Treatment of Chronic Lymphocytic Leukemia with a Hypomethylating Agent Induces Expression of NXF2, an Immunogenic Cancer Testis Antigen

Jason A. Dubovsky,1 Douglas G. McNeel,2 John J. Powers,1 John Gordon,3 Eduardo M. Sotomayor,1 and Javier A. Pinilla-Ibarz1

Abstract

Purpose: Critical to the success of active immunotherapy against cancer is the identification of immunologically recognized cancer-specific proteins with low tolerogenic potential. Cancer testis antigens (CTA), in particular, fulfill this requirement as a result of their aberrant expression restricted to cancer cells and lack of expression in normal tissues bypassing tolerogenic mechanisms against self. Although CTAs have been extensively studied in solid malignancies, little is known regarding their expression in chronic lymphocytic leukemia (CLL).

Experimental Design: Using a two-pronged approach we evaluated the immunogenicity of 29 CTAs in 22 patients with CLL and correlated these results to reverse transcriptase PCR data from CLL cell lines and patient cells.

Results: We identified IgG-specific antibodies for one antigen, NXF2, and confirmed this response by ELISA and Western blot. We found that treatment of CLL with 5-aza-2′-deoxycytidine can induce expression of NXF2 that lasted for several weeks after treatment. Treatment also increased levels of MHC and costimulatory molecules (CD80, CD86, and CD40) necessary for antigen presentation. In addition, we identified other promising antigens that may have potential immunotherapeutic application.

Conclusions: Our findings suggest that NXF2 could be further pursued as an immunotherapeutic target in CLL, and that treatment with demethylating agents could be exploited to specifically modulate CTA expression and effective antigen presentation in malignant B cells.

Chronic lymphocytic leukemia (CLL) is the most common B-cell malignancy in the western world and is characterized by a progressive accretion of long-lived mature B-lymphocytes with a low proliferation rate (1–4). CLL remains incurable using current therapies, and patients diagnosed at a young age almost invariably succumb to disease (5, 6). Recent advances in disease pathogenesis and cytogenetics have vastly improved our biological understanding and the therapeutic arsenal against this disease (3, 7). Immunotherapeutic approaches are among those novel treatments currently under investigation in CLL patients. An additional advantage is that because the majority of patients are of advanced age an immunotherapeutic approach may be more suitable than aggressive chemotherapeutic regimens (1). More notably, the emergence of novel immunomodulatory compounds has brought to light the importance of immunoregulation in the management of CLL, showing promising results that may help to alleviate the tolerogenic phenotype that characterizes this clinical malignancy (7–13).

Antitumor vaccines, also known as active immunotherapies, exemplify some of the newer targeted treatments being investigated (14). Ideal antigen candidates would have applicability to a significant percent of patients, expression restricted to the cancer, high immunogenicity, and would be essential for the survival of the CLL cell, thus restricting immune evasion (2). One particular class of antigens, the cancer-testis antigens (CTA), has been investigated for these particular characteristics (15). CTAs have expression restricted to immunologically “privileged” tissues (blood testis barrier and lacking surface expression of MHC molecule) such as germ cells, but are often aberrantly expressed in tumor cells (16, 17). Their expression is thought to be induced by global genome hypomethylation, a well-known characteristic of cancer, and it is this aberrant expression that presumably makes them available for immune recognition (18). Indeed, recent studies have shown that hypomethylating
Translational Relevance

Clinical advances in the management of chronic lymphocytic leukemia (CLL) highlight the efficacy of immunoregulatory mechanisms in the management of disease. In light of this, the discovery of immune-based strategies that complement the repertoire of drugs currently available signifies a major direction for the field. In previous studies we have helped to establish the linkage between demethylating agents and the expression of highly antigenic proteins in other solid tumors. In the current study, however, we have focused on CLL for which demethylating agents are far more translatable given the success of purine analogs in the treatment of disease. This study also provides the first focused attempt to characterize patient humoral immune response to cancer testis antigens as well as their expression in this form of cancer. Moreover, our results in in vitro models and CLL patient antigen responses allude to the potential efficacy of a "vaccine-induce" strategy that circumvents many of the tolerogenic mechanisms hindering current cancer vaccine trials.

Agents such as 5-azacytidine and 5-aza-2'-deoxycytidine (5A2) can induce expression of CTAs specifically in cancer cells for prolonged periods of time, allowing for effective administration of CTA-based antitumor vaccines (14, 19–22).

In this article, we sought to identify CTAs that are able to generate an immune response in the context of CLL and for which expression can be induced or up-regulated after treatment with a methylation inhibitor. To conduct this investigation we utilized a two-pronged approach. This approach utilized high-throughput phage immunoblot to identify immunologically recognized CTAs via identification of IgG responses in sera samples. This was followed by reverse transcriptase PCR (RT-PCR) to characterize the inducibility of these CTAs. By comparing the two datasets we have established NXF2 as an inducible CTA in CLL. Moreover, our approach has unveiled several CTAs that are frequently expressed in CLL cells and as such are amenable for immunotherapeutic interventions. Furthermore, we show that 5A2 can modulate surface expression of molecules associated with antigen presentation on CLL cells.

Materials and Methods

Subject populations. Sera and peripheral blood mononuclear cells were obtained from 22 patients with CLL (16 males and 6 females; mean age, 62 y; range, 37–77 y). Of these subjects, 10 had 13q deletions, 3 had 17p deletions, 4 had trisomy 12, and 7 had normal cytogenetics. In total, 7 patients had prior definitive treatment (Supplementary Table S1). Rai stage for the 22 patients was 0 for 5 patients, 1 for 13 patients, II for 1 patient, and IV for 3 patients. All subjects gave written institutional review board–approved informed consent for their blood products to be used for immunologic research. Blood was collected at H.Lee Moffitt Cancer Center (Tampa, FL), and sera were stored in aliquots at −80°C until used.

Phage immunoblot analysis. We previously reported the construction of a panel of λ phage encoding 29 CTAs (15). Analysis of this panel was conducted similarly to what we previously reported (14, 15). In brief, XL-1 blue MRF Escherichia coli were grown overnight, collected by centrifugation, resuspended in 10 mmol/L MgSO4, and poured on top agarose (Luria-Bertani broth/10 mmol/L MgSO4/0.2% maltose/0.7% agarose) over Luria-Bertani agar in Omniwell plates (Nunc). Phage encoding individual (9,000 pfu) CTAs were then spotted in replicates onto multiple bacterial agar lawns using a liquid handling robot (Biomek FX, Beckman). Spotted plates were allowed to sit undisturbed for 15 min and then overlaid with nitrocellulose membranes impregnated with 10 mmol/L isopropylthiogalactoside. Plates were incubated overnight at 37°C. The next day, filters were washed, blocked with TBS (50 mmol/L Tris pH 7.2, 100 mmol/L NaCl) plus 1% bovine serum albumin, and then probed overnight with human serum diluted 1:100 in blocking solution. Subsequently, the membranes were washed, and human IgG was detected with an alkaline phosphatase–conjugated antihuman IgG detection antibody (Sigma). The filters were washed again and then developed with 0.3 mg/mL nitro-blue tetrazolium chloride plus 0.15 mg/mL 5-bromo-4-chloro-3′-indolylphosphate p-toluidine salt. After development, filters were washed with deionized water and immunoreactive plaques were recorded for each filter by visual comparison with internal positive (phage encoding human IgG) and negative (empty phage encoding β-galactosidase) control plaques.

ELISA. Ninety-six–well high-binding plates (Corning) were coated with purified Glutathione-S-Transferase–linked NXF2 protein (Novus Biologicals) at 2 μg/mL or purified human IgG (Sigma) titrations starting at 2 μg/mL in 50 mmol/L sodium carbonate buffer (pH 9.6) overnight at 4°C. After blocking for 2 h at room temperature with PBS/1% bovine serum albumin, wells were filled with dilutions of CLL patient serum in blocking solution (1:25, 1:50, 1:100) and were incubated overnight at 4°C. To detect autoantibody, plates were washed three times with PBS/0.1% Tween-20 and antihuman IgG horseradish peroxidase antibody (GE-biosciences) was added at 1:1,000 in blocking buffer. Alternatively, for the IgG subtype ELISAs, biotinylated anti-IgG1, -IgG2, -IgG3, or -IgG4 (Sigma) were used followed by horseradish peroxidase–labeled streptavidin (GE-biosciences). After a three-wash step reactivity was measured using tetramethyl benzidine substrate (KPL) according to the manufacturer’s instructions.

Western blot. Lysates from NXF2 transiently transfected 293T cells were mixed 1:2 with 2× SDS Laemmli’s loading buffer (0.04 M Tris, pH 6.8, 12% glycerol, 1.25% SDS, 3% β-mercaptoethanol, 0.06% bromophenol blue) and boiled for 5 min at 100°C. Proteins were then resolved on 10% SDS-polyacrylamide gels and were electrophoretically transferred to nitrocellulose membranes. Membranes were then probed using standard immunoblot techniques, with CLL patient sera diluted 1:100 in blocking solution or NXF2-specific antibodies (Novus Biologicals). Final detection was done using ECL chemiluminescent substrate (Perkin Elmer) and autoradiography film.

Primary cell culture. CLL cells were isolated from peripheral blood by density gradient centrifugation, washed, and resuspended at 3 × 106 in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and 20 mg/mL recombinant human interleukin-4 (RDI). Isolated CLL cells (>95% purity) were cultured in 24-well tissue culture flasks atop an adherent bed of irradiated (30 Gy) CD40L (CD154)-expressing murine fibroblast L-cells. Cultures were maintained in the presence or absence of 1 μmol/L 5A2 for 72 h and were subsequently assayed via RT-PCR or flow cytometry.

RT-PCR. Total RNA was prepared from centrifugally pelleted cell cultures (RNeasy mini columns and RNAse free DNAse; Qiagen) or was commercially obtained (BioChain). RT-PCR reactions were conducted using the Qiagen one-step RT-PCR kit (Qiagen) with transcript-specific primers (Supplementary Table S2) and total RNA from various B-cell leukemia and CLL cell lines as templates (generous gifts from Dr. John Byrd at Ohio State University). RT-PCR amplification reactions were resolved on 2% agarose gels and the size of the amplified transcript was confirmed by comparison with DNA size markers (GelPilot 1 Kb Plus Ladder; Qiagen).

www.aacrjournals.org


Downloaded from clincancerres.aacrjournals.org on April 13, 2017. © 2009 American Association for Cancer Research.
Quantitative RT-PCR. Cell lines were cultured in the presence or absence of 5A2 at 1 μmol/L. After 72 h of culture, cells were washed multiple times with PBS and recultured in medium without 5A2 for up to 20 d. Total RNA obtained from these cultured cell lines (RNeasy mini columns, Qiagen) was analyzed for β-actin and NXF2 RNA by quantitative RT-PCR using a manufacturer’s standard protocol (iScript RT-PCR with SYBR green; BioRad) and NXF2, MAGE-A3, MAGE-A4, SSX1, SSX-2, or β-actin-specific gene primers (Supplementary Table S2, and β-actin-5’ TCATGAAGTGTCAGTTCCATGCT, β-actin-3’ CTTGAAACATTCGAGCCATCTG). Fluorescent amplicon signatures and cycle of transmittance values were obtained using a BioRad MyCycler and its associated software, MyQ v1.0 (BioRad). Fold changes in NXF2 expression relative to actin were calculated according to the 2−ΔΔCT method previously described (23). The reported fold change in gene expression was determined from two independent quantitative RT-PCR measurements done at different times.

Flow cytometry. Flow cytometric analysis of CLL cell lines was done using fluorochrome-labeled monoclonal antibodies (anti-HLA-A, B, C, -HLA-DR, -CD40, -CD80, -CD86, -CD19, and -CD20; Becton Dickinson and eBiosciences) and the vitality dye 4′,6-diamidino-2-phenylindole (Sigma). For carboxyfluorescein diacetate-succinimidyl ester staining, cells were resuspended in 0.5 μmol/L carboxyfluorescein diacetate-succinimidyl ester (Invitrogen) in RPMI medium for 15 min at 37°C followed by a wash and an additional 30-min incubation in serum-supplemented medium prior to resuspension in culture medium. Data were acquired on an LSRII cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Results
An IgG response specific to NXF2, a known CTA, was identified in CLL. In order to identify CTAs that may be recognized by the T-cell repertoire in the context of CLL we used high-throughput phage immunoblot, a technique we have previously described (14, 15). In brief, λ-phage encoding 29 known CTAs (Supplementary Table S2) were spotted robotically in duplicate onto a bacterial lawn and overlaid with nitrocellulose membranes impregnated with isopropylthiogalactoside to induce protein expression. These membranes were then probed with sera from 22 patients with CLL (Supplementary Table S1).

Immunoreactive plaques specific for NXF2 were identified in CLL patient 9 (Fig. 1A). This immunoreactivity was also found in serum collected from the same patient both three and nine months later, corroborating our initial data (Fig. 1B and C). These experiments were repeated in triplicate and all blots were found to be consistent. To further examine this IgG reactivity we conducted an NXF2-specific ELISA on all 22 patients that confirmed a robust IgG response in patient 9 at both available time points (Fig. 2A). Using subtype-specific ELISA this NXF2 reactivity was found to be predominantly composed of subisotype IgG1 (Fig. 2B). As further confirmation of reactivity, a Western blot was done using serum from a subset of patients (CLL 9, 1, 13, 2, 22, and 10) probing NXF2 transiently transfected 293T cell lysates that showed that the IgG response was specific for NXF2 (Fig. 2C).

Expression of CTAs is induced by demethylating agents in CLL. In addition to finding particularly immunogenic CTAs we also sought to identify methylation inhibitor inducible CTAs in CLL. To this end we designed transcript-specific primers specific for each of the CTAs described in Supplementary Table S2 and used this primer panel to profile CTA expression in a variety of CLL cells with or without 1 μmol/L 5A2 treatment using RT-PCR. The CLL cell lines MEC1, MECC2, and WaC3 were kindly provided to us by Dr. John Byrd at the Ohio State University Medical Center and are further described in (24, 25). Using these cell lines we identified multiple genes that were either induced de novo, were significantly up-regulated, or were constitutively up-regulated (Fig. 3A). Of the 29 CTAs tested, expression of NY-SAR-35, XAGE-1, GAGE-2, GAGE-7, LAGE-1, NXF2, NY-ESO-1, and SSX2 was induced de novo after treatment with 5A2. Expression of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-B2, SSX1, SSX4, TPX-1, and FATE-1 was significantly up-regulated by treatment with 1 μmol/L 5A2. Additionally, we found that mRNA levels of LIP1, MAD-CT-1, MAD-CT-2, SPA17, MAGE-B1, MAGE-E1, PAGE-5, and SPAN-XC were constitutively expressed.

To confirm the relevance of our in vitro cell culture models we examined the tumor CTA expression profile of three representative CLL patients 3, 9, and 13 using total RNA from purified CLL cells (>90% purity; Fig. 3B). We found that CTA expression was extremely similar to that of the cell lines.

![Fig. 1](https://example.com/figure1.png)

**Fig. 1.** High-throughput phage immunoblot indicates an IgG response to NXF2. Patient number 9 had a detectable response to NXF2 (A). Three months later, on a return visit for further observation, the same patient displayed reactivity to NXF2 once again, confirming the first occurrence (B). Reactivity to NXF2 was also seen at a 6-mo observation point for patient 9 (C). Immunoreactive spots are determined by visually comparing them with the negative “−” and positive control “+” spots on each filter. In the 22 screens NXF2 was the only reactive CTA discovered.
From these samples we found that NY-SAR-35 and XAGE-1 are expressed at low levels in some CLL patients. In one final layer of stringency we tested the CTA expression in cell lines from other similar B-cell lymphoproliferative disorders (Supplementary Fig. S1). In this screening the only consistently inducible CTA was NXF2, although other CTAs were inducible in particular cell lines.

Of the three classes of leukemia CTAs (inducible de novo, up-regulated, and constitutively expressed) currently the most attractive class from the prospective of CLL immunotherapy is made up of those CTAs that are not normally expressed, but upon treatment with 5A2 can be strongly induced de novo. This modulated expression may help to avoid many of the tolerogenic mechanisms utilized by CLL, as will be discussed further. Thus far, NXF2 satisfies both characteristics of our initial investigation: demonstratable immunogenicity and selective inducibility.

NXF2 is a naïve and selectively inducible CLL-specific antigen. Although NXF2 has showed expression in CLL cell lines after treatment with 5A2, aside from its initial discovery, there is scant information as to the expression in the majority of human tissues (26). Consequently, we evaluated the Oncomine microarray database, which contains data from over 20,000 human microarray studies for mRNA expression of NXF2 in normal tissues (27). In a panel of 40 normal tissues NXF2 was only significantly up-regulated in the testis, as expected (data not shown).

To confirm that NXF2 has no basal mRNA expression in CLL prior to treatment with methylation inhibitors we examined the RNA from 10 CLL patients at various stages in disease progression and found no detectable transcripts (data not shown). Because it is possible that the protein has a long half-life after initial mRNA expression, we tested several CLL patients by Western blot and again found no expression of NXF2 (data not shown). Taken together, these results indicate that the average CLL patient is likely naïve to the NXF2 antigen prior to treatment with demethylating agents.

Given that primary CLL cells do not proliferate ex vivo and thus do not incorporate 5A2 into their DNA it has previously been very difficult to study the demethylating effects of such nucleotide analogs outside of in vivo or cell culture systems. Recent research into the critical signaling networks between CLL cells and their stromal environment has led to the development of a CD40L-expressing murine fibroblast feeder cell line which, in conjunction with interleukin-4, induces limited proliferation in some human CLL primary samples. Using this system we characterized the epigenetic changes to the CTA expression pattern in CLL patient 9. As shown in Fig. 4, we saw a marked increase in FATE-1, MAGE-A4, and MAGE-A8 transcription and a de novo induction of XAGE, GAGE-4, GAGE-7, GAGE-8, SSX-1, SSX-2, PAGE-1, and NXF2 transcripts. These data served to confirm our initial antigen identification and to show NXF2 inducibility in primary cells.

**Immunogenic CTA expression can be modulated for a possible immunotherapeutic approach.** Because any potential CTA-based immunotherapy would require antigen expression to extend beyond the initial treatment period, we wanted to examine the
stability of antigen expression in the three CLL cell lines after 5A2 treatment. Cells were treated with 1 \(\mu\)mol/L 5A2 for 72 hours and then washed and cultured with serum-supplemented media only for up to 20 days. Cells were harvested at various time points after washout and assessed for NXF2 mRNA by quantitative RT-PCR. As shown in Fig. 5A, in the MEC1 cell line treatment induced a sharp increase in NXF2 expression (25-fold), which trailed off slowly yet still not completely by day 20. Surprisingly, the sister MEC2 cell line had different NXF2 expression kinetics indicating that peak expression (200-fold) came long after 5A2 treatment (around day 10) and tapered off, yet still not completely, by day 20. The WaC3 cell line up-regulated NXF2 to an extremely high level (450-fold), relative to MEC1/2. This high level of expression did not significantly change throughout the experiment, but the cell line was unable to survive beyond day 10, indicating a residual cytotoxicity induced by the 5A2 treatment.

In addition to NXF2, our screening has unveiled other promising antigens that have proven to be immunogenic in other forms of cancer. Among these antigens NY-ESO-1, SSX1, SSX2, MAGE-A1, and MAGE-A4 have received attention because they are expressed in a variety of common tumor types. Given that these antigens have shown exceptional promise, we decided to further characterize their expression in CLL after 5A2 treatment. Using the WaC3 cell line we found that mRNA expression levels for NY-ESO-1, MAGE-A1, and MAGE-A4 increased by 2.5- to 7.5-fold for each of these antigens after treatment with 5A2 (Fig. 5B).

These data indicate that in CLL there are proven antigenic CTAs expressed at high levels after 5A2 treatment, further implying the applicability of immunotherapy.

**Demethylation restores effective antigen presentation characteristics in CLL cell line.** Given that the majority of gene expression changes involved in the initiation of antigen presentation are epigenetically regulated, we wanted to investigate the role of 5A2 on the antigen-presenting cell capacity of the CLL cell. Using the representative CLL cell line MEC1 we analyzed the changes in MHC class I (HLA-A, B, C), MHC class II (HLA-DR), CD40, CD86, and CD80 by flow cytometry. As shown in Fig. 6A we found that both MHC class I and class II molecules were up-regulated after treatment. Furthermore, the costimulatory molecules CD40, CD80, and CD86 were also up-regulated. These effects were mimicked by in vitro cultured primary CLL cells, which were induced to proliferate in the presence of 5A2 using the CD40L-expressing murine fibroblasts along with interleukin-4. As an independent carboxyfluorescein diacetate-succinimidyl ester staining experiment showed, our in vitro fibroblast culture system induced proliferation in only 1% of the cells over the 72-hour treatment, thus our analysis represents a mixture of treated and untreated primary cells (Fig. 6B). These data suggest that treatment with 5A2 may aid in effective CTA presentation by the CLL cell to both CD4 and CD8 T cells.

**Discussion**

Our goal has been to identify potential CTA targets that may be pursued in the development of future immunotherapies. In previous reports we used similar high-throughput antibody screening methods to identify antigens in both prostate cancer and melanoma (14, 15). One critical difference is the two-pronged approach utilized. Hematologic malignancies such as CLL provide unparalleled access to tumor specimens via a simple blood draw, something uncommon to most solid tumors that require access to small biopsy or postsurgical resection specimens for mRNA or protein analysis. Our study has taken full advantage of this characteristic to complement...
Demethylation-induced Immunogenic CTA Expression in CLL

Fig. 1. NXF2 is among a variety of inducible CTAs in primary human CLL treated with 5A2. CLL cells cultured with interleukin-4 atop an irradiated CD40L-expressing murine fibroblast stromal layer were analyzed via transcript-specific RT-PCR for mRNA expression of CTAs with and without 1 μmol/L 5A2 treatment. Testis cDNA was analyzed as a positive control as well as a no-RNA transcript negative control, confirming the identity of the amplified products (controls displayed in Fig. 3).

our analysis. In previous reports we were only able to identify a small number of candidate antigens. We assumed that this was due to the small number of sera samples evaluated and because a smaller fraction of subjects would be expected to have an immune response to a particular antigen, even if expressed by a particular tumor (28). In the current report we started out with an even smaller number of sera samples, making this task even more difficult. Moreover, recent landmark efforts in epigenetic profiling have uncovered the methylation status of the CLL genome revealing relatively high and heterogeneous levels of global CpG methylation (29–31). In light of this evidence, it is conceivable that a variety of immunogenic proteins, including CTAs, are silenced and thus naïve to the immune system (32).

Despite these hurdles we have managed to identify NXF2 as an immunogenic CTA in a CLL patient. We confirmed this antibody response using both ELISA and Western blot. Interestingly, we were unable to confirm expression in any single patient with CLL, including patient 9 (the CLL patient with an antibody response to NXF2). It is highly likely that early progression and evolution of CLL is fashioned by immunoeediting, the repeated evasion of immune recognition (33–35). Although the exact antigen milieu that shapes CLL is still unclear because it is difficult to study the complete progression of the disease (starting at the initial transforming event) in any single patient, it is likely that antibody responses to these antigens may still be detectable because they persist years after antigen stimulation. It is postulated that these antigens already have a proven capacity to eliminate CLL clones, but their nonobligate nature has led to their eventual silencing. An additional explanation would be that patient 9 has a separate undiagnosed malignant condition which expresses NXF2 aberrantly and this served to induce this immune response. However, standard screening procedures for patients over 50 years of age were done with no evidence of any other malignant condition. Patient 9 has an indolent CLL characterized by the presence of 13q14 del., ZAP70 negative, IgVH mutated, but CD38 positive. Interestingly, patient 9 has a polyclonal IgG increase (2.1 g/dL compared with 1.5 g/dL for the upper limit of normal). This condition may have increased the likelihood of identifying a normally low-level antigen response in CLL, a disease which more commonly causes hypogammaglobulinemia. Nevertheless, because a specific IgG response requires CD4-positive T-cell help, our discovery gives us a glimpse of the functioning T-cell repertoire in the context of CLL.

Additionally, our study provides the first account of an in vitro method appropriate for treatment with DNA demethylating agents. The CD40L-expressing fibroblast stromal cell system has made it possible to isolate the epigenetic changes that occur in CLL upon treatment with such inhibitors (36–38). Furthermore, this being the first murine-based feeder cell system provides an obvious advantage when using PCR specific for a rare-expressed human gene. In our study it facilitated confirmation of NXF2 inducibility and the resulting immunophenotype of the 5A2-treated primary CLL cells. One caveat to this culture system is that it is still impossible to differentiate the expression and immunophenotype of the specific fraction of CLL cells that have incorporated the drug, leading to an observed dilution of the effects of 5A2.

NXF2 was first identified by homology to known murine spermatogonally expressed genes (26, 39). Fortunately, Loriot et al. had the foresight to attempt modulation of this gene using 5A2 at the time of discovery, a result that has shaped our current investigation. Although little attention has been paid to the antigenicity of NXF2, a few groups have investigated its function as an mRNA exporter, its interactions with cytoplasmic motor proteins, and its role in male infertility (40). It has been hypothesized that NXF2 may have multiple roles critical to the meiotic process (41). Conceivably, these roles would have wide-reaching benefits to the premalignant B cell, including the independent segregation of undesired chromosomes leading to aneuploidy and the recombination of genetic material leading to translocations and deletions (42). Moreover, Wang and coworkers have shown that expression of NXF2 is strictly temporally regulated in the early spermatocyte. This expression characteristic may also be true in the early development of leukemia.

In our study we characterized the spontaneous immune response to NXF2 as predominantly IgG1, which is consistent with the generation of a Th2 immune response that does not necessitate CD8-positive CTL activation. Although the ideal antigen would spontaneously generate a Th1 or Th17 response, it is not entirely unexpected that a patient with CLL would generate such a response given that Rossmann and colleagues have shown an association between CLL and T-cell production of Th2 bias cytokines such as interleukin-4 (43). This effect will have to be overcome in order for T-cell–based vaccines to be truly effective. However, emerging evidence implicates a CD4-positive T-cell response in significant, rapid, and antigen-specific cytotoxic responses in CLL and CTA (NY-ESO-1) vaccine trials (44, 45).

We have shown here that NXF2 mRNA can be up-regulated for extended periods of time after treatment with the methylation inhibitor 5A2. In prior studies we and others have found that transient treatment with 5A2 was able to induce prolonged
Fig. 5. NXF2 expression in CLL cell lines can be modulated by treatment with the DNA methylation inhibitor 5A2. CLL cell lines (white bars, MEC1; grey bars, MEC2; black bars, WaC3) were cultured for 72 h in the presence of 1 μmol/L 5A2. A, cells were washed and then continued to be cultured in the absence of 5A2. Cells were then collected on days 3, 5, 10, and 20 (WaC3 was unable to be cultured past day 10) and RNA was subjected to NXF2 transcript-specific quantitative RT-PCR.

B, RNA from the WaC3 cell line was subjected to quantitative RT-PCR specific for NY-ESO-1, MAGE-A4, and MAGE-A1. C, RNA from all CLL lines was subjected to quantitative RT-PCR for SSX2 and SSX1 indicating up-regulation of mRNA expression in the presence of 5A2. The fold increase in gene expression, compared with untreated cells and relative to actin, was determined using the 2^{ΔΔCT} method (23). The data shown are the mean and SD of three independent quantitative RT-PCR experiments.
and cancer-restricted expression of CTAs, and based on our results it seems as though this is true for CLL as well. It has been postulated that this is due to selective degradation of the corrective enzyme, DNA methyltransferase I, combined with an inability to up-regulate expression of this critical gene post-treatment. In our experience, however, mRNA expression of DNA methyltransferase I mRNA seems unchanged in both normal and cancer cells lending evidence to the contrary (14). Nevertheless, this characteristic will likely be essential to the generation of an effective immune response because CTAs have not proven to be obligate antigens (required for continued cancer survival or proliferation); their cancer-specific expression must be forced in order to prevent tumor escape variants from causing relapse.

In addition to NXF2, our study identified multiple novel CLL CTAs that may have varied application for an immunotherapeutic treatment of CLL. Classical antigens, antigens which are expressed constitutively in cancer cells, such as LIP1, MAD-CT-1 MAD-CT-2, SPA17, MAGE-B1, MAGE-E1, PAGE-5, and SPAN-XC as well as those antigens with lower level expression in a subset of CLL lines such as MAGE-A1, MAGE-A3, MAGE-A4, MAGE-B2, SSX1, SSX4, TPX-1, and FATE-1, have the potential to generate a robust immune response. In similar fashion, NY-ESO-1 and the MAGE family of CTAs are highly expressed in many tumor types and have shown promising results in early-phase vaccine trials (46, 47). Our results in both cell lines and primary CLL indicate that these antigens may also be effective targets in CLL.

In CLL an immunosuppressive phenotype enables the B cell to evade immune detection (2, 10, 11). Wierda and Kipps recently reviewed this topic putting together multiple strategies that have shown promising results. In the current study we

Fig. 6. Demethylation restores effective antigen presentation characteristics in CLL cells. The representative CLL cell line MEC1, or human primary CLL cells cultured using interleukin-4 and an irradiated CD40L-transfected fibroblast feeder layer, were cultured for 72 h in the presence of 1 μmol/L 5A2 and analyzed by flow cytometry for the surface expression of MHC class I molecules (HLA-A, B, C), MHC class II molecule (HLA-DR), and the antigen-presenting cell costimulatory molecules (CD80, CD86, and CD40) as compared with basal levels under an identical culture system without 5A2 (untreated; A). The 72-h proliferative capacity (and likewise 5A2 uptake capacity) is shown in carboxyfluorescein diacetate-succinimidyl ester–labeled primary human CLL cells in a separate experiment using only serum-supplemented RPMI (untreated), the CD40L fibroblast stromal layer plus interleukin-4, or the CD40L fibroblast stromal layer plus interleukin-4 plus 1 μmol/L 5A2 (B). Data show that 5A2-treated cells (black histograms) up-regulate expression of all five molecules when compared with basal expression (grey histograms) and that this trend is mimicked by primary human CLL cells despite only a small percentage of cells incorporating the drug in the in vitro system.

www.aacrjournals.org
show that surface expression of necessary costimulatory molecules as well as MHC class I and II can be increased on CLL cells using 5A2. Studies conducted by Coral et al. have shown similar results although never in a tumor arising from a semiprofessional antigen-presenting cell (48). It is conceivable this effect on a cell with antigen-presenting capacity can aid in the presentation of CLL peptides via MHC “signal 1” and the costimulation of T cells recognizing such peptides “signal 2.”

One unresolved consequence of CLL is that the current antigenic repertoire has likely generated tolerance. Theoretically this can be avoided by vaccinating towards an inducible de novo antigen that is not yet expressed followed by induction of antigen expression using hypomethylating agents. Similar experiments conducted by Guo et al. have shown that treatment of 4T1 tumor-bearing animals with 5A2, followed by adoptive transfer of P1A CTA-specific CTL, resulted in tumor-specific recognition and eradication (20).

In the current study we utilized a two-pronged approach to evaluate the immunogenicity of 29 CTAs in 22 patients with CLL and correlate these results to RT-PCR data from CLL cell lines and patient cells enumerating antigens that are both immunogenic and specific for CLL. We identified IgG specific for one novel CLL CTA, NXF2, and confirmed this response by ELISA and Western blot. In addition, we confirmed that treatment of CLL with 5A2 can induce expression of NXF2 for weeks posttreatment. Treatment also increases levels of MHC and costimulatory molecules necessary for antigen presentation. In addition, RT-PCR results identified other promising antigens that may have potential immunotherapeutic application. Our findings suggest that NXF2 could be further pursued as an immunotherapeutic target in CLL, and that treatment with demethylating agents could be exploited to specifically modulate antigen expression in a potential vaccine-induce strategy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Treatment of Chronic Lymphocytic Leukemia with a Hypomethylating Agent Induces Expression of NXF2, an Immunogenic Cancer Testis Antigen


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/10/3406

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/05/20/1078-0432.CCR-08-2099.DC1

Cited articles
This article cites 48 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/10/3406.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/15/10/3406.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.