The Spermatozoa Protein, SLLP1, Is a Novel Cancer–Testis Antigen in Hematologic Malignancies

Zhiqing Wang,[1,2] Yana Zhang,[1,2] Arabinda Mandal,[3] Jian Zhang,[1,2] Francis J. Giles,[1,2] John C. Herr,[3] and Seah H. Lim[1,2]

[1]Division of Hematology and Oncology, Texas Tech University Health Sciences Center, Amarillo, Texas; [2]Biotherapy and Stem Cell Transplant Program, Don and Sybil Harrington Cancer Center, Amarillo, Texas; and [3]Center for Research in Contraceptive and Reproductive Health, Department of Cell Biology, University of Virginia Health System, Charlottesville, Virginia

ABSTRACT

Purpose: **Neoplastic cells often aberrantly express normal testicular proteins. Because these proteins have a very restricted normal tissue expression, they may be suitable targets for immunotherapy. SLLP1 is an intra-acrosomal, nonbacteriolytic, c lysozyme–like protein recently isolated from human spermatozoa. In this study, we determined whether SLLP1 is a novel cancer–testis antigen in hematologic malignancies**

Experimental Design: **SLLP1 expression in hematologic tumor cells and normal tissues was determined using a combination of reverse transcription-PCR, real-time PCR, and Western blot analysis. The presence of antibodies against SLLP1 was determined by ELISA analysis.**

Results: **SLLP1 was aberrantly expressed in the tumor cells from 2 of 9 acute myeloid leukemia, 3 of 11 chronic lymphocytic leukemia, 4 of 14 chronic myeloid leukemia, and 6 of 17 multiple myeloma. In contrast, they were not detected in corresponding specimens from any healthy donors. SLLP1 exhibited a very restricted normal tissue expression, being found only in testis/spermatozoa. SLLP1 was expressed in some tumor cells at a level of >25%. High titer IgG antibodies against SLLP1 were also detected in the sera of some of these patients.**

Conclusions: **SLLP1 is a novel cancer–testis antigen in hematologic malignancies and is capable of eliciting B-cell immune responses *in vivo* in cancer-bearing individuals. Our results, therefore, support SLLP1 as a protein target appropriate for additional *in vitro* study to define its suitability for immunotherapy.**

INTRODUCTION

Recent advances in tumor immunology suggest that many tumor cells are immunogenic in the autologous setting (1, 2). The immunogenicity of these cells originates from either neoproteins expressed by the tumor cells as a result of gene mutation and chromosomal translocation or aberrantly expressed normal proteins. It is also apparent that neoplastic cells often aberrantly express normal testicular proteins. These proteins collectively form the new class of tumor antigen called cancer–testis antigens (3). Thus far, >44 cancer–testis gene families have been identified and their expression studied in numerous cancer types. Nineteen of 43 cancer–testis genes were found to be testis restricted, 10 of 43 tissue restricted (mRNA detected in two or fewer nongametogenic tissues), 9 of 43 differentially expressed (mRNA detected in 3 to 6 nongametogenic tissues), and 5 of 43 ubiquitously expressed (4). Not all of these antigens have been documented to be immunogenic. If they are immunogenic *in vivo* in the cancer-bearing patients, they are potentially attractive targets for immunotherapy because a subset of these antigens may be differentiation antigens showing highly selective normal tissue distribution, which is expressed primarily or exclusively in the testis.

SLLP1 is a unique nonbacteriolytic, c-lysozyme–like protein recently isolated from human spermatozoa (5). It is encoded by the gene *SPACA3* at locus *17q11.2* and the protein is localized in the acrosome of human spermatozoa. Antisera to SLLP1 blocks binding in the hamster egg penetration assay, suggesting that SLLP1 may be involved in sperm/egg adhesion. A recent study by dot blot analysis on RNA from a panel of normal tissues showed that it was expressed only in the testis and in Burkitt's lymphoma Raji cell line (5), suggesting that additional studies are warranted to determine and characterize SLLP1 expression in tumor cells, in particular, fresh tumor specimens and to investigate whether tumor-derived SLLP1 protein could elicit immune responses *in vivo* in the autologous hosts in cancer-bearing patients. Such information will form the foundation for any future *in vitro* studies into the suitability of SSLP1 as a target for immunotherapy.

In this study, we showed that SLLP1 is a novel cancer–testis antigen in hematologic malignancies. We also found the presence of antibodies against SLLP1 in the sera of these patients, suggesting the *in vivo* immunogenicity of SLLP1 in cancer-bearing individuals. Our results, therefore, support SLLP1 as a protein target appropriate for further *in vitro* studies to define its suitability as a target for immunotherapy.

Received 5/8/04; revised 6/12/04; accepted 6/22/04.

Grant support: NIH/National Cancer Institute Grants RO1 CA88434 and RO1 CA106283 (S. Lim), Child Health and Human Development Grants U54-HD29099 and RO1 HD35523 (J. Herr), the Fogarty International Center Grant D43 HD00654 (J. Herr), and by the Larry Wiley Memorial Fund (S. Lim).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Seah H Lim, Don and Sybil Harrington Cancer Center, 1500 Wallace Boulevard, Amarillo, TX 79106. Phone: (806) 359-4673; Fax: (806) 356-1928; E-mail: slim@harringtoncc.org.

MATERIALS AND METHODS

Clinical Materials. Clinical materials consisted of peripheral blood and bone marrow from patients with hematologic malignancies and healthy donors. All clinical materials were obtained after informed consents and with approval from the local ethics committee. Both presentation and relapsed samples were included.

Tumor Cell Lines. Eight multiple myeloma and one chronic myelogenous leukemia (CML) cell lines were used: ARK-B and ARP-1 [gifts from Joshua Epstein, University of Arkansas for Medical Sciences (Little Rock, AR)], H929, KMS-11, and RPMI 8226 [gifts from Raymond Comenzo, Memorial Sloan-Kettering Cancer Center (New York, NY)], IM-9 and U266 [gifts from Dharminder Chanhan; Dana-Farber Cancer Center (Boston, MA)], MM1-R [gift from Steve Rosen, Northwestern University (Evanston, IL)], and K562 (from American Type Culture Collection, Manassas, VA). All cells were maintained in liquid culture before being used for the experiments.

Reverse Transcription-PCR (RT-PCR) Amplification of SLLP1 mRNA. Total RNA was isolated using a RNAEasy kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's recommendation. RT-PCR was performed. Briefly, all RNA specimens were first treated with DNase I (Ambion, Inc., Austin, TX) to remove genomic DNA contamination. First-strand cDNA was synthesized from 1 μg of total RNA using a random hexamer primer. The PCR primers were 5′SLLP1PCR, 5′-*AAGCTCTACGGTCGTTGTGAACTG*-3′, and 3′SLLP1PCR, 5′-*CTAGAAGTCACAGCCATCCACCCA*-3′, and they amplified a SLLP1 gene fragment of 387 bp. PCR was performed using 35 amplification cycles at an annealing temperature of 66°C. Negative controls in all of the PCR reactions contained the PCR reaction mixture, except for cDNA that was substituted with water. RNA integrity in each sample was checked by amplification of a β-actin gene segment. Successful removal of genomic DNA contamination was confirmed in each sample by amplification of the RNA without prior reverse transcription reaction. PCR products were visualized on an ethidium bromide agarose gel. All results were confirmed on two independent RT-PCRs.

Real-Time PCR. A panel of normal tissue total RNA was obtained from Strategene Corp. (La Jolla, CA). Peripheral blood and bone marrow were obtained from healthy donors and patients with hematologic malignancies. Total RNA was isolated using a RNAEasy kit (Qiagen, Inc.) according to the manufacturer's recommendation. Reverse transcription was carried out using random hexamer primers on 250 ng of total RNA. All PCR reactions were carried out in triplicates in the ABI 5700 real-time PCR machine. The primers used were as follows: SLLP1/5′, 5′-*TGGGTCTGCCTTGCTTATTT*-3′, and SLLP1/3′, 5′-*TGGGTTATCTTCATGGCACA*-3′. The sequence of the TaqMan probe used was as follows: 5′-6FAM-*GACTACGAGGCTGATGGGAG*-TAMRA-3′. Thermal cycle conditions were as follows: 50°C for 2 minutes, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. All results were confirmed by two independent RT-PCRs. All results were normalized to 18S rRNA amplification (Applied Biosystems, Foster City, CA). SLLP1 cDNA in pCRII was used for constructing the standard curves. The plasmid was serially diluted, starting 10^5 plasmid copies and analyzed in triplicate. A standard curve was constructed by plotting the C_T and the known copy number on a logarithmic scale and used to calculate the copy number of SLLP1.

Generation and Purification of SLLP1 Recombinant Protein. The nucleotide sequence coding the mature SLLP1 protein (residue 88–215) was amplified from human testicular RNA. The PCR products were cloned into the TA-cloning system. The DNA was analyzed for nucleotide sequences bidirectionally and then subcloned into pQE30 vector (Qiagen, Inc.) to produce a recombinant fusion protein of SLLP1 that contained a 6-histidine peptide at the NH_2-terminal of the protein. This strategy allowed affinity purification of the recombinant protein in a Nickel Sephadex column. The recombinant plasmid was transformed into *Escherichia coli* and selected on agar plates for ampicillin resistance. Recombinant clones were selected by restriction digest for cDNA fragments of the predicted size. To generate the recombinant protein, a recombinant clone was expanded in liquid culture and induced by 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside for 4 hours. Recombinant SLLP1 protein was harvested from *E. coli* lysate by sonication. After passage through the Ni-NTA affinity column and numerous rounds of washing, the protein was eluted. Successful generation of recombinant SLLP1 protein was confirmed on SDS-PAGE by Coomassie blue staining and Western blotting.

Western Blot Analysis. Purified SLLP1 protein was fractionated in a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Successful generation of SLLP1 protein was confirmed using an antibody directed at the histidine tag and SLLP1 rat antisera (5). SLLP1 antibodies in the patients' sera (1:1000 dilution in PBS) were detected by a goat antihuman IgG alkaline phosphatase conjugate. Antibody binding was visualized by reaction with the Western Blue-stabilized substrate (Promega, Madison, WI). An equal amount of another recombinant fusion protein derived from *E. coli* (Clone 4 protein) was used as the negative control. For the detection of SLLP1 protein in tumor cell lysates, we prepared the cell lysates by repeated rounds of freezing and thawing. The lysates were then fractionated in a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. SLLP1 protein in the cell lysates was detected using the SLLP1 rat antisera.

ELISA. Antibodies directed at SLLP1 protein were detected in the patients' sera using an in-house ELISA system. Briefly, 96-well flat-bottomed microtiter plates were coated with the purified recombinant SLLP1 protein at a concentration of 5 μg/mL. After 4 hours adherence of the antigen to the plate at 37°C, the wells were washed and then blocked with 3% BSA in PBS at 37°C for 2 hours. Patients' sera were diluted 1:1000 with the blocking buffer and then dispensed into the wells in triplicates and allowed to bind overnight at 4°C. Goat antihuman IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) was then added to each well (1:1000 dilution in the blocking buffer). After 2 hours of incubation at room temperature, *p*-nitrophenylphosphate solution was added to each well and incubated at room temperature for color development. Twenty five microliters of 2 N NaOH were added to stop the reaction. Color intensity was measured on a microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed using the Softmax data analysis program. In each experiments, two different controls

were set up: one consisted of wells coated with a control *E. coli*-derived recombinant 6-histidine fusion protein and another consisted of wells coated with PBS only before the addition of the blocking buffer. All results were confirmed in two independent experiments.

RESULTS

***SLLP1* Is Expressed in Hematologic Malignancies.** Using a pair of sequence-specific primers in RT-PCR, we first investigated the expression of SLLP1 transcripts in a panel of hematologic tumor cell lines. We found that SLLP1 transcripts could be detected in five of eight multiple myeloma cell lines: ARK-B, H929, KMS-11, MM-1R, and U266 cells (Fig. 1*A*). Expression in H929, MM-1R, and U266 was strong and in ARK-B and KMS-11 weak. In contrast, SLLP1 transcripts could not be detected in ARP-1, IM-9, K562, and RPMI 8226 cells. These results, therefore, suggest that *SLLP1* may be expressed in tumor cells from some hematologic malignancies, in particular, in multiple myeloma because most of these tumor cells were derived from multiple myeloma.

We then applied the RT-PCR to RNA from fresh primary specimens to determine whether *SLLP1* expression also occurred in primary hematologic tumor cells. A total of 52 primary specimens (peripheral blood or bone marrow containing leukemia cells) was examined. SLLP1 transcripts were detected in 6 of 17 (35%) multiple myeloma, 4 of 14 (29%) CML, 3/11 (27%) chronic lymphocytic leukemia (CLL), 2 of 9 (22%) acute myelogenous leukemia (AML), and 0 of 1 hairy cell leukemia specimens (Fig. 1*B*). In contrast, SLLP1 transcripts were not detected in the peripheral blood ($n = 12$) or bone marrow ($n = 3$) from any healthy donors.

SLLP1 gene has a unique *Pvu*II internal restriction site and complete enzyme digestion of the PCR product yielded two DNA fragments of 266 and 121 bp. To confirm the specificity of the amplified DNA, all PCR products were either cloned for sequence analysis or subjected to *Pvu*II restriction digest. Sequence analysis confirmed that the PCR products were *SLLP1*. Restricted digest with *Pvu*II also confirmed the specificity of the amplified DNA fragments (data not shown). These results, therefore, indicate that *SLLP1* is expressed in hematologic malignancies. Because of the small sample size in each disease group, it was not possible to correlate *SLLP1* expression with clinical stages of the diseases.

To determine whether the SLLP1 mRNA was translated into protein in these tumor cells, we prepared cell lysates for Western blot analysis from specimens in which the tumor cells were available. Spermatozoa lysate was used as the positive control and the membranes probed with SLLP1 rat antisera. A signal at the expected molecular weight of M_r 15,000 was observed in spermatozoa lysate and in two of the five PCR+ multiple myeloma cell lines, U266 and MM-1R (Fig. 2). Probably due to low protein copy numbers, we did not observed any signal in lysates from ARK-B, H929, and KMS-11 despite positivity for the SLLP1 transcripts by RT-PCR. Similarly, positivities by Western blotting were only observed in fresh hematologic tumor cells with high RT-PCR signals. Nine AML samples (two PCR+ and seven PCR−) were evaluated. Only one of the two PCR+ AML specimens produced a M_r 15,000 band. Five CLL samples (two PCR+ and three PCR−) were evaluated, and only one of the two PCR+ CLL samples were positive in Western blot analysis. Six CML samples (two PCR+ and four PCR−) were studied and both the two PCR+ samples produced a detectable SLLP1 protein. Five multiple myeloma samples (two PCR+ and three PCR−) and SLLP1 were detected only in one of two PCR+ samples. Most importantly, all PCR− specimens were also negative in Western blot analysis. To confirm the specificity of the M_r 15,000 signals in Western blot analysis, blocking assays were carried out. We found that the positive signals could be abrogated if the SLLP1 rat antisera were preincubated with a high concentration of a SLLP1 recom-

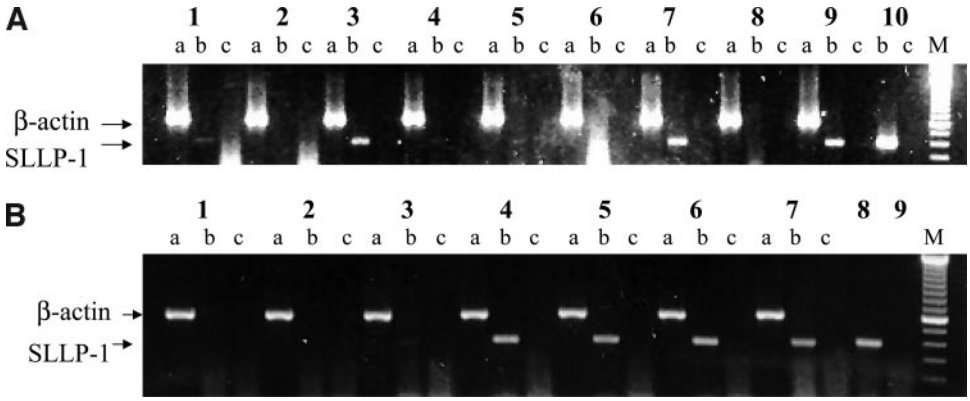

Fig. 1 SLLP1 expression by tumor cells from hematologic malignancies. *A*, RT-PCR analysis for SLLP1 gene expression in hematologic tumor cell lines, showing PCR products of the expected size in ARK-B, H929, KMS-11, MM-1R, and U266 (*Lane 1*, ARK-B; *Lane 2*, ARP-1; *Lane 3*, H929; *Lane 4*, IM-9; *Lane 5*, KMS-11; *Lane 6*, K562; *Lane 7*, MM-1R; *Lane 8*, RPMI 8226; *Lane 9*, U266; *Lane 10*, normal testis; *Lane M*, molecular marker). (*Lane a*, RT-PCR amplification of a β-actin gene segment; *Lane b*, RT-PCR for SLLP1 gene fragment; *Lane c*, PCR without reverse transcription for SLLP1 gene fragment.) *B*, RT-PCR analysis for SLLP1 gene expression in fresh hematologic tumor cells, showing PCR products of the expected size in AML, CLL, CML, and multiple myeloma (*Lane 1*, peripheral blood from a healthy donor; *Lane 2*, bone marrow from a healthy donor; *Lane 3*, hairy cell leukemia; *Lane 4*, AML; *Lane 5*, CLL; *Lane 6*, CML; *Lane 7*, multiple myeloma; *Lane 8*, normal testis; *Lane 9*, negative control; *Lane M*, molecular marker). *Lane a*, RT-PCR amplification of a β-actin gene segment; *Lane b*, RT-PCR for SLLP1 gene fragment; *Lane c*, PCR without reverse transcription for SLLP1 gene fragment.

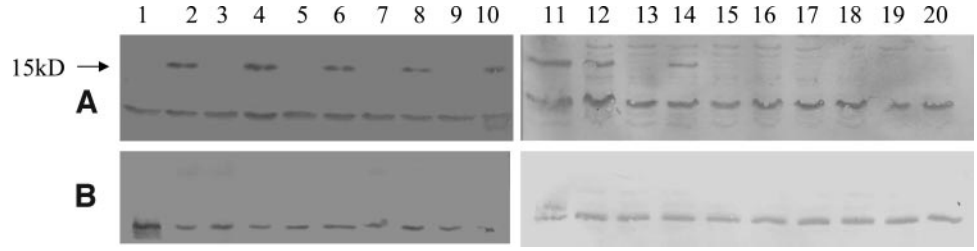

Fig. 2 Western blot analysis of cell lysates for SLLP1 protein before (*A*) and after (*B*) blocking of the SLLP1 rat antisera with a recombinant SLLP1 protein. (*Lane 1*, SLLP1− AML; *Lane 2*, SLLP1+ AML; *Lane 3*, SLLP1− CLL; *Lane 4*, SLLP1+ CLL; 5 = SLLP1− CML; *Lane 6*, SLLP1+ CML; *Lane 7*, SLLP1− MM; *Lane 8*, SLLP1+ MM; *Lane 9*, PBMCs from a healthy donor; *Lane 10*, sperm control; *Lane 11*, sperm control; *Lane 12*, U266; *Lane 13*, = RPMI 8226; *Lane 14*, MM−1R; *Lane 15*, K562; *Lane 16*, KMS-11; *Lane 17*, IM-9; *Lane 18*, H929; *Lane 19*, ARP = 1; and *Lane 20*, ARK-B).

binant protein before being used to probe the membrane, confirming the specificity of the M_r 15,000 signals. These results, therefore, indicate the expression of both SLLP1 transcripts and SLLP1 protein in hematologic malignancies.

SLLP1 Is a Cancer–Testis Antigen. Having demonstrated the expression of SLLP1 in hematologic malignancies, we then studied SLLP1 expression patterns in various tissues to determine whether SLLP1 is a cancer–testis antigen. We used 35 amplification cycles in RT-PCR on total RNA derived from a panel of 25 normal tissues. SLLP1 transcripts could not be detected in any normal tissues, except testis (data not shown), providing evidence in support of a testicular-specific expression of SLLP1. Taken together with the findings of SLLP1 expression in hematologic tumor cells, these results indicate that SLLP1 is indeed a novel cancer–testis antigen in hematologic malignancies.

Real-Time PCR Analysis of SLLP1 Transcripts. Real-time PCR was used to determine whether low copy numbers of SLLP1 transcripts could be detected in some normal tissues and to compare the levels of SLLP1 expression in tumor cells with that in normal tissues. Normal testis expressed 8206 copies/0.25 μg total RNA of SLLP1 transcripts. In contrast, none was detected in any of the normal tissues (Fig. 3), confirming the restricted normal tissue expression of SLLP1. We also determined the SLLP1 transcript copy numbers of tumor cell lines and fresh hematologic tumor cells (Fig. 3). We found that the SLLP1 mRNA copy numbers in these tumor cells were up to 2316 copies/0.25 μg total RNA, *i.e.*, >25% of the level found in normal testis. Interestingly, when we correlated these results to those on protein expression in their respective tumor cell lysates by Western blot analysis, we found that the SLLP1 transcript copy number of at least 250 copies/0.25 μg total RNA is needed for the detection of SLLP1 protein by Western blot analysis.

Successful Generation of SLLP1 Recombinant Protein. The coding sequence of SLLP1 was isolated and amplified from normal testicular RNA and expressed in *E. coli* as a recombinant protein of SLLP1 fused with a NH_2-terminal 6-histidine tag. After protein induction with β-D-thiogalactopyranoside, the bacterial lysates were fractionated by SDS-PAGE and detected using Coomassie blue staining and Western blot analysis. The recombinant SLLP1 displayed an expected molecular size of M_r ~15,000 and was visualized in Western blot analysis by anti-histidine tag antibodies and SLLP1 rat antisera (Fig. 4). The protein was subsequently purified using a Ni^+ column. The purified protein (> 95%) was used in subsequent experiments.

High Titer IgG Antibodies against SLLP1 Can Be Detected in Patients with Hematologic Malignancies. The *in vivo* immunogenicity of a protein in cancer-bearing patient is one of the prerequisite for the protein to be a suitable candidate antigen for tumor vaccine. To investigate this, we determined the presence of anti-SLLP1 antibodies in the sera of these

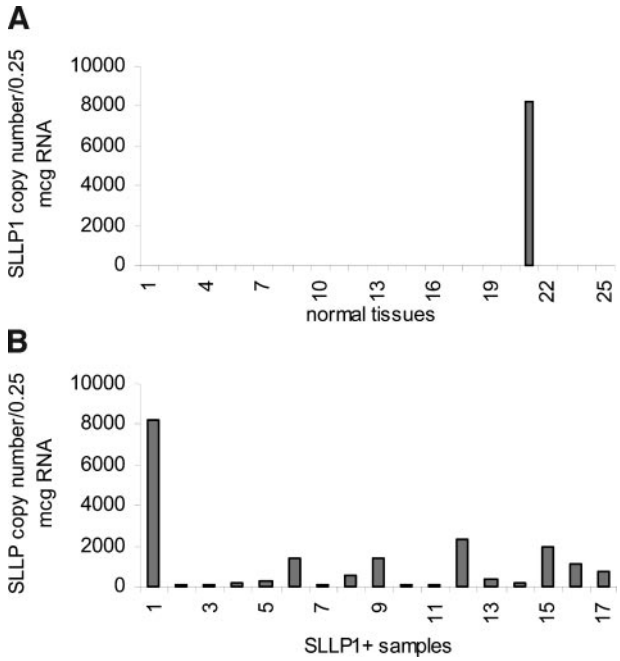

Fig. 3 Real-time PCR analysis to evaluate the levels of SLLP1 gene expression. *A.* SLLP1 transcripts were only detected in testis (*1*, adrenal gland; *2*, blood; *3*, bone marrow; *4*, brain; *5*, cerebellum; *6*, colon; *7*, fetal brain; *8*, fetal liver; *9*, heart; *10*, kidney; *11*, liver; *12*, = lung; *13*, placenta; *14*, prostate; *15*, salivary gland; *16*, skeletal muscle; *17*, spleen; *18*, stomach; *19*, small intestine; *20*, spinal cord; *21*, testis; *22*, thyroid; *23*, thymus; *24*, trachea; and *25*, uterus). *B.* SLLP1 was expressed at >25% of testicular SLLP1 mRNA level (*1*, testis; *2*, ARK-B; *3*, KMS-11; *4*, H929; *5*, U266; *6*, MM-1R; *7* to *8*, AML; *9* to *11*, CLL; *12* and *13*, CML; and *14* to *17*, MM).

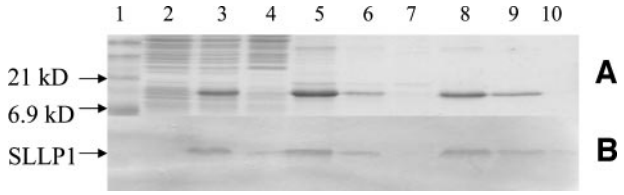

Fig. 4 Successful generation of SLLP1 recombinant protein from *E. coli*. *A*, Coomassie blue staining of SDS-polyacrylamide gel. *B*, Western blot analysis using anti-6-His antibodies showing the successful generation of a fusion protein of 6-histidine (*Lane 1*, protein marker; *Lane 2*, lysate from *E. coli* transformed with the pQE30 vector; *Lanes 3–10*, lysates from *E. coli* transformed with the pQE30-SLLP1 recombinant vector; *Lane 3*, whole lysate; *Lane 4*, soluble fraction; *Lane 5*, insoluble fraction; *Lane 6*, flow through fraction; *Lane 7*, wash through fraction; *Lanes 8–10*, different eluted fractions).

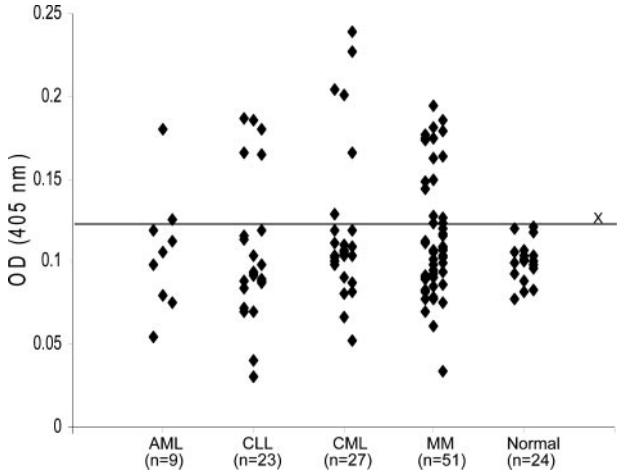

Fig. 5 ELISA analysis of diluted sera (1:1000) showing the presence of antibodies directed at SLLP1 in some patients with hematologic malignancies but not in healthy donors. X denotes the cutoff $A_{405\ nm}$ at mean +2SD from healthy donors.

patients using the purified SLLP1 recombinant protein in an ELISA system. We first established the basal signals in the ELISA system using sera from 24 healthy donors (mean ± SD at $A_{405\ nm} = 0.0995 \pm 0.0107$). Using the mean + 2SD as the cutoff signal intensities at $A_{405\ nm}$, we found that high titer IgG antibodies directed at SLLP1 protein were detectable by ELISA in some patients with hematologic malignancies (Fig. 5). SLLP1 IgG could be detected in the sera from 2 of 9 AML, 5 of 23 CLL, 6 of 27 CML, and 14 of 51 multiple myeloma patients. The sera were tested in high dilution of 1:1000 to improve the specificity of the antibody binding. In contrast, signals from the two sets of control wells in the ELISA were uniformly low and within the same levels of signal as those obtained from the healthy donors.

To additionally confirm the presence of SLLP1 antibodies in these sera, we carried out Western blot analysis, applying all of the SLLP1 antibody+ sera to the immobilized recombinant SLLP1 protein or a control recombinant 6-histidine fusion protein that is also derived from *E. coli*. Because of the decreased sensitivity of the detection system in Western blot analysis, only 1 of 2 AML, 3 of 5 CLL, 4 of 6 CML, and 7 of 14 multiple myeloma SLLP1 antibody+ sera produced a signal (Fig. 6). In contrast, sera from eight healthy donors and four each of ELISA-AML, CLL, CML, and multiple myeloma did not produce any Western blot signal.

To gain insight into the nature of the B-cell immune responses against SLLP1 in these patients, we also determined the IgG subclass of the SLLP1 antibodies from 26 patients. SLLP1 antibodies from 2 patients were IgG1, 15 were IgG2, seven were IgG3, and 2 were IgG4 (Table 1). Interestingly, SLLP1 IgG2, suggestive of a Th2 immune response, was by far the commonest IgG antibody.

Correlation between Gene Expression and Immune Responses. In 32 patients (9 AML, 7 CLL, 11 CML, and 5 multiple myeloma), paired tumor samples and sera were available to determine any correlation between SLLP1 gene expression and B-cell immune responses within these individual patients. We found that B-cell immune responses to SLLP1 predicted for SLLP1 gene expression (Table 2). SLLP1 antibodies were detected in the sera from six of the nine SLLP1+ patients but none of the 23 SLLP1− specimens ($P < 0.001$), suggesting that the SLLP1 IgG antibodies were most likely generated as a consequence of SLLP1 expression.

DISCUSSION

Using modern immunologic approaches that include serology screening of expression cDNA library (3) and bioinformatics (6–9), there are increasingly more cancer–testis antigens

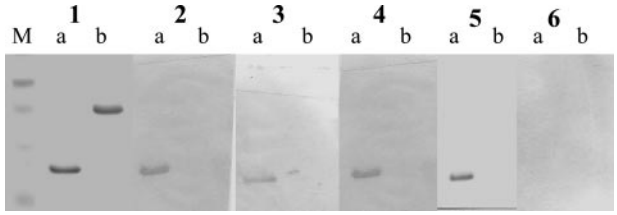

Fig. 6 Western blot analysis showing the binding of human IgG in the sera to immobilized SLLP1 recombinant protein. (*Lane M*, protein marker; *Lane 1*, Coomassie blue staining; *Lane 2*, AML; *Lane 3*, CLL; *Lane 4*, CML; *Lane 5*, MM; *Lane 6*, healthy donor.) *Lane a*, SLLP1 recombinant protein; *Lane b*, a control 6-His fusion protein derived from *E. coli*.

Table 1 SLLP1 IgG subtypes

	IgG1	IgG2	IgG3	IgG4
AML (2)	0	2	0	0
CLL (4)	0	3	1	0
CML (6)	0	4	1	1
Multiple myeloma (14)	2	6	5	1

Table 2 Correlation between GE and IRs

	GE+/IR+	GE+/IR−	GE−/IR+	GE−/IR−
AML (9)	1	1	0	7
CLL (7)	2	1	0	4
CML (11)	2	0	0	9
Multiple myeloma (5)	1	1	0	3

Note: Numbers in parenthesis represent total number of patients.
Abbreviations: GE, gene expression; IR, immune response.

being identified in hematologic malignancies. These antigens include Sperm protein 17/CT22 (7), MAGE-1 (10), NY-ESO-1/CT6 (11), SEMG1 (9), SCP1 (12), MAGE-C1/CT7 (13), SSX/CT5 (14), and SPAN-Xb/CT11 (8). Only a proportion of these antigens have been shown to induce a cellular and/or humoral immune response in humans. Identification and characterization, especially whether they are immunogenic *in vivo* in cancer-bearing patients, of many more such molecules are appropriate because they provide the opportunity for the design of polyvalent tumor vaccines that targets several antigens simultaneously to improve tumor cell lysis by increasing the *in vivo* E:T ratios and preventing tumor escape mechanisms.

Several factors determine the suitability of a protein as an antigen for tumor vaccines (15). In addition to expression in tumor cells, restricted normal tissue expression of the protein will provide specificity and hence reduced toxicity from a tumor vaccine based on the protein. Furthermore, the protein has to be immunogenic *in vivo* in cancer-bearing patients that are usually immunosuppressed. Other considerations include the appropriate processing and presentation of the antigenic peptide in association with the MHC molecules on the cell surface for T-cell recognition and optimal tumor–effector cell interaction. In the present study, we have set out to test our hypotheses that the spermatozoa protein, SLLP1, is a cancer–testis antigen in hematologic malignancies and is immunogenic *in vivo* in these patients. Proof of these hypotheses provides the foundation in support of any future *in vitro* works to additionally characterize SLLP1 as a target for tumor immunotherapy.

Using a combination of RT-PCR, sequence analysis, and/or RFLP, we demonstrated SLLP1 gene expression in the tumor cells from ~30% of patients with various hematologic malignancies. We also found that SLLP1 expression in the tumor cells occurred at both mRNA and protein levels, implying the potential clinical and therapeutic relevance of SLLP1 expression in these tumor cells.

Although a previous study showed the expression of SLLP1 transcripts restricted primarily to normal testis (5), the detection system using Northern blot analysis did not allow for the study of low copy numbers in these normal tissues. Having demonstrated the expression of SLLP1 in hematologic tumor cells, the normal tissue expression pattern of SLLP1 was investigated. Using 35 amplification cycles in RT-PCR on a large panel of 25 normal tissues, SLLP1 transcripts were detected only in testis, confirming that SLLP1 is indeed a novel cancer–testis antigen in hematologic malignancies. Real-time PCR analysis also failed to detect any SLLP1 transcript in all normal tissues studied, except testis, additionally confirming the testicular specificity of SLLP1 previously reported by Northern and dot blot analysis (5). This is in contrast to other cancer–testis antigens such as NY-ESO-1, CT15/fertilin β, and CT-16 that are also undetectable at 35 cycles of conventional RT-PCR but have been detected in a limited number of normal tissues at 40 cycles of real-time quantitative RT-PCR (6). However, the levels were also <3% of the level detected in normal testis. The biological significance of such low-level expression is unclear. It has been suggested that cancer–testis antigen genes often form families on the X chromosome (16). Interestingly, unlike the other cancer–testis antigen genes, SLLP1 is encoded by the gene, SPACA3, localized to 17q11.2.

Although the evaluation of protein expression levels is an important step toward identifying tumor-specific protein targets, other factors, such as the *in vivo* immunogenicity of the protein, are also important. In an attempt to determine the *in vivo* immunogenicity of SLLP1 in cancer-bearing patients, the sera from these patients were examined for the presence of high titer IgG antibodies against SLLP1 protein. This approach examined primarily the B-cell immunity. However, high titer IgG responses against SLLP1 generally imply that the B-cell responses have been generated with T-cell cognitive help. Therefore, the same protein would also be immunogenic to T cells. Evaluating B-cell immunity is preferred over studying *in vivo* T-cell immunity because SLLP1-reactive T cells may be difficult to detect in the peripheral blood due to their affinity for and, hence, migration to the predominant site of the tumor cells, *i.e.*, bone marrow. Even if T-cell migration to the bone marrow does not occur, these T cells may be anergized with subsequent deletion after activation because most of these tumor cells do not express significant immune costimulatory molecules. In contrast, B-cell immunity is generally long lasting. The detection of antibodies against SLLP1 will reflect the status of the immune reactivity against SLLP1 even a few months before the development of the malignant diseases. B-Cell immunity is also usually less affected by the malignant process. Using a combination of ELISA and Western blot analysis, high titer IgG antibodies directed at SLLP1 protein were in fact detected in the sera of some patients with hematologic malignancies. The close correlation between immune responses and SLLP1 gene expression suggests that the B-cell responses were most likely the result of SLLP1 expression by the tumor cells rather than a consequence of immune dysregulation and spontaneous autoimmunity because of the underlying malignant diseases. Unlike antibodies directed at SPAN-Xb (8), there was a predominant of IgG2 SLLP1 antibodies among these patients. Although IgG2 is generally associated with a Th2-type immune response, the significance of the IgG subclass differences is unclear and remains speculative.

In summary, we have provided the first evidence that SLLP1 is expressed in the tumor cells from a proportion of patients with hematologic malignancies. We have also demonstrated beyond any doubt that SLLP1 is a novel cancer–testis antigen with a very restricted normal tissue expression. SLLP1 protein is also immunogenic *in vivo* in cancer-bearing patients, suggesting that, provided the other conditions discussed earlier are fulfilled, SLLP1 may be a potential target for immunotherapy. These results, therefore, support SLLP1 as a protein target appropriate for additional *in vitro* study to define its suitability for immunotherapy. These studies should indicate whether SLLP1 protein is processed and the antigenic peptide presented in association with the MHC molecules at an adequately high concentration on the surface of the target cells for effector cell recognition and whether the E:T cell interaction that involves other surface molecules are optimal.

ACKNOWLEDGMENTS

We thank Dr. Bart Barlogie at the University of Arkansas for Medical Sciences for providing some of the multiple myeloma specimens used in this study.

REFERENCES

1. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 1995;92:11810–3.

2. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci USA 1997;94:1914–8.

3. Old LJ, Chen YT. New paths in human cancer serology. J Exp Med 1998;187:1163–1167.

4. Scanlan MJ, Simpson AJG, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun 2004;4:1.

5. Mandal A, Klotz KL, Shetty J, et al. SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. Biol Reprod 2003;68:1525–37.

6. Scanlan MJ, Gordon CM, Williamson B, et al. Identification of cancer/testis genes by database mining and mRNA expression analysis. Int J Cancer 2002;98:485–92.

7. Lim SH, Wang Z, Chiriva-Internati M, Xue Y. Sperm protein 17 is a novel cancer-testis antigen in multiple myeloma. Blood 2001;97:1508–10.

8. Wang Z, Zhang Y, Liu H, Salati E, Chiriva-Internati M, Lim SH. Gene expression and immunologic consequence of SPAN-Xb in myeloma and other hematologic malignancies. Blood 2003;101:955–60.

9. Zhang Y, Wang Z, Liu H, Giles FJ, Lim SH. Pattern of gene expression and immune responses to Semenogelin 1 in chronic hematologic malignancies. J Immunother 2003;26:461–7.

10. van Baren N, Brasseur F, Godelaine D, et al. Genes encoding tumor-specific antigens are expressed in human myeloma cells. Blood 1999;94:1156–64.

11. Dhodapkar MV, Osman K, Teruya-Feldstein J, et al. Expression of cancer/testis (CT) antigens MAGE-A1, MAGE-A3, MAGE-A4, CT-7 and NY-ESO-1 in malignant gammopathies is heterogeneous and correlates with site, stage and risk status of disease. Cancer Immun 2003;3:9.

12. Lim SH, Austin S, Owen-Jones E, Robinson L. Expression of testicular genes in haematological malignancies. Br J Cancer 1999;81:1162–4.

13. Lim SH, Bumm K, Chiriva-Internati M, Xue Y, Wang Z. MAGE-C1 (CT7) gene expression in multiple myeloma: relationship to sperm protein 17. Eur J Haematol 2001;67:332–34.

14. Tarte K, De Vos J, Thykjaer T, et al. Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. Blood 2002;100:1113–22.

15. Lim SH, Periman P, Klug P, et al. Defining tumor antigens: mRNA, protein or cytotoxicity? Trends Immunol 2002;23:236–7.

16. Scanlan JJ, Gure AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 2002;188:22–32.