The *PANE1* gene encodes a novel human minor histocompatibility antigen that is selectively expressed in B-lymphoid cells and B-CLL

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Minor histocompatibility antigens (mHAg's) are peptides encoded by polymorphic genes that are presented by major histocompatibility complex (MHC) molecules and recognized by T cells in recipients of allogeneic hematopoietic cell transplants. Here we report that an alternative transcript of the proliferation-associated nuclear element 1 *(PANE1)* gene encodes a novel human leukocyte antigen (HLA)–A*0301–restricted mHAg that is selectively expressed in B-lymphoid cells. The antigenic peptide is entirely encoded within a unique exon not present in other *PANE1* transcripts. Sequencing of *PANE1* alleles in mHAg-positive and mHAg-negative cells demonstrates that differential T-cell recognition is due to a single nucleotide polymorphism within the variant exon that replaces an arginine codon with a translation termination codon. The *PANE1* transcript that encodes the mHAg is expressed at high levels in resting CD19$^+$ B cells and B-lineage chronic lymphocytic leukemia (B-CLL) cells, and at significantly lower levels in activated B cells. Activation of B-CLL cells through CD40 ligand (CD40L) stimulation decreases expression of the mHAg-encoding *PANE1* transcript and reciprocally increases expression of *PANE1* transcripts lacking the mHAg-encoding exon. These studies suggest distinct roles for different PANE1 isoforms in resting compared with activated CD19$^+$ cells, and identify *PANE1* as a potential therapeutic target in B-CLL. (Blood. 2006;107:3779-3786)

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Introduction

Minor histocompatibility antigens (mHAg's) are peptides encoded by polymorphic genes that are presented on the cell surface by class I and class II major histocompatibility complex (MHC) molecules. In the setting of MHC-matched allogeneic hematopoietic cell transplantation (HCT), donor-recipient disparity at loci that encode mHAg's can induce T-cell responses that contribute to graft rejection, graft-versus-host disease (GVHD), and graft-versus-leukemia (GVL) activity (reviewed in Chao,[1] Falkenburg and Willemze,[2] and Bleakley and Riddell[3]). Molecular characterization of a small number of class I MHC–restricted human mHAg's recognized by CD8$^+$ T cells has established that most non–sex-linked mHAg's are created by single nucleotide polymorphisms (SNPs) in the coding sequence of autosomal genes that cause single amino acid changes in the encoded proteins. The recent identification of more than 9000 such polymorphisms in a large-scale survey of the human genome[4] suggests that the total number of human mHAg's is likely to be quite large, a finding that has important implications for the development of strategies for both preventing GVHD and enhancing GVL.

Class I MHC–restricted mHAg's exhibit diversity in the range of tissues in which they are expressed,[5,6] and multiple lines of evidence support a correlation between the tissue expression of a given mHAg and the contribution of T-cell responses against these antigens to GVHD and GVL activity.[3] MHAg's that are broadly expressed in both hematopoietic and nonhematopoietic tissues, such as the male-specific mHAg encoded by the *SMCY* gene[7-9] and the HA-8 mHAg encoded by the *KIAA0020* gene,[10,11] serve as targets for T-cell responses associated with GVHD. In contrast, T-cell responses against mHAg's such as HA-1[9,12] that are selectively expressed in hematopoietic cells, including leukemic cells, but not widely expressed in nonhematopoietic tissues, may be preferentially associated with GVL activity. Although a small number of mHAg's with the requisite tissue distribution to invoke a selective GVL response have been identified,[12-15] only a minority of allogeneic HCT recipients are eligible for immune therapy because of the allele frequency and the MHC restriction of these antigens. Thus, the identification of additional mHAg's that are selectively expressed in hematopoietic cells remains a high priority.

In this report, we have identified a novel human mHAg that is recognized by a cytotoxic T lymphocyte (CTL) clone, CTL-7A7, and selectively expressed in normal and malignant B-lymphoid cells. The antigenic peptide recognized by CTL-7A7 is encoded by

an alternative transcript of the proliferation-associated nuclear element 1 (*PANE1*) gene within an exon that is unique from all other *PANE1* transcripts. Differential recognition of donor and recipient cells by CTL-7A7 is attributable to a nonsynonymous SNP in the epitope-encoding variant exon that replaces an arginine codon with a translation termination signal. The alternative *PANE1* transcript is selectively expressed in B-lymphoid cells, with the highest levels of expression seen in resting CD19$^+$ cells and B-lineage chronic lymphocytic leukemia (B-CLL), thus suggesting that *PANE1* may be an attractive therapeutic target in B-CLL.

Materials and methods

Human subjects

Human tissue samples used in this study were obtained from adults who had provided written informed consent and were enrolled on clinical protocols approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center or the University of Pittsburgh Cancer Institute.

Cell culture

The CD8$^+$ CTL clone CTL-7A7 was isolated using techniques described previously[6] from peripheral blood mononuclear cells (PBMCs) of a female patient with acute myelogenous leukemia who received a hematopoietic cell graft from her MHC-identical brother after myeloablative conditioning. CTL-7A7 CTLs were cultured in RPMI-1640 supplemented with 25 mM HEPES (Invitrogen, Carlsbad, CA), 10% (vol/vol) heat-inactivated human serum, 1% penicillin/streptomycin, and 3 mM L-glutamine (designated CTL medium) as previously described.[16] Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines[17] (EBV-LCLs) and phytohemagglutinin (PHA)–stimulated T-cell blasts[6] (PHA blasts) were prepared from PBMCs, as previously described. T2A3 cells were a gift from Dr Peter Cresswell (Yale University, New Haven, CT). EBV-LCLs and T2A3 were maintained in RPMI-1640 with 25 mM HEPES, 10% (vol/vol) fetal calf serum (FCS), 1% penicillin/streptomycin, and 3 mM L-glutamine (LCL medium). Dermal fibroblast lines were generated from skin biopsy specimens and cultured as previously described.[18] CD19$^+$ and CD19$^-$ cells were isolated from blood of healthy human leukocyte antigen (HLA)–A*0301$^+$ donors by immunomagnetic separation with a murine monoclonal immunoglobulin G$_1$ (IgG$_1$) antibody specific for human CD19 (clone J4.119; Beckman Coulter, Hialeah, FL) and rat anti–mouse IgG$_1$ MicroBeads (Miltenyi Biotec, Auburn, CA). PBMCs from patients with B-CLL were isolated by centrifugation on Ficoll/Hypaque and depleted of monocytes by overnight adherence to plastic tissue-culture flasks. As assessed by flow cytometry, more than 95% of these cells typically coexpressed CD5 and CD19 (data not shown).

Extraction and HPLC fractionation of HLA-A3–associated peptides

HLA-A*0301 molecules were immunoaffinity purified from EBV-LCLs derived from an HLA-A*0301–homozygous individual who expressed the mHAg recognized by CTL-7A7 CTLs, using the monoclonal antibody GAP-A3,[19] and the HLA-A*0301–associated peptides were extracted as previously described.[10] Briefly, peptides were separated from class I heavy chains and β2-microglobulin (β2m) by elution in 10% acetic acid and passage through a 5-kDa cutoff filter. Peptides were fractionated on a narrow-bore HAISIL C18 column (2.1 × 40 mm, 5 μm particles, 300 Å pore size; Higgins, Mountain View, CA) on a 130A high-performance liquid chromatography (HPLC) device (Applied Biosystems [ABI], Foster City, CA). The elution gradient used was 0% to 10% solvent B in 10 minutes, 10% to 60% solvent B in the next 55 minutes, and 60% to 100% solvent B in the next 7 minutes, where solvent A was 0.1% trifluoroacetic acid (TFA) (HPLC grade; ABI) in NANOpure water (Barnstead, Dubuque, IA) and solvent B was 0.085% TFA in 60% acetonitrile (HPLC grade; Mallinckrodt, Phillipsburg, NJ). Fractions were collected every 40 seconds

at a flow rate of 200 μL/min. Active fractions were pooled and rechromatographed with the identical column and gradient, but using 0.1% heptafluorobutyric acid (HFBA) (HPLC grade; Pierce, Rockford, IL) as the ion-pairing agent. Half of the active second dimension material was used for a third-dimension fractionation on a microcapillary column[20] (280 μm outer diameter, 75 μm inner diameter packed with 25 cm of 5 μm C18 beads [YMC, Milford, MA]). TFA was used as the ion-pairing agent in solvents A and B described, and the column was eluted with a linear gradient of 0% to 100% solvent B over 40 minutes at a flow rate of 300 nL/min.

Epitope reconstitution assays

Aliquots of each HPLC fraction were incubated with 2000 ^{51}Cr-labeled donor EBV-LCL target cells and 7.5 μg/mL human β2m (Calbiochem, San Diego, CA) for 90 minutes at 26°C in 150 μL LCL medium. CTL-7A7 CTLs were added in 100 μL LCL medium at an effector-to-target (E/T) ratio of 10:1 in a standard chromium release assay.[13] Synthetic peptides were assayed using the same protocol.

Fourier transform mass spectrometry of HLA-A3–associated peptides

Mass spectrometric data were acquired on a Fourier transform ion cyclotron resonance mass spectrometer (FTMS) equipped with a nanoflow-HPLC (nano-HPLC) microelectrospray ionization (ESI) source as previously described.[21] Briefly, an aliquot of sample was loaded onto a nano-HPLC column and eluted into the FTMS using a gradient of 0% to 60% solvent B in 32 minutes and 60% to 100% solvent B in the next 3 minutes, where solvent A is 0.1 M acetic acid (Sigma-Aldrich, St. Louis, MO) in NANOpure water and solvent B is 0.1 M acetic acid in 70% acetonitrile. Full-scan mass spectra were acquired at a rate of 1 scan/second. To determine the candidate masses for the antigenic peptide, active third-dimension fractions were analyzed using FTMS and nanoflow effluent splitter technology,[22,23] with one-eighth of the column eluate sent to FTMS for mass detection and the remaining seven-eighths plated into a 96-well plate containing 25 μL NANOpure water/well for reconstitution assay and future sequencing.

Sequence analysis of candidate antigens

Collision-activated dissociation (CAD) mass spectra were recorded on selected peptide candidates using a ThermoFinnigan (Waltham, MA) LCQ ion trap mass spectrometer (MS) equipped with sheathless nanoflow HPLC-ESI.[23] Targeted CAD spectra were acquired by manually switching from MS-only mode to MS/MS mode after the chromatographic elution of a marker peptide. In MS/MS mode, the ion of interest was isolated using a 3.0 atomic mass unit (AMU) isolation window and fragmented using 35% collision energy.

Genotyping of CTL-7A7 mHAg polymorphisms

Genomic DNA was isolated from EBV-LCLs (DNA Blood Kit; QIAGEN, Valencia, CA), and a 209-bp polymerase chain reaction (PCR) fragment from the *PANE1* genomic locus encompassing the mHAg-encoding exon was amplified using the primers 5′-GGAAGGAGGAACAGCAAGTGC-3′ and 5′-AAAACCCAAGTCAGGCCACAT-3′. Cycle parameters were: denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 15 seconds, and extension at 72°C for 30 seconds; and final extension at 72°C for 5 minutes. PCR products were digested at 37°C for 2 hours with *Dde*I and analyzed on 2% agarose gels. The mHAg$^+$ allele, containing a deoxycytidine (C) at position 40 of BQ056829, was not digested under these conditions and produced a single 209-bp band, whereas the mHAg$^-$ allele containing a deoxythymidine (T) at this position produced 2 bands of 181 and 28 bp.

Taqman PCR analysis of *PANE1* transcripts *k* and *c* expression

Taqman real-time quantitative PCR was used to quantitate expression of *PANE1* transcript *k* (AceView; http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly) corresponding to GenBank no. BQ056829, which encodes the epitope recognized by CTL-7A7, and the canonical *PANE1* transcript *c*,

corresponding to GenBank no. NM_024053, in panels of cDNA from poly(A)$^+$ RNA from normal human tissues (human Multiple Tissue cDNA panels I and II, and human blood fractions; BD Biosciences/Clontech, Palo Alto, CA), and in cDNA from cultured human cells and primary malignant hematopoietic cells. For the latter samples, total RNA was prepared using Trizol (Invitrogen), and first-strand cDNA was prepared using oligo-dT, random hexamers, and Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (Invitrogen). A 6-carboxy-fluorescein (FAM)–labeled Taqman TAMRA probe (ABI) and PCR primers that specifically amplify *PANE1* transcript *k* were designed using Primer Express software, version 1.5 (ABI), as follows: *PANE1* transcript *k* probe: 5′-(FAM)-TCCACCTT-GAGCACACCAGGCAAGT-(TAMRA)-3′; *PANE1* transcript *k* forward primer: 5′-AAGTGCATGGGCCGAGTG-3′; and *PANE1* transcript *k* reverse primer: 5′-CGCCATGGTGGCCCTAA-3′. Human *GAPDH* control reagents (ABI) were used to normalize *PANE1* transcript *k* expression in each sample. A commercially available *PANE1*-specific Taqman Gene Expression Assay (Hs00608780_m1; ABI) was used according to the manufacturer's instructions to amplify *PANE1* transcript *c*.

Detection of *PANE1* transcripts and a *GAPDH* control were performed as separate reactions, but run within the same tray for each sample. All samples were run in duplicate for each gene. Reaction volume was 20 μL with 1 × Taqman Universal PCR Master Mix (ABI), 1 μM forward and reverse primers and 0.25 μM probe for *PANE1* transcript *k*, and 50 nM forward and reverse primers and 50 nM 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE) labeled Taqman/TAMRA probe for *GAPDH*. Cycling conditions were: 50°C for 2 minutes (uracil DNA glycosylase [UNG] incubation), 95°C for 10 minutes (enzyme activation), followed by 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). Reactions were run with 20 to 40 ng sample for the cultured or primary cell samples, and 2 ng sample for the prepared cDNA, in an ABI Prism 7700 Sequence Detector using SDS software, version 1.7 (ABI).

The threshold cycle (C_T) value was calculated using SDS software based on the default settings. The baseline value was calculated by averaging the fluorescence values of cycles 3 to 15 and then adding 10 times the standard deviation. C_T values for duplicate reactions were averaged. The average C_T (*PANE1* *c* or *k*) and the average C_T (*GAPDH*) were used to calculate relative expression of *PANE1* transcripts *c* and *k* using the comparative C_T method.[24]

CD40L activation of B-CLL cells

NIH3T3 cells transfected with human CD40 ligand (tCD40L) were used to generate CD40-activated CLL cells (CD40-CLL), as previously described.[25] In brief, γ-irradiated (96 Gy) tCD40L cells were cultured overnight at 37° in 5% CO_2. The medium was then removed and replaced with a suspension containing 4×10^6 CLL cells in Iscove modified Dulbecco medium (Invitrogen) supplemented with 10% human serum, 50 μg/mL transferrin (Roche Applied Science, Indianapolis, IN), 5 μg/mL insulin (Sigma-Aldrich), and 3.4 ng/mL interleukin-4 (IL-4; R&D Systems, Minneapolis, MN), and the tCD40L/CLL cells were cultured together for an additional 3 days. CD40-CLL cells were then transferred to new plates and cultured overnight to remove contaminating tCD40L cells.

Results

CD8$^+$ CTL clone CTL-7A7 recognizes a HLA-A*0301–restricted mHAg that is selectively expressed in B-lymphoid cells

The CTL clone CTL-7A7 was isolated by limiting dilution from a T-cell line generated by repeatedly stimulating posttransplantation PBMCs from a 43-year-old female myeloablative allogeneic HCT recipient with her cryopreserved pretransplantation PBMCs and EBV-LCLs. Flow cytometry revealed that CTL-7A7 CTLs have a CD3$^+$/CD4$^-$/CD8$^+$ phenotype (data not shown). CTL-7A7 CTLs showed specific recognition of EBV-LCLs derived before transplantation from the recipient, but not EBV-LCLs derived from her
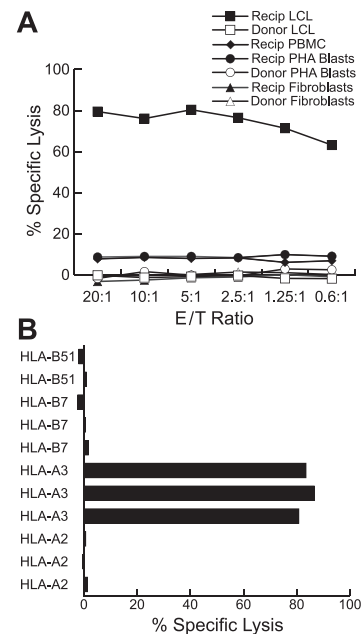
Figure 1. CTL Clone CTL-7A7 recognizes a HLA-A*0301–restricted mHAg that is selectively expressed in B-lymphoid cells. (A) The cytolytic activity of CTL-7A7 CTLs against EBV-LCLs, PHA-stimulated T-cell blasts, unfractionated peripheral blood mononuclear cells (PBMCs), and dermal fibroblasts derived from the transplant recipient (Recip) or donor was evaluated in a 4-hour ^{51}Cr release assay at the indicated effector-target ratios. (B) Cytolytic activity at E/T 10:1 of CTL-7A7 CTLs against a panel of EBV-LCLs derived from unrelated individuals, each of whom shared 1 class I MHC allele with the recipient-donor pair from whom the CTL clone CTL-7A7 was derived. The specific class I MHC allele shared with the recipient/donor by each individual is indicated.

MHC-identical donor (Figure 1A), demonstrating that the epitope recognized by this clone is a minor histocompatibility antigen. CTL-7A7 CTLs showed weak lytic activity against unfractionated recipient pretransplantation PBMCs and PHA-stimulated T-cell blasts, but no recognition of recipient dermal fibroblasts. No recognition of donor-derived PHA blasts or dermal fibroblasts was seen (Figure 1A). Collectively, these data suggest that the mHAg recognized by CTL-7A7 is expressed at high levels in recipient B cells and at much lower levels in recipient PBMCs and PHA blasts, but is not expressed in recipient fibroblasts at a level sufficient to trigger CTL lysis. When tested against EBV-LCLs derived from an extended panel of unrelated individuals, each of whom shared 1 class I MHC allele with the donor and recipient, CTL-7A7 CTLs showed recognition only of EBV-LCLs derived from individuals expressing HLA-A*0301 (Figure 1B), suggesting that the antigenic peptide recognized by CTL-7A7 is presented by HLA-A*0301.

Mass-spectrometric identification of the CTL-7A7 epitope

To identify the antigenic peptide recognized by CTL-7A7 CTLs, HLA-A*0301–associated peptides were purified from HLA-A*0301–homozygous EBV-LCLs that were recognized by CTL-7A7 CTLs in vitro. The peptides were fractionated by reverse-phase (RP)–HPLC, and the fractions analyzed for their ability to reconstitute CTL recognition in a ^{51}Cr release assay when added exogenously to donor-derived EBV-LCLs. Fractions recognized by CTL-7A7 were pooled and carried forward into sequential rounds of RP-HPLC under different conditions. Single peaks of reconstituting activity were observed through 3 rounds of fractionation (Figure 2A-C). Candidate masses for the CTL-7A7 mHAg were identified by an online effluent splitter analysis of the third-dimension active fractions using a combination of nanoflow liquid
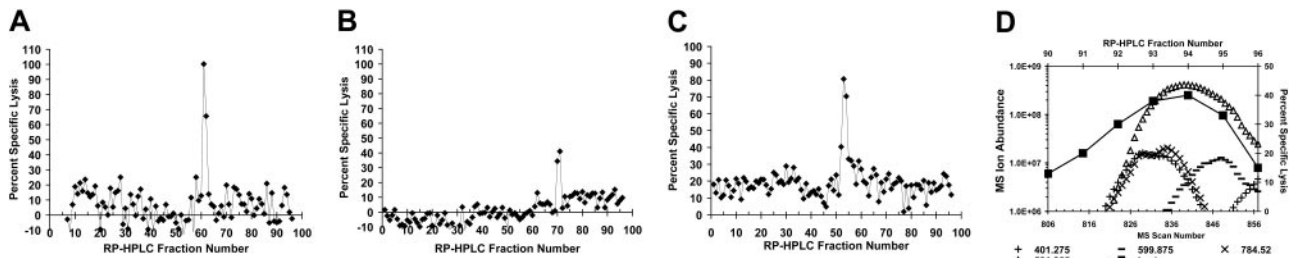
Figure 2. Reconstitution of the CTL-7A7 mHAg epitope with HPLC-fractionated peptides extracted from HLA-A3 molecules. HLA-A3–associated peptides were purified from 5×10^{10} mHAg$^+$ EBV-LCLs and fractionated by RP-HPLC as described in "Materials and methods." Aliquots of each fraction corresponding to 4×10^9 cell equivalents in panels A and B and 6×10^9 cell equivalents in panels C and D were preincubated with ^{51}Cr-labeled donor EBV-LCLs and tested for their ability to sensitize targets to lysis by CTL-7A7 CTLs. An E/T ratio of 10:1 was used. (A) First-dimension separation of extracted peptides was achieved using TFA as the ion-pairing agent. (B) Fractions 61 and 62 from panel A were pooled and rechromatographed using HFBA as the ion-pairing agent. (C) Fractions 70 and 71 from panel B were pooled and rechromatographed on a microcapillary column using TFA as the ion-pairing agent. (D) Determination of candidate peptides via mass spectrometry correlated with ^{51}Cr release assay. Fractions 52 and 53 from panel C were pooled and rechromatographed using nanoflow effluent splitter technology. Ion abundances of candidate masses within the MS scan window 806-856 were plotted and correlated to the percent specific ^{51}Cr release in that same region. Background lysis of donor EBV-LCLs by CTL-7A7 CTLs in the absence of peptide was 10% in panel A, 3% in panel B, 16% in panel C, and 10% in panel D. Lysis of recipient EBV-LCLs (positive control) was 54% in panel A, 67% in panel B, 86% in panel C, and 66% in panel D.

chromatography with ESI on an FTMS.[23] By comparing the abundance of peptide ions in spectra from wells that exhibited epitope-reconstituting activity with CTL-7A7 CTLs, 4 candidate peptides were identified (Figure 2D).

The most abundant candidate ion (m/z 591.865^{+2}) was targeted for MS/MS analysis on an ion trap mass spectrometer, and the peptide sequence was determined to be RVWDLPGVLK (Figure 3A-B). The experimental MS/MS spectra were compared with the MS/MS spectra of the synthetic peptide of the same sequence to ensure that the peptides matched. The synthetic peptide was also coeluted with the sample to further ensure that the peptides were identical (data not shown). To determine whether RVWDLPGVLK represented the antigenic epitope, the peptide was tested for its ability to sensitize donor EBV-LCL or TAP-deficient T2A3 cells to lysis by CTL-7A7 CTLs. Target cells pulsed with RVWDLPGVLK were lysed by CTL-7A7 CTLs, with half-maximal activity observed at a peptide concentration of approximately 1 nM (Figure 3C). Thus, RVWDLPGVLK defines the HLA-A*0301–restricted CTL-7A7 epitope. By comparing the mass spectrometric signal intensity of the naturally eluted peptide with a known mass of synthetic RVWDLPGVLK, we calculated that this mHAg is present at approximately 113 copies per EBV-LCL (data not shown).

The CTL-7A7 mHAg is encoded by an alternative transcript of the *PANE1* gene

A search of the known protein and translated DNA sequence databases with the peptide sequence RVWDLPGVLK identified a single perfect match with the predicted polypeptide encoded by nucleotides 34 to 63 of GenBank accession no. BQ056829, 1 of 11 alternative transcripts of the proliferation associated nuclear element 1 *(PANE1)* gene (AceView), encoded on chromosome 22 at band q13.31. This alternative transcript, designated *PANE1* transcript *k,* was identified in RNA from a lymphoma cell line, and consists of 2 exons: a small first exon that is unique from all of the other *PANE1* transcripts, and a second exon common to 5 of the longer *PANE1* transcripts, including the longest transcript, *c* (Figure 4A). The open reading frame in *PANE1* transcript *k* has a conventional ATG translation initiation codon and encodes a 58-residue polypeptide (Figure 4B), with the mHAg-encoding sequence contained entirely within the small first exon. In order to determine whether the sequence represented by BQ056829 represented the full-length cDNA clone, we performed 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR using *PANE1* locus-
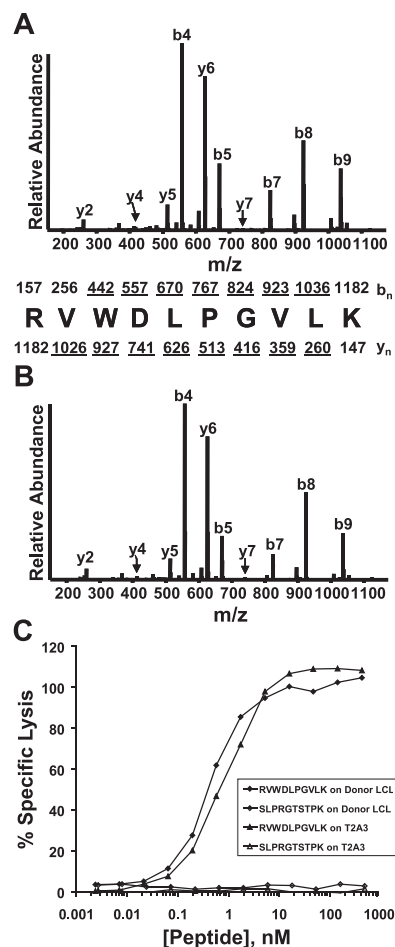
Figure 3. Identification of the antigenic peptide recognized by the CTL clone CTL-7A7. (A) CAD mass spectrum of candidate peptide (M+2H)$^{2+}$ ion with monoisotopic m/z of 591.865 as eluted from HLA-A*0301–homozygous mHAg$^+$ EBV-LCLs. (B) CAD mass spectrum of synthetic peptide RVWDLPGVLK. Mass spectra were recorded on a Finnigan LCQ ion trap MS operating with a 3.0 atomic mass unit isolation window and 35% collision energy. The b and y ions are labeled above and below the amino acid sequence, respectively. Ions observed in the spectrum are underlined. (C) CTL-7A7 mHAg epitope reconstitution with synthetic RVWDLPGVLK peptide. ^{51}Cr-labeled donor EBV-LCLs or T2A3 target cells were pulsed for thirty minutes at 37°C with the indicated concentrations of synthetic peptides RVWDLPGVLK or SLPRGTSTPK, then tested for recognition by CTL-7A7 CTLs in a 4-hour ^{51}Cr-release assay at an E/T ratio of 10:1. The control peptide SLPRGTSTPK corresponds to the HLA-A*0301–restricted epitope recognized by CTL clone DRN-7 (E.H.W., manuscript in preparation).

A

PANE1 transcript *k*

1.5 Kb

PANE1 transcript *c*

8.4 Kb

B

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                                                        M  G  R  V  W  D  L  P  G
aggcaggccacggagatacctcgtcggaaggaggaacagcaagtgcATGGGCCGAGTGTGGGACTTGCCTGGT
 V  L  K
GTGCTCAAGgtctggcaggcaggcctgggtggctgcagcacaatagcaaagggcgggttggggtggagaaat
caggaatggtggggctggatcagtcgagcctggtgggggagtcgctggaggggcttggggccagggagggatgt
ggcctgacttgggttttaaatggaccccctctggctgccgggaggagagtggactgaagggaaactagggcag
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tctttcttttttgagacagacactgggcagcatcgtgttcttccagcggacgtgtctagaacatctgtgccc
gggcctagagcagtcttgtccctctctggccccacagctccctgggcccattccttttttgatggcaccttc
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gtcaggctctggatgtctccggagggttgtttgaaggattgggcaagctgaggcagttggaagctactctgag
gtgtggcatgaatgagggcgtggctgtgggtgggcagtggtgacctgagctacccccaagtatccgccccag
ccctgctctggcagccctgcagctgtctgggtaaggggacagggccagcattctatctcactgctgttct
                                        V  E  G  F  R  A  T  M  A  Q  R  L  V  R  V  L  Q  I  C
ctgctctccccgcagGTGGAAGGCTTTAGGGCCACCATGGCGCAGCGCCTGGTGCGCGTGCTGCAGATCTGT
 A  G  H  V  P  G  V  S  A  L  N  L  L  S  L  L  R  S  S  E  G  P  S  L
GCTGGCCACGTGCCCGGTGTCTCAGCTCTGAACCTGCTGTCCCTGCTGAGAAGCTCTGAGGGCCCCTCCCTG
 E  D  L  *
GAGGACCTGTGAgggtggctggcccctgggctgccccttctcatggcttcgtgctgactccataaacattct
ctgttgaggatgtccagtcaggggcttgacaggcccaggctcagccccgccgtggctgggaaggttccctgcag
tgccagtgctgcagcagggagagctgggcagaagcagcgaggggggcccagctggcgagactgtagcccctc
ccactcccacactcactcttgcagagcctgtgtctttaagcagctggcgtgttacatctccatttaagatttt
ccttttgaacaaaaggtctgtggctaaaaaagtttaaaaatcaaaaaaaaaaaaaaaaaaaaaaaaa
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C

PANE1 isoform:

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c    MSVLRPLDKLPGLNTATILLVGTEDALLQQLADSMLKEDCASELKVHLAKSLPLPSSVNR   60
k    ------------------------------------------------------------

c    PRIDLIVFVVNLHSKYSLQNTEESLRHVDASFFLGKVCFLATGAGRESHCSIHRHTVVKL   120
k    ------------------------------------------------------------

c    AHTYQSPLLYCDLEVEGFRATMAQRLVRVLQICAGHVPGVSALNLLSLLRSSEGPSLEDL   180
k    --MGRVWDLPGVLKVEGFRATMAQRLVRVLQICAGHVPGVSALNLLSLLRSSEGPSLEDL    58
           *   ********************************************
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Figure 4. Genomic structure and gene/protein alignment of human PANE1 transcripts. (A) PANE1 transcripts *k* and *c* share a common last exon. The first exon of transcript *k* is unique from all other PANE1 transcripts, and the sequence encoding the CTL-7A7 mHAg RVWDLPGVLK is contained entirely within this first exon, as indicated by the vertical arrow. (B) PANE1 transcript *k* nucleotide sequence, and sequence of its predicted 58–amino acid isoform. The polymorphic nucleotide generating the CTL-7A7 mHAg is indicated by a vertical arrow. These sequence data are available from Entrez PubMed (http://www.ncbi.nlm.nih.gov/entrez) under accession number BQ056829. Italicized nucleotides represent additional 5′ and 3′ sequence determined by RACE PCR. (C) Comparison of the predicted protein sequences of PANE1 isoforms *k* and *c*. Asterisks indicate amino acid identity; bold type indicates CTL-7A7 epitope, with polymorphic residue indicated by an arrow.

specific-primers, and bidirectional sequencing of the RACE PCR products. We observed sequence identical to that in BQ056829, but also identified an additional 18 bp of 5′ sequence and 7 bp of 3′ sequence (italics in Figure 4B). An alignment of the predicted protein isoforms derived from PANE1 transcripts *k* and *c* reveals 100% identity between their C-terminal 46 residues, which are encoded by their common last exon (Figure 4C).

To determine the genetic basis for differential CTL-7A7 recognition of recipient and donor cells, a 209-bp region of the PANE1 genomic locus spanning the entire epitope-encoding region was amplified by PCR from recipient and donor EBV-LCL genomic D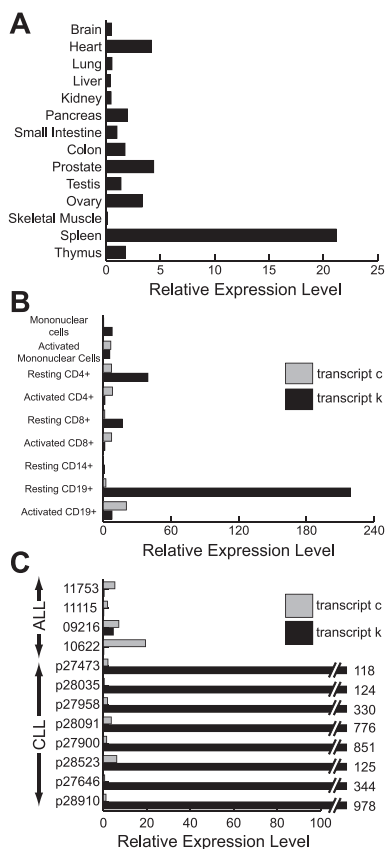NA and sequenced. Sequence analysis of the PCR products from each EBV-LCL, which included sequences amplified from both the maternal and paternal PANE1 alleles, revealed that the transplant recipient, was heterozygous for a C → T polymorphism at nucleotide (nt) 21726687 on the (−) strand of the consensus chromosome 22 sequence (GenBank no. NT_011520.10), which has a C at this position (data not shown). In contrast, the transplant donor was homozygous for T at this position. A search of the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) revealed that this SNP had previously been identified (dbSNP ref no. rs5758511). Alignment of the chromosome 22 sequence and that of BQ056829 revealed that the position of this polymorphism corresponded to nt 40 of BQ056829, and its predicted effect on the polypeptide encoded by the 58-residue open reading frame (ORF) in BQ056829 was to

replace the CGA codon for arginine at the amino terminus of the CTL-7A7 epitope with a TGA translation termination signal.

These findings suggested that the differential recognition of HLA-A*0301$^+$ EBV-LCLs by CTL-7A7 CTLs was determined by genotype at the rs5758511 polymorphism, with hetero- or homozygosity for the C allele (C/T or C/C) determining susceptibility to lysis by CTL-7A7 CTLs, and homozygosity for the T allele (T/T) determining resistance. To test this hypothesis, the rs5758511 genotype and CTL-7A7 mHAg phenotype of 63 additional HLA-A*0301$^+$ EBV-LCLs were determined by PCR/restriction fragment length polymorphism (RFLP) and by in vitro cytotoxicity assay with CTL-7A7, respectively. This analysis identified 5 individuals with a T/T genotype at rs5758511, and all of these individuals were negative (specific lysis < 5% at E/T 10:1) for expression of the mHAg recognized by CTL-7A7. The remaining 58 individuals had a C/T or C/C genotype at rs5758511, and all of these individuals expressed the mHAg recognized by CTL-7A7 (specific lysis > 50% at E/T 10:1). These data confirm that rs5758511 genotype determines phenotype for the CTL-7A7 mHAg with 100% penetrance, and demonstrate that this mHAg is specifically encoded by the allele of PANE1 transcript *k* that carries C at the position corresponding to nt 40 of BQ056829. In this population of 63 HLA-A*0301$^+$ individuals, the frequencies of the C- and T-bearing alleles of PANE1 transcript *k* were 0.75 and 0.25, respectively.

PANE1 transcript *k* is selectively expressed in resting CD19$^+$ cells and B-CLL

To determine whether the restricted recognition of hematopoietic and nonhematopoietic lineage cells by CTL-7A7 CTLs was determined by the tissue distribution of PANE1 transcript *k* or other factors, we developed a real-time quantitative PCR assay to assess the expression levels of transcript *k* in normal cell types and tissues. Among cultured cells, the highest levels of transcript *k* expression were detected in EBV-LCLs derived from the mHAg$^+$ individuals with C/T or C/C genotypes at rs5758511 (mean relative expression level, 5.3; range, 1.11-17.7). Significantly lower levels of transcript *k* expression were seen in PHA-stimulated T-cell blasts from mHAg$^+$ individuals with C/C or C/T genotypes (relative expression < 0.7), with no detectable expression in dermal fibroblasts derived from these individuals. Very low levels of transcript *k* expression were seen in EBV-LCLs derived from 5 of 5 individuals with T/T genotypes at rs5758511 (mean expression level, 0.14; range, 0.1-0.3; P = .043). Of the normal human tissues examined, the highest levels of expression were seen in spleen, with intermediate levels in heart, prostate, and ovary, and low levels in all other tissues examined (Figure 5A). Analysis of different fractions of resting and activated peripheral blood cells revealed high levels of expression in resting CD19$^+$ cells, and low levels of expression in activated CD19$^+$ cells, resting and activated mononuclear cells, resting and activated CD4$^+$ cells, resting and activated CD8$^+$ cells, and resting CD14$^+$ cells (Figure 5B). Collectively, these results suggest that the selective expression of PANE1 transcript *k* in CD19$^+$ B-lymphoid cells determines the B-lymphoid–specific expression of the CTL-7A7 mHAg.

The preferential expression of PANE1 transcript *k* in normal CD19$^+$ cells prompted us to investigate its expression in malignant CD19$^+$ cells. Expression of this transcript in a small sample of CD19$^+$ acute lymphoblastic leukemia cells was lower than in EBV-LCLs (mean expression level, 1.3; n = 4; Figure 5C). In contrast, transcript *k* was uniformly expressed in CD19$^+$ B-CLL cells obtained from 26 patients with C/C or C/T genotypes at rs5758511 at comparable or greater levels than seen in normal

Figure 5. *PANE1* transcript *k* is selectively expressed in resting CD19⁺ cells and B-CLL. (A) Relative expression levels of the CTL-7A7 mHAg-encoding *PANE1* transcript *k* in different human tissues. Expression was determined by real-time quantitative RT-PCR as described in "Materials and methods." The *GAPDH* gene was used as a standard to correct for RNA quantity and quality. (B-C) Relative expression levels of *PANE1* transcripts *c* (▨) and *k* (■) in resting and activated fractions of human peripheral blood leukocytes (B), and in primary ALL and B-CLL samples (C).

resting CD19⁺ cells (representative data in Figures 5C and 7), and greater than the levels seen in any other cell type or tissue tested. Cytotoxicity testing of purified CD19⁺ cells from HLA-A*0301⁺ healthy donors and of CD19⁺ CLL cells from HLA-A*0301⁺ CLL patients with C/C or C/T genotypes at rs5758511 confirmed that they expressed the mHAg encoded by transcript *k* and were recognized by CTL-7A7 CTLs (Figure 6).

PANE1 transcripts *k* and *c* show a reciprocal pattern of expression in resting and activated CD19⁺ cells

The finding that *PANE1* transcript *k* was selectively expressed in resting CD19⁺ cells and CD19⁺ B-CLL appeared to contradict a previous report[26] that *PANE1* is preferentially expressed in activated lymphoid cells. In view of the unique exon present in transcript *k* that is absent from the 10 other reported *PANE1* transcripts, we hypothesized that transcript *k* and other *PANE1* transcripts would show distinct expression profiles in resting compared with activated cells. We used a commercially available real-time quantitative PCR assay to evaluate the expression of *PANE1* transcript *c* in the same panel of resting and activated blood cell fractions and primary leukemic samples. This analysis revealed that *PANE1* transcript *c* was selectively expressed in activated compared with resting cells, with the highest levels of expression seen in activated CD19⁺ cells, and very low levels in resting CD19⁺ cells (Figure 5B) and primary CLL cells (representative
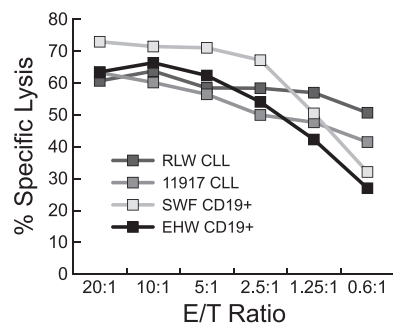
Figure 6. The CTL-7A7 mHAg encoded by PANE1 transcript *k* is expressed in normal CD19⁺ cells and CD19⁺ B-CLL. Expression of the CTL-7A7 antigen in purified CD19⁺ cells from the peripheral blood of 2 HLA-A*0301–positive healthy donors and in B-CLL cells from 2 patients as determined in a standard 4-hour ⁵¹Cr release assay at the indicated effector-target (E/T) ratios.

data in Figures 5C and 7). Thus, *PANE1* transcripts *k* and *c* show a reciprocal pattern of expression in resting and activated CD19⁺ cells and primary CD19⁺ CLL cells. To determine whether activation of CD19⁺ cells would increase expression of transcript *c* and decrease expression of transcript *k*, we evaluated the effect of CD40L stimulation on expression of the 2 transcripts in 6 primary B-CLL samples. In all samples tested, CD40L stimulation led to a profound decrease in transcript *k* expression (mean reduction, 121-fold; range, 6.4- to 377-fold), and a significant increase in transcript *c* expression (mean increase, 6.3-fold; range, 3.1- to 9.7-fold) (Figure 7).

Discussion

The majority of human mHAg's identified to date are created by SNPs in the coding sequence of cellular genes that create polymorphic amino acid sequences in the encoded proteins. MHAg polymorphisms can be classified based on their impact on either T-cell recognition or peptide presentation by MHC molecules. For several mHAg's, both the antigenic peptide and its allelic counterpart peptide are presented on the cell surface by the relevant MHC molecule, but only 1 of the 2 peptides is recognized with sufficient avidity by the T-cell receptor to trigger T-cell activation.[7,14,23,27,28] In the alternative scenario, the mHAg⁻ allelic peptide fails to be presented at the cell surface by the relevant MHC molecule, because the polymorphism affects either TAP-mediated translocation of the peptide into the endoplasmic reticulum,[10] proteasomal
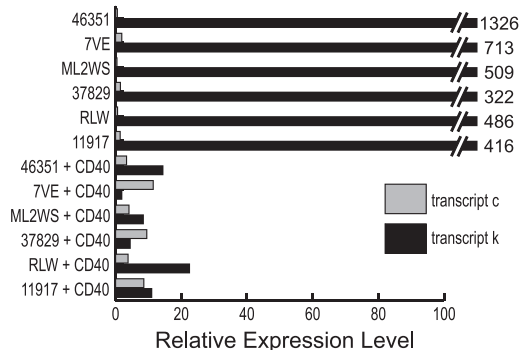

Figure 7. CD40L stimulation of B-CLL cells differentially regulates expression of *PANE1* transcripts *c* and *k*. Effect of CD40L stimulation on relative expression levels of *PANE1* transcripts *k* and *c* in 6 primary B-CLL samples. Data are representative of those from 2 independent experiments. CD40L stimulation was performed as described in "Materials and methods."

cleavage of the peptide,[29] binding of the peptide to MHC,[12,30] or protein expression.[31,32] In this study, we have identified a novel HLA-A3–restricted mHAg, the peptide RVWDLPGVLK, which is selectively expressed in B-lymphoid cells. This mHAg is encoded by 1 of 11 alternative transcripts (transcript *k*) of the *PANE1* gene, located on chromosome 22q13, and is derived from a predicted 58-residue polypeptide. The exon that encodes this mHAg is unique from all other alternative transcripts of the *PANE1* gene. Analysis of genomic DNA from EBV-LCLs of various HLA-A*0301[+], mHAg[+], and mHAg[−] individuals demonstrated that differential protein expression due to a nonsense SNP in the unique epitope-encoding exon determines the antigenicity of this locus. Quantitative RT-PCR further demonstrated that levels of transcript *k* in EBV-LCLs derived from individuals with T/T genotypes at rs5758511 were uniformly low, suggesting that in T/T homozygotes this transcript undergoes accelerated degradation due to the nonsense polymorphism (nonsense-mediated decay). This is the first mHAg whose antigenicity is due to protein truncation resulting from a SNP that creates a translation termination signal, and this may represent an important mechanism for the generation of mHAg's.

The identification of the CTL-7A7 mHAg was motivated by its selective expression in B-lymphoid cells, suggesting that it might be an attractive target for immunotherapeutic strategies designed to enhance GVL activity after allogeneic HCT without inducing GVHD. The estimated frequencies of the mHAg[+] and mHAg[−] *PANE1* alleles in a population of HLA-A*0301[+] individuals of predominantly North American and Western European ancestry were 0.75 and 0.25, respectively. Thus, donor/recipient disparity for this mHAg will be uncommon in this population, as only 6.25% of individuals would be expected to be homozygous for the mHAg[−] allele of this locus, given Hardy-Weinberg equilibrium. Data from the International HapMap Project (http://www.hapmap.org/cgi-perl/snp_details?name=rs5758511) indicate that the frequency of the null allele at this locus is higher in other populations, particularly the Han Chinese and Japanese populations, where its frequency is estimated at 0.511 and 0.398, respectively. Thus, given Hardy-Weinberg equilibrium, 26% and 16% of individuals in these 2 populations, respectively, would be expected to be homozygous for the mHAg^{−} allele, and therefore donor/recipient disparity for this mHAg would occur in a larger proportion of HLA-A*0301^{+} pairs. Analysis of CD8^{+} HLA-A*0301–restricted mHAg-specific CTL clones previously isolated from other HCT recipients identified 1 clone, 2H4,[33Tbl3] from a patient with C/T genotype at rs5758511 who received a hematopoietic cell graft from a T/T sibling donor, that also recognizes the RVWDLPGVLK peptide encoded by *PANE1* transcript *k* (Scott S. Tykodi and E.H.W., unpublished observations, July 2005). This suggests that T-cell responses against this mHAg are not uncommon in donor/recipient pairs who have appropriately discordant genotypes at rs5758511. It is also possible that the unique *PANE1* exon that encodes this mHAg also encodes peptides that bind other class I MHC alleles. If so, these peptides would not be presented by cells derived from individuals homozygous for the T allele of transcript *k*, because the translation termination signal introduced by the rs5758511 polymorphism should completely abrogate their production and thus their presentation. These peptides could potentially be antigenic in donor/recipient pairs that (1) express the appropriate class I MHC alleles; and (2) are appropriately discordant for the rs5758511 polymorphism (donor homozygous for the T allele, and recipient homo- or heterozygous for the C allele).

Transcription of the human *PANE1* gene results in the expression of 11 major mRNA isoforms, presumably as a result of either differential splicing or alternative promoter usage. Splice variant *c* encodes the longest isoform, consisting of a predicted 180 residues, whereas translation of splice variant *k* is predicted to produce a much shorter 58-residue isoform, which lacks the N-terminal 122 residues encoded by exons 1 to 5 of splice variant *c*. The observation that homozygosity for the null allele of transcript *k* occurs in an estimated 6%, 16%, and 26% of the North American, Japanese, and Han Chinese populations, respectively, indicates that loss of function of this allele is not associated with an overtly deleterious constitutional phenotype. However, loss of function of the protein product of transcript *k* may be associated with a subtle lineage-restricted phenotype in normal resting B cells and/or in B-lineage CLL cells, which have the highest levels of transcript *k* expression of any tissue or cell type. It is plausible that another transcript of the *PANE1* gene such as transcript *c*, which shares an identical terminal exon with the CTL-7A7 mHAg-encoding transcript *k*, might compensate for the severely truncated product of the null allele of transcript *k*. However, the markedly distinct expression patterns of *PANE1* transcripts *c* and *k* in resting and activated B cells and primary CLL cells are suggestive of distinct and perhaps even antagonistic functions of these 2 transcripts.

Little is known regarding the function and expression of the various *PANE1* transcripts. *PANE1* transcript *c* was originally identified as a gene highly expressed in β-catenin–transformed mouse mammary tissues,[34] but a subsequent report using semiquantitative RT-PCR and Northern blot analysis in human samples indicated preferential expression of this transcript in activated lymphoid cells and did not detect expression in cell lines of mammary origin.[26] These results are consistent with our observations using quantitative RT-PCR, although we additionally observed significant expression of transcript *c* in testis but did not assess expression in mammary cells. *PANE1* transcript *c* is evolutionarily conserved between mammals and zebrafish, and no functional motifs have been identified in the protein that it encodes.[26] Ectopic expression of murine[34] or human[26] *PANE1* transcript *c* indicated that the protein encoded by this transcript is preferentially present in the nucleus in nonconfluent cells, but is excluded from the nucleus during cell division or upon attainment of confluence. Finally, a mass-spectrometric analysis of the centromere complex from HeLa interphase cells indicated that *PANE1* colocalizes with various centromere proteins, although the identity of the *PANE1* transcript variant encoding this protein was not indicated.[35] There are presently no reports in the literature of functional characterization of the CTL-7A7 mHAg-encoding *PANE1* transcript *k*, and efforts are under way in our laboratories to further elucidate the function of this gene product and its potential interactions with other proteins in resting B cells and B-CLL.

The gene located closest to *PANE1* on chromosome 22q (12 kb from its 5′ terminus) is *TNFRSF13C*, which encodes a member of the tumor necrosis factor (TNF) receptor superfamily that, like *PANE1* transcript *k*, is selectively expressed in resting CD19^{+} B cells.[36,37] The murine homologs of *PANE1* and *TNFRSF13C* are likewise immediately adjacent to one another in the syntenic region of the mouse genome on chromosome 15.[38] The product of the human *TNFRSF13C* gene, also known as *BAFF-R*—B-cell activation factor receptor—encodes a membrane-bound receptor for BAFF (B-cell activation factor), and is the principal receptor for BAFF-mediated mature B-cell survival. Deficiency of BAFF and/or BAFF-R results in almost complete loss of follicular and marginal zone B lymphocytes in secondary lymphoid organs in

mice.[36,37] BAFF-R is uniformly expressed in B-CLL.[39-41] The great proximity of *PANE1* and *TNFRSF13C* to one another on chromosome 22, combined with the fact that they are both selectively expressed in resting as compared with activated CD19+ cells, suggests the existence of a genetic element located in that region of chromosome 22 that coordinately regulates their expression.

Our results indicate that the tissue or cell type with the highest expression of *PANE1* transcript *k* is B-CLL, and that virtually 100% of primary B-CLL samples from individuals with C/C or C/T genotypes at rs5758511 express this transcript at high levels. Recent studies indicate that BAFF-R, the product of the *TNFRSF13C* locus adjacent to *PANE1* on chromosome 22, is frequently expressed in other low-grade B-lymphoproliferative disorders, including most follicular lymphomas, mantle cell lymphomas, and marginal zone lymphomas.[40,41] Given the similarity between the reported tissue distribution of *TNFRSF13C*[36,37] and the tissue distribution of *PANE1* transcript *k* demonstrated in this study, we have initiated studies to determine whether *PANE1* transcript *k*, and the mHAg that it encodes, will be expressed at high levels in other low-grade B-lymphoproliferative disorders.

Acknowledgment

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