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Two unique HLA-A*0201 restricted peptides derived from cyclin E as immunotherapeutic targets in leukemia

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Abstract

Immunotherapy targeting leukemia-associated antigens has shown promising results. Because of the heterogeneity of leukemia, vaccines with a single peptide have elicited only a limited immune response. Targeting several peptides together elicited peptide-specific cytotoxic T lymphocytes (CTLs) in leukemia patients, and this was associated with clinical responses. Thus, discovery of novel antigens is essential. In the current study, we investigated cyclin E as a novel target for immunotherapy. Cyclin E1 and cyclin E2 were found to be highly expressed in hematologic malignancies, according to reverse transcription polymerase chain reaction and Western blot analysis. We identified two HLA-A*0201 binding nonameric peptides, $CCNE1_M$ from cyclin E1 and $CCNE2_L$ from cyclin E2, which both elicited peptide-specific CTLs. The peptide-specific CTLs specifically kill leukemia cells. Furthermore, $CCNE1_M$ and $CCNE2_L$ CTLs were increased in leukemia patients who underwent allogeneic hematopoeitic stem cell transplantation, and this was associated with desired clinical outcomes. Our findings suggest that cyclin E1 and cyclin E2 are potential targets for immunotherapy in leukemia.

INTRODUCTION

Patients with acute leukemia continue to have a poor prognosis regardless of recent treatment advances[1, 2]. Allogeneic hematopoietic stem cell transplantation (allo-SCT) provides a curative option in a subset of patients. Graft-versus-leukemia (GVL) effects are seen following allo-SCT[3, 4], and donor-derived T cells are the primary mediators. However,

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the benefits of allo-SCT often need to be balanced by graft-versus-host disease (GVHD), which is primarily a result of nonspecific immunity elicited by the donor graft. GVHD can occur in up to 50% of patients receiving allo-SCT and often accounts for high morbidity and mortality[5, 6]. This highlights the need to develop targeted immunotherapy to harness the beneficial effects of anti-leukemia immune responses while minimizing nonspecific immunity.

Numerous antigens were shown to elicit CTL immunity in leukemia patients, and some of these leukemia-associated antigens (LAAs) demonstrated clinical efficacy and promising therapuetuic potential[7–14]. Because of the heterogeneity of leukemia, vaccines with a single peptide elicited only a partial immune response. Targeting PR1 and WT1 was shown to generate peptide-specific CTLs in leukemia patients, and associated with clinical responses[15]. The fact that targeting more peptides would produce better outcomes emphasizes the need for discovery of novel antigens.

Cyclin E is highly regulated in normal cells and overexpressed in acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and solid tumors such as breast cancer, lung cancer, and gastric cancer[16–19]. Overexpression of cyclin E has been linked to high cellular proliferation, whereas drugs that downregulate cyclin E were shown to induce cell arrest[17, 20]. The cyclin E subtypes cyclin E1 and cyclin E2 are encoded by different genes, cyclin E1 by CCNE1 at 19q12 and cyclin E2 by CCNE2 at 8q22.1. These subtypes bind to cyclin-dependent protein kinase 2 in G1/S transition and activate downstream signaling by phosphorylating substrates. Further studies have identified low molecular weight (LMW) isoforms that are more active and resistant to cyclin-dependent kinase inhibitors. Moreover, LMW cyclin E subtypes are present only in malignant cells and constitutively active to promote cell division[21, 22].

Because cyclin E has been shown to be ubiquitinated and is regulated by proteasomes[23, 24], two critical steps in antigen presentation[25, 26], it is likely that antigenic peptides are generated from cyclin E subtypes that bind to MHC and are present on the cell surface, and this can shape a cyclin E-targeted immune response. We hypothesized that overexpression of these proteins might break self-tolerance and generate cyclin E1- and cyclin E2-specific CTLs. Herein, we report identification of two homologous HLA-A*0201-restricted nonomeric peptides from cyclin E1 and cyclin E2 that elicited peptide-specific CTLs, which specifically killed leukemia cells. Furthermore, cyclin E1 and cyclin E2 CTLs were increased in leukemia patients who underwent allo-SCT, and this was associated with good clinical outcomes. Taken together, our results demonstrate that cyclin E1 and cyclin E2 are potential targets for leukemia immunotherapy.

MATERIALS AND METHODS

Patient samples and cell lines

Patient and healthy donor blood and bone marrow samples were obtained after appropriate informed consent through an institutional review board-approved protocol at M.D. Anderson Cancer Center. Leukemia blast samples, peripheral blood mononuclear cells (PBMCs) from

post all-SCT patients and healthy donors were cryopreserved. U937, Jukat, K562, T2, and MCF-7 cell lines were obtained from American Type Culture Collection (ATCC).

CCNE peptide binding

All peptides were synthesized at M.D. Anderson Cancer Center with over 95% purity. Production of peptide/major histocompatibility tetramers was described[11]. Peptide/HLA-A*0201 binding assay was described previously[9]. T2 cells were incubated with peptides and β2-microglobulin, then stained with anti-HLA-A*0201 antibody BB7.2 (Becton-Dickinson , San Jose, CA) to determine the stabilization of peptide/HLA-A*0201.

Cell-mediated cytotoxicity assay

Peptide-specific CTLs were expanded by stimulating PBMCs from HLA-A*0201$^+$ donors as described[10]. T2 cells were incubated with $CCNE1_M$ or $CCNE2_L$ peptides and β2-microglobulin, then irradiated and added into PBMCs. PBMCs were re-stimulated with peptide-loaded T2 cells every 7 days with a total of 20–35 days. A standard microcytotoxicity assay was used for specific lysis as described previously[9, 27]. Target cells were stained with calcein-AM (Invitrogen, Carlsbad, CA), then co-incubated with peptide-specific CTLs. FluoroQuench (One Lambda Inc) was added and fluorescence was measured using CytoFluor II plate reader (PerSeptive Biosystems). The specific cytotoxicity percentage was calculated as: % cytotoxicity = $(1 - [\text{Fluorescence}_{\text{Target+Effector}} - \text{Fluorescence}_{\text{Target only}} - \text{Fluorescence}_{\text{media}}]) \times 100$.

CCNE tetramer staining for CCNE-CTL

Tetramer synthesis and validation was performed as described[11, 28]. The PE-conjugated tetramers included $CCNE1_M$/HLA-A*0201, $CCNE2_L$/HLA-A*0201, and pp65/HLA-A*0201. Staining against a CTL line specific for $CCNE1_M$ and $CCNE2_L$ was validated by $CCNE1_M$/HLA-A*0201 and $CCNE2_L$/HLA-A*0201 tetramers, and pp65/HLA-A*0201 tetramers were validated by staining PBMCs from a cytomegalovirus-immunized individual. The following antibodies were used in FACS[29]: FITC-anti-HLA-A2*0201 (BD), FITC-anti-CD8 (Caltag), PE-Cy7-anti-CD3 (BD), Pacific blue-anti-CD8 (BD), FITC-anti-IFN-γ (BD), PE-anti-IL-2 (BD), and tricolor-anti-CD4, -CD14, -CD16, and -CD19 (Caltag).

The absolute number of tetramer-stained cells was calculated as: (lymphocyte count per M.D. Anderson clinical laboratory report) × (frequency of CD8$^+$ T cells by flow cytometry) × (frequency of tetramer-stained cells). A 20% increase in the absolute number of circulating CCNE CTLs (per milliliter) after allo-SCT (compared with the absolute number prior to allo-SCT) was considered evidence of an immune response.

Statistical analysis

GraphPad Prism 6.0 software was used for statistical analyses and $p < 0.05$ was considered statistically significant.

RESULTS

Cyclin E1 and Cyclin E2 over expressed in hematologic malignancies

We screened cyclin E1 and cyclin E2 at the mRNA level using 28 patient samples (CML=18, AML=4, ALL=3, NHL=3) collected at the time of diagnosis and 21 samples from healthy donors. Cyclin E1 mRNA overexpression could be detected in PBMCs from 21 of 28 patients (75%) compared with 6 of 21 healthy controls (29%; $p = 0.0017$) by RT-PCR. Similar results were obtained for cyclin E2 mRNA, which was overexpressed in PBMCs from 15 of 28 patients (54%) compared with 1 of 21 healthy controls (5%; $p = 0.0005$) (data not shown). Western blot was used to confirm cyclin E1 and cyclin E2 protein expression. As shown in representative immunoblots (Figure 1A), CCNE1 and CCNE2 were overexpressed in AML and ALL patient samples. The level of expression was similar to that observed in leukemia cell lines (i.e., U937 and Jurkat cells) but barely detectable in healthy donor PBMCs. Among the 28 patients, 26 (93%) expressed CCNE1, whereas only 4 of 12 healthy donors (33%) expressed cyclin E1 ($p = 0.0002$). Likewise, CCNE2 protein overexpression was confirmed in the same PBMC samples from 24 of 28 patients (86%) compared with 1 of 12 healthy donors (8%; $p < 0.0001$). We also examined cyclin E1 and cyclin E2 protein levels in B cells, including resting cells and PHA stimulated B cells from two healthy donors; cyclin E1 LMW isoforms were overexpressed only in MCF-7 breast cancer cells (Figure 1B). Taken together, these data suggest that cyclin E1 and cyclin E2 are overexpressed in hematologic malignancies.

Two HLA-A2*0201 binding peptides can be derived from cyclin E

To identify HLA-A*0201 (HLA-A2) binding peptides, we screened both CCNE1 and CCNE2 sequences in silico against the binding motif of the HLA-A2 molecule (www.biomas.ncbi.nih.org). We identified a pair of homologous nonameric peptides based on the highest predicted binding affinities to HLA-A2*0201[33, 34], CCNE1-derived peptide $ILLDWLMEV_{144–152}$, and CCNE2-derived peptide $ILLDWLLEV_{144–152}$; these two peptides differed by a single amino acid at position 7 ($CCNE1_M$ and $CCNE2_L$), the murine CCNE1-derived peptide $VLLDWLMEV_{145–153,}$ which differed by a single amino acid at position 1 (Figure 2A). Both $CCNE1_M$ and $CCNE2_L$ peptide-loaded T2 cells showed stronger fluorescence intensities than the pp65 control, and these shifts were dose-dependent. The murine CCNE1 bound to HLA-A*0201 weakly and there were no dose-dependent effect (Figure 2B). Thus, we confirmed that the two peptides have high binding affinity to HLA-A*0201 on T2 cells.

$CCNE1_M$ is the dominant peptide and cross-recognized by $CCNE2_L$ T cell receptor

We generated $CCNE1_M$- and $CCNE2_L$-specific CTLs from HLA-A2*0201 healthy donor PBMCs. $CCNE1_M$/HLA-A*0201 and $CCNE2_L$/HLA-A*0201 tetramer staining showed that $CCNE1_M$ and $CCNE2_L$ CTL lines contained high populations of both $CCNE1_M$ / HLA-A*0201 and $CCNE2_L$/HLA-A*0201 tetramer-positive cells; irrelevant pp65/HLA-A2 tetramers did not stain positive for either $CCNE1_M$ or $CCNE_L$ CTLs (Figure 3A). Additionally, $CCNE1_M$/HLA-A*0201 tetramers stained positive for $CCNE2_L$ CTLs, and when used to stain $CCNE1_M$ CTLs, $CCNE2_L$/HLA-A*0201 tetramers bound to $CCNE1_M$

CTLs, indicating that CCNE1/2 CTLs showed cross-reactivity for the cyclin E1 and cyclin E2 tetramers.

Affinities of the peptide-specific CTL TCR for each peptide/HLA-A2 complex in each CTL line were determined by tetramer dissociation assay (Figure 3B). The fluorescence decay of tetramer dissociation over time was linear for each clone, suggesting that avidity was proportional to TCR. The time for half-maximal tetramer dissociation ($t_{1/2}$) was determined based on first-order kinetics. $CCNE1_M$/HLA-A*0201 tetramer had a longer binding $t_{1/2}$ to $CCNE1_M$ CTL ($t_{1/2}$ = 84.5 minutes) than $CCNE2_L$/HLA-A*0201 tetramer ($t_{1/2}$ = 29.5 minutes). Interestingly, the binding $t_{1/2}$ of $CCNE1_M$/HLA-A*0201 tetramer was longer even for $CCNE2_L$ CTL, in contrast with $CCNE2_L$/HLA-A*0201 tetramer ($CCNE1_M$ tetramer $t_{1/2}$ = 25.3 minutes; $CCNE2_L$/HLA-A*0201 tetramer $t_{1/2}$ = 10.7 minutes). Thus, both $CCNE1_M$ CTL and $CCNE2_L$ CTLs had increased affinity with the $CCNE1_M$/HLA-A2 complex. These data indicate that $CCNE1_M$ is the dominant peptide and is cross-recognized by the $CCNE2_L$ TCR.

$CCNE1_M$ and $CCNE2_L$ peptide-specific CTLs mediate cytotoxicity

We examined the ability of the CCNE CTLs to kill specific targets. $CCNE1_M$ CTL lines killed both $CCNE1_M$-loaded and $CCNE2_L$-loaded T2 cells, but not unloaded T2 cells or murine CCNE1 peptide-loaded T2 cells (Figure 4A), which differed by a single amino acid at position 1 ($VLLDWLMEV_{145–153}$). Similarly, $CCNE2_L$-stimulated CTL lines killed $CCNE1_M$-loaded T2 cells and $CCNE2_L$-loaded T2 cells but not the unpulsed T2 cells or irrelevant murine CCNE1 peptide-pulsed T2 cells (Figure 4B). Cross-reactive $CCNE1_M$ CTLs could be elicited from three of seven healthy donor samples and $CCNE2_L$ CTLs could be elicited from four of seven healthy donor samples. This peptide-specific cytotoxicity was blocked by anti-HLA-A2 monoclonal antibody (data not shown). This was consistent with our cytotoxicity data that indicated CCNE CTL functional cross-reactivity for CCNE1/2-positive targets.

$CCNE1_M$ and $CCNE2_L$ CTL clones preferentially kill HLA-A*0201 leukemia cells

We next examined whether CCNE CTLs can lyse primary leukemia blasts. As shown in Figure 4C, $CCNE1_M$ CTLs specifically lysed HLA-A*0201-positive AML and ALL cells, but not HLA-A*0201-negative AML cells, inactive B cells, or PHA-stimulated B cells. Furthermore, $CCNE1_M$ and $CCNE2_L$ CTLs specifically lysed HLA-$A2^+$ T-ALL cells, which overexpressed both cyclin E1 and cyclin E2 proteins (Figure 4D–E). However, these CTLs did not lyse either bone marrow cells of the same patient while in CR or HLA-$A*0201^+$ bone marrow cells of a healthy individual, which did not overexpress cyclin E1 or cyclin E2, nor did the CTLs lyse control HLA-$A2^-$ CML or AML cells that overexpressed both cyclin E1 and cyclin E2 proteins. These data demonstrate that $CCNE1_M$ CTLs and $CCNE2_L$ CTLs are specific for HLA-$A*0201^+$ and cyclin E-overexpressing leukemia targets, but not for normal, HLA-$A2^-$, or low cyclin E-expressing cells.

Functional $CCNE1_M$ and $CCNE2_L$ CTLs are detected in leukemia patients after allo-SCT

To determine whether immunity to cyclin E1 and cyclin E2 can be detected in leukemia patients after allo-SCT, we used $CCNE1_M$/HLA*0201 and $CCNE2_L$/HLA*0201 tetramers to

stain PBMC samples from leukemia patients who underwent allo-SCT. A representative flow cytometry sample demonstrating the gating strategy of $CCNE1_M$/HLA*0201 and $CCNE2_L$/HLA*0201 tetramer staining (Figure 5A). Among 25 AML patient samples collected 3–12 months after allo-SCT (Table 1), we were able to detect high frequencies of $CCNE1_M$-specific CTLs (0.46 ± 0.04, range 0.12%−1.09%) and $CCNE1_L$-specific CTLs (0.37 ± 0.03, range 0.11%−0.73%0 (Figure 5B). For the 18 patients with paired blood samples from pre- and post- allo-SCT (Table 2), we were able to compare the absolute number of tetramer-positive CTLs (Figure 5C). The mean number of $CCNE1_M$ CTLs and $CCNE2_L$ CTLs significantly increased after allo-SCT ($p = 0.0048$ for $CCNE1_M$ CTLs and $p = 0.0063$ $CCNE2_L$ CTLs), whereas the mean number of pp65 CTLs remained the same.

To determine the $CCNE1_M$ and $CCNE2_L$ CTL functionality in patients, we measured cytokine production. PBMC samples from four AML patients collected after allo-SCT were stimulated with $CCNE1_M$ or $CCNE2_L$ peptide-pulsed T2 cells. As shown in Figure 6A, the luminex assay revealed >2-fold increased IFN-γ production and >3-fold increased TNF-α production following stimulation with peptide-pulsed T2 cells for 6 hours, compared with unpulsed T2 cells. In contrast, production of Tc2 cytokines, includng IL-4, IL-5, and IL-10, following CCNE1/2 peptide-pulsed T2 stimulation was similar to that detected with unpulsed T2 cells. The cytokine-producing cells were confirmed using cytokine flow cytometry, which demonstrated increased intracellular IFN-γ and IL-2 production by $CD8^+$ T cells after stimulation with $CCNE1_M$ or $CCNE2_L$ peptide-pulsed T2 cells (Figure 6B). Additionally, when the four samples used in the cytokine analysis were stained with $CCNE1_M$ or $CCNE2_L$ HLA-A*0201 tetramers, there was a correlation between the percentage of tetramer-positive cells and IFN-γ production (data not shown). Our data indicate that $CCNE1_M$ or $CCNE2_L$ peptide-stimulated PBMCs collected from leukemia patients after allo-SCT are functional and show a Tc1>Tc2 cytokine profile.

DISCUSSION

Our results showed that two novel immunogenic epitopes, cyclin E1-derived $CCNE1_M$ and cyclin E2-derived $CCNE2_L$, bind to HLA-A*0201, and peptide-specific CTLs can be elicited from healthy donors. Each of the two peptide-specific CTL lines can also recognize the other peptide and kill both $CCNE1_M$- and $CCNE2_L$-loaded T2 cells. The cytotoxic ability of $CCNE1_M$ and $CCNE2_L$ CTLs is specific to HLA-A*0201 lymphoid and myeloid leukemia cells overexpressing the parental cyclin E1 and cyclin E2 proteins; the cytotoxicity does not target normal BM cells or $HLA\text{-}A2^-$ leukemia cells. Furthermore, $CCNE1_M$- and $CCNE2_L$-specific CTLs were detected in blood samples from allo-SCT patients, and these levels correlated with remission status following allo-SCT.

Because cell cycle proteins are expressed in many malignancies and ideal targets for cancer immunotherapy[35]. In addition to solid tumors such as breast cancer, several hematologic malignancies, including AML, diffuse large B cell lymphoma, CLL and ALL, overexpress cyclin E1 and cyclin E2[36–38]. Importantly, overexpression of cyclin E is highly predictive of relapse in early-stage breast cancer patients[17]. Treatment of primary and cultured human AML cells with epigenetic therapy led to apoptosis of the AML cells, correlating with a decrease in cyclin E expression[39]. Similar results were also obtained with the

myelomonocytic cell line U937, in which the downregulation of cyclin E was correlated with induction of G1 cell cycle arrest[40]. In CML, targeting the peroxisome proliferator-activated receptor alpha and gamma led to growth inhibition of CML cell lines, which coincided with a decrease in cell cycle proteins, including cyclin E[41]. Taken together, these reports validate the use of cyclin E targeting therapy in the treatment of leukemia.

Cellular degradation of cell cycle proteins involves the proteasome, a pathway that is also used for MHC-I antigen presentation[42, 43]. Herein, we showed that peptides derived from cyclin E1 and cyclin E2 are presented by leukemia cells. Furthermore, LMW isoforms of the full-length protein, which are more active in inducing cell cycle progression, were found exclusively in cancer cells. Recent data have shown that LMW cyclin E1 is located in the cytoplasm[23, 44], further supporting the likelihood of proteasomal degradation prior to antigen presentation, which is also localized to the cytoplasm. We reported in a breast cancer model that norepinephrine uptake can increase LMW cyclin E1 expression and enhance susceptibility to peptide-specific CTL lysis, suggesting that CCNE peptides are naturally presented on breast cancer cells[44]. Autoimmunity to cyclin E1 and cyclin E2 was demonstrated by the detection of IgG autoantibodies to cyclin proteins in the serum of leukemia patients, suggesting that cyclin E1 and cyclin E2 are immunogenic[45].

The cross-reactivity in the immune response that was noted for cyclin E1 and cyclin E2 (Figures 3 and 4) may be of great value in cyclin E-based therapy. Because the expression of cyclin E1 and cyclin E2 varies among tumor types and at different stages of the cell cycle[46–48], eliciting an immune response against either cyclin E1 or cyclin E2 would broaden the applicability of CCNE-based therapy. Furthermore, such therapy can be applied to solid tumors (i.e., breast and gastrointestinal malignancies) in addition to leukemia.

We found that the absolute number of $CCNE1_M$ and $CCNE2_L$ CTLs significantly increased in AML patients after allo-SCT, but this increase did not vary with the type of leukemia (ALL or CML), donor-recipient HLA disparity (matched or mismatched), or disease status prior to allo-SCT, according to the Fisher exact test (data not shown). In the 8 CML patients with active disease prior to allo-SCT, an immune response (≥20% increases of specific CTLs) with either $CCNE1_M$ or $CCNE2_L$ occurred more frequently in patients who achieved complete remission than in those who did not achieve complete remission after allo-SCT ($p < 0.04$). These findings were confirmed in the 7 AML patients with active disease at the time of allo-SCT. The number of $CCNE1_M$ CTLs or $CCNE2_L$ CTLs was higher in those who achieved complete remission following allo-SCT than in those who did not ($p = 0.012$ for $CCNE1_M$ CTLs and $p = 0.015$ for $CCNE2_L$ CTLs). Moreover, all 15 patients who had an immune response to cyclin E1 also had an immune response to cyclin E2, demonstrating cross-recognition of both peptides by the same CTL clone. Our results obtained from clinical samples indicated that the immune response to either CCNE1 or CCNE2 was detected more frequently in patients without acute GVHD than in patients who had acute GVHD (Supplementary Table 1). The lack of correlation between GVHD and GVL effects highlights the specificity of the cyclin E-elicited immune response to leukemia tissue. This specificity is further supported by our observations that in CML patients, immune response to CCNE1 and CCNE2 was associated with CR.

Our results provide evidence that both cyclin E1 and cyclin E2 peptides are novel LAAs. In addition, the absolute number of $CCNE1_M$ and $CCNE2_L$ CTLs increased after allo-SCT, suggesting that immune reconstitution in allo-SCT elicits $CCNE1_M$ and $CCNE2_L$ CTLs. Lastly, in view of the expression of cyclin E1 and cyclin E2 by a variety of tumors, CCNE-based therapy may be broadly applicable in the treatment of cancer.

Taken together, these results show that $CCNE1_M$ and $CCNE2_L$, self-peptides from constitutively active cell cycle proteins, are novel LAAs that could be used in immunotherapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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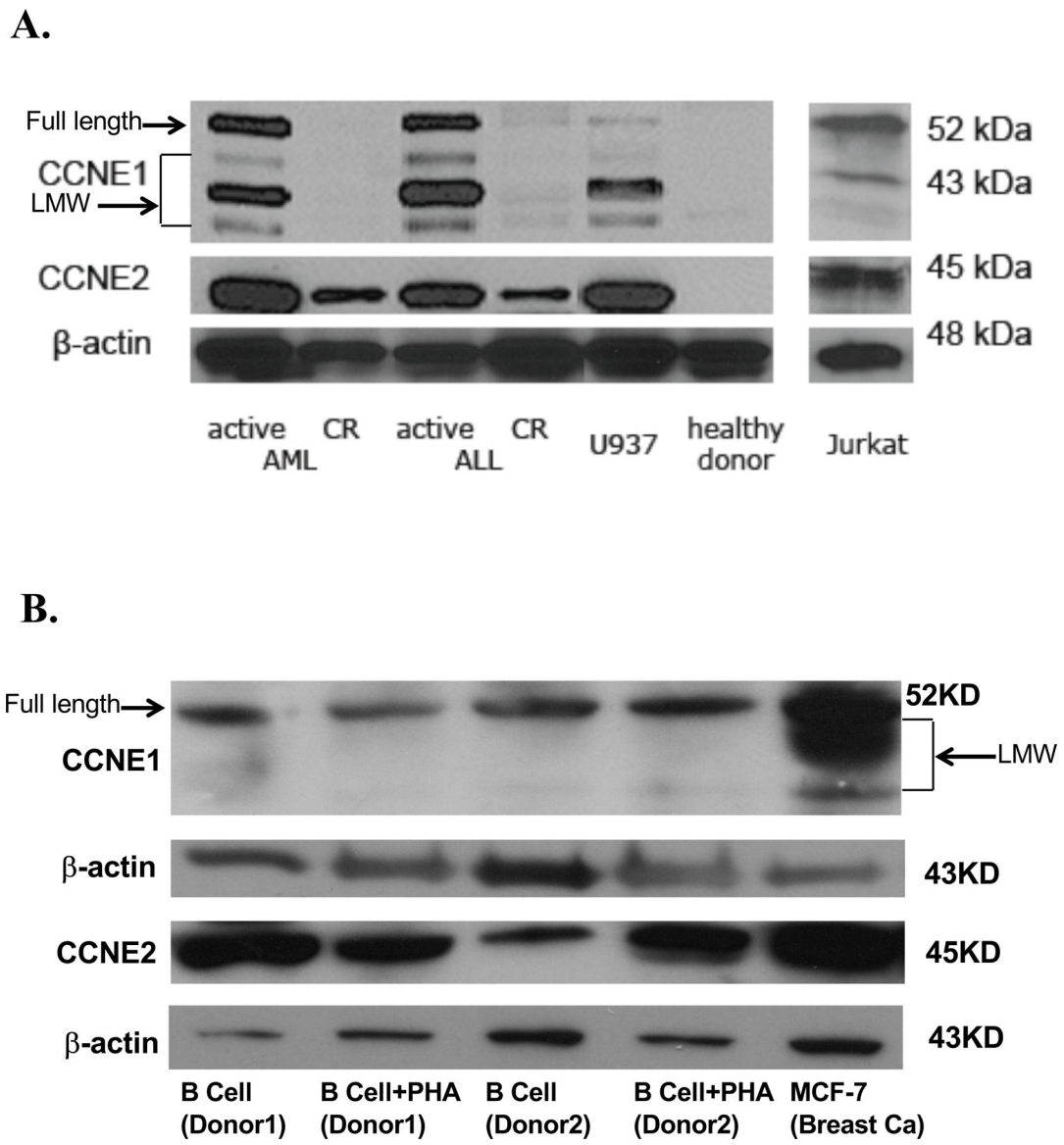

Figure 1.

Cyclin E1 and cyclin E2 were overexpressed in leukemia cells. (A) Western blot analysis of whole cell lysates (WCL) from representative samples of AML and aALL patients with active disease (active) or in complete remission (CR), U937 cells, PBMC from HD and Jurkat cells. Full-length and three LMW bands of CCNE1 were identified by the anti-CCNE1 antibody HE12 (upper panel), and CCNE2 by the anti-CCNE2 antibody A2 (middle panel) with β-actin as a loading control (lower panel). (B) Western blot analysis of WCL from resting and PHA-stimulated B cells of two healthy donor PBMC, and MCF-7 human breast cancer cells. Full-length and LMW CCNE1 with corresponding β-actin loading control are shown on the top two panels. CCNE2 with corresponding β-actin loading control are shown on the bottom two panels.

A.

$CCNE1_{144\text{-}152}$ I L L D W L M E V
$CCNE2_{144\text{-}152}$ I L L D W L L E V
Murine$CCNE1_{145\text{-}153}$ V L L D W L M E V

B.

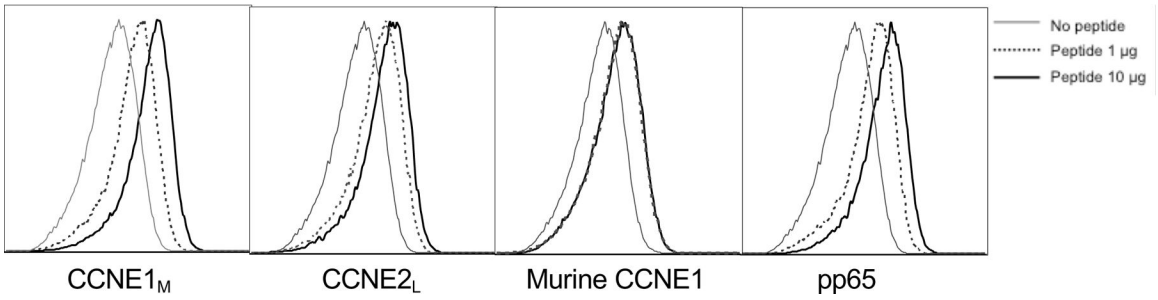

Figure 2.

Two peptides derived from cyclin E bind HLA-A*0201 with high affinity. (A) HLA-A*0201 binding epitopes were derived from a human cyclin E1-derived peptide ($CCNE1_{144–152}$) and E2-derived peptide ($CCNE2_{144–152}$) with a single amino acid difference at position 7 (M ▫ L, red), plus a murine cyclin E1-derived peptide (murine$CCNE1_{145–153}$) with a single amino acid difference at position 1(I ▫ V, violet). (B) Binding of cyclin E-derived peptides to T2 cells. Individual peptides (1 µg or 10 µg) were incubated with T2 cells in the presence of β2-microglobulin. Anti-HLA-A2.1 mAb BB7.2 was used to detect the HLA peptide complex on the cell surface. Representative histograms are shown for $CCNE1_M$ (left), $CCNE2_L$ (center), and pp65 (right). Unstained and unpulsed T2 cells were used to determine the background.

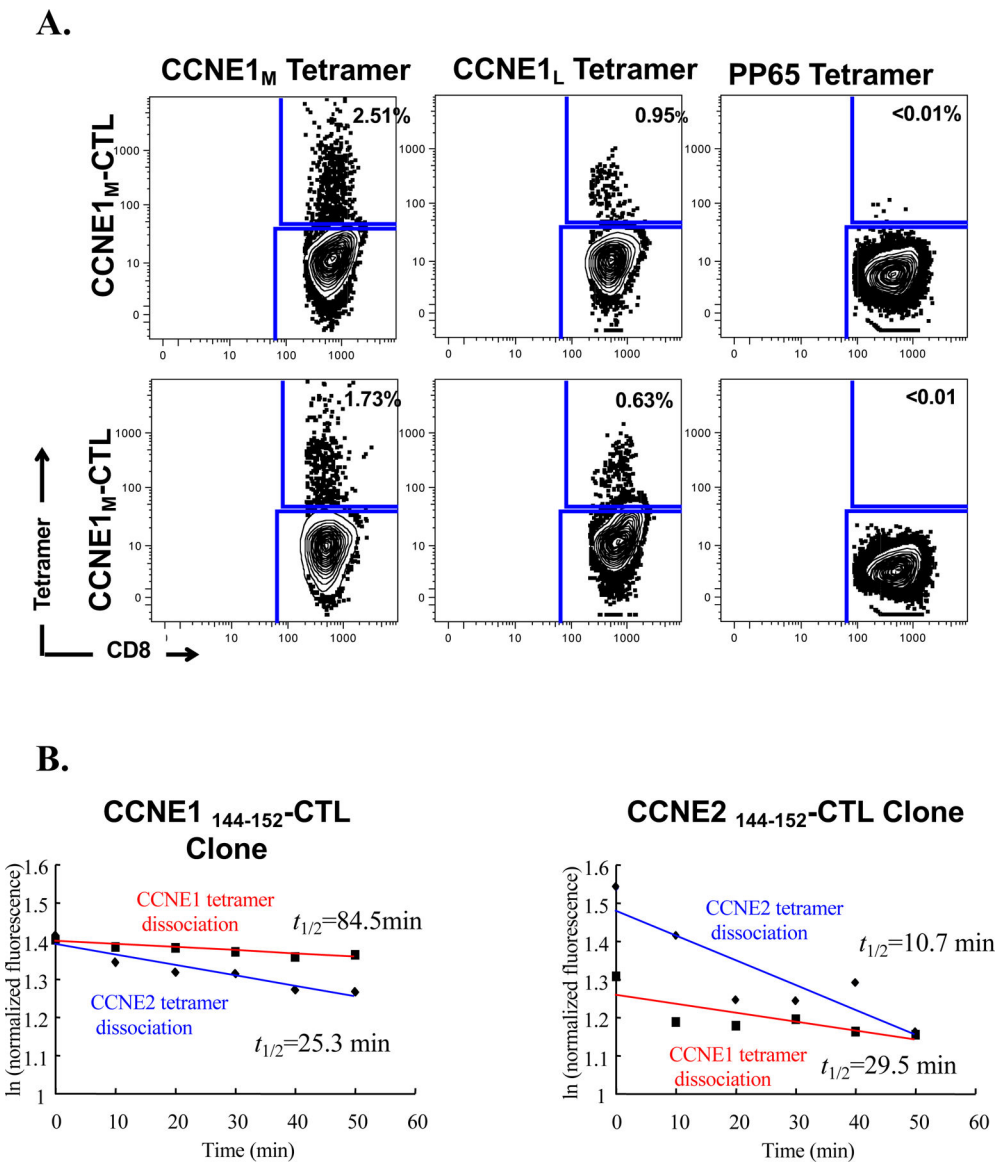

Figure 3.

$CCNE1_M$ and $CCNE2_L$ peptides elicit peptide-specific CTLs from healthy donors. (A) PBMCs from healthy HLA-A2.1$^+$ donors were stimulated with $CCNE1_M$ or $CCNE2_L$ peptide-pulsed T2 cells for 4 weeks. $CCNE1_M$/HLA-A2 and $CCNE2_L$/HLA-A2 tetramers were used to stain both $CCNE1_M$ CTL lines (upper panel) and $CCNE2_L$ CTL lines (lower panel), with irrelevant pp65/HLA-A2 tetramer as a control. Percentages of tetramer-specific CTLs among total CD8$^+$ T cells are shown on the left in representative FACS plots. (B) Tetramer dissociation assay of $CCNE1_M$ CTL lines (left) and $CCNE2_L$ CTL lines (right). Dissociation kinetics of $CCNE1_M$/HLA-A2 (filled squares) and $CCNE2_L$/HLA-A2 (filled circles) tetramer staining were determined at 4°C in the presence of saturating concentrations of BB7.2 Ab to prevent rebinding of tetramer and in the presence of PI (1 μg/mL) to eliminate dead cells from the FACS gate. This antigen-specific fluorescence was normalized to the percentage of the total fluorescence at the initial time point and

plotted on a logarithmic scale. Tetramer decay ($t_{1/2}$) was determined by plotting normalized antigen-specific fluorescence at the indicated time points for $CCNE1_M$/HLA-A2 (red) and $CCNE2_L$/HLA-A2 (blue) tetramer-stained CTLs.

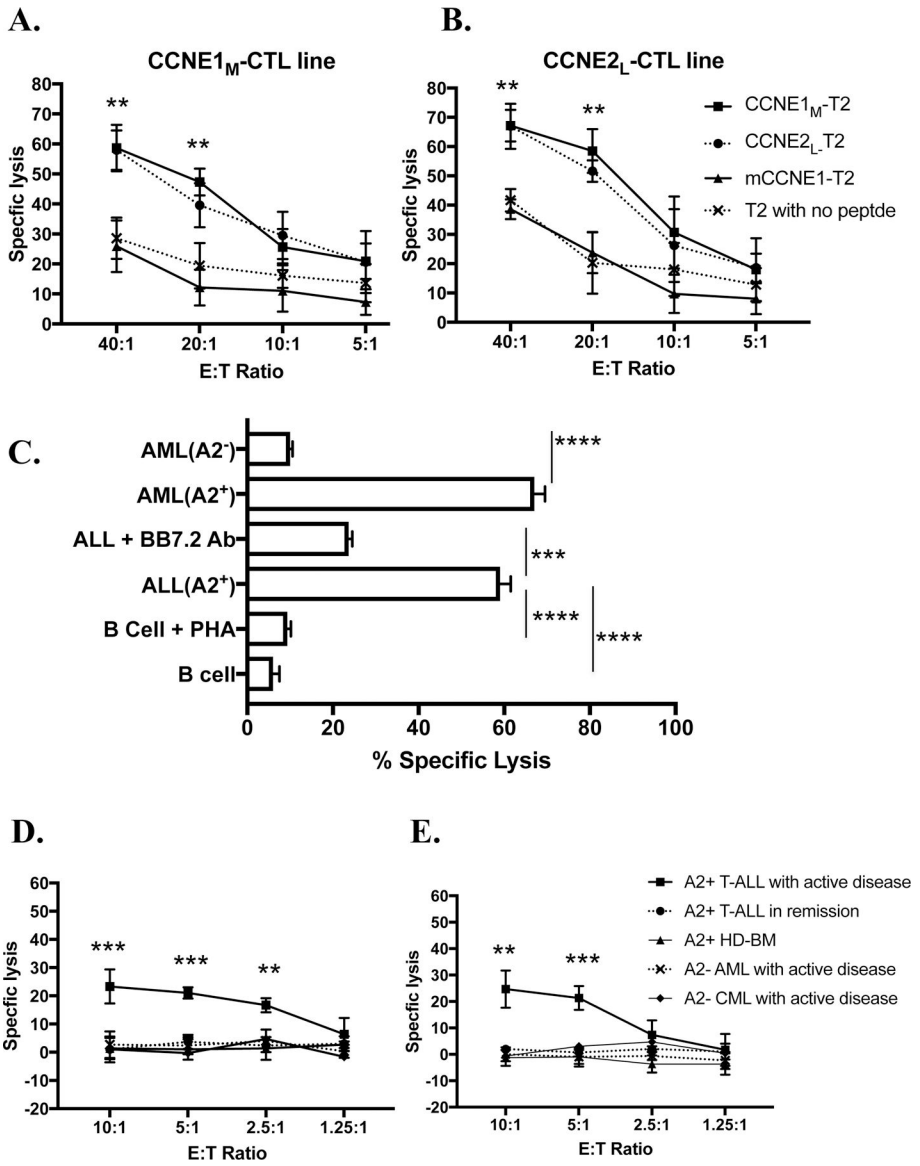

Figure 4.

Specific cytotoxicity of $CCNE1_M$- and $CCNE2_L$-CTLs. (A-B) Cytotoxicity assay using CTL lines generated from healthy donors. Both $CCNE1_M$-CTL line (A) and $CCNE2_L$-CTL line (B) were incubated with $CCNE1_M$, $CCNE2_L$, or mCCNE1 peptide-pulsed T2 cells at the indicated effector:target (E:T) ratio with T2 cells alone as control. The specific lysis percentage was determined and three replicate wells were used for each dilution of effector cells. (C) Specific cytotoxicity of $CCNE1_M$ CTLs against primary leukemia blasts. At a 20:1 E:T ratio, $CCNE1_M$ CTLs preferentially lysed HLA-A*0201-positive AML and ALL cells, but not HLA-A*0201-negative AML cells. The CTLline lysed resting B cells and PHA-stimulated B cells only at low levels. (D-E) Cytotoxicity assay showing that CCNE-CTLs specifically lysed HLA-A*0201-positive leukemia cells expressing high levels of cyclin. $CCNE1_M$-CTLs (D) and $CCNE2_L$-CTLs (E) specifically lysed HLA-A*0201 T-ALL cells that aberrantly expressed cyclin, did not lyse HLA-A2-positive normal BM

cells, nor HLA-A*0201-negative AML or CML cells. Results are the means and SD from three replicate wells. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

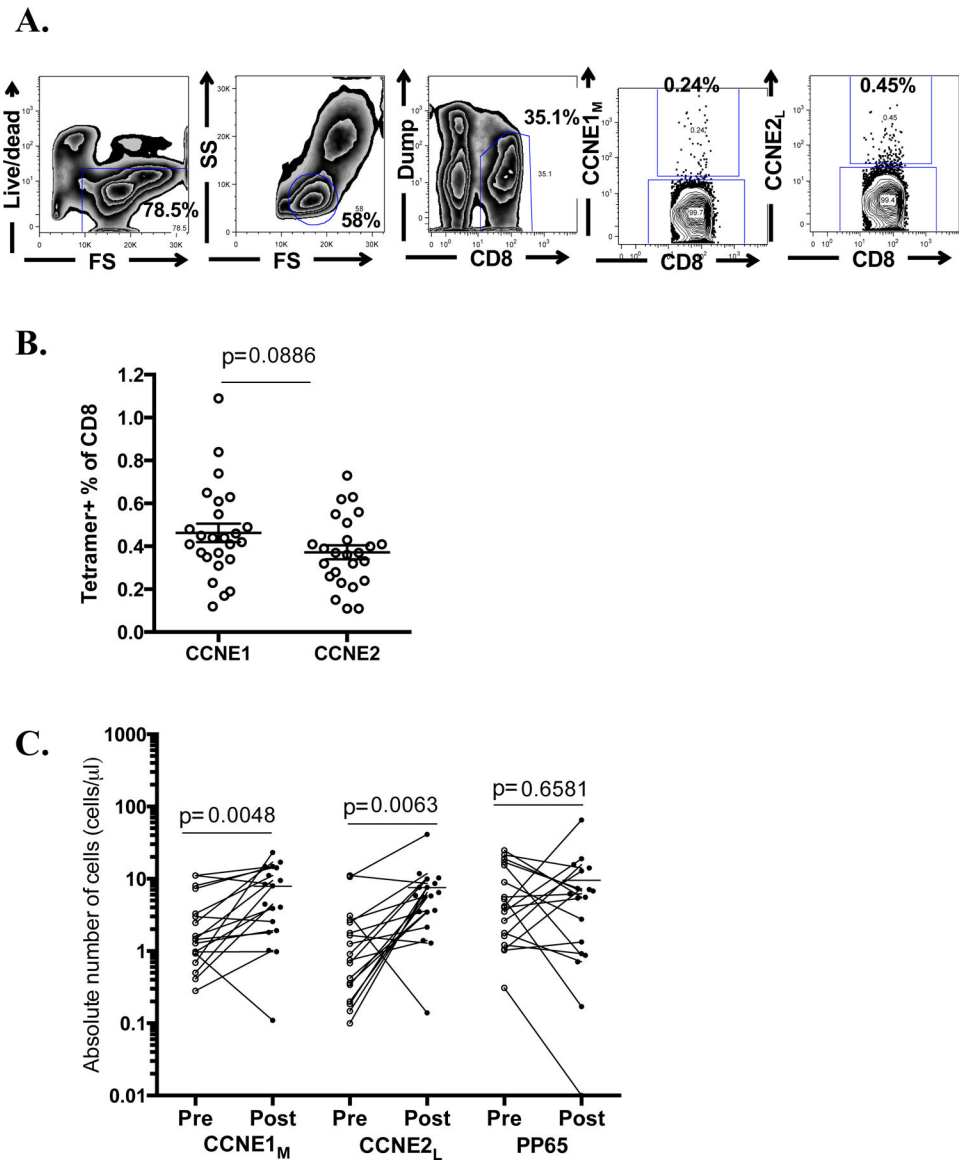

Figure 5.

High frequency of $CCNE1_M$-CTL and $CCNE2_L$-CTL in post allo-SCT leukemia patients. $CCNE1_M$/HLA-A2 or $CCNE2_L$-/HLA-A2 tetramers were used to stain the PBMC from patient samples. (A) Representative flow cytometry plots demonstrating tetramer staining of one AML patient used in the experiments. (B) Percentages of $CCNE1_M$/HLA-A2 or $CCNE2_L$/HLA-A2 tetramer-positive cells among total $CD8^+$ T cells from 25 AML patients 3–12 months after allo-SCT. Horizontal bars indicate mean values with SEM. (C) The absolute number of $CCNE1_M$-CTL, $CCNE2_L$-CTL and pp65-CTL was measured in the pre and post PBMC samples from 18 allo-SCT patients. The data are shown as the mean ± SEM. Statistically difference between groups is indicated by their *p* values.

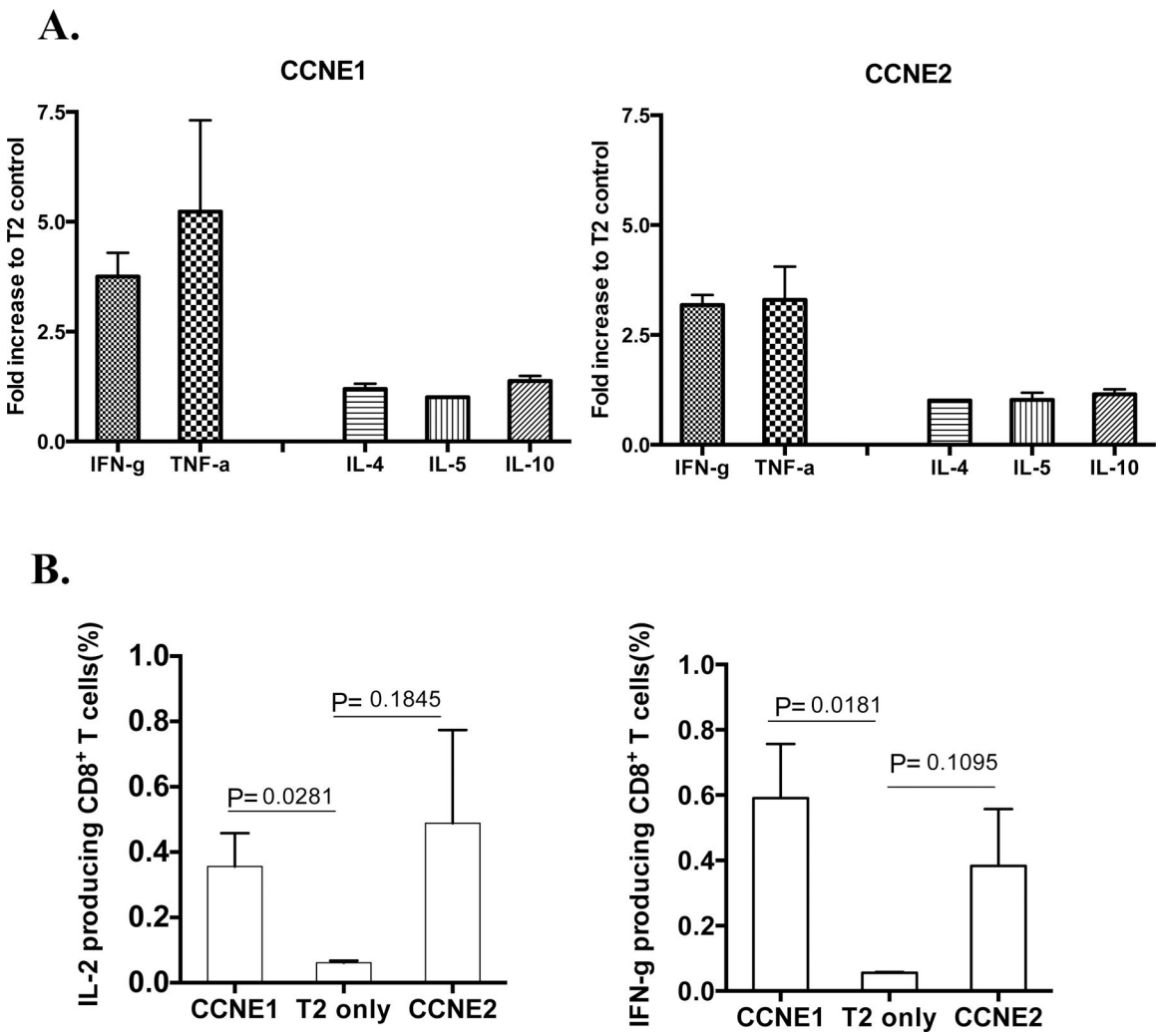

Figure 6.

CCNE1- and CCNE2-CTLs from AML patients after allo-SCT are functional. PBMC samples from four AML patients collected post allo-SCT were incubated with T2 cells pulsed with $CCNE1_M$ or $CCNE2_L$ peptide. Supernatants were collected for analysis of cytokine production by luminex assay. For intracellular cytokine analysis, cells were harvested and then processed for cytokine staining by FACS. (A) Fold change in cytokine production in the supernatant of PBMCs (n = 4) stimulated with $CCNE1_M$ (left) or $CCNE2_L$ (right) pulsed with T2 cells compared with T2 cells only. (B) IL-2 and IFN-γ-producing $CD8^+$ T cells from four post allo-SCT PBMC stimulated for 24 hours with $CCNE1_M$ or $CCNE2_L$ pulsed T2 cells or T2 cells only (control). The data are shown as means ± SEM. A *t* test was carried out to compare between two groups. Statistically significant differences between groups are indicated by *p* values.

Table 1.

AML patient characteristics

Characteristic	Patient Number(n=25)
General Information	
Median Age(rang)	47yrs(21–66)
Male/Female	13:12
Disease Status at SCT	
Remmision(CR)	18
Non-CR	7
Response to SCT(6 months after SCT)	
Maintain CR	18
Achieve Remission	5
PR/Refractory	2
Graft Source	
HLA-Matched Sibling	16
HLA-Matched Unrelated Donor	9

All the patients were HLA-A2 positive.

Abbreviations: AML Acute myeloid leukemia

Table 2.

ALL/CML patient characteristics

Characteristic	Patient Number(n=18)
General Information	
Median Age(rang)	45 yrs(29–62)
Male/Female	9:9
Diagnosis	
ALL	8
CML	10
Chronic Phase	5
Accelated Phase	3
Blast Crisis	2
Disease Status at SCT	
Remission (CR)	10
Non-CR	8
PR/Refractory	2
Partial Remission	2
Refractory to Therapy	6
Response to SCT(6 months after SCT)	
Maintain CR	10
Achieve CR	7
Non-CR	1
Graft Source	
Sibling Donor	12
HLA-Matched	7
HLA-Mismatched	5
Unrelated Donor	6
HLA-Matched	5
HLA-Mismatched	1

All the patients were HLA-A2 positive.

All patients received Tacrolimus/Methotrexate GVHD prophylaxis.

Abbreviations: ALL, Acute lymphoid leukemia;CML, chronic myeloid leukemia