indicating that PI3K signaling contributes to AKT activation (Fig. 3 F). However, BKM120 treatment only partially inhibited CCR4-mediated chemotaxis by ED40515(+) cells (Fig. 3 G). In contrast, the $G_{\alpha i}$ inhibitor pertussis toxin (PTX) abrogated AKT activation and chemotaxis in these cells (Fig. 3, F and G). Thus, CCR4-mediated chemotaxis of ATLL cells is not primarily caused by PI3K-dependent AKT signaling, but instead depends on $G_{\alpha i}$, consistent with previous work (Cronshaw et al., 2004).

Mutant CCR4 promotes ATLL expansion in the presence of ligand

Lastly, we tested whether the acquisition of *CCR4* mutations by ATLL cells imparts a selective growth advantage relative to cells with WT *CCR4*. After shRNA-mediated knockdown of endogenous CCR4 expression, ED40515(+) cells were transduced with a retrovirus expressing CCR4-WT together with GFP or with a retrovirus expressing only CCR4-Q330* (Fig. 4 A, Exp. 1). After puromycin selection of infected cells, these two cell populations were mixed in equal numbers and cultured with or without CCL22 for 12 d. The ratio of GFP^-/CCR4-Q330*–expressing cells versus GFP^+/CCR4-WT–expressing cells was monitored every 4 d by FACS (Fig. 4 B,

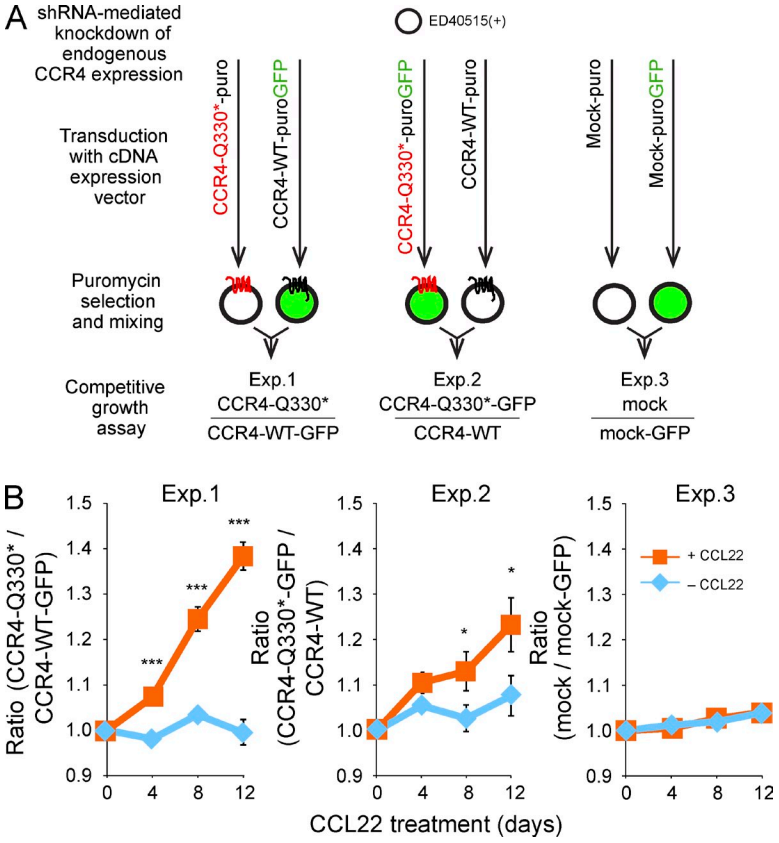

Figure 4. Growth advantage of CCR4–Q330*–reconstituted ATLL cells. (A) Schematic of the competitive growth assay. ED40515(+) cells depleted of endogenous CCR4 expression by RNA interference were transduced with the indicated CCR4-expressing vectors or mock vectors. After puromycin selection, two transduced populations, one expressed GFP and the other did not, were mixed equally and co-cultured for 12 d in vitro. The ratio of the two populations was determined by FACS every 4 d. (B) CCR4-Q330*–transduced cells have a competitive growth advantage. CCL22 was added every 2 d at 50 ng/ml. The ratios of the two populations were normalized to the value at day 0. Growth curves represent the mean of eight replicates obtained from four biologically independent experiments ± SEM. *, $P < 0.05$; ***, $P < 0.001$.

Exp. 1). Without CCL22, the ratio of the two populations did not change. In contrast, in the presence of CCL22, CCR4-Q330*–reconstituted cells preferentially expanded in a time-dependent manner. To confirm this finding, we performed a GFP-swapping experiment in which the GFP^+ cells expressed CCR4-Q330* and the GFP^- cells expressed CCR4-WT (Fig. 4 A, Exp. 2). Again, CCR4-Q330*–reconstituted cells had a selective growth advantage in the presence of CCL22 (Fig. 4 B, Exp. 2). As a negative control, we used empty vectors to create GFP^+ and GFP^- populations and observed no change in the GFP^+/GFP^- ratio in the presence or absence of CCL22 (Fig. 4, A and B, Exp. 3).

In conclusion, the present study demonstrates that mutations in *CCR4* are a frequent genetic event in ATLL and provides a mechanistic rationale for their selection in this cancer. Mutant CCR4 isoforms enhanced migration of ATLL cells toward CCL17 and CCL22 and additionally promoted PI3K/AKT activation in response to ligand engagement, leading us to propose two nonmutually exclusive hypotheses regarding the role of *CCR4* mutations in ATLL pathogenesis. First, enhanced migration of ATLL cells along a CCL17/CCL22 gradient might allow them to access a favorable microenvironmental niche that could support their proliferation and/or survival. Lymph nodes and skin are plausible ATLL niches because CCL17 and CCL22 are known to be produced by dendritic cells and M2-phenotype macrophages in lymph nodes and also by Langerhans cells and venules in skin (Campbell et al., 1999; Vissers et al., 2001; Vulcano et al., 2001; Chong et al., 2004). Previous studies showed that monocyte and M2-phenotype macrophages can promote ATLL cell growth through cell–cell interaction and by paracrine mechanisms (Chen et al., 2010; Komohara et al., 2013). Thus, the chemotaxis promoted by CCR4 mutations in ATLL might set up favorable interactions between ATLL cells and immune bystander cells and/or disrupt homeostatic mechanisms that keep the growth of these cells in check. Recently, WHIM syndrome–like *CXCR4* somatic mutations have been detected in the malignant cells of patients with Waldenström's macroglobulinemia, and these mutations correlated with bone marrow involvement as well as chemotherapy drug resistance (Roccaro et al., 2014; Treon et al., 2014). Given that CCR4 mutations and WHIM syndrome–like CXCR4 mutations share similar biological properties in terms of chemotaxis and ATK activation (Cao et al., 2014; Treon et al., 2014), it will be interesting to determine whether *CCR4* mutations affect the frequency of lymph node or skin involvement in ATLL or the clinical course.

A second hypothesis raised by our findings is that *CCR4* mutations may be selected in ATLL for their ability to enhance signaling downstream of this chemokine receptor, including activation of the PI3K/AKT signaling pathway. During physiological CCR4 signaling, ligand desensitization occurs in part because CCR4 is down-modulated from the cell surface, a process which is disrupted by the *CCR4* mutations

in ATLL. Perhaps as a result, ATLL cells bearing CCR4 mutant isoforms displayed prolonged PI3K/AKT activation in response to ligand. Given the importance of PI3K/AKT signaling to both cellular metabolism and survival, this enhanced PI3K/AKT response might provide a selective advantage for ATLL cells. Indeed, in our long-term competitive growth assay, ATLL cells expressing mutant CCR4 outgrew cells with WT CCR4 in the presence of CCR4 ligand. Finally, it is conceivable that both hypotheses detailed above may pertain. Specifically, the ability of CCR4 mutants to increase chemotaxis toward CCR4 ligands would expose them to higher ligand concentrations, which might contribute to their growth and/or survival.

Our findings provide a rationale to test whether inhibition of CCR4 signaling might have therapeutic potential for patients with ATLL. Either a CCR4 inhibitor or a PI3K inhibitor might be considered (Pease and Horuk, 2014). The anti-CCR4 monoclonal antibody KW-0761, which is showing promising results in clinical trials (Yamamoto et al., 2010; Ishida et al., 2012), was designed to promote antibody-dependent cellular cytotoxicity of ATLL cells. This antibody only inhibits chemotaxis weakly (Ishida et al., 2006), and its effect on PI3K/AKT signaling has not been evaluated. To improve the therapy of ATLL, our findings would support the development of a therapeutic anti-CCR4 antibody that both inhibits CCR4 signaling and mediates antibody-dependent cellular cytotoxicity.

MATERIALS AND METHODS

Experimental design. All experiments presented have been repeated at least two times, and consistent results were obtained. Data are depicted as means ± SEM. Statistical comparisons were made using the Student's *t* test. $P < 0.05$ was considered statistically significant.

Patient samples and cell lines. Written informed consent was obtained in accordance with the Declaration of Helsinki and was approved by the Investigational Review Board of the National Cancer Institute (NCI). Some samples were obtained before cytotoxic chemotherapy, whereas others were taken after treatment (Table S1). PBMCs were isolated from ATLL patients by Ficoll-Hypaque. In cases where the ATLL cells were <70% of the mononuclear cells, the ATLL cells were purified by an initial negative selection magnetic column method (Miltenyi Biotec) to enrich for $CD4^+$ cells followed by a positive selection on a CD25 column. The resultant population consisted of 70–95% $CD4^+CD25^+$ ATLL cells. The ATLL cell lines were provided by the following researchers: M. Maeda (Kyoto University, Kyoto, Japan; ED40515(+), ED40515(−), ED41214(+), ED41214C(−), ATL43T(+), ATL43Tb(−), ATL55T(+), and ATL42T(+)), Y. Yamada (Nagasaki University, Nagasaki, Japan; ST1, KOB, KK1, and LMY1), T. Hata (Nagasaki University; ST1), T. Naoe (Nagoya University, Nagoya, Japan; ATN1), and N. Arima (Kagoshima University, Kagoshima, Japan; Su9T01). 32Dβ (a variant of the mouse 32D myeloid cell line engineered to express the human IL-2 receptor β subunit) was also used. IL2-dependent cell lines (ED40515(+), ED41214(+), ATL43T(+), ATL55(+), ATL42T(+), KOB, KK, LMY1, and 32Dβ) were cultured with RPMI/10% FCS/penicillin-streptomycin with 100 IU/ml human recombinant IL2. Other lines were cultured with RPMI/10% FCS/penicillin-streptomycin. ED40515(+) and KOB were engineered to express ecotropic retroviral receptors and TET repressor for transduction of retroviral shRNA vectors and expression vectors as previously described (Schmitz et al., 2012).

Reagents and antibodies. Doxycycline, puromycin, ethidium bromide, and Lipofectamine 2000 were purchased from Invitrogen. Recombinant human CCL17/TARC and recombinant human CCL22/MDC were purchased from R&D Systems. Human recombinant IL2 was obtained from Roche. Anti–human CCR4 antibodies (clone 1G1) conjugated to PE and Alexa Fluor 647 fluors were purchased from BD. The following antibodies were purchased from Cell Signaling Technology: Akt, P-Akt (Ser473; D9E), P-Akt (S473)–Alexa Fluor 647 (D9E), and Alexa Fluor 647–conjugated isotype control antibody (DA1E). Anti–rabbit IgG-HRP antibody was purchased from GE Healthcare. CD8a(Lyt 2) microbeads were purchased from Miltenyi Biotec. BKM120 was purchased from Selleck Chemicals. PTX was purchased from Sigma-Aldrich. Big Dye Terminator v1.1 was purchased from Applied Biosystems. Human gamma globulin fraction II was purchased from ICN Biomedicals Inc. Paraformaldehyde was purchased from Electron Microscopy Sciences.

RNA-seq. RNA was extracted using the AllPrep kit (QIAGEN) and sequencing libraries were prepared using the TruSeq RNA sample Prep kit v2 (Illumina) according to the manufacturer's instructions. Paired-end 108-bp read sequencing was performed on a HiSeq 2000 system (Illumina). The mapping of paired-end reads and the extraction of putative single nucleotide variants (SNVs) were performed as previously described (Schmitz et al., 2012). In brief, paired-end reads were mapped to the RNA sequences in the RefSeq database (NCBI build 37) using the Burrows-Wheeler Aligner (BWA) software with default parameters. Reads that failed to map to RefSeq were mapped to RNA sequences in the Ensembl database. The remaining unmapped reads were mapped to the human genome assembly (NCBI build 37). Mutant SNV calls were declared if more than two reads were mutated and the ratio of mutant reads versus total coverage was >20%. SNVs that corresponded to single nucleotide polymorphisms in the dbSNP database (build #132), the 1000 Genomes database (May 2011 release), and the NHLBI GO Exome Sequencing Project (ESP) database (ESP5400 December 2011 release) were excluded. RNA-seq data were submitted to the NCBI Short Read Archive (SRA; accession no. SRP042199).

Sanger DNA resequencing. Genomic DNA of CCR4 exon 2 region was amplified by PCR using the primers CCR4-E2F, 5′-CTTCCCCT-CATTAGCTGCTTCTGGTTG-3′; and CCR4-E2R, 5′-CCTGAC-ACTGGCTCAGGAATCTCTTAC-3′. The purified PCR products were sequenced using a Big Dye Terminator v1.1 cycle sequencing kit and analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems). The following primers were used for sequencing: CCR4-E2.1F, 5′-CCTTC-CTGGCTTTCTGTTCAGCACTTG-3′; and CCR4-E2.1R, 5′-TGA-TTTCCAGGGAGCTGAGAACCTTCC-3′.

Sanger RNA resequencing. RNA was DNase-digested and reverse transcribed using the Omniscript RT kit (QIAGEN). The CCR4 coding region was amplified by PCR using primers CCR4-E2F and CCR4-E2R. The purified PCR products were cloned using the TOPO XL PCR Cloning kit (Invitrogen). Sequencing was performed as described in the Sanger DNA resequencing section.

CCR4 mutation search of public databases. cBioPortal and COSMIC were used.

Retroviral vectors and retroviral transduction. CCR4-WT and CCR4-Q330* cDNA was PCR amplified from ED40515(+) cDNA using the primers HindIII-CCR4-S, 5′-GGAAGCTTTTGAAAGGCACCGGGTC-3′; and CCR4-TAG-BamHI-AS, 5′-CGGGATCCCTACAGAGCATCATG-GAGAT-3′. The amplified fragments were cloned into HindII–BamHI sites of doxycycline-inducible pRetroCMV/TO/puro and pRetroCMV/TO/PG vectors. MSCV-CCR4-WT-ires-huKO or MSCV-CCR4-Q330*-ires-GFP was made by inserting HindIII (blunted)–XhoI fragments from pRetroCMV/TO/CCR4-WT-puro or pRetroCMV/TO/CCR4-Q330*-puro vectors into EcoRI(blunted)–XhoI sites of MSCV-ires-huKO or MSCV-ires-GFP (provided by S. Tsuzuki, Aichi Cancer Center Research Institute, Nagoya, Japan). shRNA targeting the 3′ UTR of CCR4 was cloned into doxycycline-inducible pRSMX-puro vector with these two annealed oligos (shRNA target

sequences are underlined): shCCR4-4A11_F, 5′-GATCCCGAATGAAGTTGTAGGTAATTTCAAGAGAATTACCTACAACTTCATTCTTTTT-3′; and shCCR4-4A11_R, 5′-AGCTAAAAAGAATGAAGTTGTAGGTAATTCTCTTGAAATTACCTACAACTTCATTCGG-3′. pBMN-shCCR4-ires-Lyt2 was made by insertion of XbaI–BsmI fragment from pRSMX-shCCR4-puro vector into XbaI–BsmI sites of pBMN-ires-Lyt2 (provided by G. Nolan, Stanford University, Stanford, CA). Retroviruses for ED40515(+) and KOB were produced by cotransfection of 293T cells with helper plasmids expressing gag-pol genes and an ecotropic pseudotyping env gene and a retroviral vector using Lipofectamine 2000 as described previously (Schmitz et al., 2012). For 32Dβ, retroviruses were produced by transfection of PlatE with retroviral vectors. Target cells were infected with the retroviruses with 8 μg/ml polybrene with spin infection at 1,290 *g* for 1.5 h. pRetroCMV/TO-puro–, pRetroCMV/TO-PG–, and pRSMX-infected cells was purified by using 2 μg/ml puromycin. pBMN-ires-Lyt2–infected cells were purified by MACS mouse CD8a (Lyt2) microbeads (Miltenyi Biotech). Doxycycline was used at 40 ng/ml for induction of shRNA and cDNA.

Chemotaxis assay. HTS Transwell 96-well permeable supports with an 8.0-μm pore polyester membrane (Corning) were used for chemotaxis assays of ED40515(+) and KOB. 235 μl of culture medium containing the indicated dose of chemokine was added to the lower chamber, followed by the addition of the Transwell inserts and the addition of cells in growth medium (75 μl) at a concentration of 5×10^5 cells/ml into the upper chamber. ChemoTx (5 μM pore; Neuro Probe) was used for chemotaxis assays of 32Dβ. 300 μl of chemokine-containing medium was added to the lower chamber, and 55 μl of cells in growth media (2×10^6 cells/ml) were added to the upper chamber. After incubation for 2 h at 37°C in 5% CO_2, the upper chamber was removed and 50 μl of 1× PBS with a 1:100 dilution of SPHERO particles (SpheroTECH) and 1:2,500 dilution of 10 mg/ml ethidium bromide was added into each lower chamber. The numbers of cells and SPHERO particles in lower chamber were quantified with a FACSCalibur (BD), and the numbers of cells that had migrated in each condition were normalized based on the SPHERO particle count.

Flow cytometry for CCR4 surface staining. Cells were washed with PBS containing 200 μg/ml of human gamma globulin (Human Gamma Globulin Fraction II; ICN Biomedicals Inc.) twice and incubated for 30 min on ice with anti–human CCR4-PE or CCR4–Alexa Fluor 647 (1G1; BD) or control isotype IgG. After washing with FACS buffer (PBS/1% FCS) twice, the cells were analyzed on a FACSCalibur (BD). For the CCR4 internalization assay, cells were exposed to CCL22 for varying lengths of time and were then treated for 1 min at 37°C to an acid buffer (pH 3.0, consisting of 50 mM glycine and 100 mM NaCl) to remove cell-bound CCL22. The cells were washed twice and then stained with anti–human CCR4–Alexa Fluor 647 on ice for 30 min, fixed with 2% paraformaldehyde (Electron Microscopy Sciences) on ice for 10 min, and analyzed on a FACSCalibur.

Immunoblot analysis. Cells were washed and resuspended in 2× SDS sample buffer at 10^6 cells/100 μl and boiled for 5 min. Samples were separated on Novex 4–12% Tris-Glycine gel (Invitrogen) and transferred to a PVDF membrane (Immobilon-P; EMD Millipore). Proteins were detected with the following primary antibodies: p-AKT(S473) (D9E) and AKT (5G3; Cell Signaling Technology). An anti–rabbit IgG-HRP antibody (GE Healthcare) was used as the secondary antibody.

AKT phospho-flow analysis. After incubation with 50 ng/ml CCL22 for the indicated time, cells were fixed in 2% paraformaldehyde for 10 min at room temperature and then permeabilized with cold methanol at −20°C overnight. Cells were washed twice in FACS buffer and stained with p-Akt (S473)–Alexa Fluor 647 (D9E) or isotype control (DA1E; Cell Signaling Technology) for 20 min at room temperature. Cells were washed and resuspended in FACS buffer for analysis on a FACSCalibur.

Online supplemental material. Table S1, included as a separate Excel file, shows Sanger sequencing analysis of exon 2 of CCR4 (NM_005508.4) in 53 cases of ATLL samples. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20140987/DC1.

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