Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA

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Leukemic B lymphocytes of a large group of unrelated chronic lymphocytic leukemia (CLL) patients express an unmutated heavy chain immunoglobulin variable (V) region encoded by *IGHV1-69*, *IGHD3-16*, and *IGHJ3* with nearly identical heavy and light chain complementarity-determining region 3 sequences. The likelihood that these patients developed CLL clones with identical antibody V regions randomly is highly improbable and suggests selection by a common antigen. Monoclonal antibodies (mAbs) from this stereotypic subset strongly bind cytoplasmic structures in HEp-2 cells. Therefore, HEp-2 cell extracts were immunoprecipitated with recombinant stereotypic subset-specific CLL mAbs, revealing a major protein band at approximately 225 kDa that was identified by mass spectrometry as nonmuscle myosin heavy chain IIA (MYHIIA). Reactivity of the stereotypic mAbs with MYHIIA was confirmed by Western blot and immunofluorescence colocalization with anti-MYHIIA antibody. Treatments that alter MYHIIA amounts and cytoplasmic localization resulted in a corresponding change in binding to these mAbs. The appearance of MYHIIA on the surface of cells undergoing stress or apoptosis suggests that CLL mAb may generally bind molecules exposed as a consequence of these events. Binding of CLL mAb to MYHIIA could promote the development, survival, and expansion of these leukemic cells. (Blood. 2008;112:5122-5129)

Introduction

The most common Western adult leukemia is B cell–type chronic lymphocytic leukemia (CLL), with an estimated 15 340 new cases and 4500 deaths occurring in the United States in 2007.[1] This incurable cancer consists of a clonal expansion of $CD5^+$, $CD19^+$ B lymphocytes characterized by a B-cell antigen receptor (BCR) or monoclonal antibody (mAb) of a defined amino acid sequence. The prognosis for CLL patients correlates with the amount of somatic mutation in the BCR sequences of the leukemic cells.[2,3] This dependence on mutation in the BCR suggests a role for antigen binding. This view is further supported by the observations that CLL mAb sequences exhibit a immunoglobulin (Ig) heavy chain variable (V) gene usage that is biased from the normal V gene repertoire[4] and that CLL cells express genes and cell surface markers consistent with antigen activation.[5-7] Furthermore, the mAb V gene sequences in groups of CLL patients are virtually identical or stereotyped.[8-11] A large survey of 1939 CLL patients showed that 27% share stereotyped mAb sequences.[12] Taken together, this evidence strongly suggests some common antigen reactivities in CLL.

We examined one particular CLL subset (known as subset 6[12] or set I[10]) characterized by a stereotypic unmutated rearrangement involving *IGHV1-69*, *IGHD3-16*, and *IGHJ3* with nearly identical heavy (H) chain complementarity-determining region 3 (HCDR3) sequence that is paired with an unmutated light (L) chain sequence having an equally restricted LCDR3 that generally involves *IGKV3-20* (Table 1). To date, the BCRs of 4 unrelated CLL patients from our center, 2 previously reported (CLL068 and CLL258) and 2 new to this study (CLL861 and CLL900), and an additional 45 CLL patients worldwide have been characterized with these HCDR3 sequences. A total of 33 of these 49 patients also have data on the associated L chain, which shows a conserved LCDR3 sequence. Based on the number of patients exhibiting this stereotypic sequence rearrangement, it is extremely improbable that random recombination of Ig V genes accounts for this occurrence. The 19-amino acid HCDR3 consensus sequence of this subset has 16 well-conserved residues, with several consensus N-region additions (shown in parentheses in Table 1) that are not found in the germ line. Two of the nonconserved residues (x) of the consensus are generally limited to I/V or S/P, further restricting the mAb sequence variability of this subset. Together, this evidence suggests a unique antigen-binding capacity for these CLL antibodies.

To isolate and identify antigens that react with these stereotypic CLL mAbs, we took advantage of the observation that these mAbs bind to cytoplasmic structures in HEp-2 cells by immunofluorescence and react with HEp-2 cell extracts by enzyme-linked immunosorbent assay.[13] Thus, using HEp-2 cell extracts, mAb from this stereotypic subset was used to immunoprecipitate an antigen that we further isolated, identified, and characterized.

Methods

CLL mAb gene sequences

Peripheral blood was collected from CLL patients 861 and 900 after informed consent obtained as approved by the Institutional Review Board

Submitted June 9, 2008; accepted August 25, 2008. Prepublished online as *Blood* First Edition paper, September 23, 2008; DOI 10.1182/blood-2008-06-162024.

The online version of this article contains a data supplement.

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Table 1. Stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearranged antibodies

Patient	Reference	HCDR3*	LCDR3*
CLL068	10	AR(GGd)YDYvWGSYR(.sN)DAFDI	QQYGSSP(..)T
CLL258	43	AR(GGi)YDYvWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL861		AR(GGe)YDYiWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL900		AR(GGd)YDYvWGSYR(.pN)DAFDI	QQYGSSP(pf)T
MF9	10	AR(GGp)YDYvWGSYR(.pN)DAFDI	QQYGSSP(p.)T
SMI	44	AR(GGn)YDYiWGSYR(.sN)DAFDI	QQYGSSP(p.)a
CLL022	45	AR(GGd)YDYvWGSYR(.pN)DAFDI	
N_28	46	AR(GGt)YDYiWGSYR(.pN)DAFDI	
N1659	47	AR(GGd)YDYiWGSYR(.as)DAFDI	QQfnnyP(..)p
P896	47	AR(GGp)YDYvWGSYR(.pp)DAFDI	QQYGSSl(g.)T
FRA-183	48	AR(GGd)YDYvWGSYR(.pN)DAFDI	QQYGSSP(n.)T
ITA16903	48	AR(GGn)YDYiWGSYR(.aN)DAFDI	QQYGSSP(y.)T
ITA16904	48	AR(GGd)YDYvWGSYR(.pN)DAFDI	QQYGSSP(g.)T
FRA-373	48	AR(GGh)YDYiWGSYR(.pN)DAFDI	mQatqfP(..)l
FRA-204	48	AR(GGi)YDYvWGSYR(.pN)DAFDI	QQYGSSP(..)l
FRA-331	48	AR(GGh)YDYiWGSYR(.pN)DAFDI	QQYGSSP(g.)s
CLL-A	11	-R(GGl)YDYiWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL-B	11	-R(GGd)YDYvWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL-C	11	-R(GGi)YDYvWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL-D	11	-R(GGd)YDYvWGSYR(.pN)DAFDI	QQYGSSP(p.)T
CLL-E	11	-R(GGg)YDYiWGSYR(.pN)DAFDI	QQYGSSP(py)T
CLL-F	11	-R(GGl)YDYiWGSYR(.pN)DAFDI	QQYGSSP(g.)s
CLL-G	11	-R(GGd)YDYiWGSYR(.sN)DAFDI	QQYGSSP(..)T
CLL-H	11	-R(GGy)YDYiWGSYR(.pN)DAFDI	QQYGSSP(pg)s
CLL-I	11	-R(GGg)YDYiWGSYR(.pN)DAFDI	QQYGnSr(i.)T
CLL-J	11	-R(GGs)YDYiWGSYR(.pN)DAFDI	QQYGSSP(pi)T
CLL-K	11	-R(GGn)YDYiWGSYR(.sN)DAFDI	QQYGSSP(sf)T
CLL-L	11	-R(GGg)YDYiWGSYR(.pN)DAFDI	QQYGSSP(l.)T
CLL-M	11	-R(GGa)YDYiWGSYR(.pp)DAFDI	QQYGSSP(l.)T
CLL-N	11	-R(GGc)YDYvWGSYR(.tN)DAFDI	QQYGSSP(ri)T
CLL-O	11	-R(GGv)YDYiWGSYR(.pN)DAFDI	QQYGSSP(..)r
CLL 24, Swe-131	49, 12	AR(GGh)YDYvWGSYR(.pN)DAFDI	QQYGSSP(g.)T
CLL 26, Swe-142	49, 12	AR(GGn)YDYvWGSYR(.pN)DAFDI	QQYGSSP(f.)T
CLL 27, Swe-208-I	49, 12	AR(GGy)YDYvWGSYR(.sN)DAFDI	QQYGSSP(g.)T
CLL 28, Swe-209-I, p239	49, 12, 24	AR(GGd)YDYiWGSYR(.pN)DAFDI	QQYGSSP(g.)T
MC67	50	AR(GGn)YDYvWGSYR(.pN)DAFDI	
MC159	50	AR(GGd)YDYiWGSYR(.pN)DAFDI	
MC160	50	AR(GGd)YDYvWGSYR(.pN)DAFDI	
MC161	50	AR(GGf)YDYiWGSYR(.sN)DAFDI	
MC162	50	AR(GGg)YDYiWGSYR(.pN)DAFDI	
MC163	50	AR(GGg)YDYiWGSYR(.sN)DAFDI	
MC164	50	AR(GGv)YDYvWGSYR(.pN)DAFDI	
F156	12	AR(GGy)YDYvWGSYR(.pN)DAFDI	
FAV-174	12	AR(GGn)YDYiWGSYR(.pN)DAFDI	
P3810	12	AR(GGy)YDYvWGSYR(.pN)DAFDI	
Swe-222	12	AR(eG.).DYvWGSYR(yra)DAFDI	
Swe-434-I	12	AR(GGy)YDYvWGSYR(.pN)DAFDI	
Swe-448-I	12	AR(GGa)YDYiWGSYR(.sN)DAFDI	
ID-7	24	AR(GGg)YDYvWGSYR(.pN)DAFDI	
Consensus		AR(GGx)YDYxWGSYR(.xN)DAFDI	QQYGSSP(x.)T
Germline	51	AR(e.y)YDYvWGSYR(.yt)DAFDI	QQYGSSP(sx)T

Germline junctional sequences assume no N additions or nucleotide trimming with the exception of a single nucleotide deletion in the D-J or V-J junctions to maintain reading frame. Uppercase letters indicate conserved sequences; "—", unknown sequence; ".", gap in sequence introduced for alignment purposes.

*CDR3 shown in single letter amino acid code with V-D, D-J, V-J junctional sequences enclosed by parentheses.

of North Shore University Hospital (Manhasset, NY) and Long Island Jewish Medical Center (New Hyde Park, NY) and in accordance with the Declaration of Helsinki. RNA from peripheral blood mononuclear cells was converted to cDNA, and the mAb V regions were sequenced as previously described[9] and deposited in GenBank (accession numbers EU202446-EU202450; Table 1).[14] The mAb V region sequences from vectors overexpressing recombinant CLL068, CLL258, and CLL412 mAbs were confirmed to be identical to those already published (GenBank accession numbers AY553640, AY574935, AY055485, AY574938, AY553648, and AY574946).

Immunoprecipitation and immunoblot

Human larynx epidermoid carcinoma cell line, HEp-2, was grown to approximately 80% to 90% confluence at 5% CO_2 and 37°C in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Crystalgen, Plainview, NY), 20 U/mL penicillin, and 20 μg/mL streptomycin (Cambrex, East Rutherford, NJ), lysed in phosphate-buffered saline (PBS; Cellgro) containing 1% Triton (Sigma-Aldrich, St Louis, MO), 0.02% phenylmethylsulfonylfluoride, and one Complete Protease Inhibitor Cocktail Mini-tablet (Roche Applied Science,

Indianapolis, IN), rotated at 4°C for 30 minutes, and centrifuged at approximately 20 000*g* for 10 minutes at 4°C to collect the cell extract. To facilitate recombinant overexpression and purification, the CLL068, CLL258, and CLL412 mAbs, which are expressed as IgMs in patients, were produced as human IgG1s.[13] Recombinant mAb or human IgG (I2511, 5 μg; Sigma-Aldrich) was bound to protein G agarose beads (50 μL settled gel; Pierce Chemical, Rockford, IL) at room temperature for 30 minutes, incubated with 500 μL HEp-2 cell extracts (precleared with protein G agarose beads alone) in 150 mM NaCl, 25 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.2, overnight at 4°C under rotation, and then centrifuged at approximately 230*g* for 1 minute at 4°C to collect supernatant and the immunoprecipitated material. Immunoprecipitated material was washed 4 times in incubation buffer. Samples were boiled 5 minutes in 0.05% sodium dodecyl sulfate (SDS), 2.5% glycerol, 1.25% 2-mercaptoethanol, 0.0125% bromophenol blue, 15.6 mM Tris, pH 6.8, and electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE), with the resulting gel stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA).[15] Alternatively, the gel was electroblotted overnight at 4°C to a 0.45-μm nitrocellulose membrane (Bio-Rad or Amersham Hybond-ECL; GE Healthcare, Little Chalfont, United Kingdom),[15] stained with Ponceau S (P7767; Sigma-Aldrich), probed with rabbit antihuman nonmuscle myosin heavy chain IIA (MYHIIA; 1:3000 dilution, BTI-561; Biomedical Technologies, Stoughton, MA), developed with 1:10 000 dilution donkey antirabbit IgG conjugated with horseradish peroxidase and the ECL Western Blotting Detection System (GE Healthcare), and exposed to film.

Protein identification

Protein bands were cut of Coomassie Blue–stained SDS-PAGE gels, trypsin-digested, and analyzed by liquid chromatography followed by sequential mass spectrometry (LC-MS/MS) as previously described.[16] Data obtained were compared with the National Center for Biotechnology Information nonredundant protein database for identification of peptide sequences by MASCOT (Matrix Science, Boston, MA), which calculated probability-based Mowse scores for ranking protein matches, with scores more than 47 being statistically significant ($P < .05$) and higher scores being more significant.

Fluorescence microscopy

HEp-2 cell-coated slides (708100; Inova Diagnostics, San Diego, CA) were incubated with 50 μg/mL CLL mAb and anti-MYHIIA antibody (1:250, BTI-561) for 1 hour at 4°C, washed in PBS, incubated with fluorescein isothiocyanate-conjugated mouse antihuman IgG (1:500; Southern Biotechnology Associates, Birmingham, AL) and Rhodamine Red-X–conjugated donkey antirabbit IgG (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature, washed in PBS, and mounted with a coverslip. Slides were imaged and acquired by a confocal laser-scanning microscope system (FluoView 300-IX; Olympus, Tokyo, Japan) using a PLAN APO 60×/1.4 oil objective lens. Images were processed and analyzed using ImageJ with colocalization plug-ins that statistically evaluate colocalization using Pearson correlation coefficient (r).[17] r varies from −1 to 1, where 1 equals complete colocalization. For MYHIIA inhibition studies, 2×10^4 human glioblastoma SNB19 cells or human breast ductal carcinoma epithelial T47D cells were cultured at 5% CO_2 and 37°C in Dulbecco minimal essential media (Cellgro) supplemented with 10% heat-inactivated FBS, 20 U/mL penicillin, and 20 μg/mL streptomycin on 12-mm circular glass coverslips in a 24-well plate for 1 day. Cells were subsequently treated overnight with 50 μM blebbistatin (Calbiochem, San Diego, CA), a direct specific inhibitor of MYHIIA ATPase,[18] or for 1 hour with 10 μM Y27632 (Calbiochem), a specific inhibitor of ROCK, a tyrosine kinase that indirectly regulates MYHIIA activity by directly phosphorylating the myosin light chain (MLC) and/or by phosphorylating the MLC phosphatase, inhibiting dephosphorylation of the MLC.[19] Then cells were fixed in 4% formaldehyde in PBS at room temperature for 15 to 30 minutes. Coverslips were washed in 0.1% Triton in PBS (PBS-T), incubated with primary antibodies in 5% FBS in PBS-T for 45 minutes at room temperature, washed 3 times with PBS-T, incubated with secondary antibodies in 5% milk (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature, washed as before, stained with 2.5 mg/mL rhodamine phalloidin (Invitrogen, Carlsbad, CA) for 10 minutes at room temperature, washed as before, mounted on slides with 70% VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA), and then examined by confocal laser-scanning microscopy.

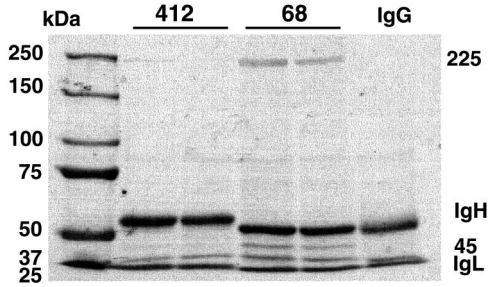

Figure 1. CLL mAb with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements immunoprecipitate observable proteins. A total of 6% SDS-PAGE of immunoprecipitate from HEp-2 cell extracts treated with mAb having stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements (CLL068, 2 different recombinant mAb preparations) or not (CLL412, 2 different recombinant mAb preparations, or IgG) is shown with protein size (kilodalton) markers. Another mAb having stereotypic *IGHV1-69, IGHD3-16,* and *IGHJ3* rearrangements (CLL258) showed similar results (Figure S1). mAb heavy chain (IgH) and light chain (IgL) as well as 225- and 45-kDa protein bands are indicated.

Short interfering RNA transfections

SNB19 cells were transfected with short interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) as previously described,[20] with the exception that cells were transfected in 6-well plates. Cells were either transfected with ON-TARGETplus SMARTpool siRNA specific to human MYHIIA (Dharmacon RNA Technologies, Lafayette, CO), Lipofectamine 2000 alone, or untreated. After 1 day of incubation, cells were split 1:6 into 24-well plates containing coverslips as in "Fluorescence microscopy" or into 6-well plates, and then incubated for 3 days. Coverslips were analyzed using antibodies and staining conditions as in "Fluorescence microscopy." Cells in each well of the 6-well plate were lysed in 300 μL for immunoblot analysis and probed with anti-MYHIIA antibody as in "Immunoprecipitation and immunoblot." The immunoblot was also probed with mouse antihuman alpha-tubulin monoclonal antibody DM1A (1:2000 dilution; Sigma-Aldrich) followed by 1:10 000 dilution sheep anti–mouse IgG conjugated with horseradish peroxidase (GE Healthcare).

Results

Stereotypic CLL mAb immunoprecipitates cellular protein(s)

CLL mAbs with stereotypic *IGHV1-69, IGHD3-16,* and *IGHJ3* rearrangements (represented by CLL068 and CLL258) immunoprecipitated protein bands of approximately 225 and approximately 45 kDa from HEp-2 cell extracts (Figure 1 and Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). In contrast, a mAb that did not have this stereotypic rearrangement, CLL412 (which has a stereotypic *IGHV3-21* and *IGHJ6* rearrangement known as subset 2 or set VI[12,13]), as well as normal human serum IgGs did not clearly immunoprecipitate these same protein bands under the same conditions. The 45-kDa protein was not always visible in experiments where the 225-kDa protein was observed, suggesting that the 225-kDa protein was the major protein reactive with this subset of CLL mAbs.

Immunoprecipitated protein(s) identified by LC-MS/MS analysis

The identity of the 225- and 45-kDa protein bands was determined by LC-MS/MS (Figure 2). The major protein in the 225-kDa band

A

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  1 MAQQAADKYL YVDKNFINNP LAQADWAAKK LVWVPSDKSG FEPASLKEEV GEEAIVELVE
 61 NGKKVKVNKD DIQKMNPPKF SKVEDMAELT CLNEASVLHN LKERYYSGLI YTYSGLFCVV
121 INPYKNLPIY SEEIVEMYKG KKRHEMPPHI YAITDTAYRS MMQDREDQSI LCTGESGAGK
181 TENTKKVIQY LAYVASSHKS KKDQGELERQ LLQANPILEA FGNAKTVKND NSSRFGKFIR
241 INFDVNGYIV GANIETYLLE KSRAIRQAKE ERTFHIFYYL LSGAGEHLKT DLLLEPYNKY
301 RFLSNGHVTI PGQQDKDMFQ ETMEAMRIMG IPEEEQMGLL RVISGVLQLG NIVFKKERNT
361 DQASMPDNTA AQKVSHLLGI NVTDFTRGIL TPRIKVGRDY VQKAQTKEQA DFAIEALAKA
421 TYERMFRWLV LRINKALDKT KRQGASFIGI LDIAGFEIFD LNSFEQLCIN YTNEKLQQLF
481 NHTMFILEQE EYQREGIEWN FIDFGLDLQP CIDLIEKPAG PPGILALLDE ECWFPKATDK
541 SFVEKVMQEQ GTHPKFQKPK QLKDKADFCI IHYAGKVDYK ADEWLMKNMD PLNDNIATLL
601 HQSSDKFVSE LWKDVDRIIG LDQVAGMSET ALPGAFKTRK GMFRTVGQLY KEQLAKLMAT
661 LRNTNPNFVR CIIPNHEKKA GKLDPHLVLD QLRCNGVLEG IRICRQGFPN RVVFQEFRQR
721 YEILTPNSIP KGFMDGKQAC VLMIKALELD SNLYRIGQSK VFFRAGVLAH LEEERDLKIT
781 DVIIGFQACC RGYLARKAFA KRQQQLTAMK VLQRNCAAYL KLRNWQWWRL FTKVKPLLQV
841 SRQEEEMMAK EEELVKVREK QLAAENRLTE METLQSQLMA EKLQLQEQLQ AETELCAEAE
901 ELRARLTAKK QELEEICHDL EARVEEEEER CQHLQAEKKK MQQNIQELEE QLEEEESARQ
961 KLQLEKVTTE AKLKKLEEEQ IILEDQNCKL AKEKKLLEDR IAEFTTNLTE EEEKSKSLAK
1021 LKNKHEAMIT DLEERLRREE KQRQELEKTR RKLEGDSTDL SDQIAELQAQ IAELKMQLAK
1081 KEEELQAALA RVEEEAAQKN MALKKIRELE SQISELQEDL ESERASRNKA EKQKRDLGEE
1141 LEALKTELED TLDSTAAQQE LRSKREQEVN ILKKTLEEEA KTHEAQIQEM RQKHSQAVEE
1201 LAEQLEQTKR VKANLEKAKQ TLENERGELA NEVKVLLQGK GDSEHKRKKV EAQLQELQVK
1261 FNEGERVRTE LADKVTKLQV ELDNVTGLLS QSDSKSSKLT KDFSALESQL QDTQELLQEE
1321 NRQKLSLSTK LKQVEDEKNS FREQLEEEEE AKHNLEKQIA TLHAQVADMK KKMEDSVGCL
1381 ETAEEVKRKL QKDLEGLSQR HEEKVAAYDK LEKTKTRLQQ ELDDLLVDLD HQRQSACNLE
1441 KKQKKFDQLL AEEKTISAKY AEERDRAEAE ARKETKALS LARALEEAME QKAELERLNK
1501 QFRTEMEDLM SSKDDVGKSV HELEKSKRAL EQQVEEMKTQ LEELEDELQA TEDAKLRLEV
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B

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  1 MDDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK DSYVGDEAQS
 61 KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE HPVLLTEAPL NPKANREKMT
121 QIMFETFNTP AMYVAIQAVL SLYASGRTTG IVMDSGDGVT HTVPIYEGYA LPHAILRLDL
181 AGRDLTDYLM KILTERGYSF TTTAERSIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY
241 ELPDGQVITI GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS TFQQMWISKQ
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Figure 2. LC-MS/MS identification of 225- and 45-kDa proteins. After LC-MS/MS analysis, 225-kDa protein band peptide sequence matches to MYHIIA (A) and 45-kDa protein band peptide sequence matches to cytoplasmic beta-actin (B) are shown in bold and are underlined.

is MYHIIA, which had the highest Mowse score (1216) and the largest number of peptide matches (43). These peptide matches cover 25% of the protein and are spread throughout the protein (Figure 2A). The calculated molecular weight (MW) of MYHIIA (226 kDa) is in close agreement with that observed in our SDS-PAGE analyses. In contrast, the next highest scoring protein match (242) had only 8 peptide matches to keratin 1, which is a common contaminant of LC-MS/MS analyses and has a different MW (66 kDa) than that identified by SDS-PAGE.

The best protein match to the 45-kDa band is cytoplasmic beta-actin, which had a Mowse score of 341 with 11 peptide matches throughout the protein with a 25% coverage (Figure 2B) and a MW (42 kDa) in close agreement with that observed. However, in this case, keratin contamination was high, with keratin 1 having the highest score (890) and the most number of peptide matches (26) despite having an incompatible MW. This suggests that cytoplasmic beta-actin was not as abundantly immunoprecipitated because of lower affinity for the CLL mAb or because it precipitated in association with MYHIIA. These possibilities are supported by the observation in "Stereotypic CLL mAb immunoprecipitates cellular protein(s)" of inconsistent actin immunoprecipitation by CLL mAb (and by subsequent immunofluorescence studies in "Treatments that alter MYHIIA expression affect stereotypic CLL mAb binding"). For this reason, we focused the remaining studies on MYHIIA.

Immunoprecipitated protein (225 kDa) confirmed to be MYHIIA

To confirm the identity of the 225-kDa band as MYHIIA, immunoprecipitated protein bands from one of these stereotypic

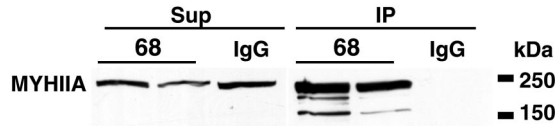

Figure 3. CLL mAb with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements immunoprecipitate MYHIIA. Antibodies from CLL068 (2 different recombinant mAb preparations) or human IgG were used to immunoprecipitate HEp-2 cell extracts. Immunoprecipitate (IP) and supernatant (Sup) samples were electrophoresed in 6% SDS-PAGE as in Figure 1. After blotting to nitrocellulose, the membrane was probed with rabbit antihuman anti-MYHIIA as shown.

mAbs (CLL068) were immunoblotted and probed with antibodies specific for human MYHIIA (Figure 3). Similar results were obtained with CLL258 mAb (C.C.C., and L.Z., unpublished data, January 22, 2007, to February 15, 2007). Anti-MYHIIA antibodies recognized the 225-kDa band immunoprecipitated by CLL068 mAb, as well as some additional smaller MW bands that were not visible in the SDS-PAGE gels (Figure 1). The smaller MW bands are probably MYHIIA degradation products. Normal human IgGs did not immunoprecipitate any protein bands recognized by anti-MYHIIA antibodies, even though the supernatant contained abundant MYHIIA protein.

Stereotypic CLL mAbs recognize MYHIIA in cell cytoplasm

CLL mAbs from this stereotypic subset (CLL068 and CLL258) recognized primarily cytoplasmic structures in HEp-2 cells.[13] The immunohistochemical staining of CLL068 colocalized with anti-MYHIIA antibody binding (Figure 4A). A merge of the images results in a color change indicating overlap of binding to the same cellular structures ($r = 0.58 \pm 0.08$). CLL068 mAb appears to bind additional nuclear structures not recognized by anti-MYHIIA. Indeed, the colocalization improves when nuclei are omitted from the analysis ($r = 0.72 \pm 0.05$). Similar results were obtained with the T47D (C.C.C. and L.Z., unpublished data, May 2, 2007, to July 30, 2007) and SNB19 cell lines (Figure 4B), indicating that this CLL mAb reactivity is not limited to a specific cell type.

Treatments that alter MYHIIA expression affect stereotypic CLL mAb binding

To further study the binding of this stereotypic CLL mAb to MYHIIA, we examined the SNB19 cell line, which can be manipulated with drugs and siRNA to alter MYHIIA expression.[20] Cytoplasmic structures containing MYHIIA can be disrupted by inhibition with blebbistatin or Y27632. CLL068 staining colocalizes with MYHIIA in untreated SNB19 cells ($r = 0.84$; Figure 4B). Treatment with blebbistatin reduces cellular levels of MYHIIA, disrupts MYHIIA cytoplasmic structures, and increases deposition of MYHIIA in membrane ruffles. CLL068 staining colocalizes very well with the blebbistatin-induced MYHIIA cellular redistribution ($r = 0.79$; Figure 4B). In contrast, CLL068 staining does not colocalize well with F-actin in its native state ($r = 0.46$; Figure 4C) or after disruption and redistribution by blebbistatin treatment ($r = 0.48$; Figure 4C). The lack of colocalization with F-actin (predominantly composed of cytoplasmic beta-actin in nonmuscle cells) supports our initial conjecture that cytoplasmic beta-actin may be immunoprecipitating in association with MYHIIA or has lower affinity for the CLL mAb. Similar results were found with SNB19 cells incubated with Y27632 (Figure S2). Thus, drug treatments that affect MYHIIA expression and localization concordantly affect the binding of this stereotypic subset of CLL mAbs.

Finally, MYHIIA expression was specifically inhibited in SNB19 cells by transfection with siRNAs specific for MYHIIA,

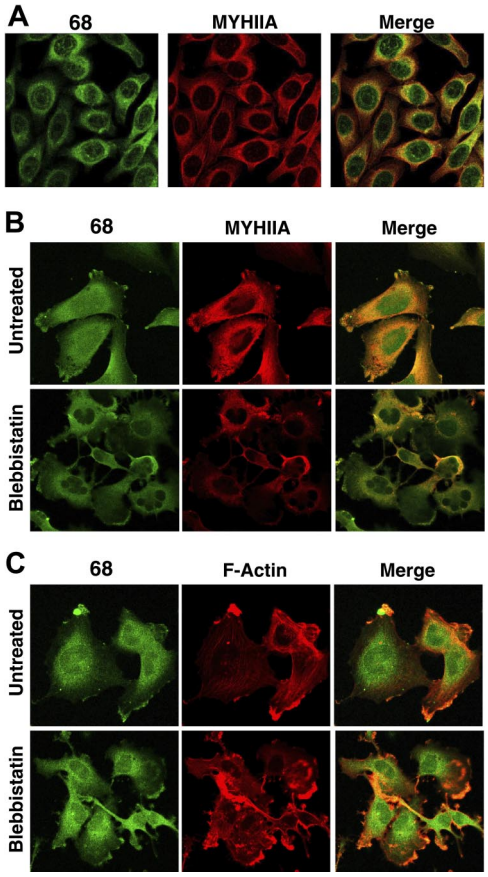

Figure 4. Binding of CLL mAb with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements colocalizes with MYHIIA. (A) HEp-2 cells stained with CLL068 antibody (green) and anti-MYHIIA antibody (red) were visualized by confocal microscopy separately and merged together (overlap in yellowish orange). Untreated or blebbistatin-treated SNB19 cells were stained with CLL068 antibody (green) and (B) anti-MYHIIA antibody (red) or (C) phalloidin-stained F-actin (red). Cells were visualized by confocal microscopy separately and merged together.

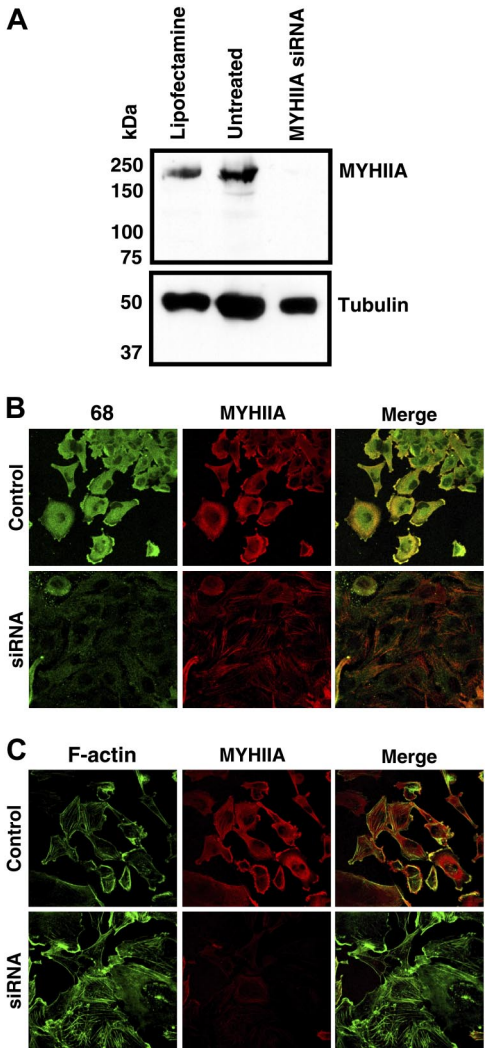

Figure 5. MYHIIA siRNA treatment removes most cellular staining by CLL mAb with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements. (A) Immunoblot of SNB19 cell extracts either untreated or after transfection with MYHIIA siRNA or Lipofectamine 2000 alone. Top part of membrane containing high MW proteins (> 75 kDa) was probed with anti-MYHIIA, and the lower part with low MW proteins was probed with anti-alpha-tubulin. MYHIIA siRNA reduced MYHIIA protein levels by 99.6% relative to Lipofectamine 2000 alone. Lipofectamine 2000 alone (Control) or siRNA-transfected SNB19 cells were stained with CLL068 antibody (green) and (B) anti-MYHIIA antibody (red) or (C) phalloidin stained F-actin (red). Cells were visualized by confocal microscopy separately and merged together.

thereby markedly reducing MYHIIA protein levels compared with untreated cells or cells exposed to transfection reagent alone (Figure 5A). In contrast, alpha-tubulin protein levels were unaffected by MYHIIA siRNA treatment. MYHIIA siRNA treatment reduced staining of SNB19 cells with anti-MYHIIA, as expected (Figure 5B). CLL068 staining was similarly reduced, suggesting that MYHIIA is the major cellular antigen recognized by this stereotypic subset of mAbs. Treatment with transfection reagent alone showed similar anti-MYHIIA and CLL068 colocalization as observed previously (Figure 4B). In comparison, F-actin staining was unaffected by MYHIIA siRNA treatment (Figure 5C). Treatment with MYHIIA siRNA or transfection reagent alone did not affect F-actin and MYHIIA staining and exhibits the anticipated low level of colocalization ($r = 0.39$ and 0.59, respectively) similar to that observed with CLL068 and F-actin (Figure 4C). Altogether, these data suggest that the stereotypic subset of CLL mAbs represented by CLL068 recognize nonmuscle cytoplasmic MYHIIA as the predominant autoantigen found in cells.

Discussion

The observation that a large number of CLL patients share stereotypic BCRs/mAbs suggests a role for antigen in this disease. Because several CLL mAbs with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements and nearly identical H and L CDR3s stain HEp-2 cells in a characteristic cytoplasmic pattern, they probably recognize a common cellular antigen. Our results demonstrate that this antigen is MYHIIA. MYHIIA is an intracellular molecule that forms transient large molecular motor complexes with cytoplasmic MLCs, actin, and other associated molecules involved in the movement and morphology of the cell and its intracellular components.[21]

How could the BCR on the extracellular surface of CLL cells interact with intracellular MYHIIA? One possibility is that MYHIIA moves extracellularly under certain situations. Indeed, during apoptosis, MYHIIA is cleaved and translocated to apoptotic blebs as shown in the human Jurkat T-cell line.[22] Thus, CLL BCRs could bind MYHIIA exposed on apoptotic blebs. Indeed, preliminary data indicate that CLL068 mAb and anti-MYHIIA antibody colocalize to the same apoptotic structures induced in Jurkat cells (C.C.C. and L.Z., unpublished data, April 18, 2008, to June 19,

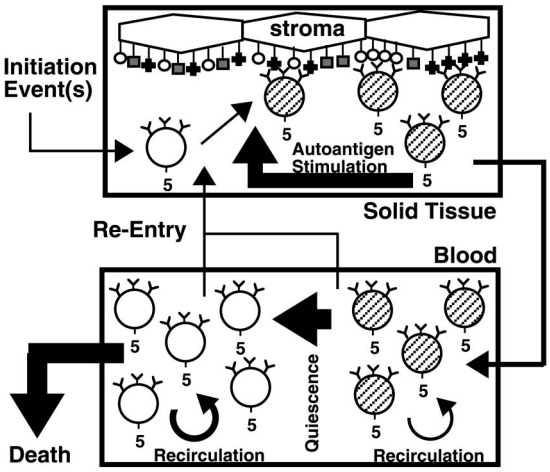

Figure 6. Model of cycle of CLL autoantigen stimulation. CLL B cell indicated by circle labeled with 3 Y-shaped antibodies and the number 5 (for CD5). Activated CLL B cells are striped. Boxes represent 2 cellular compartments: solid tissue, lymphoid organs such as bone marrow or lymph nodes, or blood. Other types of cells, possibly stromal cells as suggested in this illustration, present antigen represented by different shades and shapes. Sizes of pathway arrows indicate amount of cell traffic within and between compartments.

2008). In CLL, the source of apoptotic stimulatory material could come from normal cell turnover in the body, as well as potentially from CLL cell apoptosis. In addition, cells stressed during ischemic reperfusion also express MYHIIA on their surface.[23] Thus, CLL BCRs could also respond to MYHIIA expressed on the surface of cells undergoing stress, such as hypoxic conditions encountered during ischemia.

One model for the role of autoantigen in the maintenance and expansion of CLL clones involves cycles of autostimulation (Figure 6). After transformation by an unknown initiation event(s), the CLL B cell transits through solid lymphoid tissue, such as bone marrow or lymph node, where the cell encounters autoantigen and is activated to proliferate. Although many proliferating CLL cells are retained in solid tissue, others traffic peripherally via the blood or lymph. While circulating, these cells are less likely to encounter immunostimulatory autoantigen and therefore may become quiescent. Although many of these cells may die in circulation, some cells could transit back to solid tissue for restimulation, resuming the cycle. CLL cells could be activated by stromal or other cells in the solid tissue that present MYHIIA as a result of stress induction or as a result of trapping and display of apoptotic cell debris. CLL mAbs with stereotypic *IGHV1-69*, *IGHD3-16*, and *IGHJ3* rearrangements may be stimulated by MYHIIA by this mechanism. Purification of MYHIIA protein will be necessary to enable the direct testing of CLL BCR stimulation; this has not been accomplished by others or ourselves to date. Other stereotypic CLL subsets could potentially react with other autoantigens exposed in a similar manner. In support of this model, CLL mAbs with different Ig rearrangements have been reported to recognize vimentin, filamin B, and cofilin, all of which are cytoskeletal components that are exposed during apoptosis.[24] Similarly, we have preliminary data showing that a stereotypic *IGHV4-39*, *IGHD6-13*, and *IGHJ5* rearranged CLL mAb (known as Subset 8[9,12]) immunoprecipitates vimentin (C.C.C., L.Z., and H.M.F., unpublished data, April 16, 2007, to November 13, 2007). Furthermore, CLL mAbs can bind apoptotic cells. Thus, this model of CLL maintenance and expansion may have broad applicability.

In this CLL model, an infectious agent that binds and stimulates the CLL B cell could promote the cycle. Indeed, *Streptococcus pyogenes*, *Trypanosoma cruzi*, and cytomegalovirus infections produce antibodies that cross-react with cardiac myosin and play an important role in the subsequent development of acute rheumatic fever and heart disease, Chagas disease cardiopathy, or viral myocarditis, respectively.[25-27] Cardiac myosin is related to MYHIIA, but antibodies against either molecule do not necessarily cross-react with the other, as implied by the observation that the sequences of human antibodies reactive to cardiac myosin and *S pyogenes* protein do not exhibit amino acid similarity to this CLL mAb subset.[28] Other infectious agents could also lead to cross-reactivity with MYHIIA. Interestingly, autoantibodies against nonmuscle myosin have been reported to occur with hepatitis C virus infection.[29] Furthermore, CLL mAbs, including those in this stereotypic subset, react to lipopolysaccharide, a bacterial outer membrane component,[13] and various bacterial strains.[24,30] Finally, prior respiratory infections increase the risk of developing CLL.[31] Thus, a plausible connection exists for an infectious agent promoting the development and evolution of CLL. This type of scenario is similar to the dependence on *Helicobacter pylori* and other organisms for the development of gastric mucosa-associated lymphoid tissue lymphoma.[32] The resulting mucosa-associated lymphoid tissue lymphomas express antibodies that appear to have been antigen selected and acquired specificity for autoantigens, very similar to what is observed for CLL.[32]

Reactivity to MYHIIA and possibly some still unidentified infectious agents suggests that these CLL mAbs have some cross-reactivity or some limited polyreactivity. Indeed, some antibodies from this subset (Table 1) have been characterized to react to multiple autoantigens: CLL068 and CLL258 bind to single-stranded or double-stranded DNA, insulin, lipopolysaccharide, and HEp2 cell extracts,[13] and CLL mAb "SMI" binds to single-stranded DNA, IgG, myoglobin, thyroglobulin, and actin.[33] Furthermore, a non-CLL Ig (RIZ GVH2) with the same stereotypic characteristics (HCDR3 sequence is AR [GGn] YDYiWGSYR [sN] DAFDI in the format of Table 1) was identified based on its autoreactivity with cardiolipin.[34] Although unmutated CLL mAbs, such as this subset, are generally thought to be intrinsically polyreactive/autoreactive,[13] based on studies with the SMI mAb, this characteristic reactivity is dependent on the somatically rearranged HCDR3 region and association with a particular L chain.[33,35] This suggests that a particular antigen or group of antigens is involved in selecting the CLL mAb. Chronic stimulation of the CLL BCR with autoantigens (MYHIIA and perhaps others) and/or infectious agents could promote the transformation, development, and expansion of this leukemia.

The reactivity of this subset of CLL mAbs could be a clue to the cell of origin for this leukemia. Autoreactivity and polyreactivity are features of natural antibodies.[36] These antibodies are generally produced from B-1 cells, a subpopulation of B lymphocytes thought to be involved in the innate protective immune responses, which is especially important in neonates who do not have time to mount an adaptive immune response to infectious agents. The polyreactivity of these antibodies permits rapid reactivity to a diverse array of infections. Furthermore, the autoreactivity can stimulate these B cells because natural antibodies serve as BCRs. Natural antibody reactivity to myosin and nonmuscle myosin has been observed.[37,38] The autoreactivity of natural antibodies is also thought to help with the clearance of apoptotic cells.[39,40] Interestingly, a mouse natural antibody characterized to bind to myosin also shows binding to late apoptotic cells and altered phospholipids exposed during apoptosis.[41] In addition to our preliminary data indicating that CLL068 mAb binds to apoptotic structures (C.C.C.

and L.Z., unpublished data, April 18, 2008, to June 19, 2008), other CLL mAbs, especially those composed of unmutated IGHV segments, have been reported to bind to apoptotic cells and modified phospholipids that could be generated during apoptosis.[24,42] Reactivity with apoptotic cells fits nicely with the model (Figure 6), where apoptotic cell material, possibly from normal or CLL cell turnover, could be displayed in solid tissues by stromal or other cells and react with the BCR of CLL cells. This BCR reactivity could potentially stimulate the CLL cells to survive and expand. Thus, the autoreactivity of CLL mAbs may reveal a general mechanism for maintenance and growth of this disease.

Acknowledgments

The authors appreciate the assistance of and discussions with Marc Symons, Sal Coniglio, and Amanda Chan (Light Microscopy Facility, The Feinstein Institute for Medical Research) regarding inhibition of MYHIIA expression, Anne Bresnick (Albert Einstein College of Medicine) and Robert Adelstein (National Institutes of Health [NIH]) regarding MYHIIA biology, and Bettie Steinberg, Manuela Woelfle, and Sophia Yancopoulos (The Feinstein Institute for Medical Research) regarding manuscript preparation.

This work was supported in part by R01 grants from NIH (CA81554 and CA87956; N.C.), an M01 General Clinical Research Center grant from the NIH (RR018535; N.C.), the Intramural Research Program of NIH (H.M.F.), the Karches Foundation, the Peter Jay Sharp Foundation, the Prince Family Foundation, the Marks Foundation, the Jean Walton Fund for Leukemia and Lymphoma Research, and the Joseph Eletto Leukemia Research Fund.

Authorship

Contribution: C.C.C., R.C., X.-J.Y., L.Z., X.B.W., H.M.F., and N.C. designed research and analyzed results; R.C., K.H., X.-J.Y., L.Z., X.B.W., and H.M.F. performed experiments; C.C.C. wrote the paper and made the figures; and S.L.A., J.E.K., and K.R.R. contributed patient samples.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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