

Identification of CD19 and CD20 Peptides for Induction of Antigen-Specific CTLs against B-Cell Malignancies

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ABSTRACT

The purpose of these studies was to develop immunogenic peptides derived from the CD19 and CD20 self-antigens for the induction of antigen-specific CTLs against B-cell malignancies. A total of seven peptides were designed and examined for their HLA-A2.1 affinity and immunogenicity. Of these peptides, we identified two highly immunogenic HLA-A2.1-specific peptides, CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV), which were capable of inducing peptide-specific CTLs. The CTLs displayed HLA-A2.1-restricted and antigen-specific cytotoxicity against Burkitt's lymphoma, chronic B cell leukemia, and multiple myeloma cell lines. The CD19 or CD20 peptide-specific CTL cytotoxicity was confirmed using HLA-A2.1⁺ T2 cells presenting the appropriate peptide. No cytotoxic activity was observed against T2 cells presenting the irrelevant MAGE-3 peptide or T2 cells alone. In addition, the CTLs displayed a significant ($P < 0.05$) increase in cell proliferation and IFN- γ secretion (>830 ng/mL) following restimulation with HLA-A2.1⁺/CD19⁺/CD20⁺ tumor cells. The CTLs also displayed a distinct phenotype consisting of a high percentage of CD69⁺/CD45RO⁺ and a low percentage of CD45RA⁺/CCR7⁺ CD4⁺ or CD8⁺ T cells characteristic of effector memory cell population. Cyclic guanosine 3',5'-monophosphate culture conditions using serum-free AIM-V medium containing human AB serum, recombinant human interleukin 2 (Proleukin) and CD3/CD28 Dynabeads were developed resulting in a 35-fold expansion of CD20 peptide-specific CTLs. The expanded CD20-CTLs retained their cytotoxic activity (28-49%) against the Burkitt's lymphoma cell line. In conclusion, we report here on the identification of novel immunogenic CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptides that have immunotherapeutic potentials as peptide vaccines or targeted T-cell therapies for treating B-cell malignancies.

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INTRODUCTION

B-cell malignancies comprise a heterogeneous group of neoplasms, including acute lymphocytic leukemia, chronic lymphocytic leukemia, multiple myeloma, and B-cell lymphomas. An estimated 65,000 new cases are diagnosed annually in the United States (1). Current therapeutic strategies can be effective but the majority of patients ultimately relapse and die of their diseases (2–5). One promising approach for targeting B-cell malignancies involves the cellular immune system through activation of highly efficient T lymphocytes that mediate key functions such as cytotoxicity, cytokine production, regulation of effector cells, and induction of immunologic memory (6, 7). Earlier clinical studies have shown that dendritic cell vaccination for the priming of naive T cells can generate tumor-specific CTLs and induce remission in pretreated patients with human B-cell malignancies (8, 9). In addition, infusion of HLA-matched allogeneic T lymphocytes has been shown to induce durable long-term remissions in relapsed lymphomas, chronic B-lymphocytic leukemia, multiple myeloma (10, 11), or EBV-associated lymphoproliferative disease (12–14) after stem cell transplantation. However, only a limited percentage of patients with B-cell malignancies will achieve complete remission following donor lymphocyte infusion and the patients are at risk of developing graft-versus-host disease, which can be associated with significant morbidity and mortality. Therefore, developing peptide-based immunotherapies against specific overexpressed tumor-associated antigens offer an attractive approach for boosting patients' immune system to treat recurrent B-cell malignancies.

CD19, a 95-kDa B lineage-specific transmembrane glycoprotein, functions as a central response regulator in B cells and offers many unique characteristics that make it a relevant target for developing immunotherapeutic strategies. With the important exception of hematopoietic stem cells, CD19 is expressed during all stages of on B-cell differentiation, is down-regulated on plasma cells (15), and is maintained on cells that have undergone neoplastic transformation. It is expressed on $>95\%$ of cells in patients with B-cell lymphoma, chronic B-lymphocytic leukemia, and on the acute B-lymphocytic leukemia progenitor cells (15–21). The CD19 antigen is an attractive target molecule due to its internalization after binding to antibody (22, 23). Recent studies have also shown that CD19 expression is maintained despite loss of CD20 expression following treatment with anti-CD20 antibodies (24). Being expressed during all stages of B-cell development, except terminally differentiated plasma cells, makes CD19 a key antigen for developing cellular therapies.

CD20 is a nonglycosylated 33- to 37-kDa integral membrane phosphoprotein involved in regulation of B-cell proliferation and differentiation (25–28). It is expressed slightly later in B-cell development than CD19, is not rapidly internalized, is expressed at a high surface density on the vast majority of lymphomas, and is eventually down-regulated on terminally differentiated plasma cells (25, 29). Recently, much

of the clinical work has focused on passive therapy using Rituximab, a monoclonal antibody (mAb) directed against the CD20 antigen, either alone or coupled to a radioactive compound. Although favorable clinical responses have been observed, these antibodies alone are not curative with most responders achieving only partial remissions with a mean time to disease progression of 13.2 months following antibody treatment (30). Thus, therapies designed to activate a patient's immune system, peptide vaccination or administration of peptide induced antigen-specific T cells in conjunction with conventional therapies could improve treatment outcome for patients with B cell malignancies.

Here we report on the identification of two novel immunogenic HLA-A2.1-specific peptides, CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV), capable of inducing antigen-specific CTLs. The CTLs displayed HLA-A2.1-restricted cytotoxic activity against a broad range of malignant B cell lines including human chronic B cell leukemia (JVM-2), multiple myeloma (IM9), and Burkitt's lymphoma (ST486) cell lines, expressing the corresponding antigen from which the peptide was derived. The cytotoxic activity of CD19-CTLs or CD20-CTLs was shown as CD19₁₅₀₋₁₅₈ (KLMSPKLYV) or CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptide-specific, respectively. In addition, cell proliferation and IFN- γ secretion in response to antigen restimulation were also HLA-A2.1-restricted and CD19 or CD20 antigen-specific, thereby confirming the functional activity of the CTLs. Therefore, the immunogenic peptides derived from the CD19 and CD20 self-antigens identified in this study offers a unique and promising immunotherapeutic approaches for targeting a broad range of B-cell malignancies.

MATERIALS AND METHODS

Cell Lines

The following human cell lines were obtained from American Type Culture Collection (Manassas, VA): IM9 (multiple myeloma), ST486 and Raji (Burkitt's lymphoma), and K562 (Chronic myelogenous leukemia). The human chronic B-cell leukemia cell line JVM-2 was a gift from Dr. Smitha Sivaraman (Rush University Medical Center, Chicago, IL). The HLA-A2.1 cDNA transfected K562-A0201 cell line was a gift from Dr. W. Herr (Mainz, Germany). The T2 cell line, a human B and T-cell hybrid expressing HLA-A2.1 molecules (31), was provided by Dr. J. Molldrem (University of Texas, M.D. Anderson Cancer Center, Houston, TX) and was used as antigen-presenting cells. All cell lines were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS (Bio Whittaker, Walkersville, MD), 100 IU/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies).

Reagents

Mouse anti-human HLA-A2.1 mAb was purified from the culture supernatant of the hybridoma BB7.2 cell line (gift from Dr. J. Molldrem). Recombinant human granulocyte macrophage colony-stimulating factor was obtained from Immunex (Seattle, WA). Recombinant human interleukin 2 (IL-2), IL-4, IFN- γ , and tumor necrosis factor- α were purchased from R&D Systems (Minneapolis, MN). Mouse anti-human CD80 or CD83 mAbs conjugated with PE were purchased from Immunotech (Hialeigh,

FL). CD3, CD4, CD8, CD14, CD19, CD20, CD56, CD86, and HLA-DR mAbs conjugated with FITC, PE or PerCP were purchased from Becton Dickinson/PharMingen (San Diego, CA).

Synthetic Peptide

Influenza virus protein matrix peptide₅₈₋₆₆ (GILGFVFTL) and MAGE-3 peptide₂₇₁₋₂₇₉ (FLWGPRLV) was used as HLA-A2.1-specific peptide controls. All peptides including CD19 and CD20 peptides were synthesized (Biosynthesis, Lewisville, TX) by standard fmoc (9-fluorenylmethyl-oxycarbonyl) chemistry, purified to >90% using reverse-phase chromatography, and validated by mass-spectrometry for molecular weight. Lyophilized peptides were dissolved in DMSO (Sigma, St. Louis, MO), diluted in AIM-V medium (Life Technologies), and stored at -140°C .

Peptide Binding Assay

CD19 and CD20 peptides were evaluated for HLA-A2.1-specific binding using the T2 cell line. In the assay, T2 cells were washed, resuspended in serum-free AIM-V medium to a final concentration of 1×10^6 cells/mL and transferred into a 24-well tissue culture plate. The cells were pulsed with 100 μ g/mL of CD19 or CD20 peptide or 30 μ g/mL influenza virus protein matrix peptide plus 3 μ g human β_2 -microglobulin (Sigma) and incubated at 37°C , 5% CO_2 in humidified air. Following overnight incubation, the cells were washed, stained with mouse anti-human HLA-A2.1 mAb for 15 minutes at 4°C , washed and incubated with goat anti-mouse IgG [F(ab')₂]-FITC (Sigma) for 15 minutes at 4°C . The cells were analyzed using a FACSort flow cytometer with CellQuest v2.1 software (Becton Dickinson, San Jose, CA). The Fluorescence Index (FI = mean channel fluorescence of T2 cells pulsed with the peptide plus β_2 microglobulin / mean channel fluorescence of T2 cells pulsed with β_2 microglobulin) was calculated to determine the up-regulation of HLA-A2.1 molecules on T2 cells caused by HLA-A2.1-specific peptide binding.

Peptide Stability Assay

The CD19 and CD20 peptides were examined for their HLA-A2.1 binding stability using the T2 cell line. T2 cells were pulsed with respective peptide as described above. After overnight incubation, the cells were washed to remove unbound peptide and incubated with 10 μ g/mL Brefeldin A (Sigma) at 37°C for 1 hour to block cell surface expression of newly synthesized HLA-A2.1 molecules. The peptide/HLA-A2.1 binding stability was evaluated 0, 2, 4, 6, and 18 hours post-Brefeldin A treatment. Following the incubation period, the cells were harvested, washed, stained with mouse anti-human HLA-A2.1 mAb and goat anti-mouse IgG [F(ab')₂]-FITC (Sigma) and analyzed using a FACSort[™] flow cytometer with CellQuest v2.1 software (Becton Dickinson). The results were calculated as the dissociation complex₅₀ (dc_{50}), which is defined as the time required for a 50% loss of HLA-A2.1/peptide complexes stabilized at time = 0.

Cell Isolation

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood of normal HLA-A2.1⁺ donors by standard density gradient centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala Sweden). Peripheral blood mononuclear cells were harvested from the interface,

washed twice, and resuspended in PBS supplemented with 5 mmol/L EDTA and 0.5% human serum albumin. Informed consent was obtained from all donors and the protocol was approved by the Rush University Medical School Institutional Review Board.

Monocytes. CD14⁺ monocytes were separated from peripheral blood mononuclear cells using magnetic cell selection technology (Miltenyi Biotec, Auburn, CA). peripheral blood mononuclear cells were incubated with colloidal supraparamagnetic microbeads conjugated with anti-human CD14 mAb for 15 minutes at 4°C. After washing, the cells were passed over a selection column in a magnetic field and the positively selected CD14⁺ cells were eluted from the columns. Purity (mean ± SD) of the cells was examined by flow cytometry and was found to be 93 ± 4%.

CD3⁺ T Cells. CD3⁺ T cells were isolated from the monocyte depleted cell fractions using the Pan T cell isolation kit (Miltenyi Biotec). In brief, T-cell enrichment was accomplished by depletion of B cells, natural killer cells, early erythroid cells, platelets, and basophils by labeling with a cocktail of hapten-conjugated CD11b, CD16, CD19, CD36, and CD56 antibodies and MACs microbeads coupled to an anti-hapten mAb. The effluent (negative cell fraction) was collected from the column as enriched CD3⁺ T cells. Purity (mean ± SD) of the enriched CD3⁺ T cells was examined by flow cytometry and was found to be 95 ± 3%.

CD4⁺ or CD8⁺ T Cells. CD4 and CD8 T-cell subsets were isolated from the CD19-CTLs or CD20-CTLs using the CD8⁺ T cell isolation kit (Miltenyi Biotec). CD8⁺ T cells were enriched by direct magnetic selection (positive selection) and the CD4⁺ T cells were collected from the column flow through effluent (negative enrichment).

Generation of Mature Dendritic Cells

Fresh or frozen/thawed CD14⁺ cells were cultured in RPMI 1640 supplemented with 10% FCS, 1,000 units/mL granulocyte macrophage colony-stimulating factor, and 1,000 units/mL IL-4 to generate dendritic cells (DC). The CD14⁺ cells were fed fresh medium plus granulocyte macrophage colony-stimulating factor and IL-4 every other day, and cell differentiation was monitored by light microscopy. On day 7, the cultures were supplemented with fresh granulocyte macrophage colony-stimulating factor and IL-4 along with 1,000 units/mL IFN-α plus 10 ng/mL tumor necrosis factor-α to induce DC maturation. After an additional 3 days of incubation, the mature DCs (mDC) were harvested, phenotyped by flow cytometry and used as antigen-presenting cells in the generation of CTLs.

Induction of Peptide-Specific CTLs

To generate CTLs, CD3⁺ T lymphocytes were repeatedly stimulated with autologous mDCs pulsed with peptide. In brief, mDCs were harvested, washed with serum-free medium and pulsed overnight with 100 µg/mL of CD19₁₅₀₋₁₅₈ (KLMSPKLYV) or CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptide in serum-free AIM-V medium. The peptide-loaded mDCs were harvested, washed, irradiated at 10 Gy, and resuspended in AIM-V medium supplemented with 10% human AB serum (Bio Whittaker). Irradiated peptide-pulsed mDCs were used to prime autologous CD3⁺ T cells at a 1:20 stimulator-to-responder cell

ratio in AIM-V medium supplemented with 10% human AB serum. Cultures were restimulated every 7 days (one cycle) with irradiated peptide-pulsed mDCs, and a total of three stimulations were done. IL-2 (100 units/mL) was added to the cultures 2 days after the second stimulation and the cells were fed thrice a week with fresh medium containing human AB serum and cytokines. The CTLs were evaluated 1 week after the third peptide stimulation. Control T cells were grown under the same culture conditions in the presence of IL-2 without peptide stimulation.

Cytotoxicity Assay

The cytotoxic activities of the CD19-CTLs and CD20-CTLs were measured in a standard ⁵¹Cr-release assay. CTLs were seeded with ⁵¹Cr-labeled target cells (5 × 10³ cells per well) at various effector/target cell ratios in 96-well U-bottomed microtiter plates (triplicate wells per sample). Plates were incubated for 6 hours at 37°C, 5% CO₂. ⁵¹Cr-release was measured in 100 µL supernatant using a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Brea, CA). Maximum release was obtained from detergent-released target cell counts and spontaneous release from target cell counts in the absence of effector cells. Cellular cytotoxicity was calculated as follows: % specific lysis = [(experimental release – spontaneous release) / (maximum release – spontaneous release)].

T-Cell Proliferation Assay

Unstimulated autologous T lymphocytes, CD19-CTLs or CD20-CTLs (3 × 10⁴ per well) were cocultured with irradiated stimulator cells (malignant B cell lines, 3 × 10³ per well) in triplicate wells of 96-well U-bottomed microtiter plates. Cultures established in the absence of responder or stimulator cells were prepared to monitor background proliferation. The cells were cultured in AIM-V medium supplemented with 10% human AB serum and 100 units/mL rIL-2. After 6 days of culture, the cells were pulsed with 1 µCi [³H]-thymidine for 18 hours and harvested to measure the proliferation of the CTLs (responder cells). Cells were harvested onto filter discs using the PhD cell harvester, resuspended in liquid scintillation fluid, and evaluated for [³H]-counts per minute using a Beckman LS6500 scintillation counter.

IFN-γ ELISA

IFN-γ release by the CD19-CTLs or CD20-CTLs was measured using an IFN-γ ELISA kit from PBL-Biomedical Lab (Piscataway, NJ). Briefly, purified IFN-γ as standards or CTL culture supernatants were transferred into wells of a 96-well plate precoated with a monoclonal anti-human IFN-γ capture antibody and incubated for 1 hour in a closed chamber at 24°C. After washing the plate with PBS/0.05% Tween 20, biotin anti-human IFN-γ antibody was added to the wells and incubated for 1 hour at 24°C. The wells were washed and then developed by incubation with streptavidin horseradish peroxidase conjugate and TMB substrate solution. Stop solution was added to each well and the absorbance was determined at 450 nm with a SpectraMAX Plus plate reader (Stratagene, La Jolla, CA). The amount of cytokine present in the CTL culture supernatant was calculated based on the IFN-γ standard curve.

Expansion of CD20-CTLs

CD20-CTLs that showed functional activity were evaluated for their expansion potential to be expanded under good

manufacturing process culture conditions, a requirement to implement this approach in a clinical setting. The CD20-CTL cultures were grown in AIM-V serum-free medium containing 10% human AB serum, 50 units/mL rIL-2, and 1 μ L/mL CD3/CD28 microbeads (Dyna, Lake Success, NY) in 6-well plates. The CTLs were restimulated with fresh media, CD3/CD28 microbeads and IL-2 every 3 days during the cell expansion period.

RESULTS

Identification of HLA-A2.1-Specific CD19 and CD20 Peptides. The full-length protein sequences of the CD19 or CD20 antigens were analyzed using the search software SYFPEITHI to predict HLA-A2.1-specific peptides followed by the BIMAS program to select peptides with extended half-time disassociation rates. Based on the software analyses scores, two CD19 and five CD20 nonamers were selected and synthesized for evaluating HLA-A2.1-specific peptides. The synthesized CD19 and CD20 peptides are as follows: CD19₁₅₀₋₁₅₈ (KLMSPKLYV; extracellular domain), CD19₂₉₆₋₃₀₄ (TLAYLIFCL; transmembrane domain), CD20₁₂₇₋₁₃₅ (AISGMILSI; transmembrane domain), CD20₁₄₇₋₁₅₅ (LKMESLNFI; extracellular domain), CD20₁₅₁₋₁₅₉ (SLNFIRAHT; extracellular domain), CD20₁₅₄₋₁₆₂ (FIRAHTPYI; extracellular domain), and CD20₁₈₈₋₁₉₆ (SLFLGILSV; transmembrane domain). The HLA-A2.1 affinity/stability of these peptides was assessed using the T2 peptide-binding assay. The HLA-A2.1 peptide affinity was determined as the FI. The HLA-A2.1 stability was calculated as the dc_{50} .

Table 1 summarizes the HLA-A2.1 affinity/stability (mean \pm SE, $n = 3$) of the CD19 and CD20 peptides. Among the peptides tested, the CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptides displayed the highest HLA-A2.1 binding (CD19₁₅₀₋₁₅₈, FI = 2.66 ± 0.36 ; CD20₁₈₈₋₁₉₆, FI = 2.73 ± 0.25), which was close to the affinity of the HLA-A2.1-specific control influenza virus protein matrix peptide₅₈₋₆₆ (FI = 3.03 ± 0.55). The remaining peptides, CD19₂₉₆₋₃₀₄ (TLAYLIFCL), CD20₁₂₇₋₁₃₅ (AISGMILSI), CD20₁₄₇₋₁₅₅ (LKMESLNFI), CD20₁₅₁₋₁₅₉ (SLNFIRAHT), and CD20₁₅₄₋₁₆₂ (FIRAHTPYI) displayed minimal binding to HLA-A2.1 as shown by low FI values. The greatest HLA-A2.1 stability ($dc_{50} = 6$ hours) was found for CD19₁₅₀₋₁₅₈ and CD20₁₈₈₋₁₉₆ peptides, which correlated with their high HLA-A2.1 affinity.

Thus, the CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptides were chosen to assess their immunogenicity through the generation of CTLs targeting malignant B cell lines.

Expression of CD19, CD20, and HLA-A2.1 Molecules on Malignant B cell Lines. Malignant B cell lines were screened for their expression of CD19, CD20, and HLA-A2.1 antigens to apply them as target or stimulatory cells in the functional assays evaluating the CD19-CTLs or CD20-CTLs. Figure 1 shows that the IM9, JVM-2, and ST486 cell lines were all CD19⁺/CD20⁺/HLA-A2.1⁺. In contrast, the K562-A0201 cell line was CD19⁻/CD20⁻/HLA-A2.1⁺ and the Raji cell line was CD19⁺/CD20⁺/HLA-A2.1⁻, making them suitable as negative controls in the assays.

Antigen-Specific and HLA-A2.1-Restricted Cytotoxic Activity of CD19-CTLs and CD20-CTLs. The CTLs induced with CD19₁₅₀₋₁₅₈ (KLMSPKLYV) or CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptide pulsed autologous dendritic cells were tested in chromium release cytotoxicity assays (Fig. 2). Both the CD19 and CD20-CTLs showed effective killing of the CD19⁺/CD20⁺/HLA-A2.1⁺ malignant B cell lines, JVM-2, IM-9 and ST486. However, the CTLs did not show any significant lysis of the K562-A0201 (CD19⁻/CD20⁻/HLA-A2.1⁺) or Raji (CD19⁺/CD20⁺/HLA-A2.1⁻) negative control cell lines. In addition, the CTLs did not kill the natural killer-sensitive cell line K562 (CD19⁻/CD20⁻/HLA-A2.1⁻), confirming that the observed cytotoxicity against the malignant B cell lines was not due to an activated natural killer cell population.

Peptide-Specific Cytotoxic Activity of CD19-CTLs and CD20-CTLs. Peptide-specific cytotoxicity of the CD19-CTLs and CD20-CTLs was confirmed in ⁵¹Cr-release assays using T2 cells pulsed with HLA-A2.1-specific CD19₁₅₀₋₁₅₈ (KLMSPKLYV), CD20₁₈₈₋₁₉₆ (SLFLGILSV) or MAGE-3₂₇₁₋₂₇₉ (FLWGPALV) peptides. Figure 3 summarizes the high cytotoxic activity of the CD19-CTLs and CD20 CTLs against T2 cells pulsed with the appropriate CD19 or CD20 peptide. However, T2 cells alone or T2 cells pulsed with the irrelevant HLA-A2.1-specific MAGE-3₂₇₁₋₂₇₉ peptide were not lysed (<10% specific lysis) by either the CD19-CTLs or CD20-CTLs, thereby confirming CD19 or CD20 peptide-specific cytotoxicity by the CTLs.

Table 1 Evaluation of CD19 or CD20 peptides for HLA-A2.1 binding affinity and stability

Peptides	Peptide sequence	Protein domain	Fluorescence index*	dc_{50} (h)
Control peptide				
Influenza virus protein matrix peptide ₅₈₋₆₆	GILGFVFTL		3.03 ± 0.55	>18
CD19 peptides				
CD19 ₁₅₀₋₁₅₈ peptide	KLMSPKLYV	Extracellular	2.66 ± 0.36	6
CD19 ₂₉₆₋₃₀₄ peptide	TLAYLIFCL	Transmembrane	2.30 ± 0.31	4
CD20 peptides				
CD20 ₁₂₇₋₁₃₅ peptide	AISGMILSI	Transmembrane	1.65 ± 0.38	2
CD20 ₁₄₇₋₁₅₅ peptide	LKMESLNFI	Extracellular	1.53 ± 0.45	4
CD20 ₁₅₁₋₁₅₉ peptide	SLNFIRAHT	Extracellular	1.03 ± 0.09	2
CD20 ₁₅₄₋₁₆₂ peptide	FIRAHTPYI	Extracellular	1.62 ± 0.39	2
CD20 ₁₈₈₋₁₉₆ peptide	SLFLGILSV	Transmembrane	2.73 ± 0.25	6

*Mean \pm SE.

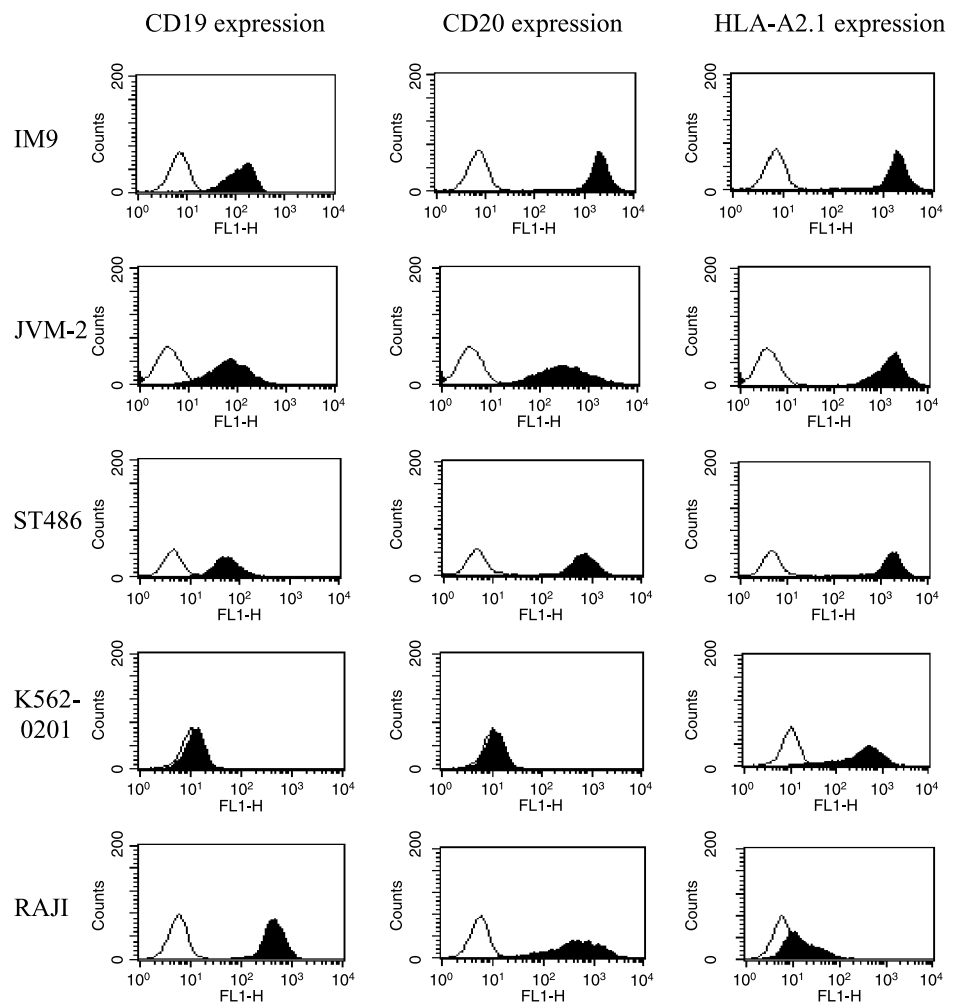


Fig. 1 Phenotype of malignant B cell lines. Malignant B cell lines were analyzed for CD19, CD20, or HLA-A2.1 antigen expression by flow cytometry. The results show that the IM9, JVM-2, and ST486 cell lines were CD19⁺/CD20⁺/HLA-A2.1⁺, making them useful as target cells in evaluating the CD19-CTLs and CD20-CTLs.

Antigen-Specific and HLA-A2.1-Restricted Induction of CD19-CTLs and CD20-CTLs Proliferation. Proliferation of CD19-CTLs and CD20-CTLs in response to stimulation with various malignant B cell lines was measured by ³[H]-thymidine incorporation (Fig. 4). No significant CTL proliferation ($P > 0.05$) was observed in response to the CD19⁺/CD20⁺/HLA-A2.1⁻ Raji or the CD19⁻/CD20⁻/HLA-A2.1⁺ K562-A0201 cell lines. CTL proliferation was significantly higher ($*P < 0.05$) in response to CD19⁺/CD20⁺/HLA-A2.1⁺ ST486 or IM9 cells stimulation compared with the Raji or K562-A0201 cells. Stimulation with the Raji or K562-A0201 cells did not result in proliferation of the CD19- or CD20-CTLs. These results show that the CTL proliferation was CD19 or CD20 antigen specific and HLA-A2.1 restricted, which was also observed in the cytotoxicity assays described previously (Figs. 2 and 3). Overall, the CD20-CTLs showed a higher proliferative response to the CD19⁺/CD20⁺/HLA-A2.1⁺ stimulator cells compared with the CD19-CTLs.

The Specific IFN- γ Secretion by the CD19-CTLs and CD20-CTLs. Further confirmation of the antigen specificity and HLA-A2.1 restriction of the CD19-CTL and CD20-CTL was provided by the induction of IFN- γ secretion following overnight stimulation with the various malignant B cell lines.

Both the CD19-CTL and CD20-CTL showed a significant increase ($*P < 0.05$) in IFN- γ secretion following stimulation with CD19⁺/CD20⁺/HLA-A2.1⁺ ST486 or IM9 cells compare to the stimulation with CD19⁺/CD20⁺/HLA-A2.1⁻ Raji or CD19⁻/CD20⁻/HLA-A2.1⁺ K562-A0201 cells (Table 2). These results offer further evidence of an antigen-specific and HLA-A2.1-restricted response by our CD19-CTL and CD20-CTL.

Higher Level of Cytotoxicity by CD8⁺ T Subset rather than CD4⁺ T Subset within CD19-CTLs and CD20-CTLs. To identify the effector cell population with the higher cytotoxic activity, CD4⁺ or CD8⁺ cells were isolated from the CD19-CTLs and CD20-CTLs using Miltenyi cell separation technology. The purity of enriched CD4⁺ or CD8⁺ cells was >94% (data not shown) following the isolation procedure. Both effector subsets displayed cytotoxic activity against IM9 and JVM cells. However, the CD8⁺ T cell subsets from both the CD19-CTLs and CD20-CTLs displayed a higher cytotoxic activity against the target cells compared with the CD4⁺ cell population (Fig. 5).

Ex vivo Expansion of CD20-CTLs. Using 10×10^6 of starting number of CD3⁺ T cells, about 3 to 4×10^6 CTLs were obtained after three peptide stimulation and they showed the

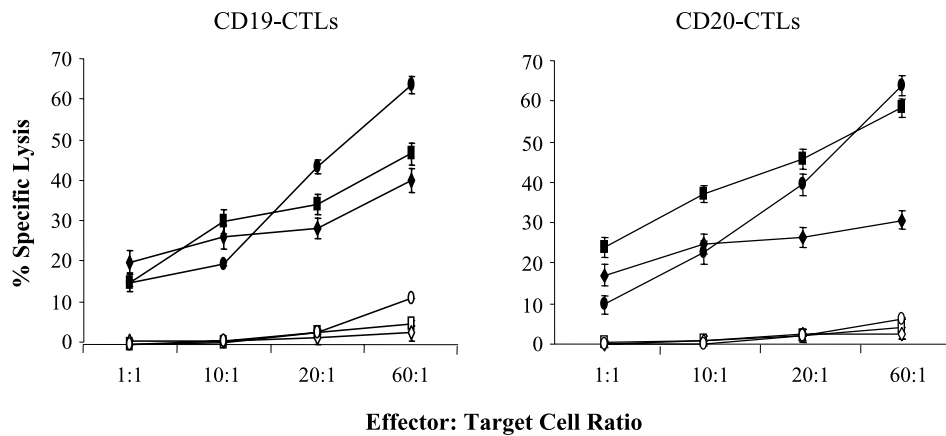


Fig. 2 HLA-A2.1-restricted and antigen-specific cytotoxicity of the CD19-CTLs or CD20-CTLs. CTLs were generated by repeated stimulation of normal donor HLA-A2.1⁺ T lymphocytes with autologous mDCs pulsed with either CD19₁₅₀₋₁₅₈ (KLMSPKLYV) or CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptide. The data displays standard ⁵¹Cr-release cytotoxicity assay results done one week after the third stimulation. Both the CD19-CTLs and CD20 CTLs displayed significant cytotoxic activity against the CD19⁺/CD20⁺/HLA-A2.1⁺ JVM-2 (●), IM9 (■), or ST486 (◆) cell lines, but not the CD19⁺/CD20⁺/HLA-A2.1⁻ K562-A0201 (□), CD19⁺/CD20⁺/HLA-A2.1⁻ Raji (◇), or natural killer-sensitive K562 (○) cell lines. Points, mean specific lysis from three experiments; bars, ±SE.

characteristics of antigen specificity in cytotoxicity, cell proliferation, IFN- γ secretion, and phenotypes. Clinical application of antigen-specific CTLs requires large-scale expansion of these cells under good manufacturing process conditions. We therefore evaluated the expansion potential of the CD20 antigen-specific CTLs by culturing the cells (1×10^6 starting cells) in AIM-V medium supplemented with 10% human AB serum and 100 units/mL rIL-2 (Proleukin) in the absence or presence of CD3/CD28 microbeads. Figure 6A shows a 10-fold cell increase by day 30 of culture in the presence of IL-2 and CD3/CD28 beads. The greatest CTL expansion occurred between day 30 and 40, with a total of 35×10^6 cells obtained at the end of the culture period. CD20-CTLs grown in IL-2 alone died off after 10 days of culture (Fig. 6A), indicating that stimulation of the cells through CD3 and CD28 was

crucial for supporting expansion of the CTLs. The expanded CTLs were tested for their ability to maintain their cytotoxic activity. Figure 6B shows a comparable level of cytotoxicity between the initial (day 0) and the expanded CD20-CTLs against the CD19⁺/CD20⁺/HLA-A2.1⁺ ST486 cell line.

DISCUSSION

Adoptive immunotherapy targeting antigens overexpressed on tumor cells is a developing field for treating patients with a variety of malignancies. Current treatment modalities include mAb therapy, vaccine strategies, and/or the infusion of antigen-specific CTLs targeting the tumor cells. To date, cellular-based therapies have been difficult to develop due to limitations in identifying tumor-specific antigens. Alternatively, self-proteins

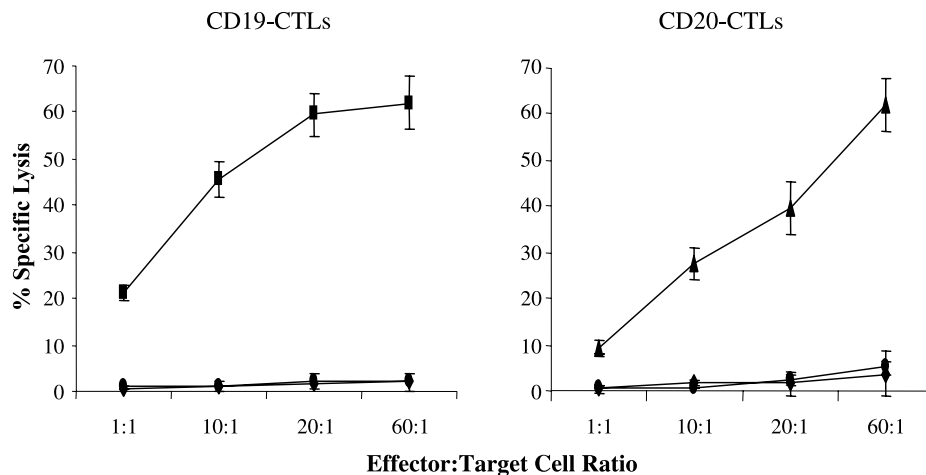
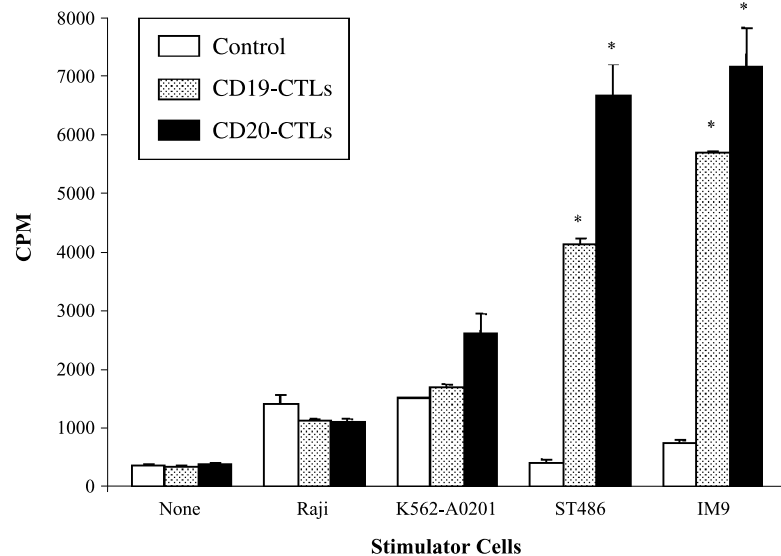


Fig. 3 Peptide-specific cytotoxicity of CD19-CTLs or CD20-CTLs. CTLs were tested 1 week after the third stimulation of normal HLA-A2.1⁺ T lymphocytes with mDCs pulsed with either HLA-A2.1-specific CD19₁₅₀₋₁₅₈ (KLMSPKLYV) or CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptide. CD19- or CD20-CTL cytotoxic activity was measured in a standard ⁵¹Cr-release assay against CD19 peptide (■), CD20 peptide (▲), irrelevant HLA-A2.1-binding MAGE-3 peptide (●) or no peptide (◆) pulsed T2 target cells. The cytotoxicity of the CD19-CTLs and CD20-CTLs is peptide-specific against T2 cells presenting the appropriate CD19 or CD20 peptide. No cytotoxic activity was observed against T2 cell presenting the irrelevant MAGE-3 peptide or T2 cells alone. Points, mean % specific lysis from three experiments; bars, ±SE.

Fig. 4 Induction of antigen-specific and HLA-A2.1-restricted proliferation of CD19-CTLs and CD20-CTLs. Proliferation of the CD19-CTLs and CD20-CTLs in response to antigen restimulation by [³H]-thymidine incorporation on day 7 of culture. Columns, mean; bars, \pm SE. CTLs alone were used to determine the background proliferation and unstimulated T cells were used as control effector cells. The CD19-CTLs and CD20-CTLs did not show a significant level of proliferation in response to CD19⁺/CD20⁺/HLA-A2.1⁻ Raji or CD19⁻/CD20⁻/HLA-A2.1⁺ K562-A0201 cells but showed a significant proliferation activity against the CD19⁺/CD20⁺/HLA-A2.1⁺ ST486 or IM9 cell lines. A statistically significant difference (*, $P < 0.05$) in cell proliferation was observed between the CD19-CTLs or CD20-CTLs stimulated with the ST486 or IM9 cell lines and the CTLs stimulated with the Raji or K562-0201.



overexpressed on tumor cells can offer unique target antigens for inducing the tumor-specific CTLs. Recently, CD45 (32), CD52 (33, 34), p53 (35–37), HER-2/*neu* (38–40), and CD33 (41) self-antigens were shown to be effective in eliciting CTLs *in vivo* and *in vitro*. Therefore, the development of an immunotherapeutic strategy targeting overexpressed B-cell self-antigen(s) could potentially bring new treatment options to a broad range of B-cell malignancies.

In this study, we identified nonameric peptides for the generation of CTLs specific to immunogenic epitopes derived from the CD19 and CD20 self-antigens that are overexpressed on malignant B cells of non Hodgkin's lymphoma or chronic B-lymphocytic leukemia. CD19 and CD20 are coexpressed during B-cell differentiation until they are down-regulated on plasma cells (42). Only the CD19 antigen is expressed on hematopoietic progenitor cells of the B cell lineage with the CD20 antigen appearing later during B-cell development. Our strategy was to identify HLA-A2.1-specific CD19 and CD20 immunogenic peptides that would allow targeting cells of the B lineage that have undergone neoplastic transformation including leukemia and lymphomas. Initially, we screened the full-length of CD19 and CD20 protein sequences to predict HLA-A2.1-specific peptides by using algorithms contained within the SYFPEITHI and BIMAS software programs. Based on the prediction using the software programs and examination of HLA-A2.1 anchor amino acid residues, peptides were synthesized and tested for HLA-A2.1

affinity/stability. Among the peptides tested, the CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV) peptides were shown to have the highest HLA-A2.1 affinity (FI > 2.6) and stability ($dc_{50} = 6$ hours).

The challenge in targeting CD19 and CD20 antigens is to break T-cell tolerance to these B cell-specific self-antigens, yielding CTLs that have strong and specific immune reactivity. Previously, other investigators have shown that immunization with CD20 peptides induces an active and antigen-specific immune response in mice (43). Our data show that repeated stimulation of human T lymphocytes with HLA-A2.1-specific CD19₁₅₀₋₁₅₈ or CD20₁₈₈₋₁₉₆ peptide pulsed autologous mDCs generated the antigen-specific CTLs. Functional assays, including IFN- γ ELISA, lymphoproliferation assays, and cytotoxicity assays confirmed the antigen-specific and HLA-A2.1-restricted responses of the CTLs to various malignant B cell lines *ex vivo*.

To develop an optimal strategy for a B cell-directed immunotherapy, a better understanding of the interplay among diverse cell types of the immune system, particularly MHC class I CD8⁺ CTLs and MHC class II CD4⁺ T helper cells, is desired. Although the peptides were MHC class I-specific, the CD19-CTLs and CD20-CTLs contained both CD4⁺ and CD8⁺ T cells. Our observation is in agreement with previous studies showing that malignant B cell-associated antigens contain tumor-specific protein sequences that stimulated both CD4⁺ and CD8⁺ T cells (44–46).

Table 2 IFN- γ secretion by the CD19-CTLs or CD20-CTLs in response to restimulation with malignant B cell lines

Stimulator cells	Responder cells		
	Control T cells (IFN- γ secretion, ng/mL)	CD19-CTLs (IFN- γ secretion, ng/mL)	CD20-CTLs (IFN- γ secretion, ng/mL)
None	122 \pm 3.5*	540 \pm 20	524 \pm 20
Raji	71 \pm 0.5	594 \pm 49	605 \pm 39
K562-A0201	93 \pm 4	489 \pm 17	479 \pm 4
ST486	133 \pm 17	846 \pm 14 [†]	1065 \pm 16 [†]
IM9	94 \pm 5	1449 \pm 80 [†]	1445 \pm 82 [†]

*Mean \pm SE ($n = 3$).

[†] $P < 0.05$.

The CD19-CTLs and CD20-CTLs were isolated into CD4⁺ and CD8⁺ T subsets (purity > 94%) using Miltenyi magnetic cell separation technology for further characterization of phenotype and cytotoxic activity. The CD8⁺ T-cell subset showed a higher level of target cell lysis than CD4⁺ T cells, demonstrating that CD8⁺ T cells would be stronger effector cells in potential immunotherapeutic applications. However, the presence of CD4⁺ T helper cells may provide critical support for the survival and expansion of CD8⁺ CTLs in long-term culture (47). Phenotypic analyses revealed a higher percentage of the CD8⁺ T cells in the CD19-CTLs and CD20-CTLs compared with the control T cells. We also showed a decrease in the percentage of CD4⁺ or CD8⁺ cells having a CD45RA⁺/CCR7⁺ (naive) or CD45RA⁻/CCR7⁺ (central memory) phenotype and a corresponding increase in the percentage of cells having a CD45RA⁻/CCR7⁻ (effector memory) and CD69⁺/CD45RO⁺ (activated memory) phenotype (data not shown). These observations confirm results by other investigators showing that *ex vivo* peptide-specific CTLs have distinct phenotypes (48–50).

The immunotherapeutic approach of this study can be translated into two different clinical applications. Peptides, especially derived from melanoma or chronic myeloid leukemia, have been successfully used in clinical trials (51–54). Based on this history, the identified CD19 and/or CD20 peptides could be used directly as a tumor vaccine, possibly supported by an adjuvant and IL-2 administration. Alternatively, the *ex vivo* generated peptide-specific CTLs could be given to patients as an

adoptive T-cell immunotherapy (55, 56). This strategy is of particular interest in the context of HLA-identical sibling transplants where donor lymphocyte infusions have shown to induce remission. The advantage of infusing allogenic over autologous CTLs is related to the observation that autologous CTLs can be tolerant to the malignant cells due to the T-cell defects. To examine the potential immunotherapeutic CTL approach, we tested whether they can be successfully expanded using the cyclic guanosine 3',5'-monophosphate reagents including AIM-V media, human AB serum, rIL-2 (Proleukin), and CD3/CD28 microbeads. Our results showed that this could be accomplished and it is also important to note that the cells retain their antigen-specific cytotoxicity without having to be restimulated with CD20 peptide-pulsed mDCs.

Because nonmalignant CD19⁺ and CD20⁺ B cells will be subject to recognition by redirected CTLs, the persistence of the adoptively transferred CD19- or CD20-specific CTLs has the potential to result in prolonged B-cell autoreactivity. However, *in vivo* persistence of the specific T cells can be limited by coexpression of a suicide gene, such as thymidine kinase of herpes simplex virus, which would allow the elimination of the antigen-specific CD19-CTLs or CD20-CTLs with ganciclovir treatment if necessary (57–59). In addition, the clinical sequel of temporary B-cell lymphopenia may be an acceptable side effect of CD19- or CD20-directed immunotherapy, especially because prolonged ablation of normal CD20⁺ B cells in patients receiving rituximab therapy does not seem to result in clinically significant

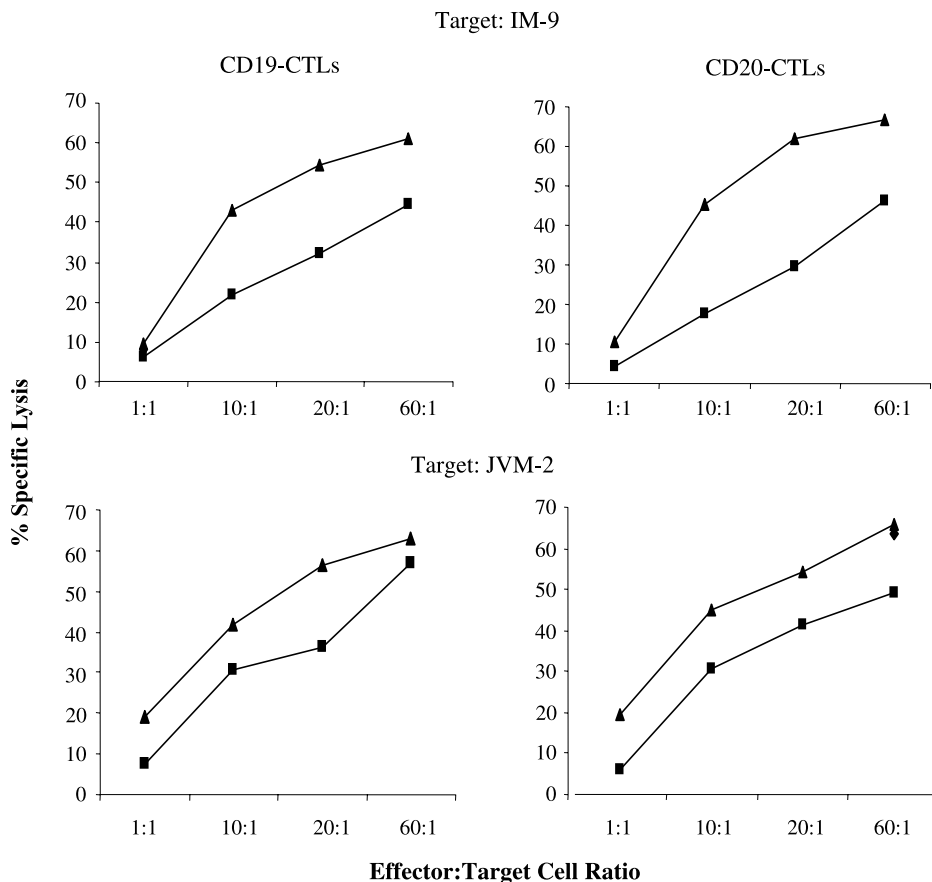


Fig. 5 Cytotoxicity of CD8⁺ or CD4⁺ cells isolated from CD19-CTLs or CD20-CTLs against malignant B cell lines. CD8⁺ (▲) or CD4⁺ (■) cells isolated from the CD19-CTLs and CD20-CTLs by magnetic cell separation (Miltenyi Biotec) were examined as effector cells in a standard ⁵¹Cr-release assay. The isolated CD8⁺ T cells from both CTLs showed a higher level of cytotoxicity against the CD19⁺/CD20⁺/HLA-A2.1⁺ IM9 or JVM-2 cell lines compared with CD4⁺ effector cells.

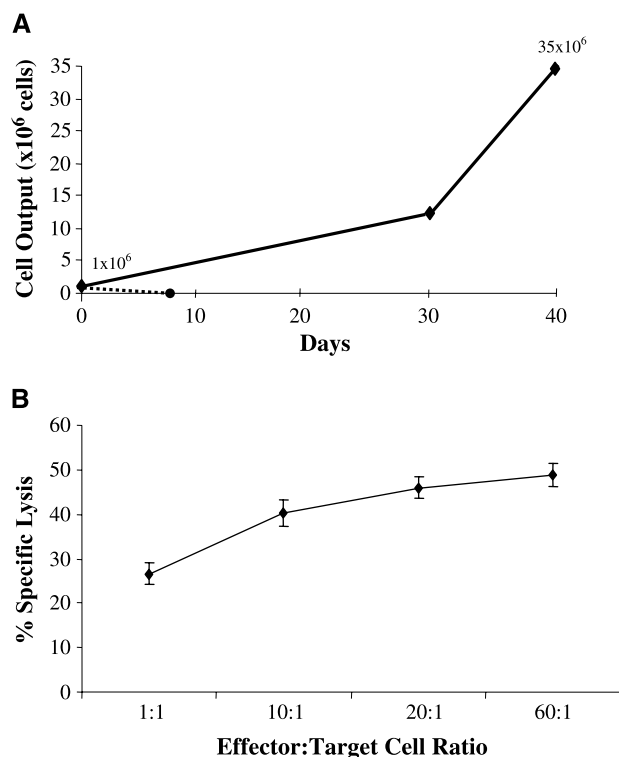


Fig. 6 Expansion and cytotoxic activity of CD20-CTLs grown under cGMP conditions. *A*, level of CD20-CTLs expansion was evaluated in the presence (◆) or absence (●) of Dynal CD3/CD28 microbeads. The CTLs were expanded for total 40 days in AIM-V medium containing 10% human AB serum and 50 units/mL rIL-2. The CD20-CTLs cultured with the CD3/CD28 microbeads showed continuous cell expansion. *B*, expanded CD20-CTLs were evaluated for their antigen-specific cytotoxicity. The CTLs maintained their cytotoxicity against the CD19⁺/CD20⁺/HLA-A2.1⁺ ST486 cell line.

complications attributable to depleted numbers of normal B cells (60, 61).

In conclusion, we have identified the novel CD19₁₅₀₋₁₅₈ (KLMSPKLYV) and CD20₁₈₈₋₁₉₆ (SLFLGILSV) HLA-A2.1-specific immunogenic peptides for generating functional human CTLs against malignant B cells. Our data suggests that the CD19- or CD20 peptides can be applied *ex vivo* with antigen-specific adoptive T-cell immunotherapy or *in vivo* for peptide vaccination. Future studies will investigate the clinical relevance of using these peptides either for infusion of the peptide-specific CTLs or vaccination.

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